Mushroom Biology and Mushroom Products

Edited by
Shu-ting Chang, John A.Buswell and Siu-wai Chiu

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MUSHROOM BIOLOGY AND MUSHROOM PRODUCTS

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Mushroom Biology and Mushroom Products

Proceedings of the First International Conference on Mushroom Biology and Mushroom Products 23–26 August 1993, The Chinese University of Hong Kong, Hong Kong

Edited by

Shu-ting Chang, John A. Buswell and Siu-wai Chiu

Department of Biology

The Chinese University of Hong Kong

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FOREWORD

The bringing together of scientists with an interest in mushrooms from the standpoint of any subdivision of biology and persons having practical economic concerns with mushrooms and mushroom products is essential if there is to be an open exchange of information of common interest. The First International Conference on Mushroom Biology and Mushroom Products was created to achieve this objective. The location of the conference in Hong Kong is especially appropriate since The Chinese University of Hong Kong has previously been the host of many gatherings of mushroom specialists for workshops and conferences, as well as being an active center for mushroom research by faculty and students.

It should be recognized at the outset that when we speak of Mushroom Biology as the subdiscipline of mycology that is concerned with the scientific study of mushrooms, a broad definition of the term mushroom has been taken; namely, the mushroom is considered as a macrofungus with a distinctive fruiting body large enough to be seen with the naked eye and to be picked up by hand. Thus defined, a mushroom is not restricted to the class Basidiomycetes; it may be either epigeous or hypogeous; and it is not necessarily fleshy or edible.

The total worldwide production of cultivated mushrooms has increased dramatically during the past 10 to 15 years. A production of approximately 1,100,000 metric tons (fresh weight) in 1980 increased to over 4 million tons in 1992. The factors responsible for this tremendous acceleration of growth in mushroom production are many and include:

1. **Expansion of production in a large number of countries.** This has frequently occurred as a cottage type enterprise rather than as a highly mechanized industry. This has been especially important for developing countries, because a good profit can frequently be realized in a short time with a low capital outlay.
2. **Cultivation of many species other than *Agaricus bisporus* in increased amounts.** Examples are *Lentinus edodes, Pleurotus* spp., and *Auricularia* spp.
3. **Plastic bag cultivation of many species.** This has increased the biology efficiency, shortened the period from spawning to harvesting and made possible mushroom production on waste materials.
4. **A great increase in production of *Volvariella volvacea* has resulted from the use of a compost made of cotton wastes from the textile mills and/or cotton seed hulls which contain a higher proportion of cellulose than the traditional paddy straw substrate.** This same compost is also used successfully for the bag cultivation of *Pleurotus* and *Lentinus*.
5. **Demonstration of the existence of medicinally valuable substances in many**

mushrooms. Examples include the polysaccharides: krestin, lentinan, and schizophyllan which are produced by *Coriolus, Lentinus,* and *Schizophyllum*, respectively. These compounds are indicated for treatment of various types of cancer and as immuno-regulators that stimulate the host's immune system.

6. **Increased consumption of mushrooms as a consequence of their use in popular foods such as pizza, and increased consumption by many because of the recognition of mushrooms as a health food that is high in protein content and low in caloric value.**
7. **The overcoming of social and cultural taboos** in some countries about eating mushrooms through education, promotion and as a consequence of increased travel.
8. **Research advances in both basic and applied aspects of mushroom biology.**

This conference has as its goal the exchange of information about new findings in the various aspects of mushroom biology and mushroom products, including cultivation technology, nutritional and medicinal aspects of mushrooms, bioconversion and composting, genetics and breeding. In addition to lectures, oral presentations, and poster demonstrations on these topics, round table discussions have been arranged on various specialized topics, such as: protein-bound polysaccharides of medicinal importance, post-harvest processing, contamination of mushrooms and mushroom products, and the current status of biological knowledge and cultivation practices in *Volvariella volvacea*.

Representatives from 41 countries have registered for the conference. In addition to established mushroom scientists, the conference, through the support of UNESCO and other organizations, was enhanced by the attendance of enthusiastic younger scientists from a number of countries. Two years from now, when the Second International Conference on Mushroom Biology and Mushroom Products is held, it is anticipated that an equally interesting and exciting scientific menu will await mushroom biologists.

June, 1993

Philip G. Miles
Professor of Biology
State University of New York
at Buffalo

PREFACE

Mushrooms are familiar items that have long been consumed as a food of high nutritive value and as a contribution to a healthy diet. Mushroom cultivation has traditionally served as a viable bioconversion process for the utilization of a wide range of abundantly available organic wastes. Mushrooms are produced today not only for their food value but also for their metabolic products, many of which possess antitumour, immunoregulatory and hypotensive functions without apparent adverse side effects. Therefore, it is clear that advances in mushroom cultivation technology and a better understanding of mushroom biology will bring long-term benefits to humankind.

At an early stage in the planning of the First International Conference on Mushroom Biology and Mushroom Products, held between 23-26 August 1993 in Hong Kong, we were most fortunate to receive rapid and positive guarantees of financial and moral support from the United Nations Educational, Scientific & Cultural Organization (UNESCO) Network for Microbial Resource Centres (MIRCENS), Concord Health Foods Limited (Hong Kong) and The Chinese University of Hong Kong, which were crucially important for the implementation of the Conference. To them, we express our special gratitude and thanks. Our sincere thanks also go to the UNESCO Regional Network for Microbiology in Southeast Asia, the International Society for Mushroom Science (ISMS), the International Union of Microbiological Societies (IUMS), The Croucher Foundation (Hong Kong), Carl Duisberg Gesellschaft - SEAPO (Thailand), Wakan Shoyaku Laboratory Company (Japan), Beijing-Hong Kong Academic Exchange Centre (Hong Kong), Giorgio Foods Inc. (USA) and Tak Fat Trading Company (Hong Kong) for their financial support.

The Conference provided a forum for the presentation of recent advances in the field, a meeting place for the discussion and exchange of ideas and, hopefully, served to promote future research and collaboration among researchers in both developed and developing countries.

The following topics are covered in this series of contributions:

(i) Fundamental Aspects of Mushroom Biology
(ii) Mushroom Cultivation and Bioconversion
(iii) Postharvest Processing and Quality Control
(iv) Nutritional and Medicinal Attributes of Mushrooms
(v) Future Prospects for Research and Development

These Proceedings are designed to give readers a cross-section of current research in the area and to explain how this research impacts on mushroom production and mushroom products. We extend our sincere thanks to all the contributing authors for their endeavours. In addition, we thank the staff of The Chinese University Press for their hard work in expediting the publication of this

volume in time for distribution during the Conference period. It is our hope that these Proceedings, in addition to providing information on recent advances in the areas of mushroom biology and mushroom products, will serve also to bring pleasant memories to all those scientists, some of whom travelled thousands of miles, who attended the Hong Kong Conference.

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Part I

MUSHROOM BIOLOGY - FUNDAMENTAL ASPECTS

CHAPTER 1

MUSHROOM BIOLOGY: THE IMPACT ON MUSHROOM PRODUCTION AND MUSHROOM PRODUCTS

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1. INTRODUCTION

According to the fossil record, mushrooms have existed since the Lower Cretaceous Period (approximately 130 million years ago), long before human beings evolved on this planet. It is probable that, from his earliest beginnings, man has utilized mushrooms as a food and, when we observe present day animals running in the wild and consuming mushrooms, it is easy to believe that prior to man's advent on the planet, animals were consuming mushrooms with relish and enthusiasm. In nature, mushrooms have not only been a source of food for man and other animals, but also have played an important role in the cycling of carbon and other elements through the breakdown of lignocellulosic plant residues and animal dung, which serve as the substrates for these saprophytic fungi. In this way, mushroom species, as agents of decay, help keep the environment from being overwhelmed by the dead organic debris of plants and animals. Simultaneously, mushrooms can produce a wide range of enzymes that degrade complex substrates, following which they absorb the soluble substances so formed for their own nutrition (Wood & Fermor, 1982; Wood, 1984). This absorptive nutrition is a characteristic of mushrooms. These complex substrates, which are generated annually in huge quantities, are agricultural, industrial, and forest waste products. Examples are: cereal straws, coconut and coffee waste products, sugar cane bagasse, sawdust and cotton wastes, etc. (Chang & Miles, 1989). Much of this material is either burned, shredded or used as landfill or for improvement of soil quality, even though these wastes constitute a potentially valuable resource and can be recycled for the production of edible food for man. Although physical and chemical technologies may, in some cases, play important associated roles for handling these wastes, biotechnical approaches are essential for the emergence of practical conversion processes which can be applied to situations in developing countries throughout the world where large-scale capital intensive operations are inappropriate.

Today, we understand much better than we did a few decades ago, the importance and widespread nature of the symbiotic mycorrhizal association that involves fungi with the roots of plants. The growth of these plants is enhanced by minerals received from the fungus which actively

absorbs them from the soil, and, in return, the fungus obtains sugars from the plant. Therefore, when we define a mushroom as a macrofungus with a distinctive fruiting body that is large enough to be seen with the naked eye and to be picked up by hand (Chang & Miles, 1992), we must also add that there are some mycorrhizal fungi which are mushroom species.

Although mushrooms were long appreciated because of their flavor and texture, and some for medicinal or tonic attributes, the recognition that they are nutritionally a very good food is much more recent. It is now known that mushrooms have a high protein content of good quality. Furthermore, mushrooms have a high proportion of unsaturated fatty acids, are a good source of several vitamins, fiber and minerals, and they are low in calories, sodium, fat, and cholesterol. In addition, their nucleic acid content is not high enough to limit their daily use as vegetable (Li & Chang, 1982).

Our present conference is concerned with the impact of mushroom biology upon mushroom production (mushroom science) and mushroom products (mushroom biotechnology). Broadly considered, mushroom biology includes any aspect of biology involving species which produce mushrooms as previously defined. Thus, mushroom biology provides the central knowledge for both mushroom science and mushroom biotechnology. It is our hope that this conference will not only provide for the exchange of information, but will also provoke thoughtful planning for future research and encourage collaborative efforts aimed at higher mushroom production and better use of mushroom products and their derivatives.

2. MUSHROOMS AND MUSHROOM BIOLOGY

According to the definition previously stated, mushrooms need not be Basidiomycetes, nor aerial, nor fleshy, nor edible. Mushrooms can be Ascomycetes, grow underground, have a non-fleshy texture and need not be edible. In other words, mushrooms can be roughly divided into four categories: (1) those which are fleshy and edible fall into the edible mushroom category, e.g., *Agaricus bisporus*; (2) mushrooms which are considered to have medicinal applications, and which are referred to as medicinal mushrooms, e.g., *Ganoderma lucidum*; (3) those which are proven to be, or suspected of being poisonous and which are called poisonous mushrooms, e.g., *Amanita phalloides*; and (4) a miscellaneous category which includes a large number of mushrooms whose properties remain less well defined. These may tentatively be grouped together as 'other mushrooms'. Some mushrooms fall into more than one category; for example, mushrooms that are not only edible, but also possess tonic and medicinal qualities. Out of approximately 69,000 described species of the estimated 1,500,000 species of fungi (Hawksworth, 1991), there are about 10,000 known species of fleshy macro-fungi, of which only a handful are lethal (Kendrick, 1985).

The historical records of intentionally cultivated mushrooms are shown in Table 1 and Figure 1. It is estimated that the first mushroom was cultivated around 600 A.D. This was *Auricularia auricula*. The mushroom which is produced in the greatest amount today, *Agaricus bisporus*, was not cultivated until 1600. Of the leading mushrooms of today that were cultivated before 1900, *Agaricus* is the only one that was not first cultivated in China. Note that *Pleurotus ostreatus* was first cultivated in the USA, and several other species of *Pleurotus* were initially cultivated in India.

Mushroom biology is the discipline concerned with the scientific study of mushrooms. It includes not only cultivation but also deals with every aspect of mushrooms, such as: taxonomy, development, nutrition, physiology, genetics, medicinal and tonic attributes, edibility, toxicity, etc. (Chang & Miles, 1992). Through improvement of, for example, taxonomic methods, the discovery and characterization of a great number of new species has recently occurred worldwide. The conservation of mushroom germplasm as part of the conservation of the world's biological diversity

TABLE 1. Historical record of commonly cultivated mushrooms.

Species	Record first cultivated (Est.)	Source
Agaricus bisporus	1600	Atkins (1979)
Agaricus bitorquis	1961	Singer (1961)
Agrocybe cylindracea	1950	Huang (1984)
Amanita caesarea	1984	Zhu & Xie (1984)
Armillaria mellea	1983	Zhang & Lu (1983)
Auricularia auricula	600	So (659)
Coprinus comatus	1984	Wang & Kang(1984)
Dictyophora duplicata	1982	Lin *et al.*, (1982)
Flammulina velutipes	800	Han (1590)
Ganoderma spp.	1621	Wang (1621)
Gloestereum incarnatum	1989	Zhang *et al.*,(1989)
Grifola frondosus	1983	Zhao & Yang (1983)
Hericium coralloides	1984	Xu & Li (1984)
Hericium erinaceus	1960	Chen (1988)
Hohenbuehelia serotina	1982	Liu & Guo (1982)
Hypsizigus marmoreus	1973	Zhang & Wang (1992)
Lentinus edodes	1000	Wang (1313)
Lentinus tigrinus	1988	Wu & Wei (1988)
Lyophyllum ulmarium	1987	Wang & Zhang (1987)
Morchella spp.	1986	Ower *et al.*,(1986)
Oudemansiella radicata	1982	Ji *et al.*, (1982)
Pholiota nameko	1958	Kaga & Kondo (1958)
Pleurotus citrinopileatus	1981	Shen (1981)
Pleurotus cystidiosus	1969	Miller (1969)
Pleurotus ferulae	1958	Mou & Cao (1986)
Pleurotus flabellatus	1962	Bano & Srinvatava (1962)
Pleurotus florida	1958	Block *et al.*,(1958)
Pleurotus ostreatus	1900	Falck (1917)
Pleurotus sajor-caju	1974	Jandaik (1974)
Poria cocos	1232	Zhou (1232)
Sparassis crispa	1985	Sun *et al.*,(1985)
Tremella fuciformis	1800	Chen (1983)
Tremella mesenterica	1985	Liu (1985)
Tricholoma gambosum	1991	Tian & Yang (1991)
Tricholoma lobayense	1990	Ganeshan (1990)
Tricholoma mongolicum	1991	Tian & Yang (1991)
Volvariella volvacea	1700	Yuen (1822)

has long term worldwide implications. A majority of mushrooms are described as saprophytic, obtaining nutrients from non-living organic materials, so-called compost. The presence of thermophilic fungi in mushroom compost has been shown to be important in promoting growth of mushroom

1991 *Tricholoma monolicum; Tricholoma gambosum*
1990 *Tricholoma lobayense*
1989 *Gloestereum incarnatum*
1988 *Lentinus tigrinus*
1987 *Lyophyllum ulmarium*
1986 *Morchella* spp.
1985 *Tremella mesenterica; Sparassis crispa*
1984 *Coprinus comatus; Amanita caesarea; Hericium coralloides*
1983 *Armillaria mellea; Grifola frondosus; Pleurotus sapidus*
1982 *Dictyophora duplicata; Oudemansiella radicata; Hohenbuehelia serotina*
1981 *Pleurotus citrinopileatus*
1974 *Pleurotus sajor-caju*
1973 *Hypsizygus marmoreus*
1969 *Pleurotus cystidiosus*
1962 *Pleurotus flabellatus*
1961 *Agaricus bitorquis*
1960 *Hericium erinaceus*
1958 *Pleurotus florida; Pleurotus ferulae; Pholiota nameko*
1950 *Agrocybe cylindracea*
1900 *Pleurotus ostreatus*
1800 *Tremella fuciformis*
1700 *Volvariella volvacea*
1621 *Ganoderm* spp.
1600 *Agaricus bisporus*
1232 *Poria cocos*
1000 *Lentinus edodes*
800 *Flammulina velutipes*
600 *Auricularia auricula*

FIGURE 1. Historical record of cultivated mushrooms.

mycelium and for a good yield of fruit bodies (Straatsma *et al.*, 1991 ; Straatsma & Sanson, 1993). Those that obtain nutrients from living organisms are called parasitic. Others associated with roots of plants are mycorrhizal mushrooms. According to their nutritional and ecological habits, mushrooms can be divided into two big groups: ectomycorrhizal and non-ectomycorrhizal. Within non-ectomycorrhizal mushrooms are five groups based upon the nature of the substrate upon which they grow - wood, straw, dung, soil, insects (Table 2).

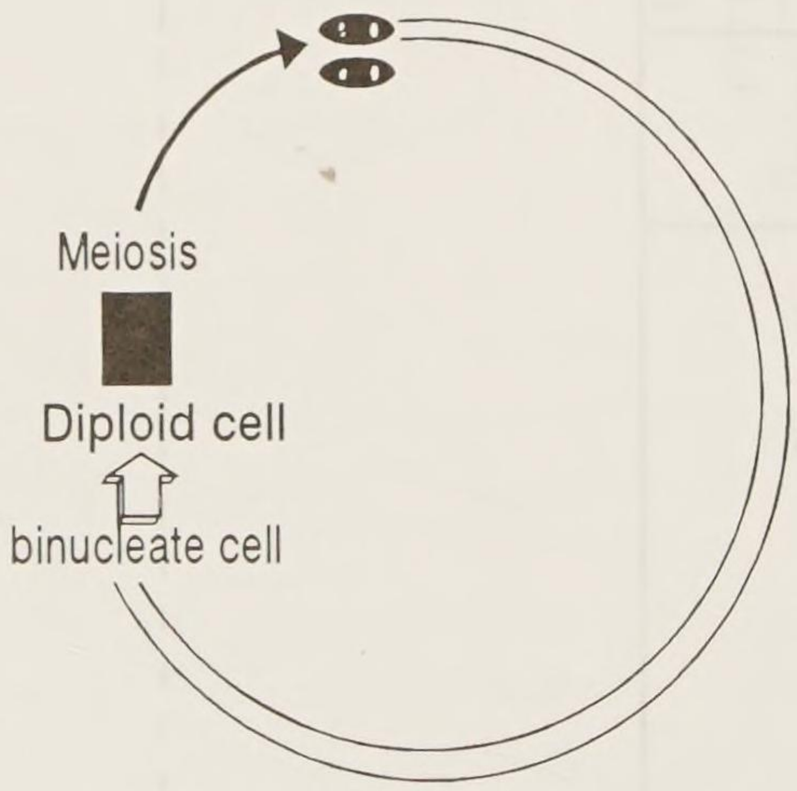
TABLE 2. The ecological habitats and the breeding selection of mushrooms.

Ecological habitats			Important genera	Fruiting by artificial cultivation
Non-ectomycorrhizal mushrooms	Above ground	Wood	*Pleurotus* *Lentinus* *Auricularia* *Tremella* *Hericium* *Pholiota* *Kuehneromyces*	easy
		Straw	*Volvariella*	
	On earth	Dung	*Coprinus* *Agaricus* *Agrocybe* *Stropharia*	
		Soil	*Lepiota* *Dictyophora* *Lepista* *Melanoleuca* *Morchella*	
		Insects	*Cordyceps* *Termitomyces*	
Ectomycorrhizal mushrooms	With roots	Mycorrhizal	*Tricholoma* *Ramaria* *Cantnarellus* *Boletus* *Suillus* *Gomphidus* *Lactarius* *Russula* *Amanita* *Cortinarius* *Rhizopogon* *Terfezia*	difficult

Due to a better understanding of their biological nature and the development of advanced cultivation techniques, more and more new species of mushrooms have been domesticated. About 80 out of 2,000 species which are regarded as prime edible mushrooms have been experimentally grown, 40 economically cultivated, around 20 commercially cultivated, and 5 to 6 have reached an industrial scale in many countries (Chang, 1993). Furthermore, by applying breeding techniques, cultivated strains can be developed that are uniform, of stable yield, specialized for quality, grow on a substrate of a particular composition and are adapted to specific environments. It is worth noting that the breeding program of any organism involves processes central to its reproductive genetics. In mushrooms, breeding systems involve both the mating system and post-meiotic nuclear behaviour. The nuclear behaviour determines whether an outbreeding or substantially inbreeding (self fertile) system controlled by mating-type factors is operative (Fig. 2). Outbreeding results from matings between two compatible single-spore isolates (Takemaru, 1954; Tanaka & Koga, 1972; Raper, 1976). Enforced inbreeding due to spores possessing two mating-type compatible nuclei is called secondary homothallism (Elliott, 1972; Tommerup *et al.*, 1991), in contrast to primary homothallism due to a uninucleate spore which can complete the life cycle without mating (Chang & Yau, 1971). Classical genetics demonstrated the genes that control mating type in these mushrooms. The fructification is directly or indirectly regulated by mating types (Miles, 1993; Wessels, 1993). Molecular cloning techniques demonstrated in *Coprinus cinereus* can identify the proteins coded by the mating type genes (Kues & Casselton, 1992). This analysis can gain insight into how these determine cell type and regulate sexual development. On the one hand, mushroom biology is the basis of mushroom cultivation and production, and it is also the very core of mushroom biotechnology. On the other hand, a knowledge of mushroom biology can enhance many aspects of human endeavour and it can lead on to a variety of other interests such as mushroom hunting, painting and photography.

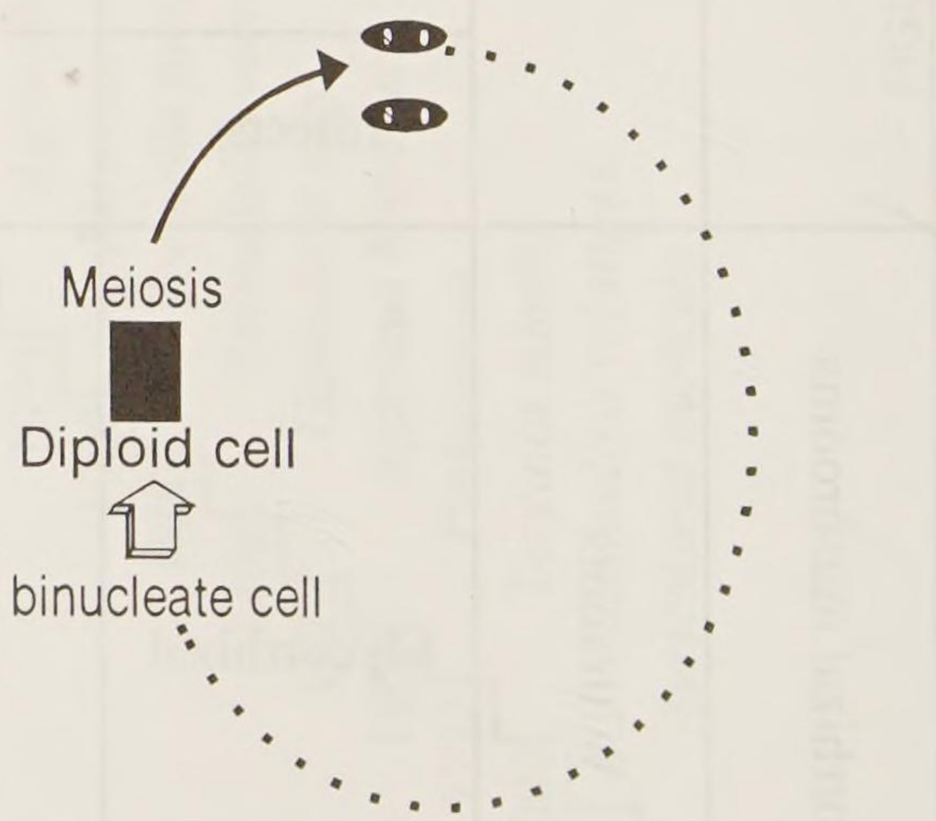
I. Enforced inbreeding types

(a): The pre-diploid binucleate cell is derived from dikaryotic hyphae by a binucleate spore



Laccaria fraterna

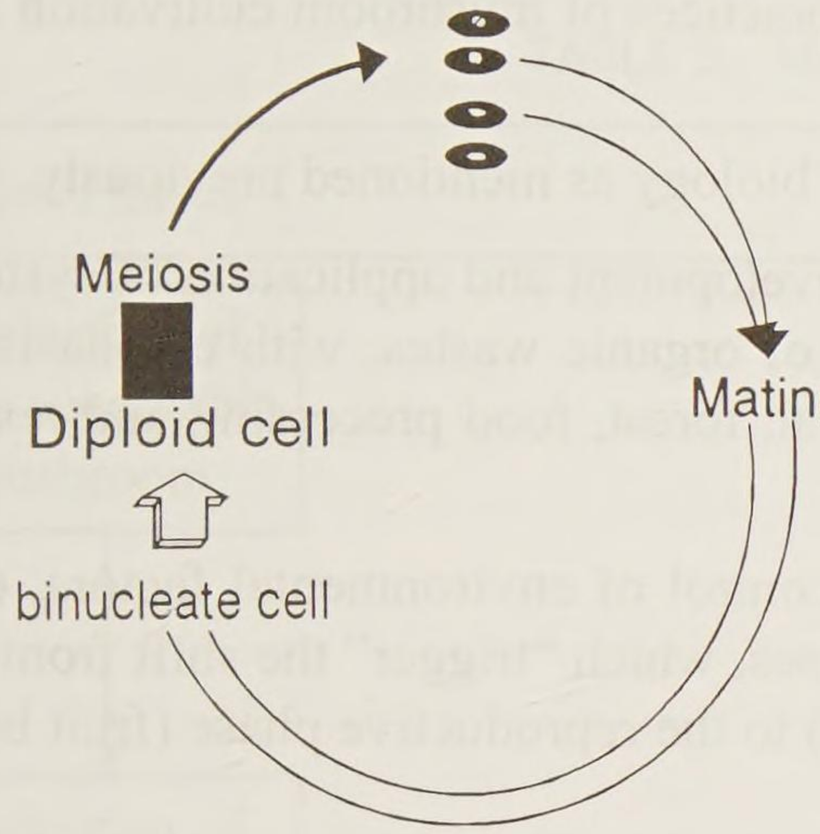
(b): The pre-diploid binucleate cell is derived from multinucleate hyphae by a binucleate spore



Agaricus bisporus

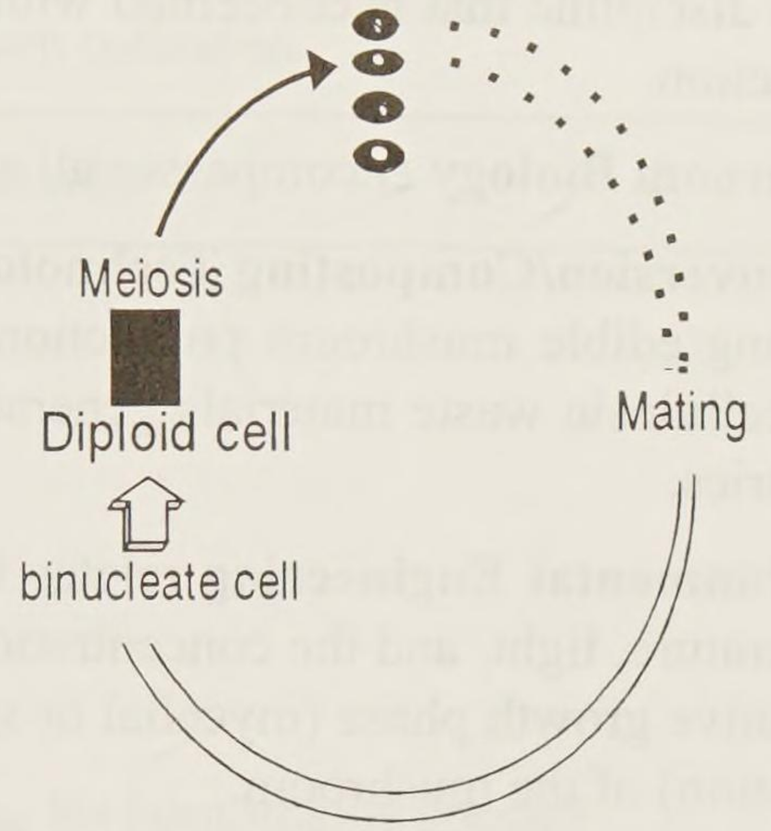
II. Outbreeding types

(a): The pre-diploid binucleate cell is derived from dikaryotic hyphae by mating two compatible monokaryons from uninucleate spores



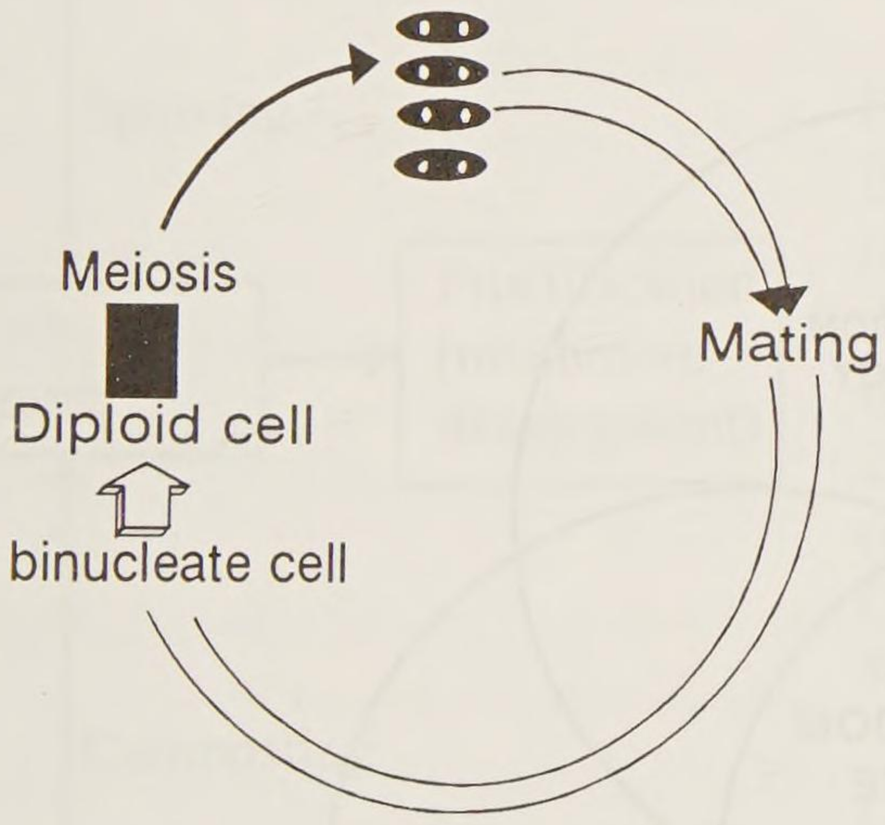
Lentinus edodes

(b): The pre-diploid binucleate cell is borne on multinucleate mycelium resulting from mating two compatible multinucleate hyphae of uninucleate spore origin



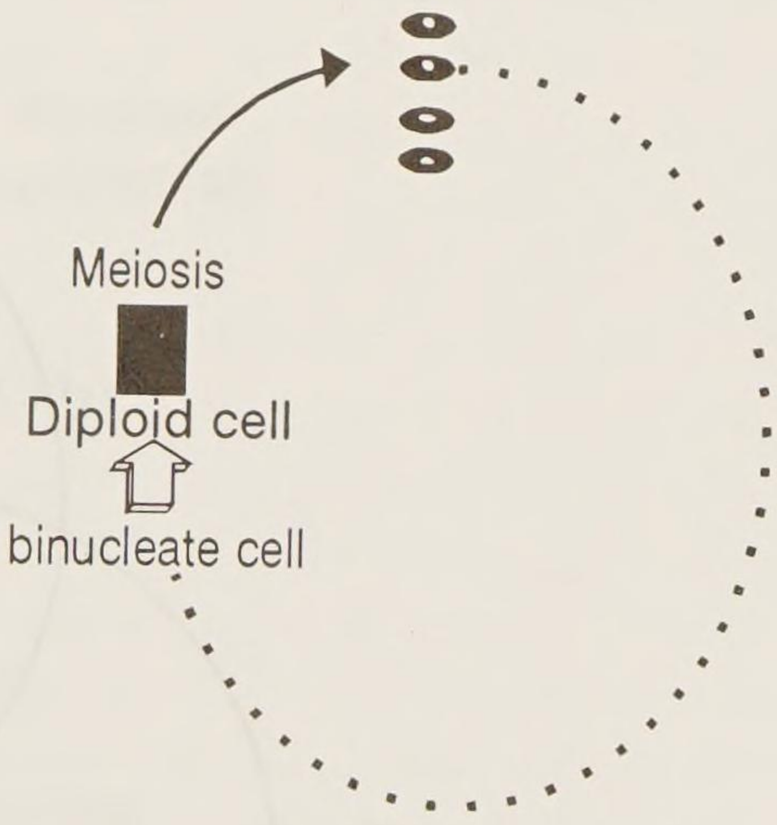
Agaricus bitorquis

(c): The pre-diploid binucleate cell is derived from dikaryotic hyphae which result from mating two compatible monokaryons from binucleate spores



Flammulina velutipes

III. Self-fertile types



Volvariella volvacea

FIGURE 2. Breeding systems of some selected mushrooms. The nuclear behaviour in a basidium is followed: from a binucleate cell to a transient diploid which is immediately followed by meiosis up to spore formation. The nuclear condition in different phases of a mushroom is illustrated.
▬▬▬ diploid; ——— monokaryon; ═══ dikaryon; ▪▪▪▪▪▪▪▪ multinucleate.

3. MUSHROOM SCIENCE

Mushroom science deals with **mushroom production** and encompasses **Mushroom Biology**, **Bioconversion/Composting Technology** and **Environmental Engineering** (Fig. 3). The term mushroom science already exists, but it is generally restrictive in the sense that it has been defined as the discipline that is concerned with the principles and practices of mushroom cultivation and production.

Mushroom Biology encompasses all aspects of mushroom biology as mentioned previously.

Bioconversion/Composting Technology comprises the development and application of systems coupling edible mushroom production and the utilization of organic wastes, with emphasis on lignocellulosic waste materials generated by the agricultural, forest, food processing and textile industries.

Environmental Engineering relates to the analysis and control of environmental factors, e.g., temperature, light, and the concentration of atmospheric gases, which "trigger" the shift from the vegetative growth phase (mycelial or spawn-running phase) to the reproductive phase (fruit body formation) of the mushroom.

Mushroom production is a complicated business. It involves a number of different operations, including: the selection of an acceptable fruiting culture of the mushroom, preparation of spawn and compost, inoculation of the compost, crop care, harvesting, preservation of the mushroom, and marketing. Each of these operations consists of many sequential steps which are equally crucial and important if success is to be achieved in the mushroom business as diagrammed in Table 3. In order to maintain a reasonably high and stable yield of mushrooms, both fundamental knowledge of mushroom science and the accumulative information of practical experience are required.

In Western countries, cultivation of *Agaricus* - the most popular edible mushroom, which is variously known as the white mushroom, button mushroom, champignon, or simply the common

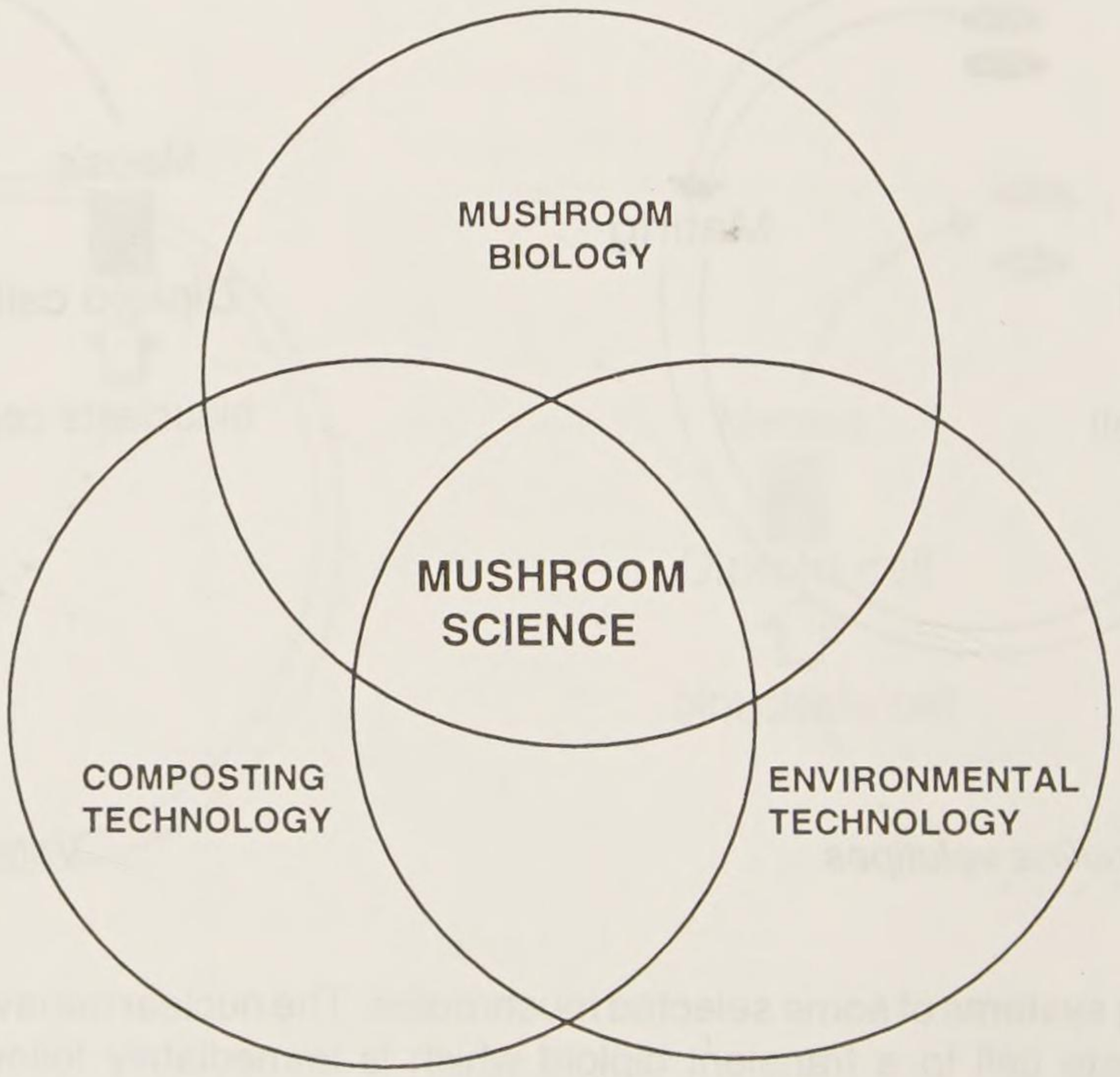


FIGURE 3. Mushroom science: concerned with mushroom production.

cultivated mushroom - has developed over the past 50 years from a beginning as a risky venture to a largely predictable and controllable industrial process, e.g., fifty years ago the yield was less than 5 kg/m^2 in more than twelve picking weeks of harvest. The picking was done by hand. Today, the yield can be 50 kg/m^2 in four picking weeks. The harvesting can be done by cutting machines.

TABLE 3. Major phases of mushroom cultivation.

Major Phases	Main points of consider
Selection of an acceptable mushroom	Location Climate Raw materials Acceptability
Selection of a fruiting culture	Tissue culture Spore culture (a) without mating for homothallic species (b) mating with compatible isolates for heterothallic species Mixture culture Preservation
Development of spawn	Substrate Vigorous growth Free of contamination Avoid use of senescent and degenerate spawn Good survival in storage
Spawing	Establishment of mycelium Environmental requirements (a) temperature (b) light (c) aeration (O_2, CO_2) 2(d) pH (e) moisture Casing Watering and care
spawn running	
Fructification (mushroom development)	
Composting	Concept of composting Microbial activity Softening of substrate for ease of colonization Physical characteristics Chemical components Aeration Water content
Preparation of compost	

Source: Chang (1991).

Deeptroughs, developed in the UK and adopted in Czechoslavakia with technical refinements, now achieve average yields of 60 kg/m^2 (Noble, 1989). Another technological development in *Agaricus* has broken the barriers of climate and geography. This mushroom in nature is a temperate climate mushroom, produced in Western countries where the production involves the most advanced and highly mechanized technology in the mushroom industry. But in 1965, Taiwan, with a subtropical/ tropical climate, emerged as the third leading producer of *Agaricus*. This was notable for three reasons. In the first place, Taiwan did not use a highly mechanized technology for the production of *Agaricus* on a relatively few large mushroom farms, but developed the industry as a cottage type enterprise on thousands of farms with small mushroom houses constructed of bamboo frames and banana leaf and/or straw and plastic for the roofs and sidings. In the second place, *Agaricus* had not been grown in a subtropical country previously, but it was found that this was possible on a seasonal basis with a single crop being grown in the period from September through March. Lastly, in the absence of a plentiful supply of horse manure, a synthetic compost was developed with rice straw as the main ingredient (Chang & Miles, 1989).

Lentinus edodes has for many years been grown on wood logs. It usually takes at least 8-12 months for the first flush to occur with the biological efficiency not exceeding 15%. Since being introduced in the early 1970's, the "plastic bag" method (sometimes known as "synthetic logs") has been used. The plastic bag method utilizes a sawdust based substrate contained in autoclavable plastic bags. This method has a shorter production cycle and gives higher yields. The cultivation also usually takes place in controlled environments, which facilitates consistent year-round production at the expense of higher energy costs compared with that of other mushrooms simply because of the longer production cycles (Cho & Nair, 1987; Chang & Miles, 1989).

One of the main drawbacks of the existing "plastic bag" method is that the quality of the mushroom obtained is usually inferior to those cultivated by the log method. The method of inoculating individual bags using either grain or sawdust based spawn is also laborious, and thorough spawning by shaking after inoculation or by the use of a "spawning channel" is not easily accomplished.

Volvariella volvacea is referred to as a "warm mushroom" because it can grow at relatively high temperatures, i.e., vegetative growth at about 32 to 34°C. It is a fast growing mushroom; the time required from spawning to harvesting is only about 8 to 10 days. No other vegetables or cultivated mushrooms can be served as a table dish within such a short time from its planting; but, under favorable conditions, *V. volvacea* can do this. However, the ability of its mycelium to colonize the substrate is rather weak; therefore, the mycelial network in the substrate is easily broken and disconnected if the inoculated compost is disturbed. The yield of mushrooms can be reduced drastically by mismanagement or improper care.

V. volvacea can use cellulose materials more effectively than other cultivated mushrooms; e.g., the optimum C/N ratio for *V. volvacea* is about 40 to 60:1, for *Agaricus bisporus* it is 17 to 18:1, and for *Lentinus edodes* it is 20 to 25:1 during the mycelial running stage. It can grow quickly and easily in uncomposted substrates such as paddy straw and cotton wastes or other highly cellulosic organic waste materials. Under favorable environmental conditions and growth medium, the primordium of the mushroom can be formed 4 to 5 days after spawning. Due to the above-mentioned special biological characteristics of the mushroom, it is considered to be one of the easiest mushrooms to cultivate, but it should be noted that the biological efficiency of this mushroom is lower than other commonly cultivated mushrooms.

With technical advances during the past few decades, cultivation of edible mushrooms has spread all over the world. Since cultivated mushrooms can be grown under different climatic

conditions and on agricultural and industrial wastes, they can be used as an aid in solving many problems of global importance including protein shortage, resource recovery and re-use, and environmental management.

In the overall view, world production of cultivated edible mushrooms was 2,176 thousand tons and 3,794 thousand tons in 1986 and 1989/90, respectively (Table 4). In those 3 years, mushroom production increased by 74.4% at an annual increase of 24.5% (Chang & Miles, 1991). A comparison of production between 1986 and 1989/90 reveals that all cultivated mushroom species increased during that period, ranging from 19% for *Agaricus* up to 437% for *Pleurotus*. The next big increase was 236.1% for *Auricularia*. However, the percentage of total world production of *Agaricus* and *Lentinus* mushrooms decreased as a consequence of the increase in production of the other cultivated edible mushroom species, particularly *Pleurotus* species.

TABLE 4. Comparison of 1986 and 1991 world production of cultivated edible mushrooms.

Unit: (metric ton x 1000)

Species	Common Name	1986		1991		
		Fresh wt.	%	Fresh wt.	%	% increase
Agaricus bisporus/ bitorquis	Button mushroom	1,215	55.8	1,590	37.2	30.9
Lentinus edodes	Shiitake or oak mushroom	320	14.7	526	12.3	64.4
Volvariella volvacea	Straw mushroom or Chinese mushroom	178	8.2	253	5.9	42.1
Pleurotus spp.	Oyster mushrooms	169	7.8	917	21.5	442.6
Auricularia spp.	Wood-ear	119	5.5	465	10.9	290.8
Flammulina velutipes	Winter mushroom	100	4.6	187	4.4	87.0
Tremella fuciformis	White Jelly fungus/ or "Silver Ear"	40	1.8	140	3.3	250.0
Pholiota nameko	"Nameko" or Viscid mushroom	25	1.1	40	0.9	60.0
Hericium erinaceus	Monkey head mushroom or Hedgehog fungus	-	-	66	1.5	
Hypsizygus marmoreus	Shimeji	-	-	32	0.7	
Grifola frondosus	Sitting-hen mushroom or Limuo, Maitaka	-	-	7.6	0.2	
Others		10	0.5	49.4	1.2	
Total		2,176	100.0	4,273	100.0	96.4

4. MUSHROOM BIOTECHNOLOGY

Mushroom biotechnology concerns **mushroom products** and encompasses **Mushroom Biology, Fermentation Technology** and **Bioprocessing**, and **Marketing and Management** (Fig. 4). Mushroom biotechnology, both as a technology and as the basis for new mushroom products requires industrial development. This kind of industry, like many bioscience industries, operates at the cutting edge of science, and involves numerous regulatory issues. It requires individual or institutional investors who have the background and resources necessary to develop and support the industry.

Mushroom Biology in this context would include, as an additional feature, the identification of medicinals (e.g., anti-tumour, hypocholesterolaemic and immuno-potentiating agents) and other added-value compounds (e.g., flavourants and colourants for the food and beverage industries, additives for the cosmetic industry).

Fermentation Technology and Bioprocessing embodies the development of large-scale systems for the production of added-value compounds and the conversion of mushrooms into other retail products (myco-meat, mushroom-based health drinks, soups, etc.).

Marketing and Management will develop and provide training in business expertise and marketing techniques which are essential to profit-oriented biotechnological enterprises and which constitute a key factor in the commercial success of mushroom-based products. Mushroom market opportunities must be identified in relation to domestic/regional/international markets.

Although various mushroooms have been highly valued as food, as tonics and, in some cases,

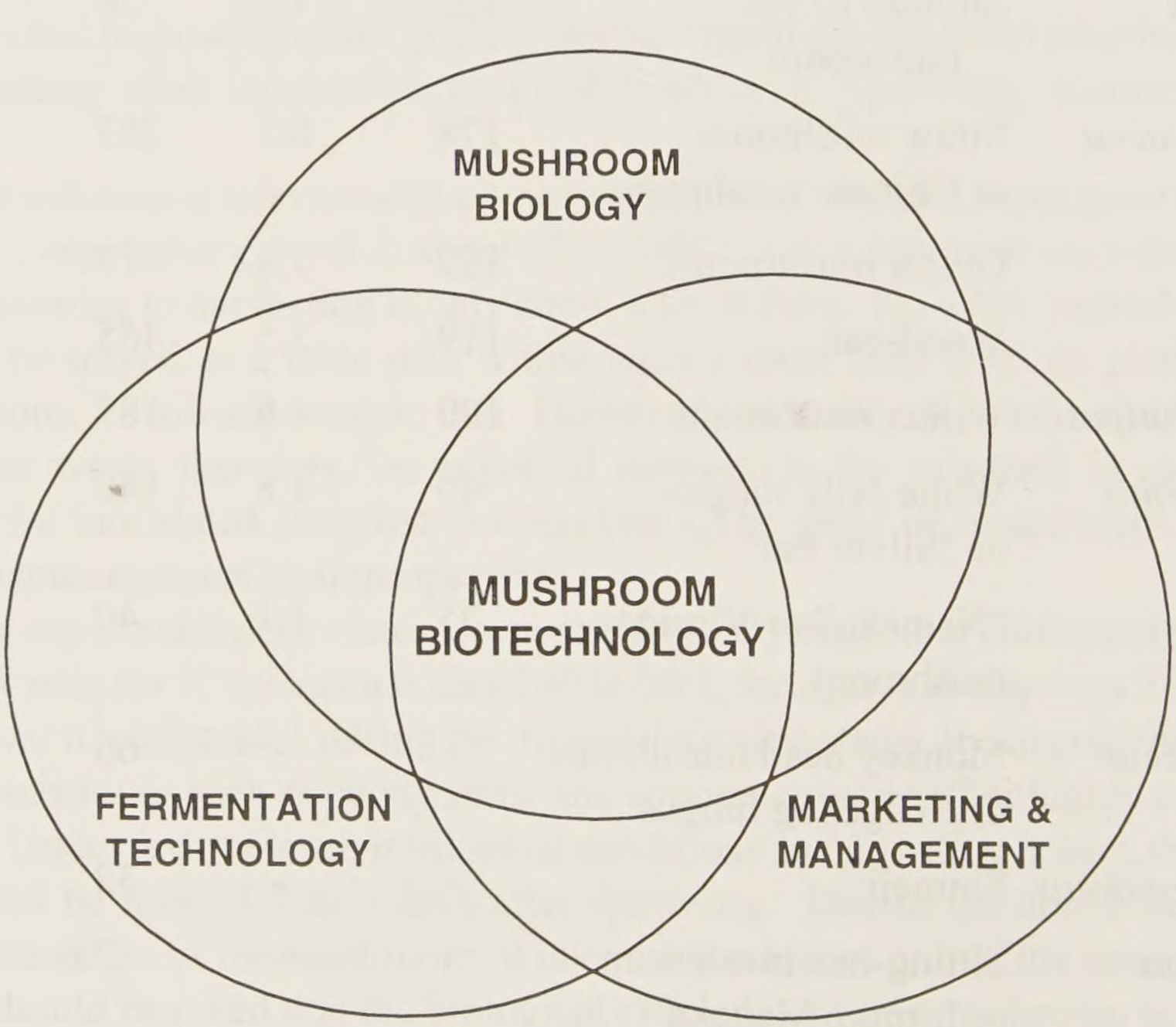


FIGURE 4. Mushroom biotechnology: cencerned with mushroom products.

as a medicine for a long period of time, mushrooms have become even more popular in recent years, as can be witnessed by the increased demands for higher production volumes. Their popularity is derived from three highly desirable characteristics as food: (1) they have remarkable taste and flavour; (2) they are nutritious, not only because they contain high contents of protein with significant amounts of lysine and methionine (which are low in plants), fibres, minerals, and vitamins, but also for what they do not have (high calories, sodium, fat, and cholesterol); (3) they can be easily processed, dried, pickled and canned to allow maximum storage and transportation.

In addition to these unique characteristics, many edible mushrooms have been traditionally used in China and Japan for their medicinal and tonic properties. Chihara (1992) reported that hot-water extracts of some basidiomycetes, such as *Ganoderma applanatus*, *Coriolus versicolor*, etc. markedly inhibited the growth of sarcoma 180. *Lentinus edodes* induced complete tumor regression in 6 out of 10 mice, whereas *Pleurotus ostreatus* was effective in 5 out of 10 mice. Similar degrees of activity were noted in hot-water extracts of many other edible mushrooms, such as *Tricholoma matsutake, Pholiota nameko*, and *Flammulina velutipes* (Table 5). In most cases, active components were β-glucans, although some variations were noted. Furthermore, PSK, a protein-bound polysaccharide preparation (Fukushima, 1989), and PSP, a peptide-bound polysaccharide preparation (Yang *et al*., 1989), have been extracted from *Coriolus versicolor* which is one of the well-known medicinal mushrooms. In Japan, PSK is reported as the top-selling anticancer drug and its annual sales in 1987 reached 358 M dollars, and accounted for 25.2% of the total sales of anticancer drugs which were worth about 1,000 million dollars per year (Table 6). PSK is widely prescribed for cancers of digestive organs (stomach, oesophagus, colon and rectum). *In vivo* and *in vitro* experiments

TABLE 5. Antitumor activity against sarcoma 180 of extracts from various basidiomycetes and edible mushrooms.

Basidiomycetes or edible mushrooms	Complete regression	Tumor inhibition (%)
Ganoderma applanatum	5/10	64.9
Coriolus versicolor	4/8	77.5
Coriolus hirsutus	2/10	65.0
Trametes gibbosa	1/10	49.2
Lenzites betulina	0/8	23.9
Daedaleopis tricolor	4/7	70.2
Fomitopsis semilaccata	3/10	44.2
Rigidoporus geotropus	0/7	44.8
Hirschioporus fusco-violaceus	1/10	45.5
Phelinus linteus	7/8	96.7
Lentinus edodes	6/10	80.7
Flammulina velutipes	3/10	81.1
Pleurotus ostreatus	5/10	75.3
Amanita rubescens	0/8	72.3
Pholiota nameko	3/10	86.5
Tricholoma matsutake	5/9	91.8
Auricularia mesenterica	0/9	42.6

Source: Chihara (1992).

conducted by Yang *et al.* (1992) have shown that the inhibitory effect of PSP on tumor cells was essentially similar to PSK. It is believed that there is a huge amount of economic potential not only for PSK and PSP, but also for other protein-bound polysaccharide preparations from other mushrooms. Cosmetic products and some healthy beverages have also been produced from the mushrooms of *Ganoderma*. A variety of proprietary products, including health drinks and foods, have also become available on the markets, a trend which is expected to increase with reported satisfaction and acceptability.

5. CONCLUDING REMARKS: LEARN FROM HISTORY

It is important to know how and to what extent the benefits of both mushroom production and mushroom products can be maximized. No matter whether you want to increase mushroom production through the advancement of mushroom science or to enhance the yield of mushroom products through improvement of mushroom biotechnology, it is always important to keep in mind what the past benefits were and the ways by which these benefits were achieved. If we can identify those events and discoveries which have led to the current status of importance of modern mushroom cultivation and mushroom products, is it not reasonable to expect that we can apply this knowledge and make greater leaps and advancements in the mushroom industry, which, in turn, would contribute more to the welfare of humankind? Moreover, if we make the assumption that mushroom science concerns mushroom production and mushroom biotechnology concerns mushroom products, then mushroom science and mushroom biotechnology are the two legs of the mushroom industry. In the past, the mushroom industry concentrated mainly on the production of fresh, canned and dried mushrooms. This means the industry had only one leg. At the present time, in addition to the value of the world mushroom crop estimated to be 7.5 billion dollars in 1990 (Chang & Miles, 1992), a large

TABLE 6. The top-ten best-selling anti-cancer drugs in Japan and the United States,1987.

	Japan			United States		
	Drug	Sales ($ x 10^6)	Percentage of market share	Drug	Sales ($ x 10^6)	Percentage of market share
1.	PS-K	358	25.2	Doxorubicin	86	16.3
2.	OK-432	191	13.4	Cisplatin	79	15.0
3.	Tegafur uracil	177	12.5	Tamoxifen citrate	68	12.9
4.	5-Fluorouracil	104	7.3	Etoposide	52	9.8
5.	Tegafur	101	7.1	Cyclophosphamide	31	5.9
6.	Tamoxifen citrate	63	4.4	Methotrexate	29	5.5
7.	Interferon-β	40	2.8	Megestrol acetate	27	5.1
8.	Lentinan	31	2.2	Mitomycin C	25	4.7
9.	Carmofur	26	1.9	Bleomycin	20	3.8
10.	Estramustine phosphate sodium	25	1.8	Vincristine sulphate	18	3.4

Source: Fukushima (1989).

amount of money is also generated from mushroom products, e.g., in 1987, the value of pharmaceuticals developed from three mushrooms: *Coriolus versicolor, Lentinus edodes* and *Schizophyllum commune* was reported to be 769 million dollars annually in Japan (Buswell & Chang, 1993). Surely, there are several other mushroom products e.g., those from *Ganoderma* manufactured and used in China, Korea and Taiwan that are also worth many millions of dollars. The mushroom industry with these two strong legs can now walk steadily. There should be no surprise when, in the next century, the industry starts to run. The bioscience industry is already one of the fastest growing investment sectors in the world.

It is clear from the above that mushroom biology is the very core of both mushroom science and mushroom biotechnology. These two aspects of the mushroom industry will not compete but they will complement each other. To achieve this goal, it is essential that there be cooperation in the exchange of information and technology concerning mushroom production and mushroom products among research scientists, industry, regulatory agencies, governments, and the public. It is hoped that all individual achievements can be shared and spread to all participants at this, the First International Conference on Mushroom Biology and Mushroom Products, and that this will be the first of a series of such international conferences.

REFERENCES

ATKINS, F.C. (1979). Research and the mushroom grower. *Mushroom Science* **10**, 7-8.

BANO, Z.& SRINVATAVA, H.C. (1962). Studies on the cultivation of *Pleurotus* species on paddy straw. *Food Science* **11**, 363-365.

BLOCK, S.S., TSAO, G. & HAN, L.H. (1958). Production of mushroom from sawdust. *Journal of Agricultural and Food Chemistry* **6**, 923-927.

BUSWELL, J.A. & CHANG, S.T. (1993). Edible mushrooms: Attributes and applications. In *Genetics and Breeding of Edible Mushrooms*, pp. 297-324. Edited by S.T. Chang, J.A. Buswell and P.G. Miles. Philadelphia: Gordon and Breach Science Publishers.

CHANG, S.T. (1991). Cultivated mushrooms. In *Handbook of Applied Mycology, Vol. 3: Foods and Feeds*, pp. 221-239. Edited by D.K. Arora, K.G. Mukerji and E.H. Marth. New York: Marcel Dekker Inc.

CHANG, S.T. (1993). Mushrooms and mushroom biology. In *Genetics and Breeding of Edible Mushrooms*, pp. 1-13. Edited by S.T. Chang, J.A. Buswell and P.G. Miles. Philadelphia: Gordon and Breach Science Publishers.

CHANG, S.T. & MILES, P.G. (1989). *Edible Mushrooms and Their Cultivation*, 345pp. Baca, Raton, Florida: CRC Press.

CHANG, S.T. & MILES, P.G. (1991). Recent trends in world production of cultivated mushrooms. *The Mushroom Journal* **503**, 15-18.

CHANG, S.T. & MILES, P.G. (1992). Mushroom biology - a new discipline. *The Mycologist* **6**, 64-65.

CHANG, S.T. & YAU, C.K. (1971). *Volvariella volvacea* and its life-history. *American Journal of Botany* **58**:552-561.

CHEN, G.L. (1988). *Hericium erinaceus* (Bull) Pers. In *Cultivation Techniques of Mushroom of China*, pp. 475-488. Edited by X.M. Yang. Beijing: Agricultural Press.

CHEN, S.Y. (1983). *General Account of Edible Mushroom*. China: Jangsu Science and Technology Press.

CHIHARA, G. (1992). Immunopharmacology of Lentinan, a polysaccharide isolated from *Lentinus edodes*: Its application as a host defense potentiator. *International Journal of Oriental Medicine* **17**, 57-77.

CHO, K.Y. & NAIR, N.G. (1987). Advances in the biotechnology of mushroom cultivation. *Australian Journal of Biotechnology* **1**, 53-57.

ELLIOTT, T.J. (1972). Sex and the single spore. *Mushroom Science* **8**, 11-18.

FALCK, R. (1917). Uber dir Waldkultur des Austernpilzes (*Agaricus ostreatus*) auf Laubhozstubben. *Z. Forst-Jagdwes* **49**, 159-165. (cited from Zadrazil, 1978).

FUKUSHIMA, M. (1989). The overdose of drugs in Japan. *Nature* **342**, 850-851.

GANESHAN, G. (1991). Cultivation of *Tricholoma lobayense* Heim on paddy straw substrate. *Mushroom Journal for the Tropics* **10**, 31-33.

HAN, O. (1590). T'ang Dynasty 618-907 *Sou Shih Tsuan*, copy of version published in China (1961).

HAWKSWORTH, D.L. (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research* **95**, 641-655.

HUANG, N.L. (1984). *Agrocybe cylindracea* - A new species of mushroom. *Edible Fungi* **4**, 1-3.

JANDAIK, C.L. (1974). Artificial cultivation of *Pleurotus sajor-caju* (Fr.) Singer. *Mushroom Journal* **22**, 405.

JI, D.G., LI, D.F. & SONG, M.J. (1982). *Oudemansiella radicata* and its domestication characteristics. *Edible Fungi* **1**, 11.

KAGA, T. & KONDO, H. (1958). Effect of climatic factors on the yield of nameko mushroom in a given forest region. *Proceeding of 68th Annual Meeting of the Japanese Forestry Society.* pp.256-258.

KENDRICK, B. (1985). *The Fifth Kingdom*, 364pp. Waterloo: Mycologue Publication.

KUES, U. & CASSELTON, L.A. (1992). Fungal mating type genes - regulators of sexual development. *Mycological Research* **96**, 993-1006.

LI, G.S.F. & CHANG, S.T. (1982). The nucleic acid content of some edible mushrooms. *European Journal of Applied Microbiology Biotechnology* **15**, 237-240.

LIN, J.N., ZHENG, C.R., ZHANG, Q.L. & LIU, Q.M. (1982). Studies on the artificial cultivation of *Dictyophora diuplicata* (Bosc) Fisch. *Mushroom Newsletter for the Tropics* **2**, 14-15.

LIU, F.C. & GUO, Y.C. (1982). Studies on the cultivation of *Hohenbuehelia sertina*. *Edible Fungi* **1**, 12.

LIU, P. (1985). Studies on artificial cultivation on logs of *Tremella mesenterca*. *Edible Fungi of China* **2**, 6-8.

MILES, P.G. (1993). Biological background for mushroom breeding. In *Genetics and Breeding of Edible Mushrooms*, pp. 37-64. Edited by S.T. Chang, J.A. Buswell and P.G. Miles. Philadelphia: Gordon and Breach Science Publishers.

MILLER, O.K. (1969). A new species of *Pleurotus* with a coremioid imperfect stage. *Mycologia* **61**, 887-893.

MOU, C.J. & CAO, Y.Q. (1986). The domestication characteristics of *Pleurotus ferulae* of Xinjiang. *Edible Fungi* **5**, 4-5.

NOBLE, R. (1989). Growing deep in Czechoslovakia. *The Mushroom Journal* **203**, 352-353.

OWER, R., MILLS, G. & MALACHOWSKI, J. (1986). Cultivation of *Morchella*. U.S. Patent No.4,594,809.

RAPER, C.A. (1976). Sexuality and life cycle of the edible, wild *Agaricus bitorquis*. *Journal of General Microbiology* **95**, 54-66.

SHEN, J.H. (1981). The Cultivation of *Pleurotus citrinopileatus*. *Edible Fungi* **1**, 21.
SINGER, R. (1961). Mushroom and truffles. New York: Intersciences Publishers.
SO, J. (or SO, GUANG). (659). *T'ang Pen Tsao.*
STRAATSMA, G., GERRITS, J.P.G., GERRITS, T.M., OP DEN CAMP, H.J.M. & VAN GRIENSVEN, L.J.L.D. (1991). Growth kinetics of *Agaricus bisporus* mycelium on solid substrate (mushroom compost). *Journal of General Microbiology* **137**, 1471-1477.
STRAATSMA, G. & SAMSON, R.A. (1993). Taxonomy of *Scytalidium thermophilum*, an important thermophilic fungus in mushroom compost. *Mycological Research* **97**, 321-328.
SUN, P., WANG, X. & LIU, P. (1985). Studies on the domestication cultivation of *Sparassis crispa*. *Edible Fungi of China* **3**, 7-8.
TAKEMARU, T. (1954). Genetics of *Collybia velutipes*. II. Dedikaryotization and its genetics implication. *Japanese Journal of Genetics* **29**, 1-7.
TANAKA, R. & KOGA, I. (1972). Karyological studies on *Lentinus edodes*, a Basidiomycete. *Japanese Journal of Botany* **47**, 289-296.
TIAN, S.Y. & YANG, F.M. (1990). The techniques of domestication and cultivation of *Tricholoma* spp. *Edible Fungi of China* **10(5)**, 21.
TOMMERUP, I.C., BOUGHER, N.L. & MALAJCZUK, N. (1991). *Laccaria fraterna*, a common ectomycorrhizal fungus with mono- and bi-sporic basidia and multinucleate spore: comparison with the quadristerigmate, binucleate spored *L. laccata* and the hypogeous relative *Hydnangium carneum*. *Mycological Research* **95**, 689-698.
WANG, B. & ZHANG, M.J. (1987). Experiment of domestication of *Lyophyllum ulmarium*. *Edible Fungi of China* **10(4)**, 17-18.
WANG, C. (1313). *Book of Agriculture*.
WANG, S.L. & KANG, Z.L. (1984). The domestication of cultivation of *Coprinus comutus* (or *ovatus*). *Edible Fungi* **5**, 7-8.
WANG, X.J. (1621). *Qun Fang Pu.*
WESSELS, J.G.H. (1993). Fruiting in the higher fungi. *Advances in Microbial Physiology* **34**, 147-201.
WOOD, D.A. (1984). Microbial processes in mushroom cultivation: a large scale solid substrate fermentation. *Journal of Chemical Technology and Biotechnology* **34B**, 232-240.
WOOD, D.A. & FERMOR, T.R. (1982). Nutrition of *Agaricus bisporus* in compost. *The Mushroom Journal* **114**, 194-197.
WU, Z.Y. & WEI, X.M. (1988). *Lentinus tigrinus* and its domestication cultivation. *Edible Fungi* **1**, 12.
XU, J.B. & LI, A.Q. (1984). *Hericium coralloides* - One of the wild mushroom in Changbaishan of China. *Edible Fungi* **4**, 6.
YANG, Q.Y., JONG, S.C., LI, X.Y., ZHOU, J.X., CHEN, R.T. & XU, L.Z. (1992). Antitumor and immunomodulating activities of the polysaccharide-peptide (PSP) of *Coriolus versicolor*. *EOS - Journal of Immunology and Immunopharmacology* **12**, 29-34.
YANG, Q.Y., YONG, S.C. & YANG, X.T. (1989). The physico-chemical characteristics of the polysaccharide-peptide (PSP) of *Coriolus versicolor* (Yun-Zhi). In *Recent Advances in Cancer 1989*, pp.7-17. Edited by E.C. Chew, D.P. Huang, W.C.T. Shiu and S.Y. Tsao. Cancer Research Group, CUHK, Hong Kong.
YUEN, Y. (1822). *Kuangtung Tung Chin.*
ZHANG, J.X., GUO, F.Y. & LI, B. (1989). Biological characteristics and cultivated techniques of *Gloestereum incarnatum*. *Edible Fungi of China* **5**, 23-25.

ZHANG, Y.F. & WANG, L. (1992). The biological characteristics and cultivation of *Hypsizygus marmoreus*. *Edible Fungi* **14(4)**, 13-14.
ZHANG, Y.X. & LU, G. (1983). The cultivation of *Armillara mella*. *Edible Fungi* **1**, 9.
ZHAO, G.N., CHONG G. & MAO, X.L. (1990). *Handbook of the Cultivation of Chinese Mushrooms*, 510pp. Beijing: Popular Science Publisher. (in Chinese).
ZHAO, Z.G. & YANG, X.L. (1983). *Polyporus* (or *Grifola*) *frondosus*. *Edible Fungi* **5**, 8-9.
ZHOU, M. (1232). *Kui Xin Za Shi.*
ZHU, D.X. & XIE, G.L. (1984). The domestication of *Amanita caesarea*. *Edible Fungi* **3**, 6.

CHAPTER 2

IDENTIFICATION, NAMES AND NOMENCLATURE OF COMMON EDIBLE MUSHROOMS

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1. TAXONOMY IS FUNDAMENTAL TO MUSHROOM SCIENCE

Taxonomy is fundamental to biological science and is the basis of information exchange. Names are the key to all knowledge about a species. Increased taxonomic knowledge of edible mushrooms benefits research on cultivation technology and breeding. For example, an awareness of current taxonomy will help to focus breeding strategy, avoiding crosses between two apparent 'species' (strains bearing different names) that are taxonomically identical.

Name changes typically derive from improved scientific knowledge of an organism in comparison with related taxa. Other reasons for changes include the existence of an earlier name which has priority, the replacement of an incorrect name (e.g. a homonym), and misidentification. The literature on systematic mycology is extensive and diverse, and is likely to be consulted infrequently by applied mushroom researchers. Taxonomists have a responsibility to transfer important information on taxonomic reassessment to relevant users in a form that can be understood and applied. Name changes of fungi of economic importance are explained in a series of papers prepared by the International Commission on the Taxonomy of Fungi, although only one mushroom species, shiitake, has been treated (Cannon, 1986).

International conferences allow a multidisciplinary approach to mushroom science in which the contributions of taxonomists can be assimilated by applied scientists. This paper considers the species concept in the basidiomycete fungi, and reviews recent clarification and current usage of names for cultivated edible mushroom species of the seven most important genera (Chang, 1991). The genera are treated in order of total production. The lack of taxonomic consensus for names of certain species, and unresolved variability in other species is discussed.

2. THE SPECIES CONCEPT IN THE BASIDIOMYCETES

The application of fungal names is determined by means of permanently preserved type specimens, a foundation Principle of the governing International Code of Botanical Nomenclature

(the Code) and the cornerstone of nomenclatural stability.

Morphology of the fungal fruit-body is the traditional basis for classification in fungi. Macroscopic features, in isolation, are rarely adequate to support taxonomic conclusions. Cap colour, for example, can vary considerably. Albinism has been reported in *Auricularia cornea*, *Lentinula edodes*, *Pleurotus ostreatus* and *P. salmoneostramineus*, arising spontaneously from mutation of a single recessive gene (Murakami & Takemaru, 1990). Microscopic features - in particular of spores, sterile elements of the spore-bearing region, and hyphal arrangement - provide more reliable characters.

Intercompatibility studies (Boidin, 1986) have frequently resolved questions about morphological variability within a single 'species', and apparent similarities between 'species' which carry different names. Monokaryotic strains derived from single spores or by neohaploidisation of dikaryotic strains (e.g. Petersen, 1993) are mated. In species with clamp connections at septa of dikaryotic hyphae, intercompatibility is judged by the presence of clamp connections on stable mycelia of mated colonies. True 'interfertility', however, requires successful fruiting of the mated colony and determination of the viability of progeny (Boidin, 1986). Intercompatibility is frequently sufficient to infer species relationships, though possible misinterpretations, in particular resulting from use of too few strains, have been discussed by Boidin (1986). Intercompatiblity studies assist taxonomists in determining whether collections should be regarded as representing different species or grouped into a single species. Partial intercompatibility may indicate speciation in progress.

The biological species concept, based on genetic isolation between species, is widely accepted (Hallenberg, 1987). Hallenberg (1987) divided species (of corticioid basidiomycetes) into three groups: a majority of species with uniform morphology distinct from that of other species; a second group of 'species complexes' where a species circumscription suggests that more than one biological species may be present, though these entities are not easily separated by morphology; and a third group where a species is considered to be morphologically uniform but interincompatible groups (cryptic or sibling species) are discovered among strains through intercompatibility studies. Examples of the second and third groups among the edible mushrooms include the *Pleurotus ostreatus* 'complex', recently resolved, and the siblings of *Tremella fuciformis*, respectively. The nomenclatural treatment of siblings varies. Hallenberg (1987) is followed here in requiring at least some distinguishing features of morphology, biochemistry, ecology or distribution before detected sibling entities receive Latin binomials.

3. NAMES OF COMMONLY CULTIVATED EDIBLE MUSHROOMS

3.1. *Agaricus*

The choice of name for the white button mushroom remains open to debate, following the reports of Malloch (1976) and Malloch *et al.* (1987) that *Agaricus bisporus* (Lange) Imbach, the basionym of which was described in 1926, is a synonym of the earlier name *A. brunnescens* Peck, described in 1900. Applying the principle of priority, the earlier name *A. brunnescens* is nomenclaturally correct. Neither name was originally applied to the cultivated mushroom (Malloch *et al.*, 1987). Comparison of type material of the two names is unfortunately not possible since there is no extant holotype material of *A. bisporus*.

Singer (1984) compared fresh collections with the holotype of *A. brunnescens* and with published descriptions of *A. bisporus* and *A. brunnescens*, and concluded that the names refer to two distinct species separated by fine morphological differences including colour change in context

tissue and presence of 4-spored basidia in young fruit-bodies of *A. brunnescens*. Malloch *et al.* (1987) reported that a fresh Canadian collection was both conspecific in morphology with the holotype of *A. brunnescens* and, on the evidence of mitochondrial and nuclear DNA restriction patterns, conspecific with cultivated *Agaricus* strains. While questioning the validity of Singer's distinguishing characters, Kerrigan (1987) did not emphatically support synonymy. Rather, he suggested that synonymy be accepted as a 'working hypothesis' and that the name *A. bisporus* be considered for conservation, against *A. brunnescens*, because of its long-established use. A formal proposal for conservation has not yet been made.

The other important cultivated *Agaricus* species, *A. bitorquis* (Quél.) Sacc., is clearly separated from *A. bisporus/brunnescens* (Smith, 1978; Malloch *et al.*, 1987). In addition to morphology, electrophoretic analysis of isozymes of extracellular laccases can reliably separate *Agaricus* species (Kerrigan & Ross, 1988).

3.2. *Pleurotus*

Pleurotus production increased over four-fold between 1986 and 1989/90 (Chang, 1991). The taxonomic confusion so apparent in the genus (Rajarathnam & Bano, 1987) may be due, in part, to the rapid development of cultivation of several species and the misapplication and misinterpretation of names of newly cultivated strains. Commercial strains may carry ambiguous or incorrect names, thus perpetuating early errors in identification.

Only recently has significant taxonomic analysis brought necessary clarification. The genus is taxonomically difficult because of variability in fruit-body morphology in several species, resulting in species being described under more than one name, especially from different regions of the world. Through intercompatibility studies, a number of apparently morphologically distinct taxa have been found to belong to a common biological species. Similarly, intercompatibility studies have enabled discrimination of taxa for which distinguishing morphological characters were lacking or apparently inconsistently present in different geographic regions.

Other means of discriminating species of *Pleurotus* include electrophoretic analysis of enzymes (Magae *et al.*, 1990), and differences in nuclear DNA content correlated with different chromosome number and ploidy (Bresinsky *et al.*, 1987). Allozyme analyses of May & Royse (1988) suggest that a number of strains labelled *P. ostreatus* have been misidentified.

The following list cites epithets of commonly cultivated *Pleurotus* species, with references to synonymy and descriptions. Currently accepted names are in bold. Names followed by an asterisk are discussed further:

abalonus* Han *et al.* - (Han *et al.* 1977; Hilber 1982).

*citrinopileatus** Singer = ***P. cornucopiae*** var. ***citrinopileatus*** (Singer) Ohira - (Hilber, 1982; Ohira, 1990; Petersen, 1993).

columbinus Quél. = ***P. ostreatus*** var. ***columbinus*** (Quél.) Quél. - (Hilber, 1982, 1989).

cornucopiae* (Paul.) Roll. - (Hilber, 1982; Boekhout, 1990).

cystidiosus* O.K. Miller - (Miller, 1969; Hilber, 1982; Guzmán *et al.*, 1991).

djamor* (Fr.) Boedijn - (Corner 1981; Pegler 1986).

eryngii (DC.) Quél. - (Hilber, 1982; partially inter-incompatible varieties or ecotypes - Hilber, 1982; Bresinsky *et al.*, 1987; Boekhout, 1990).

flabellatus Berk. & Br. = ***P. djamor*** - (Corner, 1981).

fossulatus (Cooke) Sacc. - (Pegler, 1976).

nebrodensis (Inzenga) Sacc. = ***P. eryngii*** var. ***nebrodensis*** (Inzenga) Sacc. - (Hilber, 1982).
opuntiae (Dur. & Lév.) Sacc. - (Hilber, 1982; Pegler, 1977).
ostreatoroseus Singer =? ***P. djamor*** - (Petersen & Hughes, 1993).
ostreatus* (Jacq.) Kumm. - (Hilber, 1982; Boekhout, 1990).
pulmonarius* (Fr.) Quél. - (Ohira & Matsumoto, 1980; Hilber, 1982; Boekhout, 1990).
sp. 'florida'* (unpublished and ambiguous name) = ***P. ostreatus*** and ***P. pulmonarius*** - (Hilber, 1989).
*sajor-caju** (Fr.) Singer (misapplied name) = ***Lentinus sajor-caju*** (Fr.) Fr. - (Pegler, 1983; Hilber, 1989).
salignus (Pers.) Kumm. = ***P. ostreatus*** - (Hilber, 1982, 1989; Boekhout, 1990).
salmoneostramineus L. Vassilieva = ***P. djamor*** - (Corner, 1981; Neda *et al.*, 1988).
*sapidus** (Schulz.) Sacc. = ***P. cornucopiae*** - (Hilber, 1989).
spodoleucus Fr. = ***P. ostreatus*** - (Magae *et al.*, 1990).
squarrosulus (Mont.) Singer = ***Lentinus squarrosulus*** Mont. - (Pegler, 1977).

3.2.1. Pleurotus abalonus* and *P. cystidiosus. Both species have an anamorphic (asexual) fruiting phase characterised by production of coremia. Macro- and microscopic differences between the species and their geographical separation support distinction (Han *et al.*, 1977; Hilber, 1989), even though monokaryons from each showed partial intercompatibility (Hilber, 1982). Intercompatible crosses produced fertile fruit-bodies and viable spores (Bresinsky *et al.*, 1987). The two species are considered to be distinct, the partial intercompatibility suggestive of allopatric (geographically separated) speciation in progress.

3.2.2. Pleurotus cornucopiae. This species, described from Europe, is characterised by pale cream to pale brown, funnel-shaped caps. *P. citrinopileatus* Singer, from eastern Asia, was separated from *P. cornucopiae* by its bright yellow caps, an often branched stipe, and smaller basidiospores. Corner (1981) reported specimens of the former as *P.* aff. *cornucopiae*. Ohira (1990) and Petersen & Hughes (1993) found complete intercompatibility between monokaryotic strains of both species. Fruit-bodies produced from these crosses were yellow, and basidiospores, intermediate in size between those of the parents, were able to germinate (Ohira, 1990). In recognition of a single biological species containing morphologically and geographically separated variants, *P. citrinopileatus* was reduced to varietal status as *P. cornucopiae* var. *citrinopileatus* (Singer) Ohira.

3.2.3. Pleurotus djamor. Corner (1981) divided this variable species into six varieties reflecting differences in cap and gill colour, form of stipe, and substrate. *P. salmoneostramineus* (as *P. incarnatus* Hongo) was considered to be a synonym of var. *roseus*, and this was confirmed by intercompatibility between strains of *P. djamor* and *P. salmoneostramineus* (Neda *et al.*, 1988).

3.2.4. Pleurotus pulmonarius and P. ostreatus. *P. pulmonarius* (grey oyster mushroom, phoenix-tail mushroom) is arguably the species with the most frequently misapplied name. It has been repeatedly confused with the morphologically similar *P. ostreatus*. *P. pulmonarius* is the correct name for some strains labelled *Pleurotus* sp. 'florida' and for others misidentified as *P. sajor-caju*.

Mycologists have differing opinions on the usefulness of morphological characters for discrimination of *P. pulmonarius* from *P. ostreatus*. Discriminating characters relevant for European collections (Hilber, 1982; Bresinsky *et al.*, 1987; Boekhout, 1990) and Japanese collections (Ohira & Matsumoto, 1980) include:

P. pulmonarius - pale coloured cap, smaller size (laterally cap <10-13 cm), stipe typically more

eccentric than lateral, fruiting in late spring to early autumn, sweet odour like anise, under humid conditions upper surface of cap glabrous, thinner pileipellis (40-50 µm thick), and presence of sclerified hyphae.

P. ostreatus - dark grey-brown cap (sometimes with blue tint), larger size (laterally cap usually 12-18 cm), stipe typically more lateral than eccentric, fruiting in autumn until early spring, fungoid odour, under humid conditions upper surface of cap pubescent-tomentose, thicker pileipellis (90-120 µm thick), and usual absence of sclerified hyphae.

Petersen & Hughes (1993) reported that in North America the two species could not be separated by these characters. For example, North American *P. ostreatus* has tan to pale tan caps, and could be considered an allopatric 'morphotaxon' distinguishable morphologically from the European form.

The two species are, however, distinct genetically and are separate biological species. A complete barrier to hybridisation has been documented by several intercompatibility studies using single spore strains sourced from widely separated geographic regions, while intraspecific crosses within each species are uniformly intercompatible (Bresinsky *et al.*, 1977, 1987; Hilber, 1982; Petersen & Hughes, 1993).

Exceptions noted were two strains of *P. pulmonarius* found to be completely intersterile with all other strains of *P. pulmonarius* (Bresinsky *et al.*, 1987), possibly representative of an independent species, and partial intercompatibility between monokaryons of a strain of *P. pulmonarius* and one of *P. ostreatus* (Magae *et al.*, 1990). A high similarity index was also reported by Magae *et al.* (1990) from enzyme analyses between the two species. While regarding the two taxa as variants of a single species, they acknowledged that further strains of *P. pulmonarius* should be studied before their conclusion could be substantiated.

Both species lack a type specimen on which to base taxonomic decisions. For *P. pulmonarius*, Petersen & Hughes (1993) propose a 'representative specimen', to serve in the interim until a neotype can be designated. The neotype should be from the same host, *Betula*, and geographic area as was originally described for the species, and should be the source of single spore tester strains intercompatible with the *P. pulmonarius* intersterility group. Typification of *P. ostreatus* has not yet been proposed.

3.2.5. Pleurotus sajor-caju. This is a synonym of *Lentinus sajor-caju*, a very common species in tropical forests. The *Pleurotus* combination has been cited frequently in error in literature on cultivated mushrooms. Kurtzman & Zadrazil (1982) reported differences in morphology between published descriptions of the species (Corner, 1981; Pegler, 1983) and a commercially available cultivated strain labelled *P. sajor-caju* (ITCCF 1725 = ATCC 32978). Several commercial strains labelled *P. sajor-caju* have proved to be misidentified and are intercompatible with *P. pulmonarius* (Hilber, 1989). Another misidentification is suggested from the description of a synnematous anamorph from a strain of *P. sajor-caju* (Nair & Kaul 1980), probably based on either *P. cystidiosus* or *P. abalonus* or a related species.

3.2.6. Pleurotus sapidus. This is a synonym of *P. cornucopiae*. Usage of the name in North America has only recently been clarified (Hilber, 1989). Eugenio & Anderson (1968) reported a common decay fungus on hickory, sugar maple and American elm as *P. ostreatus*. Anderson *et al.* (1973), in error, redetermined this species as *P. sapidus*. Hilber (1989) found that Eugenio & Anderson's material was intercompatible with *P. ostreatus*. The fungus from aspen, which Anderson *et al.* (1973) mistakenly identified as *P. ostreatus*, is now distinguished as a new species, to be published as *P. populinus* (Hilber, 1989).

3.2.7. Pleurotus sp. 'florida'. Though never published nor intended as a binomial, the name appears frequently in literature on cultivated mushrooms. The origin and history of the name were reviewed by its originator, G. Eger, in Eger *et al.* (1979). 'Florida' strains were found to be compatible with *P. ostreatus* (Eger et al. 1979), while Bresinsky et al. (1977) had previously reported intercompatibility with *P. pulmonarius.* These results were misinterpreted by Eger *et al.* (1979) to indicate that *P. pulmonarius* and *P. ostreatus* were conspecific. Bresinsky *et al.* (1987) and Hilber (1982) concluded that strains available under the name 'florida' represent two taxa, a majority being *P. ostreatus* and at least one strain being *P. pulmonarius*. Clearly, strains should not be designated 'florida' in future scientific literature. The unfortunate confusion surrounding this name has called into question several scientific results, e.g., Yoo *et al.* (1986), where interspecific hybrids were reported between *P. ostreatus* and '*P. florida*'. Hilber (1989) noted that *P. floridanus* Singer is unrelated to the two species represented by *P. 'florida'*.

3.3. *Lentinula*

Lentinula, sensu Pegler (1975), and *Lentinus,* sensu Singer (1986), are both currently used to accommodate the shiitake mushroom, as *Lentinula edodes* (Berk.) Pegler and *Lentinus edodes* (Berk.) Singer.

According to Pegler hyphal composition and gill form separate *Lentinula* from *Lentinus* and *Pleurotus*, both genera that have formerly accommodated shiitake. Hyphal composition of the fruit-body is an important taxonomic character for classification of polypore fungi, to which *Lentinus* and *Pleurotus* are related. Hyphae are not inflated and septa are not constricted. *Lentinula,* in contrast, is considered to belong in the agaric family Tricholomataceae (Pegler, 1975), with a monomitic hyphal system (one type of hypha) of inflated, thick-walled hyphae with constricted septa. The gills of *Lentinula* species do not extend down the stipe (non-decurrent), unlike those characteristic of species in the other two genera. Pegler (1972) separates *Lentinus* from *Pleurotus* on the basis of the former being dimitic and the latter monomitic.

Singer (1986) listed *Lentinula* as a synonym of *Lentinus.* He accepted both *Lentinus* and *Pleurotus* as including species with monomitic or dimitic hyphal systems, separated by differences including the structure of gill hyphae (trama) underlying the spore-bearing layer. Hyphal systems were considered by Singer to be useful to delimit sections within these two genera, but not the genera themselves.

Corner (1981) accepted *Lentinula*, while including within *Pleurotus* species with a monomitic or a dimitic hyphal system, thus blurring Pegler's distinction between *Pleurotus* and *Lentinus*.

3.4. *Auricularia*

auricula (L.) Underwood - (Lowy, 1952).
auricula-judae (Bull.) Schröt. = ***A. auricula*** - (Lowy, 1952).
cornea (Ehr.) Ehr. ex Endl. - (Wong & Wells, 1987).
fuscosuccinea (Mont.) Farl. - (Lowy, 1952).
polytricha (Mont.) Sacc. = ***A. cornea*** - (Wong & Wells, 1987).
tenuis (Lév.) Farl. = ***A. cornea*** - (Wong & Wells, 1987).

Morphological characters of basidiocarps, especially the nature of internal, hyphal, cross-sectional zones and hairs of the upper surface, were used as discriminating characters for species of *Auricularia*, the wood ear fungi (Lowy, 1951, 1952; Cheng & Tu, 1978; Quimio & de Guzman, 1982). Duncan (1982) reported that within a sample of 7 strains of *A. polytricha*, a strain from the

U.S.A. was inter-incompatible with all others; among the latter group intercompatibility varied from complete to partial. Wong & Wells (1987) found that morphological variability in *A. cornea, A. polytricha* and *A. tenuis* prevented discrimination. Monokaryotic strains of these species from Hawaii were fully intercompatible, and basidiocarps arising from 'interspecific' crosses conformed to *A. polytricha* (Wong & Wells, 1987). These authors concluded that described morphological distinctions between species were environmentally induced, and that the earliest name, *A. cornea*, should be adopted for *A. polytricha* and *A. tenuis*, and for an albino form (Wong, 1989). Formal synonymy, however, awaits designation of a neotype for *A. cornea* in the absence of available type material (Wong, 1989).

3.5. *Volvariella*

The cultivated paddy straw mushroom is widely accepted as *Volvariella volvacea* (Bull.: Fr.) Singer (Chang, 1978; Kurtzman & Chang-Ho, 1982), with the varietal name *masseei* Singer applicable to most strains grown in southeast Asia (Samarawira & Fernando, 1973; Singer & Harris, 1987). Orton (1986) questioned whether the cultivated fungus of Asia is conspecific with *V. volvacea* from the U.K., on the basis of reported differences in spore size. The genus lacks a recent monographic treatment. Further taxonomic comparison of the temperate and tropical species of *Volvariella*, using morphological, biochemical and genetic characters, would benefit clarification of species names.

3.6. *Flammulina*

Flammulina velutipes (Curt.: Fr.) Sing. (winter mushroom, enokitake) is a variable species. Bas (1983) distinguished from within the *velutipes* complex a new, morphologically distinct European species, *F. fennae* Bas. Monokaryons of *F. velutipes* and *F. fennae* were inter-incompatible (Bas, 1983; Lamoure, 1989), and the two species have different enzyme activities (Klán & Baudisová, 1992). *F. velutipes* was itself divided into two macroscopically distinct varieties, *F. velutipes* var. *lactea* (Quél.) Bas, and var. *velutipes*. Within the latter, Bas distinguished two forms, forma *longispora* and forma *velutipes,* separated by different spore dimensions and periods of fruiting. Lamoure (1989) reported incomplete genetic isolation between monokaryons of the two forms, and inconclusive results in intercompatibility studies between var. *lactea* and var. *velutipes*, indicating that speciation may be in progress. Monokaryons from two collections of var. *lactea* were typically inter-incompatible (Lamoure 1989), supporting Bas's (1983) suggestion that var. *lactea* could also be divided into two forms.

Field strains of *F. velutipes* from Japan were reported to be intercompatible with strains from New Zealand, western and eastern U.S.A., the U.K., the Netherlands, and South Korea, but inter-incompatible with strains from boreal and Northern Hemisphere alpine regions (Yokoyama, 1991). The latter were considered to belong to a separate species, which differed from *F. velutipes* in having a lower optimal temperature for mycelial growth, a higher optimum for fructification and more rounded spores.

It is not clear from published literature available to me which of the varieties and forms of *F. velutipes* are represented among cultivated strains.

3.7. *Tremella*

While the name *Tremella fuciformis* Berk. is taxonomically well accepted, intraspecific

differences have been recently documented. Fox & Wong (1990) found both homothallic and heterothallic strains from different geographic locations, and incomplete intercompatibility between some geographically separated heterothallic strains. These results were interpreted by Fox & Wong to suggest that *T. fuciformis* comprises either two morphological species, or else a single species in which homothallic forms have arisen through deletion of mating factors.

4. 'LISTS OF NAMES IN CURRENT USE', IMPROVING STABILITY OF NAMES?

Nomenclature of fungi is governed by the Principles, Rules and Recommendations of the Code, which provide order and stability through precise definition of requirements for valid publication of names and provisions by which names can be changed. Measures allowing for conservation and rejection of names are built into the Code to avoid name changes judged to be disadvantageous.

Criticism of the frequency of name changes and the resulting disruption and confusion caused, from both users of taxonomy and practitioners, has stimulated considerable recent debate towards improvement (Hawksworth, 1991). In 1989 the General Committee of the International Association for Plant Taxonomy established a committee to coordinate discussion and preparation of 'Lists of Names in Current Use' for all plant groups covered by the Code, beginning with the estimated 36,500 generic names. At the XVth International Botanical Congress (IBC) in Tokyo (August 1993) delegates will vote on the principle of such lists, the resulting necessary changes to the Code, and the procedure for production of lists.

Opinions among mycologists are strongly divided over the criteria for exclusion of names from the list, and the need for 'protected status' of listed names against those excluded. Protected status is a direct attack on Principle III of the Code: 'The nomenclature of a taxonomic group is based upon priority of publication'. The 'digging-up' of old and long forgotten names to replace names in current use is cited by Hawksworth (1991) as the main cause of nomenclatural change, hence justifying the diminution of the priority rules, but this view is contested by Korf (1991). In agreement with Korf, mushroom name changes discussed in this paper, with the exception in part of the debate surrounding *Agaricus bisporus/brunnescens*, have arisen from new knowledge of species enabling better discrimination and are reflected in the establishment of a more accurate and stable nomenclature. Such advances in systematic science are essential to improved understanding of fungi and their interrelationships, and should not in any way be limited by the proposed lists. It is generally agreed that the lists must cater for alternative taxonomies where such taxonomies are currently employed. Gams & Kuyper (1991) favour comprehensive computerised lists which include all typified fungal names, excluding only those names with insufficient description or lacking authentic type material. Any expectation among users that the lists will effectively freeze existing names is unwarranted (Anderson, 1991).

A draft list of the estimated 64,000 accepted fungal species names, coordinated from the International Mycological Institute, U.K., is expected to be completed during 1993, but will not be ready for formal adoption at the Tokyo IBC. Whether names on the list are given protected status or not, the list once vetted and approved will serve as a reference document to standardise spelling of names, authorship and place of publication. This will provide tangible benefits to all users.

5. RECOMMENDATION

Authors in mushroom science are encouraged to obtain authoritative identification of mushroom

strains before publication, and to use validly published species names. Spawn suppliers are similarly encouraged to confirm identification of commercial strains, as use of incorrect and invalid names can enter and confuse the scientific literature. Culture strains should be deposited in an internationally recognised culture collection, ensuring long-term preservation and availability to other workers. The International Collection of Micro-organisms from Plants (ICMP), Auckland, for example, provides an international holding facility for commercial strains of edible mushrooms, with the supplier retaining security of ownership.

Accuracy in the application of mushroom names will benefit mushroom science by reducing confusion and duplication of effort, and improving the reliability and reproducibility of published results.

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REFERENCES

ANDERSON, N.A., WANG, S.S. & SCHWANDT, J.W. (1973). The *Pleurotus ostreatus-sapidus* species complex. *Mycologia* **65**, 28-35.

ANDERSON, W.R. (1991). Should we change the Code? Concerns of a working taxonomist. In *Improving the Stability of Names: Needs and Options*, pp. 95-103. Edited by D.L. Hawksworth. *Regnum Vegetabile* No. 123. Königstein: Koeltz Scientific Books.

BAS, C. (1983). *Flammulina* in Western Europe. *Persoonia* **12**, 51-66.

BOEKHOUT, T. (1990). 1. *Pleurotus*. *Flora Agaricina Neerlandica* **2**, 20-24.

BOIDIN, J. (1986). Intercompatibility and the species concept in the saprobic Basidiomycotina. *Mycotaxon* **26**, 319-336.

BRESINSKY, A., HILBER, O., & MOLITORIS, H.P. (1977). The genus *Pleurotus* as an aid to understanding the concept of species in basidiomycetes. In *The Species Concept in Hymenomycetes*, pp. 229-258. Edited by H. Clémençon. Vaduz: J. Cramer.

BRESINSKY, A., FISCHER, M., MEIXNER, B. & PAULUS, W. (1987). Speciation in *Pleurotus*. *Mycologia* **79**, 234-245.

CANNON, P.F. (1986). International Commission on the Taxonomy of Fungi (ITCF): name changes in fungi of microbiological, industrial and medical importance, Part 1. *Microbiological Sciences* **3**, 168-171.

CHANG, S.T. (1978). *Volvariella volvacea*. In *The Biology and Cultivation of Edible Mushrooms*, pp. 573-603. Edited by S.T. Chang, S.T. & W.A. Hayes. New York: Academic Press.

CHANG, S.T. (1991). Mushroom biology and mushroom production. *Mushroom Journal of the Tropics* **11**, 45-52.

CHENG, S. & TU, C.C. (1978). *Auricularia* spp. In *The Biology and Cultivation of Edible Mushrooms*, pp. 605-625. Edited by S.T. Chang, S.T. & W.A. Hayes. New York: Academic Press.

CORNER, E.J.H. (1981). The agaric Genera *Lentinus, Panus,* and *Pleurotus* with particular reference to Malaysian species. *Beihefte zur Nova Hedwigia* **69**, 1-169.

DUNCAN, E.G. (1982). Population diversity and speciation in *Auricularia polytricha*. In *Tropical Mushrooms - Biological Nature and Cultivation Methods*, pp. 409-425. Edited by S.T. Chang, & T.H. Quimio. Hong Kong: The Chinese University Press.

EGER, G., LI, S.F. & LEAL-LARA, H. (1979). Contribution to the discussion on the species concept in the *Pleurotus ostreatus* complex. *Mycologia* **71**, 577-588.

EUGENIO, C.P. & ANDERSON, N.A. (1968). The genetics and cultivation of *Pleurotus ostreatus*. *Mycologia* **60**, 627-634.

FOX, R.D. & WONG, G.J. (1990). Homothallism and heterothallism in *Tremella fuciformis*. *Canadian Journal of Botany* **68**, 107-111.

GAMS, W. & KUYPER, T.W. (1991). Some notes of caution regarding names in current use. In *Improving the Stability of Names: Needs and Options*, pp. 257-266. Edited by D.L. Hawksworth. *Regnum Vegetabile* No. 123. Königstein: Koeltz Scientific Books.

GUZMÁN, G., BANDALA, V.M. & MONTOYA, L. (1991). A comparative study of teleomorphs and anamorphs of *Pleurotus cystidiosus* and *Pleurotus smithii*. *Mycological Research* **95**, 1264-1269.

HALLENBERG, N. (1987). On speciation in Corticiaceae (Basidiomycetes). *Windahlia* **17**, 19-25.

HAN, Y.H., CHEN, K.M. & CHENG, S. (1977). Characteristics and cultivation of new *Pleurotus* in Taiwan. *Mushroom Science* **9**, 167-173.

HAWKSWORTH, D.L. (1991). Lists of names in current use: a new initiative to address a continuing problem. *Mycotaxon* **40**, 445-458.

HILBER, O. (1982). Die Gattung *Pleurotus*. *Bibliotheca Mycologica* **87**, 1-448.

HILBER, O. (1989). Valid, invalid and confusing taxa of the genus *Pleurotus*. *Mushroom Science* **12**, 241-248.

KERRIGAN, R.W. (1987). What's in a name: the chaetaceous case of the chaste champignon. In *Developments in Crop Science 10, Cultivating Edible Fungi*, pp.141-154.Edited by P.J. Wuest, D.J. Royse & R.B. Beelman. Amsterdam: Elsevier.

KERRIGAN, R.W. & ROSS, I.K. (1988). Extracellular laccases: biochemical markers for *Agaricus* systematics. *Mycologia* **80**, 689-695.

KLÁN, J. & BAUDIOVÁ, D. (1992). Cultural, enzyme and genetic studies in the genus *Flammulina* Karst. *Mycotaxon* **43**, 341-350.

KORF, R.P. (1991). Shall we abandon the principle of priority (and other nomenclatural caveats) for the sake of expediency? *Mycotaxon* **40**, 459-468.

KURTZMAN, R.H. JR & CHANG-HO, Y. (1982). Physiological considerations for cultivation of *Volvariella* mushrooms. In *Tropical Mushrooms - Biological Nature and Cultivation Methods*, pp. 139-166. Edited by S.T. Chang & T.H. Quimio. Hong Kong: The Chinese University Press.

KURTZMAN, R.H. JR & ZADRAZIL, F. (1982). Physiological and taxonomic considerations for cultivation of *Pleurotus* mushrooms. In *Tropical Mushrooms - Biological Nature and Cultivation Methods*, pp. 299-348. Edited by S.T. Chang, S.T. & T.H. Quimio. Hong Kong: The Chinese University Press.

LAMOURE, D. (1989). Species concept in the *Flammulina velutipes* group. *Opera Botanica* **100**, 163-167.

LOWY, B. (1951). A morphological basis for classifying the species of *Auricularia*. *Mycologia* **43**, 351-358.

LOWY, B. (1952). The genus *Auricularia*. *Mycologia* **44**, 656-692.

MAGAE, Y., HAGA, K., TANIGUCHI, H. & SASAKI, T. (1990). Enzymes of strains of *Pleurotus* species (Basidiomycetes) compared by electrophoresis. *Journal of General and Applied*

Microbiology **36**, 69-80.
MALLOCH, D. (1976). *Agaricus brunnescens*: the cultivated mushroom. *Mycologia* **68**, 910-919.
MALLOCH, D., CASTLE, A. & HINTZ, W. (1987). Further evidence for *Agaricus brunnescens* Peck as the preferred name for the cultivated *Agaricus*. *Mycologia* **79**, 839-846.
MAY, B. & ROYSE, D.J. (1988). Interspecific allozyme variation within the fungal genus *Pleurotus*. *Transactions of the British Mycological Society* **90**, 29-36.
MILLER, O.K. (1969). A new species of *Pleurotus* with a coremioid imperfect stage. *Mycologia* **61**, 887-893.
MURAKAMI, S. & TAKEMARU, T. (1990). Genetic studies of *Pleurotus salmoneostramineus* forming albino basidiocarps. *Reports of the Tottori Mycological Institute* **28**, 199-204.
NAIR, L.N. & KAUL, V.P. (1980). The anamorphs of *Pleurotus sajor-caju* (Fr.) Singer and *Pleurotus gemmellarii* (Inzeng.) Sacc. *Sydowia* **33**, 221-224.
NEDA, H., FURUKAWA, H. & MIGAYI, T. (1988). Two *Pleurotus* species from Okinawa. *Proceedings of the 32nd Annual Meeting, Mycological Society of Japan*. p.51.
OHIRA, I. (1990). A revision of the taxonomic status of *Pleurotus citrinopileatus*. *Reports of the Tottori Mycological Institute* **28**, 143-150.
OHIRA, I. & MATSUMOTO, T. (1980). A new record of *Pleurotus pulmonarius* Fr. in Japan. *Reports of the Tottori Mycological Institute* **18**, 129-132.
ORTON, P.D. (1986). *British Fungus Flora - Agarics and Boleti. vol. 4. Pluteaceae: Pluteus & Volvariella.* Edinburgh: Royal Botanic Garden.
PEGLER, D.N. (1972). Lentineae (Polyporaceae), Schizophyllaceae et espèces lentinoïdes et pleurotoïdes des Tricholomataceae. *Flore illustrée des champignons d'Afrique Centrale, Fasc.* **1**, 1-26.
PEGLER, D.N. (1975). The classification of the genus *Lentinus* Fr. (Basidiomycota). *Kavaka* **3**, 11-20.
PEGLER, D.N. (1976). *Pleurotus* (Agaricales) in India, Nepal and Pakistan. *Kew Bulletin* **31**, 501-510.
PEGLER, D.N. (1977). A Preliminary Agaric Flora of East Africa. *Kew Bulletin Additional Series* **6**, 1-615.
PEGLER, D.N. (1983). The genus *Lentinus*. *Kew Bulletin Additional Series* **10**, 1-281.
PEGLER, D.N. (1986). Agaric Flora of Sri Lanka. *Kew Bulletin Additional Series* **12**, 1-519.
PETERSEN, R.H. (1993). Neohaploidisation and neohaplont matings as a means of identification of *Pleurotus* cultures. *Mycosystema*, in press.
PETERSEN, R.H. & HUGHES, K.W. (1993). Intercontinental interbreeding collections of *Pleurotus pulmonarius*, with notes on *P. ostreatus* and other species. *Sydowia*, in press.
QUIMIO, T.H. & DE GUZMAN, R. (1982). Taxonomy and basidiocarp development of *Auricularia* mushrooms. In *Tropical Mushrooms - Biological Nature and Cultivation Methods*, pp. 383-395. Edited by S.T. Chang & T.H. Quimio. Hong Kong: The Chinese University Press.
RAJARATHNAM, S. & BANO, Z. (1987). *Pleurotus* mushrooms. Part I A. Morphology, life cycle, taxonomy, breeding, and cultivation. *CRC Critical Reviews in Food Science and Nutrition* **26**, 157-223.
SAMARAWIRA, I. & FERNANDO, S.T. (1973). Antigen analysis of the cultivated paddy straw mushroom of Thailand, Philippines and Ceylon. *Annuals of Botany* **37**, 371-374.
SINGER, R. (1984). *Agaricus brunnescens* Peck and *Agaricus bisporus* (Lange) Imbach. *Mycotaxon* **20**, 479-482.
SINGER, R. (1986). The Agaricales in Modern Taxonomy. Königstein: Koeltz Scientific Books.

SINGER, R. & HARRIS, B. (1987). *Mushrooms and Truffles, Botany, Cultivation, and Utilisation*. Königstein: Koeltz Scientific Books.

SMITH, A.H. (1978). Morphology and classification. In *The Biology and Cultivation of Edible Mushrooms*, pp. 3-34. Edited by S.T. Chang & W.A. Hayes. New York: Academic Press.

WONG, G.J. (1989). Compatibility and fruiting studies of an albino form of *Auricularia cornea*. *Mycotaxon* **34**, 259-266.

WONG, G.J. & WELLS, K. (1987). Comparative morphology, compatibility, and interfertility of *Auricularia cornea, A. polytricha,* and *A. tenuis*. *Mycologia* **79**, 847-856.

YOKOYAMA, K. (1991). Distribution and speciation in *Flammulina velutipes*. Abstract. 'Ascomycete and Basidiomycete Studies Now'. *Proceedings of the International Minisymposium of the Research Center for Pathogenic Fungi and Microbial Toxicoses*, Chiba University.

YOO, Y.-B., YOU, C.-H., PARK, Y.-H. & PEBERDY, J.F. (1986). Genetic analysis of the life cycle in interspecific hybrids of *Pleurotus ostreatus* and *Pleurotus florida* following protoplast fusion. *Korean Journal of Mycology* **14**, 9-15.

CHAPTER 3

CONTROL OF PATTERN AND FORM IN MUSHROOM MORPHOGENESIS

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1. INTRODUCTION

Major tissue domains in the mushroom fruit body (pileus, stipe, basal bulb, etc.) are established very early in the development of the structure, and the controlling events which determine tissue differentiation and distribution are completely obscure.

Some observations provide evidence for control of patterning by diffusion of chemical morphogens in morphogenetic fields around differentiating cells. Even the generation of the agaric gill can be understood in terms of organising centres which interact with one another by production of (and response to) freely diffusing activator and inhibitor molecules.

A major problem is our ignorance of the anatomy of the fruit body. Recent analyses of the stipe in *Coprinus* will be described to illustrate the new sorts of information which can be obtained through application of simple numerical methods to otherwise conventional microscopic analysis. Knowledge of the seat of the growth which drives morphogenesis and appreciation of the balance between physical and biological phenomena are also necessary. Research on these aspects shows that (a) agaric gills grow at their base, not their margins; and (b) agaric gills are initially convoluted, being stressed into their regular radial arrangement later.

We have chosen stipe gravitropism as a model representing a controllable morphogenetic change. Some of the new findings and the new ideas which are beginning to emerge from this work will be discussed.

2. PATTERNS IN THEORY

Morphogenesis is the development of the shape, form and structure of an organism. The most extensive research has been done with animals and from this a vocabulary has been developed which describes morphogenetic events without prejudging the mechanisms which may be involved. It is important to appreciate that the basic 'body plan' of the developing structure (whether fungal, plant

or animal) is not specified all at once. Rather, the shape and form arises as a result of a sequence of developmental 'decisions'. Each decision is irreversible within its morphogenetic sequence although often reversible by some gross disturbance; e.g. differentiated cells being put into tissue culture, nuclear & cell transplants, regeneration after injury, etc.

The states which precede terminal differentiation each embody reduction of potential compared with the previous state. Each decision is made by cells already ***specified*** by earlier decisions to belong to a particular developmental pathway. Consequently, each decision is made from among progressively smaller numbers of alternatives until the particular structure to which the cell will contribute is finally ***determined***. Classic embryological transplantation experiments revealed these states. Where the explant differentiated in accord with its old position then it was said to have been determined prior to transplantation. If it developed in accord with its new position, then it had not been determined, but may have been specified.

At the cellular level, the competent tissue becomes differentially determined in response to chemical signals from other regions of the developing structure. These chemicals (none truly identified yet) may be termed organisers, inducers or morphogens, and seem to inhibit or stimulate entry to particular states of determination. They may contribute to a ***morphogenetic field*** around a structure (cell or organ) which permits continued development of that structure but inhibits formation of another structure of the same type within the field.

All of these phenomena contribute to the ***pattern formation*** which characterises the 'body plan' or, more formally, the distribution of differentiated tissues in the structure (organ or individual). Pattern formation depends on ***positional information***, which allows the cell to differentiate in a way characteristic of its position in the structure. Positional information is usually understood to be imparted by the concentration of one or more morphogens emitted from one or more spatially distinct organisers. Effectively the cell 'triangulates' on the incoming signals and adjusts its morphogenetic response in accord with its position relative to the controlling organisers. Populations of cells which respond like this are said to show ***regional specification***. The operation can be divided into an ***instructive process*** which imparts positional information, and an ***interpretive process*** in which the competent tissue responds.

The basic rules of pattern formation seem to be that regional specification (directed by organisers producing morphogens) occurs first, regulating gene activity in ways specifically geared to morphogenesis so that particular cells are first specified (a state which is still flexible) and then determined (a state which is inflexible) to their differentiated fates. Cell differentiation is a consequence of these events - cells which are either specified or determined are not necessarily morphologically different from their neighbours or predecessors.

These statements highlight the major events contributing to animal and plant morphogenesis. Our challenge is to establish whether evidence exists for such mechanisms in the development of fungal structures. The great bulk of the published research on fungal morphogenesis has been done with taxonomic intentions. It has great value for its descriptive and comparative content, but precise developmental accounts are extremely rare and ***experimental*** approaches rarer still. Nevertheless, as I hope to show in what follows, there is a *prima facie* case for similar events (and perhaps similar mechanisms) being involved in establishing the patterns which result in formation of defined tissues in mushroom fruit bodies.

3. PATTERNS IN TIME

Descriptions and definitions of development, differentiation and morphogenesis always

emphasise ***change***. Morphogenesis has a time dimension and it is crucial to appreciate that developmental processes are dynamic ones which involve interactions in time as well as space. In the beginning, the normal invasive growth of vegetative mycelium is modified. Hyphae grow towards one another and a population of cells is assembled from contributions of a number of co-operating hyphal systems to form the community of hyphae and their branches which creates the mass of undifferentiated prosenchymal tissue described as the ***fruit body initial***.

Microscope sections of even extremely small fruit body initials can be resolved into regions of recognisable pileus and stipe and, since the creation of such histologically distinct regions requires that some organisation is imposed upon the homogeneous prosenchyma, these images provide *prima facie* evidence for regional specification. Reijnders (1948, 1963, 1979) has stressed the importance of: (a) development and nature of the veil and **pileipellis** (the 'epidermis' of the pileus) in relation to covering the developing hymenophore (the **hymenophore** carries the **hymenium**, a cell layer responsible for eventually producing the basidiospores); (b) the sequence of development of the stipe, pileus and hymenophore, which are the major functional zones of the basidiome; (c) the mode of development of the hymenophore. The terminology has been discussed by Watling (1985). The most highly differentiated cells are found at the boundaries of tissue regions (Williams, 1986). In the youngest specimens these boundaries are occupied by layers of parallel hyphae (called meristemoids by Reijnders, 1977) which seem to be involved in rapid cell formation in the sense that the distance between successive hyphal cross walls is minimised. But these are **NOT** meristems. Meristems do not occur in fungi.

Major tissue domains are demarcated very early in fruit body development. For example, in *Coprinus cinereus*, fruit body initials only 800 µm tall are clearly differentiated into pileus and stipe (Moore *et al.*, 1979) though this size represents only 1% of the size of a mature fruit body. As the different tissues do not grow uniformly, the ***differential growth*** of the primordium as it matures causes inevitable geometrical changes. As a typical fruit body of *C. cinereus* grows from 1 to 34 mm in height (i.e. a vertical linear change of 34x), the circumference of the stipe increases 9x, the outer circumference of the pileus increases 15x, but the volume increases more than 3000x.

The mechanical consequence of primordium enlargement for relationships between tissue layers which are often concentrically arranged is extremely important. For example, the internal structure of the *Coprinus* primordium is uniformly solid at the time that gills begin to arise, so gills and gill space arise together, but the enormous increase in size of the fruit body primordium helps explain where the space comes from to form a gill cavity. When the opposing hymenia of neighbouring gills differentiate they form a fracture plane which can be opened out into a cavity when the expansion of the underlying tissue puts tension across the 'fracture' and pulls the hymenia apart. This is a process I call 'cavitation' (Moore, 1994) and the argument applies to cavitation in all differentially expanding cellular structures. Variations on the theme can be imagined in other organisms. If the 'fracture planes' form an annulus around the top of the stipe (one tissue layer might be the stipe apical meristemoid, the other the hymenophore meristemoid), then an annular cavity could arise before gill formation.

The meristemoid of the developing hymenophore of *Coprinus* is a protohymenium in which probasidia which proceed to karyogamy and initiate the meiotic cycle ending with sporulation. There is a defined temporal sequence: probasidia appear first and then paraphyses arise as branches from sub-basidial cells and insert into the hymenium (Rosin & Moore, 1985b).

4. PATTERNS IN SPACE

About 8% of the hyphal tips in the protohymenium of *C. cinereus* become cystidia, but when a cystidium does arise, it inhibits formation of further cystidia within a radius of about 30 µm (Horner & Moore, 1987); this is the cystidial morphogenetic field. Its influence is limited strictly to the hymenium from which the cystidium arises and it determines the distribution of cystidia over the hymenial surface.

Perhaps the most obvious pattern, though, is the distribution of the gills of agaric fungi. Essentially the gills are plates suspended from the fruit body pileus tissue. In *Coprinus*, the pileus of the fruit body primordium encloses the top of the stipe and gills are formed as essentially vertical plates arranged radially around the stipe. There are two types of gill: primary gills which, from formation, have their inner, tramal tissue in continuity with the outer layers of the stipe, and secondary (and lesser ranked) gills in which the hymenium is continuous over the gill edge (Reijnders, 1979; Rosin & Moore, 1985a; Rosin *et al.*, 1985; Moore, 1987).

Primary gills are connected with pileus tissue at their outer edge and with the stipe at their inner edge; the tendency to widen as the stipe circumference increases is compensated by gill replication, and specifically by formation of a new gill cavity and its bounding pair of hymenia ***within*** the trama of a pre-existing gill. This clearly sets the direction of development as outwards ***from the stipe***; i.e. gills in the *C. cinereus* fruit body grow radially outwards, their roots extending into the undifferentiated tissue of the pileus context.

The formative element is an **organiser** in the tissue at the extreme end of the gill cavity. The gill organiser is responsible for the progression of the gill cavity radially outwards, away from the stipe. It directs the prosenchyma/protohymenium transition - an increase in branch frequency to produce branches of determinate growth which are mutually 'attracted' so that they form the opposing palisades of a fracture plane (see above). Pileus expansion separates the two protohymenia, thus extending the gill cavity. Since they are progressing radially outwards, neighbouring organisers become further and further separated from one another as development proceeds and as the distance between neighbouring organisers increases a new one can arise between them (Rosin & Moore, 1985a); when a new gill organiser emerges, the margin of a new (but 'secondary') gill is formed. It is extended not by growth of its margin, but by continued radial outward progression of the two gill organisers on either side of its root.

In the origin of the gills we seem to have operating two classic components of theoretical morphogenesis - activation and inhibition by diffusing morphogens. First, we can suggest that diffusion of an activating signal along the fruit body radius assures progression of the gill organiser along its radial path. Second, each organiser can be assumed to produce an inhibitor which prevents formation of a new organizer within its diffusion range (i.e. the gill organiser uses this inhibitor to control its morphogenetic field). As radial progression into the extending pileus context causes neighbouring organisers to diverge, a region appears between them which is beyond the range of their inhibitors -- at this point a new organizer can arise in response to the radial activating signal. Interaction between the diffusion characteristics of the activator and the inhibitor is all that is necessary to control gill spacing, gill number, gill thickness, and the radial orientation of the gill field.

Gill development of *Volvariella bombycina* seems to be exactly homologous with the process in *Coprinus*; i.e. growth of any one gill occurs by outward progression of gill organisers on either side of the root of the gill into outwardly expanding pileus context. We have demonstrated that the gill margins remain essentially intact by painting black ink marks on the tissues in a primordium (Chiu & Moore, 1990a). During further fruit body development, ink marks placed on the pileus margin and those placed on the edges of the gills ***remained at the margin or the gill edges, respectively***. The

growth increment here is quite considerable, the radius of the pileus increasing from 0.5 to 2.5 cm and the depth of the gills from 1.5 to 5 mm. If growth of the pileus and gill margins resulted from apical growth of the hyphal tips which occupied the margin, then ink particles placed on those hyphal tips would be left behind as the hyphal apices extended which would consequently have resulted in the ink marks being buried beneath 4 to 20 mm of newly formed tissue by the end of the experiment. It follows, therefore, that the hyphal tips which first form the pileus margin, and those which form the gill margin, always remain ***at*** the margin. They do not continue to grow apically to extend the margin radially, nor are they overtaken by other hyphae; instead they are 'pushed' radially outwards by the press of fresh growth behind, and they are joined by fresh branches appearing alongside as the circumference of the margin is increased.

In both *C. cinereus* and *V. bombycina* gills are formed as convoluted plates (Chiu & Moore, 1990b). A sinuous, labyrinthiform hymenophore appears to be a normal 'embryonic' stage in basidiome development in agarics, yet a regular radial arrangement of the gills is characteristic of the mature basidiome. How this is achieved is a function of the expansion of the maturing primordium generating stresses between tissue layers which stretch (in *C. cinereus*) or inflate (in *V. bombycina*) the convoluted gills into strict radii.

Until recently there was surprisingly little information concerning the structure of the stipe of the *Coprinus* fruit body. Hammad, Watling & Moore (1993) have now demonstrated that the stipe contains both narrow and inflated hyphae. Narrow hyphae (cross-sectional area $<20\ \mu m^2$) always comprise a significant numerical proportion (23% to 54%) of the cells seen in microscope sections of stipe tissue, although they only contribute 1% to 4% to the overall cross-sectional area of the stipe. During normal stipe growth the greatest cell expansion is seen in the inflated cells situated between the mid-cortex and the lumen rather than at the periphery of the stipe. Such a distribution of expansion would actually generate the lumen in the first instance and would obviously contribute to the stretching mechanisms (remember that the pileus surrounds the stipe in *Coprinus*) referred to in the previous paragraph.

Nearest neighbour analysis of cell distributions in *Coprinus* stipes shows that inflated hyphae are evenly rather than randomly distributed so, presumably, there is some sort of control over the pattern of inflation. This is yet another example of indirect evidence for local control of morphogenesis to be added to those provided by the distribution pattern of cystidia, the formation of groups of paraphyses around basidia, and the patterning process in gill development. Very similar aggregations of hyphae, termed hyphal knots (Reijnders, 1977) have been observed in a wide range of species (Reijnders, 1993). The common features of Reijnders' hyphal knots seem to be a central hypha (which remains hyphal) and an immediately-surrounding family of hyphae which differentiate in concert. Perhaps, in all multihyphal fungal structures, the ultimate morphogenetic regulatory unit is the Reijnders hyphal knot - a little community comprising an induction hypha (or hyphal tip, or hyphal compartment) and the immediately surrounding hyphae (or tips, or compartments) which can be brought under its influence. Larger scale morphogenesis could be co-ordinated by 'knot-to-knot' interactions.

Unfortunately, there are no clues to the nature of the **morphogens** which might serve as the growth factors involved in these phenomena. Also, ***lateral*** contacts between fungal hyphae are extremely rare, being represented only by lateral hyphal fusions. The constituent cells of plant and animal tissues are interconnected laterally by frequent plasmodesmata, gap junctions and cell processes. The absence of similar structures connecting adjacent hyphae suggests that any morphogens which do exist are likely to be communicated exclusively through the extracellular environment (Reijnders & Moore, 1985). Although it is abundantly clear that co-ordination of developmental processes is successfully achieved in fungal multicellular structures, the evidence for chemicals able

to perform the signal communication involved is sparse and disappointingly unconvincing (discussed in Moore, 1991).

5. PATTERNS IN EXPERIMENT

Most of the phenomena described so far are poor candidates for experiment because their control processes are entirely endogenous. We have recently begun to experiment with gravitropism in *Coprinus* as a morphogenetic model system. Mushrooms have a very sensitive gravity detection system and if reoriented they regain the vertical by bending the stem. The gravitropic response is a simple developmental pattern forming process; its control demands that the organism has a gravity perception system and a means of coupling this to stipe growth. The perception system must establish a new morphogenetic pattern to which the organ is caused to adjust by differential growth. Study of gravitropism is therefore a natural, non-invasive means of generating a particular morphogenetic change on demand in a specific location.

We have used video recording and computer-based video-image analysis to complete the first kinetic analysis of mushroom stem gravitropism (Kher *et al*., 1992). Completion of meiosis in the cap coincides with the stem becoming competent to react gravitropically. Within 30 min of disorientation the negatively gravitropic bend initially appears within the apical 15% of its length. The bend then becomes more acute and progresses basally, traversing 40% of the initial length of the stem. Gravitropic bending is most likely the result of asymmetric distribution of growth, as represented by cell expansion, being stimulated by a diffusing, extracellular growth factor produced by the apical region of the stem. Continued exposure to the unilateral gravity vector is necessary for continued bending (Hatton & Moore, 1992). Bending raises the apex and as this approaches an angle of about 35° to the horizontal curvature compensation begins to adjust the degree of bending so that the apex can be brought exactly vertical. Curvature compensation requires that the apical region is free to move towards the vertical. The mechanism involved is uncertain, but it may involve a second diffusing signal.

Further work will concentrate on the nature of the signalling processes, biochemical, structural and ultrastructural aspects of curvature and curvature compensation, and sensitivity to gravitational acceleration.

REFERENCES

CHIU, S. W. & MOORE, D. (1990a). Development of the basidiome of *Volvariella bombycina*. *Mycological Research* **94**, 327-337.

CHIU, S. W. & MOORE, D. (1990b). A mechanism for gill pattern formation in *Coprinus cinereus*. *Mycological Research* **94**, 320-326.

HAMMAD, F., WATLING, R. & MOORE, D. (1993). Cell population dynamics in *Coprinus cinereus*: narrow and inflated hyphae in the basidiome stipe. *Mycological Research* **97**, 269-274.

HATTON, J. P. & MOORE, D. (1992). Kinetics of stem gravitropism in *Coprinus cinereus*.: determination of presentation time and 'dosage-response' relationships using clinostats. *FEMS Microbiology Letters* **100**, 81-86.

HORNER, J. & MOORE, D. (1987). Cystidial morphogenetic field in the hymenium of *Coprinus cinereus*. *Transactions of the British Mycological Society* **88**, 479-488.

KHER, K., GREENING, J. P., HATTON, J. P., NOVAK FRAZER, L. A. & MOORE, D. (1992). Kinetics and mechanics of gravitropism in *Coprinus cinereus*. *Mycological Research* **96**, 817-824.

MOORE, D. (1987). The formation of agaric gills. *Transactions of the British Mycological Society* **89**, 105-108.

MOORE, D. (1991). Perception and response to gravity in higher fungi - a critical appraisal. *New Phytologist* **117**, 3-23.

MOORE, D. (1994). Tissue formation. In *The Growing Fungus*, Edited by N. A. R. Gow & G. M. Gadd. chapter 21, in press. London: Chapman & Hall.

MOORE, D., ELHITI, M. M. Y. & BUTLER, R. D. (1979). Morphogenesis of the carpophore of *Coprinus cinereus*. *New Phytologist* **83**, 695-722.

REIJNDERS, A. F. M. (1948). Études sur le développement et l'organisation histologique des carpophores dans les Agaricales. *Recuil des Travaux Botaniques Néerlandais* **41**, 213-396.

REIJNDERS, A. F. M. (1963). *Les problèmes du développement des carpophores des Agaricales et de quelques groupes voisins.* The Hague: Dr W. Junk.

REIJNDERS, A. F. M. (1977). The histogenesis of bulb and trama tissue of the higher Basidiomycetes and its phylogenetic implications. *Persoonia* **9**, 329-362.

REIJNDERS, A. F. M. (1979). Developmental anatomy of *Coprinus*. *Persoonia* **10**, 383-424.

REIJNDERS, A. F. M. (1993). On the origin of specialised trama types in the Agaricales. *Mycological Research* **97**, 257-268.

REIJNDERS, A. F. M. & MOORE, D. (1985). Developmental biology of agarics - an overview. In *Developmental Biology of Higher Fungi*, pp. 333-351. Edited by D. Moore, L. A. Casselton, D. A. Wood & J. C. Frankland. Cambridge, U.K.: Cambridge University Press.

ROSIN, I. V., HORNER, J. & MOORE, D. (1985). Differentiation and pattern formation in the fruit body cap of *Coprinus cinereus*. In *Developmental Biology of Higher Fungi*, pp. 333-351. Edited by D. Moore, L. A. Casselton, D. A. Wood & J. C. Frankland. Cambridge, U.K.: Cambridge University Press.

ROSIN, I. V. & MOORE, D. (1985a). Origin of the hymenophore and establishment of major tissue domains during fruit body development in *Coprinus cinereus*. *Transactions of the British Mycological Society* **84**, 609-619.

ROSIN, I. V. & MOORE, D. (1985b). Differentiation of the hymenium in *Coprinus cinereus*. *Transactions of the British Mycological Society* **84**, 621-628.

WATLING, R. (1985). Developmental characters of agarics. In *Developmental Biology of Higher Fungi*, pp. 281-310. Edited by D. Moore, L. A. Casselton, D. A. Wood & J. C. Frankland. Cambridge, U.K.: Cambridge University Press.

WILLIAMS, M. A. J. (1986). *Studies on the structure and development of Flammulina velutipes (Curtis: Fries) Singer*. Ph.D. Thesis, University of Bristol.

CHAPTER 4

PHYSIOLOGY, CYTOLOGY AND GENETICS OF MUSHROOMS

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1. INTRODUCTION

Mushrooms are familiar macrofungi being well-known of or having potential for nutritive, medical and industrial importance. Regardless of this, emphasis is in most cases placed on the cultivation technology of these mushrooms (Chang & Hayes, 1978; Chang & Quimio, 1982; Chiu & Chang, 1992) with scarce information on other aspects which are fundamental and basic. In this way, unlike other crops, breeding in edible mushrooms is handicapped, and conventional means, such as single spore isolation, mass pairing and screening, is employed to hunt for a good hybrid (Chang *et al.*, 1993). It is not the intention to say that such conventional method does not work. Yet with our better knowledge on mushrooms, we can explain the phenomena encountered in handling mushrooms, and it hopefully enables us to devise a better and more directed strategy for improving strains, obtaining higher yield of mushrooms or mushroom products. Thus, mushroom biology serves us.

For *Agaricus bisporus*, the most popular cultivated mushroom in the world, researches fall on various aspects including genetics, cytology, physiology and cultivation (Flegg *et al.*, 1985; Moore *et al.*, 1985; Wuest *et al.*, 1987; Kerrigan *et al.*, 1993), with the result that commerical strains are patented using molecular markers, and a computerized system has been developed to control composting to guarantee good yield (Leendertse, 1988). However, a protocol of directed breeding of a strain with desirable traits is not available. In comparison, less attention and research activities are paid to other mushrooms. I, therefore, utilize this opportunity to review some information on various fundamental aspects of these so-called specialty mushrooms and their significance and possible impacts on applied field.

2. DEVELOPMENTAL PLASTICITY - A CHALLENGE TO TAXONOMISTS

Developmental plasticity is an inborn property of an organism to adapt to the fluctuating environment and is enabled by the differential expression of the genome according to the immediate

needs. Spontaneous fruitbody plasticity is widely observed during mushroom cultivation although mushroom farmers usually regard the variant form as abnormal. Unlike abnormal forms caused by infections (Burden, 1992), the variant forms just reflect the response towards a certain stimulus, e.g. the uprolling of the *Pleurotus* caps exposing the gills or the premature exposure of the fruitbody proper (hymenophore) from a basal bulb of *Volariella* to the stagnant air, and the tumor outgrowth on the caps of *Agaricus bisporus* induced by the fuming oil in the mushroom farm. Once when the environmental conditions change or become better, normal fruitbodies appear again. In *Volvariella bombycina*, spontaneous variants in the forms of exposed cap, morchelloid forms, supernumerary gills, inverted caps, enclosed form (gasteromycetoid form) and even the agaric form without basal volva which is a characteristic feature of genus *Volvariella* were found together with the normal agaric form (Chiu *et al*., 1989). All of these normal and variant fruibodies except the morchelloid form matured with spore formation. Yet there were some irregularities: gills were heavily covered with mucilage, asynchronous maturation of spores borne on the same basidium, the number of spores/sterigmata borne on a basidium ranged from 2 to 4, and the basidiospore shape which is also of taxonomic value was not egg-shaped but heart-shaped (Chiu *et al*., 1989). By *in vitro* and *in vivo* studies using gills of *Coprinus cinereus*, basidial maturation can be disturbed by exogenous factors leading to arrestment of development without spore formation, a basidium with fewer than 4 spores/sterigmata, a mosaic basidium bearing both spore and hyphal tips and giant spherical spores (Chiu & Moore, 1988 & 1990a). By carrying out a developmental study with *V. bombycina*, some of the variants were found to be actually the embryonic form which under favourable conditions will transit into the normal agaric form (Chiu & Moore, 1990b). Therefore, care is needed to differentiate this developmental plasticity with the disease symptoms caused by pathogens. The latter requires an environmental study isolating the causative agent. In addition, it is common to find the different morphological forms when a fungus is subcultured onto different media, the same old phenomenon named plasticity. Therefore, when variant fruitbodies are encountered, the critical thing to do is to check for any unfavourable environmental conditions and contaminants present.

Taxonomists in the old days stressed on morphological features (phenotype in genetic term) for classification purpose but a developmental biologist regards the phenotype which is the product of interaction between environment and genotype. No wonder why nowadays, taxonomists are collecting information at the DNA level in addition to the morphological and physio-chemical features of organisms. These information collected in addition to the strains preserved in a culture collection centre would surely reflect and conserve the fungal biodiversity on earth and provide a rich resource for human goodness.

3. CYTOGENETICS - A CHALLENGE TO MUSHROOM BREEDERS

Today, breeding through protoplast fusion technology is available although transformation system using protoplasts has not been well established for edible mushrooms.

3.1. Karyotype Analysis

Fungal chromosomes are minute and at the limit of resolution by conventional light microscopes. With the development of pulsed field gel electrophoresis (PFGE) (Lai *et al*., 1989), the karyotypes of various mushroom species showing molecular size and numbers of chromosomes have been worked out. We successfully devised a freeze-grinding method to prepare the intact chromosomal DNAs of the yeast *Saccharomyces cerevisiae* which is commonly employed as the size marker

(Kwan *et al.*, 1991) but such method is found causing too much mechanical damage to most mushroom chromosomal DNAs of size greater than 2Mb (million base pairs).

The importance of karyotype for identification of species is not justified as chromosomal polymorphism among strains is now known to be a widespread phenomenon. Yet electrophoretic karyotype is a tool to reveal chromosomal aberration during growth and development if any in fungi. Strain instability and sectoring are commonly encountered especially for old cultures but no one has looked at this phenomenon from this aspect, perhaps simply because of the lack of funding for basic research.

The fungal karyotype can also be revealed by chromosome spread at the meiotic prophase stage. Up to now such technique first developed in plant system has only been applied to the mushroom *Coprinus cinereus* (Pukkila & Lu, 1985). This protocol requires good preparation of chromosome spread and the equipment scanning electron microscope for best resolution. In comparison, electrophoretic karyotype requires high yield of protoplasts which are then embedded in agarose, *in-situ* lysis of protoplasts to release the chromosomal DNAs which are protected from mechanical shear by the agarose and an equipment to do PFGE. PFGE is an important tool for gene mapping and for gene cloning especially when using vectors such as yeast artifical chromosome which can harbour DNA up to 1Mb. Therefore, the latter technique should have more widely uses. All these in future will build up into a transformation system involving a particular gene or chromosome for strain improvement.

3.2. Molecular Markers

Although a particular agaric is by convention characterized by the specific morphological properties of the fruitbodies (Buchanan, this proceedings), it is impractical to always fruit different strains in order to distinguish among them, especially with our poor knowledge on the fruiting initiation mechanism and the encounter of sterile strains. Therefore, DNA fingerprinting techniques were attempted. Currently, by using polymerase chain reaction, different strains (no matter it is of the same or different species) can be differentiated by arbitrarily-primed polymerase chain reaction (Williams *et al.*, 1990; Kwan *et al.*, 1992; Chiu *et al.*, 1993). When 2 or more primers are used, the differences can be revealed definitely. The isozyme pattern, another molecular marker commonly used (Royse, this proceedings), has a drawback that enzyme expression and so the detection is in some cases dependent on the physiological conditions of the strains. i.e. Before an enzyme can be used, the isozyme pattern of a particular strain should not show temporal or spatial specificity. Also, isozyme pattern only samples the expression of the genome while DNA markers reveal indiscriminately various sequences of the genome. In comparison, there are fewer isozyme markers than DNA markers.

Similar DNA fingerprinting technique using RAPD (random amplified polymorphic DNA markers) (Welsh & McClelland, 1990; Kerrigan *et al.*, 1993) is commerically employed to patent *Agaricus bisporus* strains. This way of strain characterization can be routinely employed for culture collection centre and can have great potential for being incorporated for any hybridization/breeding experiments for labelling the parental strains and characterizing hybrid formation. In addition, when the segregation pattern of the amplified markers are followed in the progeny, some of them will be found to be genetic markers (Chiu *et al.*, 1993; Kerrigan *et al.*, 1993). Linkage relationship will then be established between phenotypic traits and DNA markers. These information will facilitate the direct cloning of genes.

3.3. Sexuality & Life History

Chang in this proceedings gives a general grouping of sexuality patterns found in mushrooms. For self-sterile cross-fertile species such as *Lentinus edodes*, *Pleurotus sajor-caju*, strain improvement can be done by mating with a good homokaryon, hoping for hybrid vigor to occur. Yet when two homokaryons are of the same mating types or different species are involved, only protoplast fusion can bring the two genomes into the same cytoplasm. Although many research groups succeed in performing protoplast fusion, commercialization of the hybrid is rare. The reasons may be as follows: auxotrophic mutants instead of the cultivated strains are used; protoplast fusion does not guarantee genetic recombination in the way desired by an experimenter. With the availability of DNA markers in addition to other biochemical and morphological markers, hybridization using wild type strains is now made possible to be traced and is in progress in many laboratories. However, no one study follows what has happened after protoplast fusion in terms of the genetic makeup and the nuclear-cytoplasm compatibility, indicating our poor knowledge on the selection process. Therefore, protoplast fusion has not yet been a directed strategy. Heterologous gene expression is possible for a foreign gene from one organism be transformed and expressed in another organism. Perhaps this is the way to which breeding can de directed.

For self-sterile species such as *Volvariella volvacea* and *V. bombycina* (Chang & Yau, 1971; Li & Chang, 1978; Chang *et al*., 1981; Chiu & Chang, 1987a), heterokaryon formation is possible by pairing two homokaryons as demonstrated by isozyme markers (Royse *et al*., 1987). Based on morphological features, fruiting ability and growth rate, variation is observed in the progenies of the two species. Recently, microspectrophotometric study on the DNA content and nuclear behaviour of *V. bombycina* has shown that a primary homothallic species bears a uninucleate not binucleate hymenial cell initial which if develops into a basidium, will synthesize its DNA content at prophase I instead of prekaryogamy stage contrasting with those of heterothallic species (Chiu & Moore, 1993). Although we now realize more differences between heterothallic and homothallic species in terms of the nuclear cycle, we still do not know how *Volvariella* species create variation.

4. MODULATING MUSHROOM GROWTH & DEVELOPMENT - A CHALLENGE TO FARMERS

Both spores and vegetative mycelium are commonly used for seeding mushroom cultures. Also, in conventional breeding programmes, single spore isolates are commonly used. Basidiospores of some mushrooms germinate readily when plated onto nutrient media but some, such as *Agaricus bisporus*, *Volvariella volvacea* and *V. bombycina* (Chiu & Chang, 1987b), require a heat soak treatment and the presence of an actively growing mycelium for stimulation. Once when a spore germinates, and it establishes into a mycelial mat. Various attempts have been made to heighten the mycelial/metabolite yields using solid-state-fermentation and submerged fermentation technologies. In addition to the classical method by searching for the best C- and N-sources, nutrient supplementation is another common method used (Batterley, 1989). Addition of lipid/waste tea leaves was found to enhance biomass gain (Nair *et al*., 1990; Li *et al*., 1992). Lipid and waste tea leaves not only add more nutrients to the medium but also promote production of substrate-utilizing enzymes (Nair *et al*., 1990; Chiu *et al*., unpublished result). Supplementation with waste tea leaves and slow-release nutrients were found to increase fruiting yield (Batterley, 1989; Tan & Chang, 1989). However, the triggering factors for fruiting remain obscure. In *Coprinus cinereus*, the monokaryotic mutant with an elevated level of endogenous cAMP produced fruitbodies but exogenous cAMP did not induce fruiting in

Schizophyllum commune and other mushrooms (Uno & Ishikawa, 1971; Schwalb & Miles, 1978). Although in many mushrooms, endogenous glycogen elevated at time of fruiting (Moore *et al.*, 1985; Chiu & Cheng, 1991), at least in *C. cinereus* and *Pleurotus sajor-caju*, by using differnt approaches, endogenous glycogen was found not a trigger for fruiting (Chiu & To, 1993; Ji & Moore, 1993). We really are in great need for financial support and research activities to examine the fruiting pathway in details.

5. CONCLUSION

A brief account on some current aspects of mushroom biology is presented. From it we can find that:

i. Financial support is urgently needed for mushroom research and development;
ii. Basic studies using model mushrooms, such as *Coprinus cinereus* and *Schizophyllum commune* can provide us good and relevant information for cultivated mushrooms although specificity does exist in some cases; and most of all,
iii. Basic and fundamental research is necessary and essential for mushroom application.

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REFERENCES

BATTERLEY, D.A. (1989). Supplementation of *Agaricus* and *Pleurotus* substrate - an overview. *Mushroom News* **37**, 10-16.

BURDEN, J.L. (1992). Mushroom malaise. *Mushroom Journal* **506**, 16-21.

CHANG, S.T., BUSWELL, J.A. & MILES, P.G. (1993). *Culture Collection and Breeding of Edible Fungi*. Philadelphia: Gordon & Breach Pub., Inc.

CHANG, S.T. & CHIU, S.W. (1992). Mushroom production - an economic measure in maintenance of food security. In *Microbial Technology: Economic and Social Aspects,* pp.110-141. Edited by DaSilva, E.J., Ratledge, C. & Sasson, A. Cambridge: Cambridge University Press.

CHANG, S.T. & HAYES, W.A. (1978). *The Biology and Cultivation of Edible Mushrooms*. New York: Academic Press.

CHANG, S.T., MILES, P.G. & WAI, C.C. (1981). A study of monosporous isolates of *Volvariella volvacea*. *Mushroom Science* **11**, 603-619.

CHANG, S.T. & QUIMIO, T.H. (1982). *Tropical Mushrooms: Biological Nature and Cultivation Methods*. Hong Kong: The Chinese University Press.

CHANG, S.T. & YAU, C.K. (1971). *Volvariella volvacea* and its life history. *American Journal of Botany* **58**, 552-562.

CHIU, S.W. & CHANG, S.T. (1987a). *Volvariella bombycina* and its life history. *Mushroom Journal for the Tropics* **7**, 1-12.

CHIU, S.W. & CHANG, S.T. (1987b). Activation of spore germination in *Volvariella bombycina*. *Mushroom Journal for the Tropics* **7**, 61-66.

CHIU, S.W. & CHENG, H.P. (1991). Involvement of endogenous glycogen in fruiting of *Volvariella volvacea* and *Pleurotus sajor-caju*. *Mycological Research* **95**, 632-634.

CHIU, S.W., KWAN, H.S. & CHENG, S.C. (1993). Application of arbitrarily-primed polymerase chain reaction in molecular studies of mushroom species with emphasis on *Lentinula edodes*. *Culture Collection and Breeding of Edible Fungi*, pp.265-284. Edited by Chang, S.T., Buswell, J.A. & Miles, P.G. Philadelphia: Gordon & Breach Pub., Inc.

CHIU, S.W. & MOORE, D. (1988) Ammonium ions and glutamine inhibit sporulation of *Coprinus cinereus* basidia assayed *in vitro*. *Cell Biology International Reports* **12**, 519-526.

CHIU, S.W. & MOORE, D. (1990a). Sporulation in *Coprinus cinereus*: use of *in vitro* assay to establish the major landmarks in differentiation. *Mycological Research* **94**, 249-253.

CHIU, S.W. & MOORE, D. (1990). Development of the basidiome of *Volvariella bombycina*. *Mycological Research* **94**, 327-337.

CHIU, S.W. & MOORE, D. (1993). Cell form, function and lineage in the hymenia of *Coprinus cinereus* and *Volvariella bombycina*. *Mycological Research* **97**, 221-226.

CHIU, S.W., MOORE, D. & CHANG, S.T. (1989). Basidiome polymorphism in *Volvariella bombycina*. *Mycological Research* **92**, 69-77.

CHIU, S.W. & TO, S.W. (1993). Endogenous glycogen is not a trigger for fruiting in *Pleurotus sajor-caju*. *Mycological Research* **97**, 363-366.

FLEGG, P.B., SPENCER, D.M. & WOOD, D.A. (1985). *The Biology and Technology of the Cultivated Mushrooms*. Chichester: John Wiley & Sons.

JI, J. & MOORE, D. (1993). Glycogen metabolism in relation to fruitbody maturation in *Coprinus cinereus*. *Mycological Research* **97**, 283-289.

KERRIGAN, R.W., ROYER, J.C., BALLER, L.M., KOHLI, Y., HORGEN, P.A. & ANDERSON, J.B. (1993). Meiotic behaviour and linkage relationships in the secondarily homothallic fungus, *Agaricus bisporus*. *Genetics* **133**, 225-236.

KWAN, H.S., CHIU, S.W., PANG, K.M. & CHENG, S.C. (1992). Strain typing in *Lentinula edodes* by polymerase chain reaction. *Experimental Mycology* **16**, 163-166.

KWAN, H.S., LI, C.C., CHIU, S.W. & CHENG, S.C. (1991). A simple method to prepare intact yeast chromosomal DNA for pulsed field gel electrophoresis. *Nucleic Acids Research* **19**, 1347.

LAI, E., BIRREN, B.W., CLARK, S.M., SIMON, M.I. & HOOD, L. (1989). Pulsed field gel electrophoresis. *BioTechniques* **7**, 34-42.

LEENDERTSE, I.K.D.K. (1988). The Agrisystems-Sohm indoor composting system: is this the future? *Mushroom News* **36**, 10-13.

LI, S.F. & CHANG, S.T. (1978). Variation in the homothallic basidiomycete, *Volvariella volvacea*. *Mushroom Science* **10**, 171-184.

LI, Y., CHO, K.Y., WU, Y.Z. & NAIR, N.G. (1992). The effect of lipids and temperature on the physiology and growth of *Volvariella volvacea*. *World Journal of Microbiology and Biotechnology* **8**, 621-626.

MOORE, D., CASSELTON, L.A., WOOD, D.A. & FRANKLAND, J.C. (1985). *Developmental Biology of Higher Fungi*. Cambridge: Cambridge University Press.

NAIR, N.G., HOLLEY, M.P., SONG, C.H. & CHO, K.Y. (1990). Lipid metabolism of *Pleurotus sajor-caju*. *Annual of Applied Biology* **114**, 167-176.

PUKKILA, P.J. & LU, B.C. (1985). Silver staining of meiotic chromosomes in the fungus, *Coprinus cinereus*. *Chromosoma* **91**, 108-112.

ROYSE, D.J., JODON, M.H., ANTONIO, G.G. & MAY, B.P. (1987). Confirmation of intraspecific crossing and single and joint segregation of biochemical loci of *Volvariella volvacea*. *Experimental Mycology* **11**, 11-18.

SCHWALB, M.N. & MILES, P.G. (1978). *Genetics and Morphogenesis in the Basidiomycetes*. New York: Academic Press.

TAN, Y.H. & CHANG, S.T. (1989). Effect of growth regulators, enzyme inhibitors and stimulatory additives on the vegetative development and fruitification of *Lentinus edodes*. *Mushroom Science* **12**, 267-271.

UNO, T. & ISHIKAWA, T. (1971). Chemical and genetical control of induction of monokaryotic fruiting bodies in *Coprinus macrorhizus*. *Molecular and General Genetics* **113**, 228-239.

WELSH, H. & MCCLELLAND, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213-7218.

WILLIAMS, J.G.K., KUBELIK, A.R., LIVAK, K.J., RAFALSKI, J.A. & TINGEY, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

WUEST, P.J., ROYSE, D.J., & BEELMAN, R.B. (1987). *Cultivating Edible Fungi*. Amsterdam: Elsevier.

CHAPTER 5

MOLECULAR GENETIC ANALYSIS OF DIVERSITY IN POPULATIONS OF EDIBLE MUSHROOMS

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1. INTRODUCTION

Over 25 years ago, Lewontin and Hubby (1966) first demonstrated high levels of molecular polymorphisms in the fruit fly *Drosophila pseudoobscura*. The discovery of allozymic protein variation in this species revolutionized our ability to study evolution. The availability of this technology stimulated molecular genetic studies of many other organisms including fungi.

Most early isozyme studies of fungi focused primarily on general protein patterns, utilizing some specific enzymes (Shechter *et al*., 1973; Mouches *et al*., 1979; Paranjpe *et al*., 1979). The electrophoretic phenotypes were evaluated on the presence or absence of particular bands without regard to the genetic bases of these bands. One early study, however, on an imperfect fungus (*Neurospora intermedia*) established a genetic basis for the protein variants observed (Speith, 1975). With allozyme analysis, unambiguous codominant genotypes can be recognized—a must for successfully conducting genetic studies of fungi.

Relatively few population studies of edible fungi have been conducted using molecular genetic markers. Natural populations of *Agaricus campestris* (May & Royse, 1982), *Pleurotus* spp. (Boisselier-Dubayle, 1983), *Agaricus bisporus* (Kerrigan & Ross, 1989; Kerrigan, 1990; Kerrigan *et al*., 1993a), and *Morchella* spp. (Gessner *et al*., 1987; Yoon *et al*., 1990) have been examined by multilocus enzyme electrophoresis (Royse & May, 1993). Wild *A. bisporus* populations recently have been examined using DNA restriction site variation (Kerrigan *et al*., 1993a).

2. METHODS USED IN FUNGAL MOLECULAR GENETICS

2.1. Allozyme Analysis

Enzymes differing in electrophoretic mobility as the result of allelic differences in a single gene are called allozymes. Informative data sets are allowed by allozyme analysis because the phenotypic

differences in electrophoretic banding patterns between individuals can be directly correlated to genotypic differences. Thus, allozyme electrophoresis provides unambiguous codominant genotypes that can be used successfully in conducting genetic studies of fungi.

The methods used for allozyme electrophoresis have been reviewed by Royse and May (1993) and May (1993). The review by Royse and May (1993) included a summarization of buffer systems, enzymes, abbreviations, Enzyme Commission numbers, encoding loci, protein subunit composition, and a list and discussion of mushroom species where allozymes have been used for genetic studies.

More recently, Kerrigan *et al.*, (1993a) have provided evidence that a wild California population of *A. bisporus* may comprise two or more ancestral elements. Using seven allozyme markers and 14 restriction fragment length polymorphisms (RFLPs) they were able to evaluate the California isolates relative to " a comprehensive sample of cultivar isolates likely to be of European ancestry". Such a comparison revealed that the California isolates could be placed either in the cultivar group or in a group associated with a cypress habitat. The discovery of an intermediate or less distinct group of isolates was suggestive of spontaneous hybrids between the two ancestral groups or of lines with other affinities. Studies such as the one cited above are crucial to our understanding of genetic behavior of isolates of a cultivated species in the wild. As natural habitats are lost and man-made habitats are created, genetic markers will be crucial in monitoring the changing population of this commercially important mushroom. Allozyme analysis will continue to play a role in this assessment.

2.2. DNA-DNA Hybridization

There are two basic methods used for DNA-DNA hybrid molecule tests: thermal stability and cross-hybridization. Almost all studies on fungi have tested the percentage of cross-hybridization between total DNA extracts rather than the thermal stability of hybrid molecules (Burns *et al.*, 1991). One study, however, by Horgen et al. (1984) in which some wild and cultivated mushrooms were examined by thermal elution, revealed a 7.7 to 11.6% mismatch between unique DNAs of *A. bisporus* and *A. bitorquis*. This study confirms earlier contentions that these two species are closely related as inferred from cultivation and habitat studies.

2.3. Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs result from the presence or absence of particular restriction sites in DNA. Polymorphisms can be identified because they alter the length of characteristic restriction fragments. These DNA fragments can be separated by electrophoresis, in a manner similar to that used to separate allozymic proteins. RFLP analysis can be accomplished in two ways: 1) by using radioactive probe DNA molecules containing the nucleotide sequences of interest, or 2) through enzymatic amplification of DNA from specific target sites in the genome followed by treatment with restriction enzymes.

2.3.1. RFLP analysis with probe DNA. In 1975, Southern developed a procedure to identify DNA fragments through a special procedure known as a Southern blot (Southern, 1975). The key element in a Southern blot is the use of a probe DNA molecule. This probe molecule contains the nucleotide sequence of interest, which is obtained from a gene that has been cloned into bacterial cells. In the Southern procedure, DNA fragments are transferred to and immobilized on a filter, hybridized to the labeled DNA probe, and visualized by autoradiography. The number of observed bands depends on the number of loci in the genome homologous to the probe and the number of restriction sites within these DNA sequences.

This method has been used to analyze wild populations (Kerrigan *et al.*, 1990, 1993a) and isolates (Summerbell *et al.*, 1989; Loftus *et al.*, 1988) of *A. bisporus* and *A. campestris* and *A. bitorquis* (Hintz *et al.*, 1989). The number of RFLP markers is effectively unlimited because any cloned, low-copy-number piece of DNA can be used as a probe. This may be combined with several restriction enzymes to identify RFLPs with each probe.

2.3.2. RFLP analysis with enzymatic amplification of DNA. Enzymatic amplification (via the polymerase chain reaction; PCR) of DNA is now a routine procedure in genetic studies of fungi (White *et al.*, 1990; Kohn, 1992). Restriction analysis of PCR-amplified DNA allows analysis without Southern blotting and use of radioactive probes. Population studies on cultivated edible mushrooms using this approach have yet to be published. Vilgalys and Hester (1990) have shown that this technology is not only faster but it avoids the potential complication of RFLPs caused by methylation differences (Burns *et al.*, 1991). In addition, this methodology allows limitation of the population survey to a portion of the DNA that is evolving at the appropriate rate. The use of this methodology in systematic studies of edible mushrooms is likely to increase due to the relative ease of enzymatic amplification of DNA and the commercial availability of over 500 restriction endonucleases.

2.4. Random Amplified Polymorphic DNA (RAPD)

A PCR-based method, RAPDs produce fragments similar to RFLPs via random amplification of DNA with a single short primer of arbitrary sequence. These fragments have been demonstrated to behave as simple Mendelian loci (Williams *et al.*, 1990; Kerrigan *et al.*, 1993b). One of the major problems encountered with use of RAPDs, however, is that they are dominant. Alternative alleles are null so that in diploid fungi it is not possible to distinguish heterozygous and homozygous individuals. One other reported problem with this methodology is that multiple fragments of a single RAPD sometimes map to the same or nearly the same locus and so may not indicate independent loci (Williams *et al.*, 1990). In the future, however, these problems should be overcome (Burns *et al.*, 1991) because this methodology offers speed and convenience relative to many of the above methods.

2.5. Sequencing

Nucleic acid sequencing is a relatively new approach for systematic studies (Hillis *et al.*, 1990). Direct sequencing by PCR is more efficient than cloning techniques and thus, will probably increase for applications in fungal population genetics. Sequencing is expensive and more time consuming, however, than RFLP analysis and information generated may be difficult to analyze especially where nucleotide alignments are highly subjective.

Sequencing in cultivated edible fungi has already begun (Kwan *et al.*, 1992). Ribosomal RNA genes (rDNA) and their intergenic spacer regions appear to be good targets for many systematic and population studies. Differences in the rate of evolution for each region in the rDNA repeat allows for selection of the appropriate region for the comparison of interest. Many of the sequences already determined for various organisms including fungi are available from several databases. GenBank, perhaps the best known of the databases, contains over 108 base pairs and is doubling every 1 to 2 years (Hillis *et al.*, 1990).

2.6. Electrophoretic Karyotyping

Pulsed-field electrophoretic separation of chromosomal-length DNAs has been applied to many species of fungi (Mills & McCluskey, 1990). Most of these studies have revealed a high frequency of chromosomal-length polymorphisms within a species. In *A. bisporus*, Southern hybridizations to contour-clamped homogeneous field (CHEF) electrophoretic separations of chromosomal DNAs showed polymorphisms between parental homokaryons used in a cross (Kerrigan *et al.*, 1993a). In addition, 13 chromosomes were observed with the rDNA repeat mapping exclusively to chromosome VII. These studies also help to clarify the pattern of marker transmission in *A. bisporus* and demonstrate that determinants of traits such as homokaryon vigor can be mapped to chromosomal-length DNAs.

3. OUTLOOK

As our knowledge continues to increase with regard to the evolutionary relationships among and between taxa, perhaps, in the future, more emphasis will be placed on estimates of a time frame for divergence. A phylogenetic tree with times of divergence for all cultivated edible fungi and their close relatives, would allow a new perspective on the way we view the evolutionary relationship of each species. Such a tree also might provide insight on species that are the most likely candidates for genetic and economic exploitation.

REFERENCES

BOISSELIER-DUBAYLE, M. C. (1983). Taxonomic significance of enzyme polymorphism among isolates of Pleurotus (basidiomycetes) from umbellifers. *Transactions of the British Mycological Society* **81**, 121-127.

BURNS, T. D., WHITE, T. J. & TAYLOR, J. W. (1991). Fungal molecular systematics. *Annual Reviews of Ecological System* **22**, 525-64.

GESSNER, R. V., RAMANO, M. A. & SCHULTZ, R. W. (1987). Allelic variation and segregation in *Morchella deliciosa* and *M. esculenta*. *Mycologia* **79**, 683-687.

HINTZ, W. E., ANDERSON, J. B. & HORGEN, P. A. (1989). Relatedness of three species of *Agaricus* inferred from restriction fragment length polymorphism analysis of the ribosomal DNA repeat and mitochondrial DNA. *Genome* **32**, 173-178.

HORGEN, P. A., ARTHUR, R., DAVY, O., MOUM, A., HERR, R., STRAUS, N. & ANDERSON, J. (1984). The nucleotide sequence homologies of unique DNAs of some cultivated and wild mushrooms. *Canadian Journal of Microbiology* **30**, 587-593.

HILLIS, D. M., LARSON, A., DAVIS, S. K. & ZIMMER, E. A. (1990). Nucleic acids III: sequencing. In *Molecular Systematics*, pp. 318-369. Edited by HILLIS, D. M. & MORITZ. Sunderland, Massachusetts: Snauer Associates.

KERRIGAN, R. W., HORGAN, P. A. & ANDERSON, J. B. (1993a). The California population of *Agaricus bisporus* comprises at least two ancestral elements. *Systematic Botany* **18**, 123-136.

KERRIGAN, R. W., ROYER, J. C., BALLER, L. M., KOHLI, Y., HORGEN, P. A. & ANDERSON, J. B. (1993b). Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics* **133**, 225-236.

KERRIGAN, R. W. (1990). Evidence of genetic divergence in two populations of Agaricus

bisporus. *Mycological Research* **94**, 721-733.
KERRIGAN, R. W. & ROSS, I. K. (1989). Allozymes of a wild Agaricus bisporus population: new alleles, new genotypes. *Mycologia* **81**, 433-443.
KWAN, H. S., PANG, K. M., CHIU, S.W. & CHENG, S. C. (1992). Nucleotide sequence of the 5.8 S ribosomal RNA gene of *Lentinula edodes*. *Nucleic Acids Research* **20**, 610.
KOHN, L. M. (1992). Developing new characters for fungal systematics: an experimental approach for determining the rank of resolution. *Mycologia* **84**, 139-153.
LEWONTIN, R. C. & HUBBY, J. L. (1966). A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics* **54**, 595-609.
LOFTUS, M. G., MOORE, D. & ELLIOTT, T. J. (1988). DNA polymorphisms in commercial and wild strains of the cultivated mushroom, *Agaricus bisporus*. *Theoretical and Applied Genetics* **76**, 712-718.
MAY, B. (1992). Starch gel electrophoresis of allozymes. In *Molecular Genetic Analysis of Populations*. Edited by A. R. Hoelzel. Oxford: IRL Press at Oxford University Press.
MAY, B. & ROYSE, D. J. (1982). Genetic variation and joint segregation of biochemical loci in the common meadow mushroom, *Agaricus campestris*. *Biochemical Genetics* **20**, 1165-1173.
MILLS, D. & MCCLUSKEY, K. (1990). Taxonomic structure of *Phytophthora cryptogea* and *P. drechsleri* based on isozyme and mitochondrial DNA analysis. *Mycological Research* **95**, 31-48.
MOUCHES, C., DUTHIL, P., BOVE, J. M., VALJALO, J. & DELMAS, J. (1979). Characterisation des champignons superieurs par electrophorese de leurs proteins. *Mushroom Science* **10**, 491-503.
PARANJPE, M. S., CHEN, P. K. & JONG, S. C. (1979). Morphogenesis of *Agaricus bisporus*: changes in proteins and enzyme activity. *Mycologia* **71**, 469-478.
ROYSE, D. J. & MAY, B. (1993). Multilocus enzyme electrophoresis for the genetic analysis of edible mushrooms. In *Genetics and Breeding of Edible Mushrooms,* pp. 225-248. Edited by S.T. Chang, J.A. Buswell & P.G. Miles. Philadelphia: Gordon and Breach Science Publishers.
SHECHTER, Y., HALL, R., OELSHLEGAL, JR., F. S., STAHMAN, R. D. SNIDER, M. A., FRANKE, R. G., HOLMAN, R. W., REDDY, M. M. & GARBER, E. D. (1973). Symposium on the use of electrophoresis in the taxonomy of algae and fungi. *Bulletin of the Torrey Botanical Club* **100**, 253-312.
SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.
SPEITH, P. T. (1975). Population genetics of allozyme variation in *Neurospora intermedia*. *Genetics* **80**, 785-805.
SUMMERBELL, R. C., CASTLE, A. J., HORGEN, P. A. & ANDERSON, J. B. (1989). Inheritance of restriction fragment length polymorphisms in *Agaricus brunnescens*. *Genetics* **123**, 293-300.
YOON, C-S., GESSNER, R. V. & RAMONO, M. A. (1990). Population genetics and systematics of the *Morchella esculenta* complex. *Mycologia* **82**, 227-235.
VILGALYS, R. & HESTER, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**, 4238-4246.
WHITE, T. J., BURNS, T. D., LEE, S. & TAYLOR, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide To Methods and Applications,* pp. 315-322. Edited by M.A. Innis, D.H. Gelford, J.J. Sninsky & T.J. White. New York: Academic Press.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

CHAPTER 6

NEW PERSPECTIVES ON THE GENETICS OF *PLEUROTUS*

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1. INTRODUCTION

The production of cultivated mushrooms has a long history and up until the recent past has not been greatly influenced by the major developments of biotechnology that have occurred in the past two decades. However, research on the genetics and molecular biology, and on biologically important metabolites has started to have an impact on this major multi-million dollar earning industry.

The production of *Pleurotus* spp. (the Oyster mushroom) along with all other forms has increased significantly in the last seven years. In 1990 a 538% increase in production took *Pleurotus* to the second position in world production (Table 1). To meet demand it has been necessary to exploit the metabolic and physiological diversity of the fungus with respect to substrate utilisation. Utilisation of these materials is clearly dependent on the ability of *Pleurotus* to secrete a range of enzymes including peroxidases, laccases, cellulases, hemicelluloses, xylanases and ligninases (Toyama & Ogawa, 1974; Ulezlo *et al*, 1975; Dauglis & Bone, 1977).

TABLE 1. World production of the major edible mushrooms in 1989/1990.

Species	Fresh Weight of Mushrooms*		% Increase
	1986	1989/90	
Agaricus bisporus	1,227	1,424	16.1
Lentinula edodes	314	393	25.5
Volvariella volvacea	178	207	16.3
Pleurotus spp.	169	909	537.9
Auricullaria spp.	119	400	336.1
Flammulina velutipes	100	143	43.0

* Metric tons x 1000
(After Chang, personal communication).

Improved substrate utilisation and conversion leading to higher mushroom yields are clear goals in all areas of mushroom production. Whilst empirical strategies undoubtedly play a role the existence of a natural system for breeding based on the reproductive process (of which the mushroom itself is a key part) provides an obvious rational alternative. In the medium to longer term other strategies using recombinant DNA technology may also play a role particularly in enzyme engineering to improve substrate utilisation.

The reproductive strategies of several basidiomycetes, including some commercial species, have been investigated. Three fertility mechanisms have been described (Table 2). With the exception of *Agaricus bisporus* the other edible species that have been studied exhibited a heterothallic system. Thus *Pleurotus ostreatus* and *P. cystidiosus* are typically tetrapolar with two multi-allelic loci. In such fungi crosses between compatible mycelia derived from single spores can provide a practical method for hybridisation and strain improvement. Fruiting of the dikaryon is an obvious indication of fertility and the progeny of successful crosses may be propagated by tissue culture from the fruit bodies. Interstrain crosses using compatible monokaryons derived from fruit bodies from divergent sources provide an obvious source of potentially useful hybrids.

The advent of protoplast technology in fungi in the early 1970's (Peberdy, 1979) led two useful areas of applications in the basidiomycetes. Isolation and subsequent regeneration of protoplasts derived from mycelium of dikaryons gave rise to a mixed population of dikaryotic and monokaryotic progeny. In the latter case, although the progeny were of only two mating types diversity in genotype was observed. Such de-dikaryotisation has been shown to be a useful procedure in generating genetically variable monokaryons for crossing purposes. Dikaryons derived as protoplast regenerants may have some advantages over the original strain. Magae *et al.* (1985) showed that some regenerants from *P. ostreatus* yielded a 5.7 - 8.1% increase in fruit body weight.

Protoplast fusion has been used as a means of establishing crosses in several edible mushrooms. The value of this technique lies in the potential to generate hybrids. Several reports of hybrids produced in this manner have been published, however, the realisation that some of the so-called species used can be mated by conventional techniques (see later) casts doubt on their validity as interspecies hybrids. Furthermore better substantiation of hybridity is necessary and is possible

TABLE 2. Genetic structures of mating systems identified in agaric basidiomycetes.

Fertility Mechanism	Polarity	Structure	Examples
Primary Homothallism			*Coprinus steritirus* (Raper, 1966)
Secondary Homothallism	Bipolar	1 locus/2 alleles	*C. sassii* (Kemp, 1975)
	Bipolar	1 locus/multiple alleles	*C. bisoporus* (Kemp, 1980) *Agaricus bisporus* (Elliott, 1978)
	Tetrapolar	2 loci	*C. plagioporus* (Fincham & Day, 1971)
Heterothallism	Bipolar	1 locus/multiple alleles	*Pholiota nameka* (Arita & Takemaru, 1962)
		2 loci/multiple alleles	*Pleurotus ostreatus* (Eugenio & Anderson, 1968)
			Lentinula edodes (Takemaru, 1961)

TABLE 3. Mating-type alleles in *Pleurotus species*.

Species	Number of Alleles		Reference
	A	B	
P. ostreatus	17	20	Eugenio & Anderson, 1968
P. sapidus	31	36	Anderson *et al,* 1973
P. cystidiosus	6	6	Moore, 1985

through the use of a range of molecular markers such as RFLPs and RAPDs, and the use of molecular karyotypes generated by pulsed-field electrophoresis techniques.

2. MATING TYPE ANALYSIS IN *PLEUROTUS SAJOR-CAJU*

Previous studies of *P. sajor-caju* and other *Pleurotus* species (Eugenio & Anderson, 1968; Roxon & Jong, 1977) indicated that the genus had a typical tetrapolar mechanism of incompatibility with mating factors designated A and B. The number of alleles found for the two mating factors varies in different species (Table 3). However, the numbers obtained will reflect the number of dikaryons analysed. Roxon and Jong (1977) described the mating system of *P. sajor-caju* with only two A and two B alleles. We therefore initiated a study to understand further the mating system of this fungus as a basis for future genetic improvement.

Sixteen dikaryotic strains derived from Europe, North America, the Far East and South East Asia were used in this investigation (Table 4). Mating type testers A_1 B_1, A_2 B_2, A_1 B_2 and A_2 B_1 were obtained from the ATCC. The dikaryons were fruited on wheat straw based substrates and at least 30 single basidiospores were isolated from one sporocarp. Germination of spores was never less than 60%.

The individual monokaryons were identified for mating type and the basis of mating reactions with the master strains on agar plates. Clamp connections were seen in the contact zone of compatible mating after 7-10 days. The formation of clamp connections was consistent with all compatible matings as confirmed by random fruiting trials.

Of the sixteen strains examined, 14 proved to have the A_1 A_2 B_1 B_2 genotype. Strains designated 32-8 and 32-15 in our collection, from Thailand and Bhutta (India) respectively were both of different genotypes and therefore indicating the existence of at least 6A and 6B alleles.

In non-complementary crosses several reactions were observed. False clamp connections or pseudoclamps were frequently found in barrage zones where common B alleles were introduced as described by Roxon and Jong (1977) but also in some common A matings as has also been described in *P. ostreatus* (Eger, 1978). The flat mycelium described for common A matings in *Schizophyllum commune* (Raper, 1966) was found in both common A and common B matings in *P. sajor-caju.*

The segregation frequencies of the four mating types was very variable in spore progeny from fruit bodies. In one extreme case, strain 32-4 obtained from France, segregants with the A_2 B_2 alleles were either non-viable, or as in the case of A_2 B_1 of very low viability. This suggests that the A_2 allele may be linked to a deleterious or lethal gene.

The identical nature of the mating type alleles of the majority of the strains investigated raises the question of their origin from a single common progenitor. This perception was confirmed by

TABLE 4. Mating-type analysis of *P. sajor-caju.*

Strain No	Origin	Mating-type
32-1	Hong Kong	$A_1A_2B_1B_2$
32-2	Korea	$A_1A_2B_1B_2$
32-3	France	$A_1A_2B_1B_2$
32-4	France	$A_1A_2B_1B_2$
32-5	Brunei	$A_1A_2B_1B_2$
32-6	Malaysia	$A_1A_2B_1B_2$
32-7	Taiwan	$A_1A_2B_1B_2$
32-8	Thailand	$A_3A_4B_3B_4$
32-9	China	$A_1A_2B_1B_2$
32-10	Korea	$A_1A_2B_1B_2$
32-11	China (Shanghai)	$A_1A_2B_1B_2$
32-12	India	$A_1A_2B_1B_2$
32-13	Italy	$A_1A_2B_1B_2$
32-14	Italy	$A_1A_2B_1B_2$
32-15	India	$A_5A_6B_5B_6$
32-16	Canada	$A_1A_2B_1B_2$

studies on isozyme profiles (Hanifah, 1993) and molecular karyotypes (Jia, J.H. unpublished data). For enzymes previously identified as showing allozyme variation in *Pleurotus* (May & Royse, 1988) were investigated by polyacrylamide gel electrophoresis. In the cases of alcohol dehydrogenase, malate dehydrogenase and lactate dehydrogenase the band patterns obtained were identical for all sixteen strains. With esterase, however, some polymorphism was evident; the two strains with different mating type alleles (32-8 and 32-15) presented different isozyme patterns. Of the strains with $A_1A_2B_1B_2$ mating type all were identical for esterase except 32-4 which had a unique profile.

3. BREEDING STRATEGY IN *PLEUROTUS SAJOR-CAJU*

Several of the dikaryons produced in the course of mating-type allele analysis were investigated as part of an assessment of the potential of recombination as a breeding tool in this mushroom. Comparisons were made of colony radial growth rate, spawn run, mushroom yield and computed biological efficiency for several of the hybrid dikaryons. The data (Table 5) shows that even in such a limited trial some improvement was possible through such matings. It was interesting to observe that crosses between monokaryons derived from dikaryons from the presumed common progenitor (i.e. 32-1 and 32-4) also gave some improved progeny.

4. MATING INTERACTIONS BETWEEN *PLEUROTUS* SPECIES

There are several reports concerning interspecies crosses involving *Pleurotus* species based on protoplast fusion (see Yoo & Cha, 1993 for review; Ogawa, 1993). In view of the uncertainties in the taxonomy of the genus the status of some of these crosses as interspecies crossed has to be

TABLE 5. Characteristics of *P. sajor-caju*.

Parents Hybrids	Colony Growth[1] (cm)	Spawn Run[1] (days)	Yield[1] (g)	Biological Efficiency[3] (%)
32-1 x 32-2				
32-1	3.68	27a[2]	238.9b	59.7
32-2	3.82	27a	210.0bc	52.5
H1	4.27	46de	114.4fg	28.6
H2	3.90	28ab	119.5fg	29.9
H3	2.90	30ab	106.7g	26.9
H4	4.90	28ab	81.9hi	20.5
32-1 x 32-4				
32-4	4.13	28ab	230.76	57.7
H9	4.50	35bc	235.36	58.8
H10	4.37	29ab	275.2a	68.7
H11	4.07	28ab	231.96	58.0
32-1 x 32-5				
32-5	4.60	32ab	159.2de	39.8
H12	4.18	33abc	47.3ij	11.8
H13	3.18	40cd	31.9j	8.0
H14	2.45	45de	141.9efg	35.5
H15	3.30	55f	109.9g	27.5
32-1 x 32-8				
32-8	2.33	26a	242.4b	60.6
H24	7.93	25a	215.0c	53.7
H25	5.80	27a	256.9b	64.2
H26	6.10	25a	283.0a	70.7
H27	7.63	25a	218.4c	56.0

[1] All measured values are means of 3 replicates

[2] Mean values sharing similar letters (ie a,b, c etc) in the same column do not differ significantly at $P<0.05$.

[3] Biological efficiency was calculated as the percentage of fresh mushroom yield over the dry weight (400g/bag) of the wheat straw/bran substrate.

questioned. There are also several reports of hybrids between supposed species being produced by hyphal anastomosis thus raising the question whether the strains involved are in fact distinct species.

Recent experiments (Jia, J.H. unpublished data) have been directed at the generation of dikaryons between strains identified as different species, and an assessment of the fertility of the products. Differences in these results (Table 6) compared to the published data emphasise the importance of the criteria used in defining speciation in *Pleurotus*. The formation of basidiocarps by a dikaryon must imply that the monokaryotic progenitors are in fact the same species. Based on the results of these experiments we conclude that the strains of *P. ostreatus*, *P. florida*, *P. columbinus* and *P. sapidus* are isolates of a single species.

TABLE 6. Compatibility of interspecific crosses in *Pleurotus*.

	P.ostreatus	*P.florida*	*P.sajor-caju*	*P.pulmonarius*	*P.colombinus*	*P.sabidus*
P.ostreatus	+	+	-	-	+	+
P.florida		+	-	-	+	+
P.sajor-caju			+	-	-	-
P.pulmonarius				+	-	-
P.colombinus					+	+
P.sapidus						+

+ Compatible and fertile mating.
- Incompatible mating.

TABLE 7. Estimated chromosome size of *Pleurotus* spp. in megabase pairs

Chromosome	*P.ostreatus*	*P.florida*	*P.sajor-caju*	*P.pulmonarius*	*P.colombinus*	*P.sapidus*
IX	5.7	5.1	5.7	5.7	5.7	5.5
VIII	5.3	4.7	5.1	5.3	4.7	4.6
VII	4.6	4.1	3.1	5.1	4.3	4.3
VI	4.3	3.8	2.5	4.5	3.6	3.8
V	3.6	2.7	2.0	3.1	3.1	3.3
IV	3.3	2.2	1.6	2.7	2.5	2.3
III	1.8	1.6		2.0	1.8	1.4
II	1.6	1.1		1.6	1.4	0.9
I	1.1	0.7				
Genome size (Mb)	31.3	26.0	20.0	30.0	27.1	26.1

5. MOLECULAR KARYOTYPES IN *PLEUROTUS*

In view of the observations made in the mating experiments between presumptive different species of *Pleurotus* it was of interest to re-examine and compare the karyotype patterns of strains that proved to be compatible when crossed. Furthermore improvements in the pulsed-field gel electrophoresis procedure, as compared to that developed by Fox (1991) and discussed in Peberdy & Fox (1993), has enabled better resolution of the putative chromosome bands. Compared to the earlier report it has now been possible to resolve more DNA bands in several species of *Pleurotus* thereby obtaining a more reliable estimate of the size of the genome. Of the species (strains) investigated (Table 7) the smallest genome (20Mb) was found in *P. sajor-caju*. Several isolates of this species, of presumed common genetic ancestry, gave identical karyotypes. The three compatible species, *P. florida, P. columbinus* and *P. sapidus* had very similar karyotypes further supporting the conclusion that these isolates could be the same species. However, the genome of the fourth compatible species, *P. ostreatus* is larger. If these are the same species then this observation records possible polymorphism at the gross chromosome level. Current work is directed to the development of a physical map for these species which can then be used to analyze the homology between the different chromosomes and the potential for their recombination.

6. FUTURE OUTLOOK

Unlike other agricultural and horticultural crops most of the edible mushrooms have not been developed through extensive breeding programmes. Conventionally new strains were obtained using tissue culture isolation techniques to recover dikaryons from fruit bodies which had been selected for specific desirable characteristics. More recently the situation has changed and in the last decade the potential of breeding has been recognised for many edible mushrooms including *Agaricus bisporus*, *Lentinula edodes* (Ellingboe, 1993), and *Flammulina velutipes* (Kitamoto *et al*, 1993; Kinugawa, 1993). Clearly with the recent developments in fungal biotechnology and gene technology, combined with a breeding programme based on sexual recombination, a broader strategy for strain development in many edible fungi is now conceivable. Breeding provides the only possibility, certainly in the short to medium term, for improvement of multigenic properties, e.g. growth rate, gross nutritional value, but for more specific characteristics such as improved substrate utilisation, recombinant DNA approaches through the manipulation of specific enzymes involved in the process, has greater potential. The importance of high value medically useful products from the basidiomycete fungi will also provide an impetus for new developments based on genetic engineering strategies.

REFERENCES

ARITA, I. & TAKEMARU, T. (1962). Some problems on the mating system of *Pholiota nameko*. *Report of the Tottori Mycological Institute (Japan)* **2**, 1-10.

DAUGLIS, A. J.& BONE, D. H. (1977). Submerged cultivation of edible white rot fungi on tree bark. *European Journal of Applied Microbiology* **4**, 156-168.

EGER, G. (1978) Biology and breeding of *Pleurotus*. In *The Biology and Cultivation of Edible Mushrooms,* pp. 497-519. Edited by S.T. Chang & W.A. Hayes. New York: Academic Press.

ELLIOTT, T. J. (1978). Comparative sexuality in *Agaricus* species. *Journal of General Microbiology*

107, 115-122.
EUGENIO, C. P. & ANDERSON, N. A. (1968). The genetics and cultivation of *Pleurotus*. *Mycologia* **60**, 627-634.
FINCHAM, J. R. S. & DAY, P. R. (1971). *Fungal Genetics* 3rd ed. Oxford: Blackwell.
HANIFAH, A. H. M. (1993). Studies on the mating biology of *Pleurotus sajor-caju*. M.Phil. Thesis, University of Nottingham.
KEMP, R. F. O. (1975). Breeding biology of *Coprinus* species in the section *Lanatuli*. *Transactions of the British Mycological Society* **75**, 29-35.
KEMP, R.F.O. (1980). Bifactorial incompatibility without clamp connections in the *Coprinus patouillardii* group. *Transactions of the British Mycological Society* **74**, 357-362.
KINUGAWA, K. (1993). Physiology and the breeding of *Flammulina velutipes*. In *Genetics and Breeding of Edible Mushrooms,* pp.86-109. Edited by S.T. Chang, J. A.Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
KITAMOTO, K., NAKAMATA, M. & MASUDA, P. (1993). Production of a novel white *Flammulina velutipes* by breeding. In *Genetics and Breeding of Edible Mushrooms,* pp.65-86. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
MAGAE, Y., KAKIMOTO, Y., KASHIGAWI, Y. & SASAKI, T. (1985). Fruiting body formation from regenerated mycelium of *Pleurotus ostreatus* protoplasts. *Applied and Environmental Microbiology* **49**, 441-442.
MAY, B. & ROYSE, D. J. (1988). Interspecific allozyme variation within the fungal genus *Pleurotus*. *Transactions of the British Mycological Society* **90**, 29-36.
OGAWA, K. (1993). Interspecific and Intergeneric hybridization of edible mushrooms by protoplast fusion. In *Genetics and Breeding of Edible Mushrooms,* pp.193-206. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
PEBERDY, J. F. (1979). Fungal protoplasts: isolation reversion and fusion. *Annual Review of Microbiology* **33**, 21-39.
PEBERDY, J. F. & FOX, H. M. (1993). Protoplast technology and edible mushrooms. In *Genetics and Breeding of Edible Mushrooms,* pp. 125-155. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
RAPER, J. R. (1966). *Genetics of Sexuality in Higher Fungi*. New York: Ronald Press.
ROXON, J. E. & JONG, S. C. (1977). Sexuality of an edible mushroom, *Pleurotus sajor-caju*. *Mycologia* **69**, 202-205.
TAKEMARU, T. (1961). Genetical studies on fungi. IX. The mating system of *Lentinus edodes* (Berk.) Sing. *Report of the Tottori Mycological Institute (Japan)* **1**, 61-68.
TOYAMA, N. & OGAWA, K. (1974). Comparative studies on cellulolytic and oxidising enzymes of edible and iedible wood rotters. *Mushroom Science* **9**, 745-760.
ULEZLO, I. V., UPCROVA, V. & FENISKOVA, R. (1975). Oxidative enzymes of the lignin-degrading fungus, *Pleurotus ostreatus*. *Microbiology* **11**, 535-538.
YOO, Y.B. & CHA, D. Y. (1993). Gene transfer in edible fungi using protoplasts. In: *Genetics and Breeding of Edible Mushrooms,* pp.157-192. Edited by. S.T. Chang, J.A.Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.

CHAPTER 7

GENETICS AND BREEDING OF SPORE-DEFICIENT STRAINS IN *AGROCYBE CYLINDRACEA* AND *LENTINUS EDODES*

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1. INTRODUCTION

Spores discharged from cultivated mushrooms may give rise to several problems: (a) respiratory allergies among mushroom growers which are known to be caused by antigens present on the spore surface, (b) reduced commercial value because of spores deposited on mushrooms, (c) increased populations of mites and other microorganisms feeding on fungal spores in the cultivation rooms, (d) erosion of genic variation in natural populations of the mushroom species. To solve these problems, spore-deficient strains may be useful.

In this paper, the cytology and genetics of a sporeless mutant of *Lentinus edodes* are presented (Hasebe *et al*., 1991). In relation to this mutant, problem (d) is discussed in relation to the large-scale outdoor cultivation of shiitake. Secondly, induction of spore-deficient mutants of *Agrocybe cylindracea* by UV-irradiation and their use in breeding is described. In addition, spore deficiency resulting from matings between geographically separated strains of *A. cylindracea* (Japanese and European) is introduced.

2. SPORELESS MUTANT OF *LENTINUS EDODES*

During genetical analysis of a *Lentinus edodes* mutant bearing aberrant clamp connections (Murakami *et al*., 1987), a spontaneously occurring sporeless mutant (TMI-1597) was found. Its sporeless fruitbodies were of normal morphology but a spore print could not be collected. When examined under scanning electron microscope , basidia on a hymenium of a wild type fruitbody matured asynchronously (Fig. 1). The basidia usually formed four sterigmata whose apices subsequently swelled and developed into basidiospores. In the sporeless mutant, basidia at different developmental stages were also present, but only up to the stages of sterigma formation; sterigmata developed but their distal ends rarely swelled (Fig. 2). The majority of autolyzed basidia bore

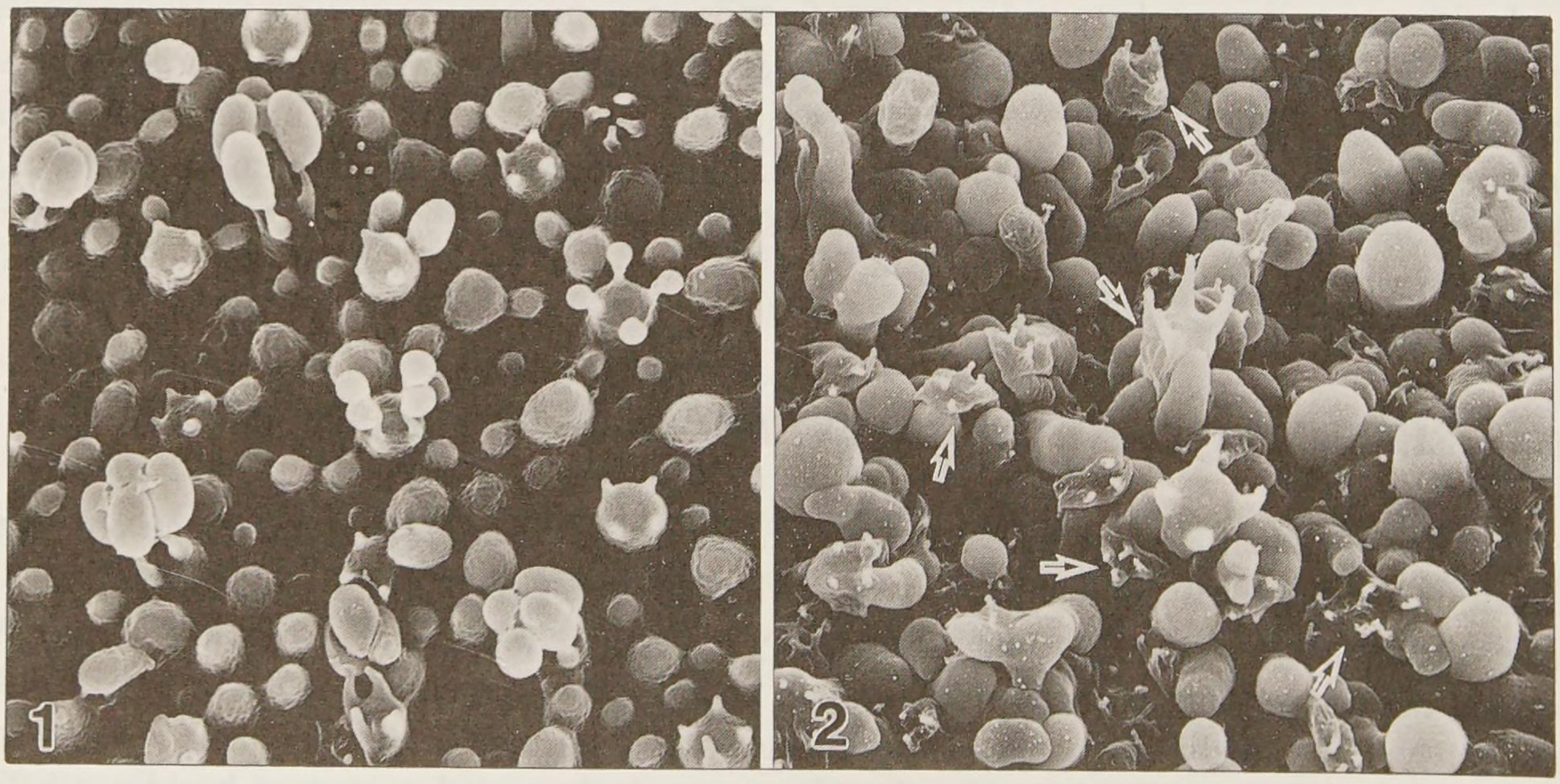
remnants of sterigmata only (Fig. 2).

Nuclear behaviour during basidiospore formation in the sporeless mutant was studied under the light microscope, using the HCl-Giemsa staining protocol. Nuclear fusion and meiosis in the mutant were completed normally ending in a stage with tetrads of nuclei. After meiosis, sterigmata developed on the basidium but swelling of the distal ends was not usually evident. Murakami & Takemaru (1985) reported that a post-meiotic mitosis took place in *L. edodes* corresponding to the pattern C defined by Duncan and Galbraith (1972): a single post-meiotic mitotic division occurs in each basidiospore while the spore is still attached to the sterigma, and then one of the daughter nuclei will move back to the basidium.

We also examined the post-meiotic nuclear behaviour in abortive basidia of normal fruitbodies and found that the post-meiotic mitosis took place within basidia which thus became eight-nucleate. However, we could not find any eight-nucleate basidia in the sporeless mutant. It is apparent that during the period between meiosis and post-meiotic mitosis, the cytoplasm of the basidium degenerated to such an extent that the tetrads of nuclei were unable to undergo further division.

When the component homokaryons (Sp1 and Sp2) of the sporeless mutant were each mated to the wild-type homokaryon #9, both the resulting dikaryons formed normal basidiocarps bearing numerous basidiospores. From a basidiocarp of Sp1 x #9 dikaryon, 193 single spore isolates were taken, and all of these homokaryons were crossed with Sp2 to make 193 dikaryons. One hundred of these dikaryons produced sporeless basidiocarps and 93 produced normal ones (Table 1). These results indicated that deficiency in sporulation is caused by a single recessive gene, *spo*. Linkage analysis showed that the *spo* gene was not linked to either A or B incompatibility factor.

Respiratory allergies caused by the inhalation of shiitake spores have been reported (Kondo, 1969; Sastre *et al.*, 1990), and this problem appears to have escalated with the increase in indoor cultivation of shiitake. On the other hand, the large-scale outdoor cultivation of shiitake by the bedlog method may reduce the extent of genetic variation in natural populations. Circumstantial evidence



FIGURES 1 & 2. Scanning electron micrographs of gill surfaces of a wild strain and the sporeless mutant of *Lentinus edodes*. 1. Wild type. Various stages of sporulation are shown. 2. Sporeless mutant. Arrows point to autolyzed basidia bearing remnants of sterigmata.

for this problem is provided by the observation that the number of alleles at both A and B incompatibility loci is significantly lower when calculated using data from western Japan (A = 40-65, B = 63-100) (Tokimoto *et al.*, 1973) compared to values based on data collected in Hokkaido (A = 130-152, B = 192-205). Traditionally, there have been many large-scale shiitake farms in western Japan, but only a few in Hokkaido. We speculate that genic variation of *L. edodes* has been significantly reduced by the spores discharged from major cultivars. Use of sporeless cultivars would, therefore, be effective in reducing these problems.

3. BREEDING SPORE-DEFICIENT STRAINS OF *AGROCYBE CYLINDRACEA*

This breeding programme is aimed primarily at reducing problems (a) and (b) referred to in the Introduction, because this mushroom produces numerous brown-pigmented basidiospores.

3.1. Induction of Spore-deficient Mutants

Dikaryotic hyphal fragments from a strain (2493) were irradiated with a Toshiba 10-watt

TABLE 1. Genetic analysis of the basidiospore progeny derived from the cross between Sp1 (genotype: *spo A1 B1*), one of the component homokaryons of the sporeless mutant, and the wild-type homokaryon #9 (genotype: + *A2 B2*) of *Lentinus edodes.*

Mating type	Sporeless	Wild type	Total
A1B1	20	19	39
A2B2	36	26	62
A1B2	19	19	38
A2B1	17	17	34
A1Brec	6	4	10
A2Brec	2	8	10
Total	100	93	193

Analysis:

					X^2 (1:1)	P
Segregation of						
Sporeless	*spo*	100	+	93	0.25	0.750 - 0.500
A-factor	*A1*	87	*A2*	106	1.87	0.250 - 0.100
B-factor	*B1*	73	*B2*	100	4.21	0.050 - 0.025
Recombination between						
		Parental		Recomb.		
spo and *A*		96		97	0.01	- 0.900
spo and *B*		82		91	0.47	0.500 - 0.250
A and *B*		101		72	4.86	0.050 - 0.025
B-factor*		173		20	121.29	0.005 -

* Recombination value = 10.4%.

germicidal lamp for 30-40 sec at a distance of 10cm. The irradiated fragments were then plated on CY-2 medium containing 20g glucose, 0.5g $MgSO_4 \cdot 7H_2O$, 0.46g KH_2PO_4, 1g K_2HPO_4, 2g polypeptone, 2g yeast extract and 20g agar per litre of water, and incubated at 25°C. The UV treatment and the incubation were performed in darkness. Lethal rate was more than 95%. Viable colonies were then isolated and transferred on to fresh CY-2 medium. A few days later, the recovered colonies were examined for clamp connections. The isolates obtained were inoculated on slants of CY-2 medium and placed under conditions suitable for fruiting. The fruitbodies produced were examined for production of basidiospores. This fungus produced fruitbodies on CY-2 medium; the fruitbodies were very small but otherwise normal in their morphology and spore productivity. However, since germination of basidiospores did not occur on CY-2 medium, PSA medium (extract of 200gm fresh pototoes, 20 gm sucrose and 20gm agar per liter of water) was used for germination tests. Final assessment of the morphology of fruitbodies, cultural characteristics and deficiency of spore formation were done using plastic bottles filled with sawdust medium (400g of air-dried sawdust wetted evenly with a liter of tap water, containing 80gm of rice bran). From 11,500 colonies treated with UV irradiation, 24 spore-deficient mutants and 233 strains with reduced sporulation were detected. Imbernon & Labarere (1989) reported that UV treated strains of *Pleurotus ostreatus* and *P. pulmonarius* exhibited some abnormality in morphology and yields of fruitbodies. In our experiment, pilei of the spore-deficient mutants were either very thin at the margin or small in size, or their stipes were slender and became bent.

3.2. Strain Improvement

In several fungi, di-mon mating is useful for introducing one nucleus of a dikaryon into another homokaryon. However, in *A. cylindracea*, this method is not useful because of lack or delay of the nuclear migration through the homokaryotic mycelia. The sporeless mutants obtained produced very few basidiospores, which did not germinate well (< 2%). About a thousand basidiospore progenies gathered from all these mutants were mated with four wild tester homokaryons having different mating types derived from the strain 2943. Resultant dikaryons were then grown on sawdust medium and examined for spore deficiency. It was found that 153 progenies carried the spore deficient characters, indicating that these spore deficiencies were dominant characters. From the result obtained, several spore-deficient homokaryons which had the ability to produce normal fruitbodies were selected.

3.3. Hybrid Construction

To change the colour of fruitbodies according to the consumer's preference is one of the important aims for breeders. When the basidiospore progeny derived from a strain 2944 was crossed with the homokaryon from strain 2943, the fruitbodies produced from the resultant dikaryons were divided into two types: yellowish or brownish, at the ratio of 1:1 (unpublished data). The selected homokaryons from strain 2944 were then mated with previously selected spore-deficient homokaryons. Fruitbodies of resultant dikaryons were examined for their yield and morphology. Figure 3 shows the results obtained with selected strains (#43 and #44) and the control strain 2943. There were no significant differences between the three strains in their fruitbody yields.

Spore-deficient strains are now available for *A. cylindracea* and matings between spore-deficient progeny and homokaryons derived from a wild-type strain have improved cultural characteristics.

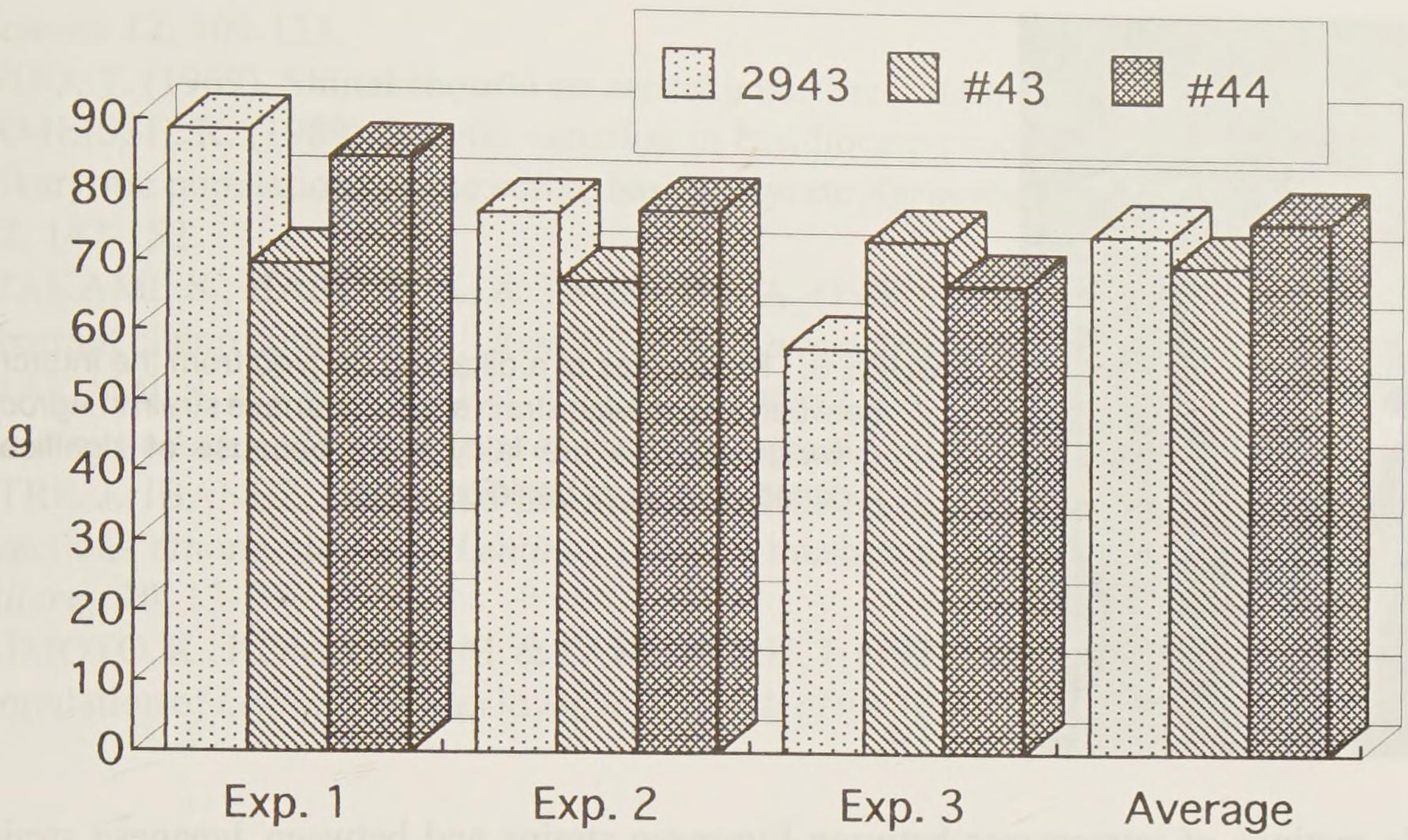
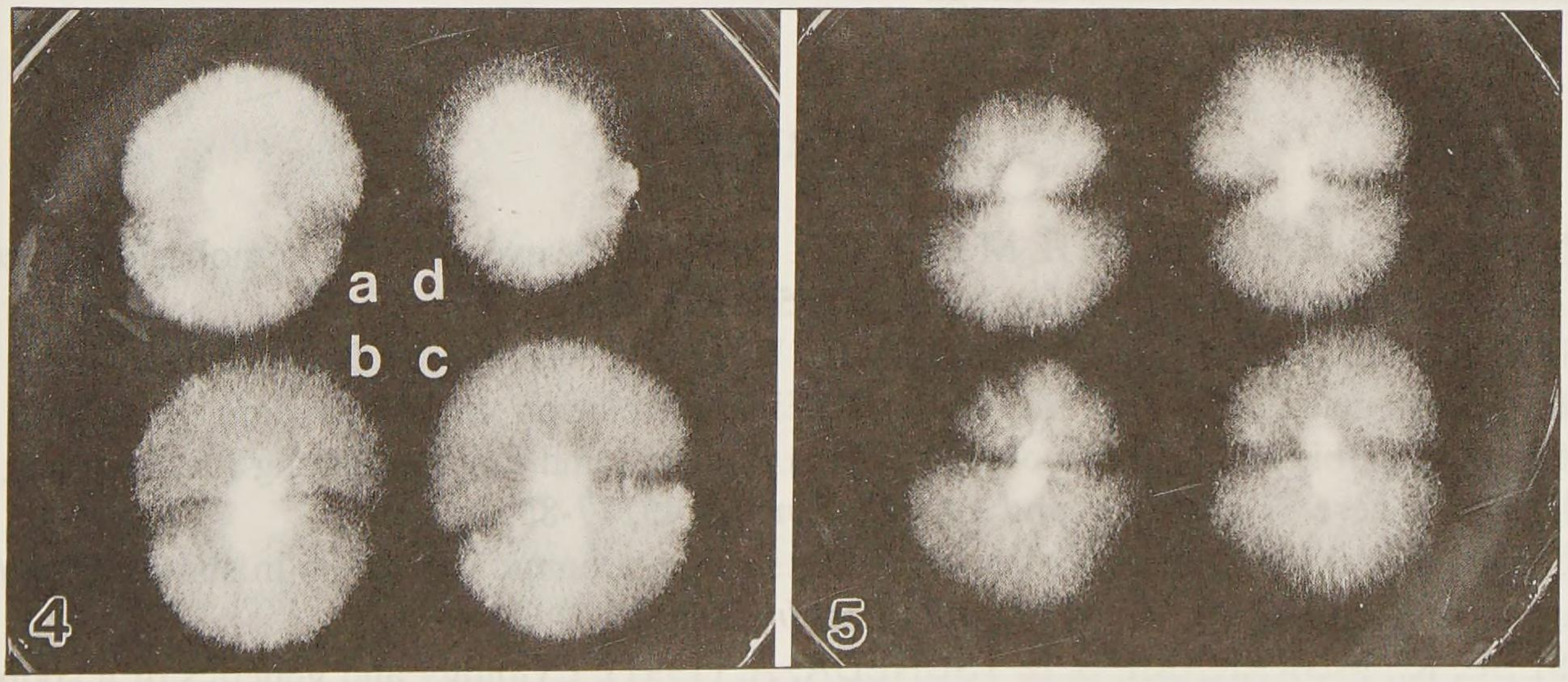


FIGURE 3. Fruitbody yield of selected strains (#43, #44) and control strain (2943) in *Agrocybe cylindracea*. The Y axis indicates mean fresh weight per 800ml bottle. Eight duplicates for respective strain in each yield experiment.

3.4. Spore Deficiency Resulting from Matings between Geographically Separated Strains

Marmisse (1989) examined the genetic variation in basidiocarp production within several strains of *A. aegerita* (= *A. cylindraea*). We also carried out similar experiments using several European and Japanese strains. During this study, we found curious and interesting phenomena. We confirmed the bifactorial incompatibility system in this mushroom (Esser *et al.*, 1974). As shown in Fig. 4, fully compatible matings were visibly distinguishable from other non-compatible matings by the denser and white, but relatively smaller, dikaryotic colony formed in the contact zone.



FIGURES 4 & 5. Mating reactions in *Agrocybe cylindracea*. 4. Intrastrain crosses. a: common-AB, b: common-A, c: common-B, d: compatible. 5. Compatible mating reactions in the interstrain crosses between European and Japanese strains.

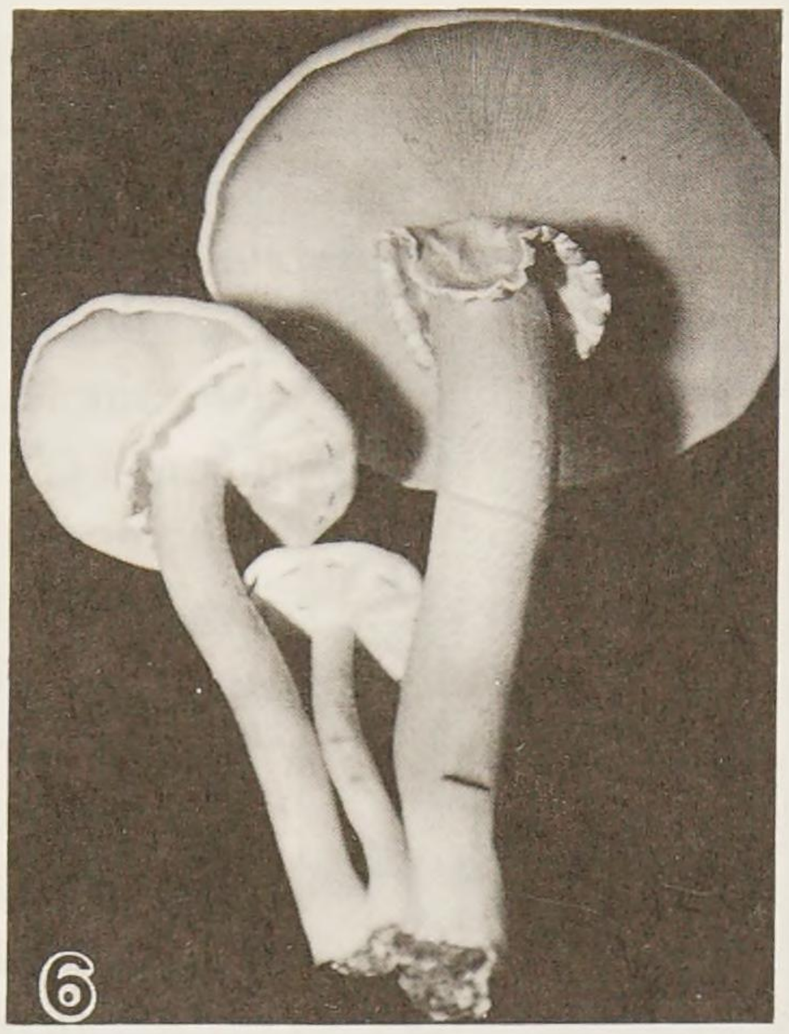


FIGURE 6. Fruitbodies of a dikaryon derived from the intercross between a European strain and a Japanese strain in *Agrocybe cylindracea*. Gills are fairly white because of significantly reduced number of basidiospores.

Compatible matings of intracrosses between European strains and between Japanese strains all showed this response. However, in all intercrosses between European and Japanese strains, the compatible mating reaction was very different. In such crosses, barrage like reaction, characterized by a contact zone of scanty mycelium bordered by somewhat more congested hyphae, was always observed (Fig. 5). Clamped dikaryotic hyphae were not observed in the margin of these colonies. When the central regions of the contact zone were cut and reinoculated on to fresh medium, most of the inocula produced dikaryotic hyphae.

Most of the resultant dikaryons readily produced fruitbodies of normal morphology but with significantly reduced numbers of basidiospores. The degree of spore production of fruitbodies varied with the combination of homokaryons. As shown in Fig. 6, gills of some dikaryotic strains were white because of much reduced spore production. The germination frequency of the basidiospores derived from all of these basidiocarps was relatively high, but most of the germ tubes soon ceased to grow. Thus less than 4% of the germinated spores formed colonies. The reduction in compatibility, spore production and viability in the basidiospore progeny stated above, may have an important implications for microevolution in fungi.

REFERENCES

DUNCAN, E.G. & GALBRAITH, M.H. (1972). Post-meiotic events in the homobasidiomycetes. *Transactions of British Mycological Society* **58**, 387-392.

ESSER, K., SEMERDZIEVA, M. & STAHL, U. (1974). Genetische Untersuchungen an dem Basidiomyceten *Agrocybe aegerita*. 1. EineKorrelation zwischen dem Zeitpunkt der Fruchtkorperbildung und monokaryotischem Fruchten und ihre Bedeutung fur Zuchtung und Morphogenese. *Theoretical and Applied Genetics* **45**, 77-85.

HASEBE, K. (1991). Genetic studies on mutants and agronomic characters in shiitake, *Lentinus edodes*. *Reports of the Tottori Mycological Institute* **29**, 1-69.

HASEBE, K., MURAKAMI, S. & TSUNEDA, A. (1991). Cytology and genetics of a sporeless mutant of *Lentinus edodes*. *Mycologia* **83**, 354-359.

IMBERNON, M. & LABARERE, J. (1989). Selection of sporeless or poorly spored induced mutants from *Pleurotus ostreatus* and *Pleurotus pulmonarious* and selective breeding. *Mushroom*

Science **12**, 109-123.
KONDO, T. (1969). Shiitakehoushi no zensoku no. 1 rei. *Allergy* **18**, 81-123.
MARMEISSE, R. (1989) Genetic variation in basidiocarp production within wild and controlled dikaryotic populations of the edible basidiomycete *Agrocybe aegerita*. *Mycological Research* **92**, 147-152.
MURAKAMI, S., HASEBE, K. & TSUNEDA, A. (1987). A mutant of *Lentinus edodes* forming aberrant clamp connections. *Reports of the Tottori Mycological Institute* **25**, 49-55.
MURAKAMI, S. & TAKEMARU, T. (1985). Nuclear behaviour during basidiospore formation in *Lentinus edodes*. *Transactions of the Mycological Society of Japan* **26**, 253-260.
SASTRE, J., IBANEZ, M.D., LOPEZ, M. & LEHRER, S.B. (1990). Respiratory andimmunological reactions among shiitake (*Lentinus edodes*) mushroom workers. *Clinical and Experimental Allergy* **20**, 13-20.
TOKIMOTO, K., KOMATSU, M. & TAKEMARU, T. (1973). Incompatibility factors in the natural population of *Lentinus edodes* in Japan. *Reports of the Tottori Mycological Institute* **10**, 371-376.

Part II

MUSHROOM CULTIVATION AND BIOCONVERSION TECHNOLOGY

CHAPTER 8

BIOLOGY AND CULTIVATION TECHNOLOGY OF *VOLVARIELLA VOLVACEA*

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1. INTRODUCTION

Although more than 100 species, subspecies and varieties of *Volvariella* have been described throughout the world (Shaffer, 1957), four species, including *V. esculenta* Massee, *V.bakeri* Dennis, *V. displasia* Singer and *V. volvacea* Singer, are reported to be cultivated in Asia and Africa. The most cultivated forms of *Volvariella* in Southeast Asia are probably members of the species *V. volvacea* (Bull. ex Fr.) Sing. *V. volvacea*, commonly known as the straw mushroom, belongs to the family Pluteaceae Kotl. & Pouz. of the Basidiomycetes. It is an edible mushroom of the tropics and subtropics. The cultivation of this fungus can be traced back to 1822 in China (Chang, 1977). It was introduced into the Philippines, Malaysia and other Southeast Asian countries about 1932-35 (Baker, 1934; Benemerito, 1936). The importance of this mushroom is reflected by the steady increase in world production every year (Fig. 1). Among the countries cultivating *V. volvacea*, China is the top producer, followed by Thailand, Indonesia and Vietnam (Table 1).

V. volvacea is also referred to as a "warm mushroom" because it grows at the relatively high temperature range of 30-36°C. The high temperature requirement for cultivation makes it suitable for South Asian countries where it is especially important as a source of protein to the rural populace. However, the inability to enhance its post-harvest life by ordinary refrigeration at 5°C, a temperature at which it autolyzes, is one of the major setbacks to big-scale commercial exploitation except where facilities for a superb marketing network exist.

The natural growth substrate of *V. volvacea* is paddy straw. With rice being the major crop in Southeast Asian countries, the source of substrate for *Volvariella* cultivation is not restricted. Other plant cellulosic residues, including cotton waste, bagasse, banana leaves, oil palm pericarp, sorghum straw and fibre crop wastes, can also be used for growing *V. volvacea*. Growth of this fungus from spawning to harvesting can be completed within 10 days. It is considered to be one of the easiest mushrooms to cultivate, with a short cropping period of 14 days, yet it is also the most difficult where it comes to obtaining high, consistent yields.

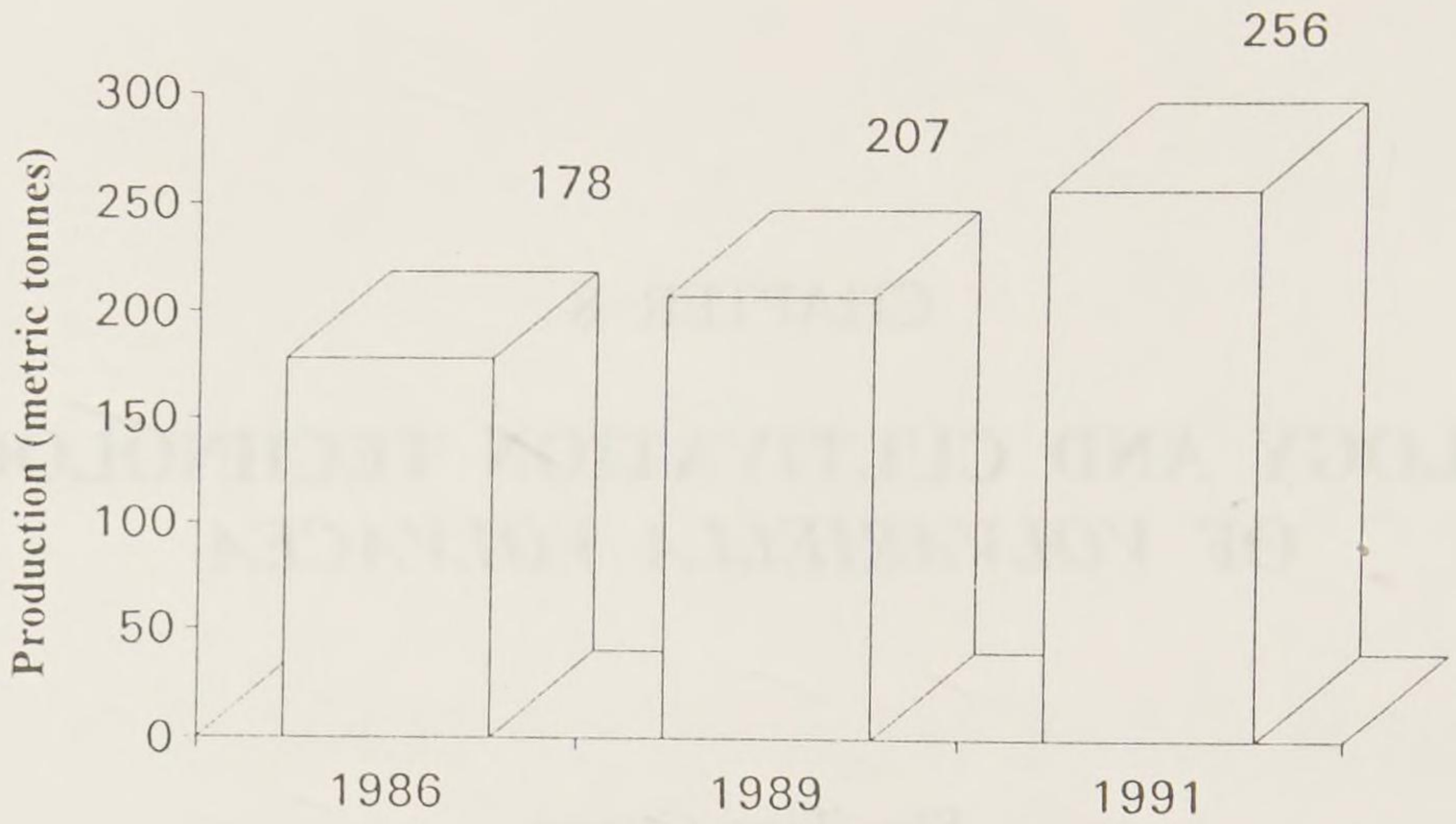

FIGURE 1. Comparison of world production of *V. volvacea* in 1986, 1989 and 1991.

2. PHYSIOLOGICAL NATURE

It is impossible to obtain good crops without good cultures. To obtain and maintain good cultures, an understanding of the biological nature of the mushroom is important. It is clear that mushrooms are fungi which are characterized by their heterotrophic mode of nutrition. Digestive enzymes are liberated from the fungal cells into the immediate environment where organic matter is broken down into simpler molecules which in turn pass into the fungal cells.

The diversity of substrates on which *V. volvacea* grows and its wide distribution may indicate a physiological and nutritional adaptibility within the species. Such attributes are also shown by other cosmopolitan fungi. In terms of nitrogen source, the lignocellulosic materials on which *V. volvacea* is cultivated are usually low in nitrogen. Various studies have shown that *V. volvacea* frequently responds better to organic than inorganic nitrogen. Ofosu-Asiedu *et al.*, (1984) reported that the best yields were obtained on media containing peptone or potassium nitrate. Urea, glutamic acid and

TABLE 1. World production of *V. volvacea* in 1991 (fresh weight, metric tonnes).

Country	Production	%
China	150,000	58.6
Thailand	63,000	24.6
Indonesia	35,000	13.7
Vietnam	3,500	1.4
Taiwan	3,000	1.2
Philippines	800	0.3
India	400	0.2
Others	400	0.2
	256,100	100.0

TABLE 2. Growth of four isolates of *V. volvacea* on different nitrogen sources (maximum mg mycelial dry weight per culture).

Nitrogen source	Isolate			
	V1	42	130	132
Control	32*	36	37	31
$(NH_4)_2SO_4$	55	49	71	46
KNO_3	250	202	253	166
Urea	186	177	157	138
Glutamic acid	156	175	186	198
Asparagine	230	142	191	200
Peptone	193	294	261	229

Source: Ofosu-Asiedu *et al.,* (1984).

* Values presented are biomass harvested on the modified complete medium without nitrogen source (Raper & Miles, 1958) included separately with different nitrogen compounds.

asparagine were also good nitrogen sources. Ammonium sulphate was a poor source (Table 2). At low levels of nitrogen, however, the response to organic and nitrate nitrogen is similar to that reported by Chang-Ho & Ho (1979).

In terms of carbon source, the results obtained by Ofosu-Asiedu *et al.,* (1984) show that *V. volvacea* utilizes starch and glucose better than other carbon sources. None of the isolates tested could grow on lignin (Table 3). On the other hand, Chakravarty & Mallick (1979) obtained considerably more growth with glucose than with starch. Although good growth of *V. volvacea* can be observed on such simpler carbohydrates, the monomeric sugars and starch are available in limited amount in the natural substrates of *V. volvacea*. In general, the different isolates of *V. volvacea* show similar physiological behaviour although some differences have been observed. The discrepancies between two subcultures, V1 and V2, which were derived from the same isolate, may be attributed to the different culture methods used in the laboratories of origin. It should be noted that the growth of mushrooms is based on the hydrolysis of polymeric lignocellulose. That is, mushrooms can produce a range of extracellular hydrolytic and oxidative enzymes including lignocellulolytic and oxidative enzymes. The primary function of hydrolytic enzymes is to break down the macromolecular polymer into more-readily assimilated low molecular weight carbohydrates. For example, the cellulolytic and hemicellulolytic enzymes produced by mushrooms can degrade respectively the polysaccharide components into hexoses (e.g., glucose) and pentoses (e.g., xylose). The simpler sugars resulting from enzymic hydrolysis support the growth of mushrooms. Glucose, which is the basic unit of the cellulose component in the lignocellulosic substrate, was the most favourable carbon source for the growth of *V. volvacea* (Table 4). Xylose also served as a carbon source although to a lesser extent. Therefore, the ability of the mushroom to produce cellulose- and hemicellulose-degrading enzymes is an important factor for fungal growth.

Lignocellulosic substrates that are commonly used in mushroom cultivation include cellulose, hemicellulose and lignin. The proportion of each component varies with different lignocellulosic materials from nearly zero lignin and high cellulose substrate (e.g., cotton waste) to high lignin content substrate (e.g., wood residues). For example, the lignocellulose in paddy straw consists of 36% cellulose, 18% hemicellulose and 8.5% lignin (Dale, 1987). The cellulose and hemicellulose

TABLE 3. Growth of four isolates of *V. volvacea* on different carbon sources (maximum mg mycelial dry weight per culture).

Carbon source	Isolate			
	V1	42	130	132
Control	16	18	15	15
Glucose	160	241	197	137
Galactose	30	33	35	33
Xylose	28	29	27	29
Sucrose	32	36	32	32
Cellulose	47	63	54	52
Xylan	43	59	64	50
Lignin	14	18	16	15
Starch	146	274	343	162

Source: Ofosu-Asiedu *et al.*, (1984). As indicated in Table 2, the modified complete medium without carbohydrates.

components of the plant cell wall are intimately associated with the lignin moiety. The cellulose component is generally enveloped by a lignin-hemicellulose matrix (Kirk, 1985). The heterogeneity and complexity of lignocellulosic materials impart a very stable resistance to microbial attack. For those species possessing ligninolytic ability, such as many white-rot basidiomycetes, the lignin-

TABLE 4. Growth of *V. volvacea* on different reducing sugars and short chain polysaccharides as carbon source.

Carbon source	Growth (%)*
arabinose	34(9)
cellobiose	63(0)
fructose	96(4)
galactose	35(12)
glucose	100
lactose	15(8)
maltose	90(4)
mannose	94(7)
raffinose	72(8)
saccharose	88(7)
xylose	34(26)
soluble starch	88(7)

(Cai, Buswell & Chang, unpublished data)

*Values are expressed as a percentage of the growth recorded over the growth on glucose and represent the mean of three replicates. Figures in parentheses refer to the percentage variation about the mean. The growth rate of *V. volvacea* and the values presented are those recorded after 4 days of incubation. In all cases, the density of fungal colonies was similar when the values shown were taken.

hemicellulose matrix will not be a barrier to fungal growth since the lignin component would be degraded by lignin-transforming enzymes produced by such fungi (e.g., *Pleurotus sajor-caju* and *Lentinus edodes*). Degradation products (e.g., simpler phenolic compounds) would be further modified by a phenol-oxidizing system. However, for those species lacking ligninolytic activity, the lignified complex of lignocellulose would restrict the access of mushroom hyphae to the cellulose component. Consequently, the ability of a mushroom to produce oxidative enzymes, including phenol oxidases and lignin-transforming enzymes, are also important to fungal growth when the fungus is cultured on lignocellulosic substrate.

When cultured on different lignocellulosic substrates, including paddy straw and cellulose, *V. volvacea* exhibited a general inability to synthesize phenol-oxidizing and lignin-transforming enzymes. However, the mushroom produces several of the enzymes necessary for the hydrolysis of cellulose and hemicellulose. These include endo- and exogluconases, β-glucosidase, xylanase and β-xylosidase (Cai *et al*., to be published). In this respect, *V. volvacea* behaves like a brown-rot fungus. Brown-rot fungi have been defined as being able to degrade cellulose and hemicelluloses, leaving the lignin more or less intact as a brown layer (Deacon, 1980).

Lignin-related components are known to repress fungal growth and the activity of enzymes hydrolyzing cellulose and hemicellulose. In the work of Cai *et al.,* (1993), several lignin-related phenolic monomers and tannin derivatives were reported to inhibit the growth of *V. volvacea* (Tables 5 & 6). Recently, the activity of cellulose-degrading enzymes produced by *V. volvacea* was also found to be inhibited by lignin-related and tannin phenolic compounds (Cai, Buswell & Chang, unpublished data). Tannin was also reported to inhibit the growth of *V. volvacea* by Yu and Chang (1989).

Recent results suggest that the growth of *V. volvacea* is more sensitive to lignified substrates because of the lack of ability to produce phenol-oxidizing and lignin-transforming enzymes. Inhibition of polysaccharide-degrading enzymes (e.g., cellulase) by phenolic compounds present in the straw might explain why the formation of mushrooms on paddy straw is limited, giving a low biological efficiency. The means to produce simpler sugars for growth might be inhibited by the phenolic compounds existing in the straw. It has been shown that cellulase production in cultures of *V. volvacea* grown on rice straw was lower than cultures grown on pure cellulose (Cai, Buswell & Chang, unpublished data). In general results suggest that when *V. volvacea* was cultured on a rich cellulosic substrate such as cotton waste, in which the amount of lignified components is negligible, the cellulose-degrading activity of the fungus was not inhibited and fungal growth was less disturbed. As a result, production yields were increased. It has been shown in the case of some fungi that the production of extracellular enzymes involved in lignocellulose degradation is closely coupled to fungal growth and colonization as well as to the formation of the sporophore (fruiting body).

According to preliminary research results, *V. volvacea* can also tolerate sodium chloride. There are numerous plants that grow in saline and alkaline soils and they are widely distributed throughout the world, especially along seashores and in saline areas in various continents. Many of them are known as "salt bushes" and "salt grasses" and could perhaps be used in the preparation of composted substrate for the growth of *V. volvacea*.

Several fatty acids and sunflower oil were found to enhance the fungal growth of *V. volvacea* (Li *et al*., 1992). More recently, a complex of fatty acids was found to increase the production of cellulases and hemicellulases in *V. volvacea* cultures (Cai *et al*., to be published).

TABLE 5. Effect of lignin-related phenolic compounds on the growth of *V. volvacea*.

Phenolic compound	Concentration (mM)	Growth (%)*
4-hydroxybenzoic acid	1	80(3)
	2	76(7)
	5	68(3)
	10	45(3)
Vanillic acid	1	109(2)
	2	115(2)
	5	97(3)
	10	81(5)
Syringic acid	1	92(3)
	2	93(3)
	5	97(3)
	10	94(2)
p-Coumaric acid	1	83(6)
	2	78(3)
	5	53(8)
	10	32(7)
Caffeic acid	1	107(3)
	2	103(4)
	5	94(2)
	10	73(3)
Ferulic acid	1	92(3)
	2	74(9)
	5	45(4)
	10	23(16)
p-Hydroxybenzaldehyde	1	69(1)
	2	48(11)
	5	0
Vanillin	1	34(16)
	2	0

Source: Cai *et al.*, (1993).

* Values are expressed as a percentage of the growth recorded in the absence of lignin-related phenolic compounds and represent the mean of three replicates. Figures in parentheses refer to the percentage variation about the mean. The growth rate of *V. volvacea* and the values presented are those recorded after 42 hours of incubation. In all cases, fungal growth rates were linear with time when the values shown were taken.

3. PROSPECTS FOR GENETIC MANIPULATION IN THE BREEDING OF *VOLVARIELLA* MUSHROOMS

V. volvacea is regarded as a homothallic mushroom species (Chang & Yau, 1971). Each basidiospore is uninucleate when first formed but it gives rise to a multinucleate mycelium. There is no dikaryotic phase, and no clamp connections have been observed. Karyogamy and meiosis take

TABLE 6. Effect of tannin derivatives on the growth of *V. volvacea*.

Phenolic compound	Concentration (%,v.v)	Growth (%)*
Gallic acid	0.01	93(5)
	0.02	88(3)
	0.05	78(6)
	0.10	68(3)
	0.15	64(5)
Tannic acid	0.01	91(6)
	0.02	74(16)
	0.05	20(29)
	0.10	0
Catechin	0.01	90(3)
	0.02	102(7)
	0.05	90(5)
	0.10	77(13)
	0.15	69(13)

Source: Cai *et al.*, (1993).

* Values are expressed as a percentage of the growth recorded in the absence of the tannin derivative and represent the mean of three replicates. Figures in parentheses refer to the percentage variation about the mean. The growth rate and the values presented are those recorded after 42 hours of incubation. In all cases, fungal growth rates were linear with time when the values shown were taken.

place in the basidium and four basidiospores are produced. With reference to that, some single spores can give rise to fruit bodies. The technique of single spore selection might still be the best way to maintain good strains of *V. volvacea*; yet the great variation of many characteristics found among the single spores of one progeny cannot be properly explained. It is hoped that much will soon be clarified since this is a great handicap for the breeding work and strain improvement of the straw mushroom.

The cellulase complex appears to be involved in the utilization of the polymeric substrate for fungal growth and is probably related to the further production of fruit bodies. Therefore, increasing the ability of *V. volvacea* to produce cellulase would be a potential target in strain improvement. Wong *et al.*, (1988) reported that two *Cellulomonas fini* cellulase genes, *cenA* (encoding an endoglucanase) and *cex* (encoding an exoglucanase) have been cloned and transformed into *Saccharomyces cerevisiae* through plasmids. This displays the possibility of using recombinant DNA techniques to engineer co-expression of cellulases. By such molecular biological techniques it would be possible, through enforcing the ability of *V. volvacea* to produce substrate-utilizing enzymes, or introducing the genes encoding lignin- and phenol- transforming enzymes, to obtain improved strain(s) of *V. volvacea* with adaptation to a wider range of substrates and with higher production yields.

Obtaining fusion products between *V. volvacea* and other species by protoplast fusion demonstrates another possibility for achieving the improvement of *Volvariella* strains (Zhao & Chang, unpublished data). This approach of hybridization may open a new channel for breeding programmes.

4. CULTIVATION

The traditional method of cultivating *Volvariella* mushrooms in Southeast Asia is by straw beds in the open field or, more recently, by the use of wooden frames. Although this is an inexpensive method, it is subject to pests and diseases and to the vagaries of nature as environmental conditions for fruiting are difficult to control. The most recent development of *Volvariella* culture in Southeast Asia is indoor cultivation utilizing mushroom houses under a controlled environment. Pasteurization and heating facilities are incorporated. The use of cotton waste in place of rice straw as substrate resulted in a significant increase (two to three fold) of the biological efficiency (B.E. = fresh weight of harvest mushroom over the air dried weight of substrate X 100 expressed as percentage) and more stable production yield. This technique proved to be a turning point in the history of straw mushroom cultivation (Chang, 1974). Although the B.E. of this species was significantly increased when the solid-state culture was grown on cotton waste in place of paddy straw, it is still lower than other cultivated mushroom species (Table 7).

Several techniques have been adopted for the cultivation of *V. volvacea*, which thrives in the temperature range of 28-36°C and a relative humidity of 75-85%. Detailed descriptions of the various methods are given in the Food and Agriculture Organization Plant Production and Protection Paper (FAO) No.106: "Technical Guidelines for Mushroom Growing in the Topics" by Quimio *et al.*, (1990) and will not be repeated here. Choice of technologies usually depends on personal preference and on the availability of substrates and the amount of resources available. While the more sophisticated indoor technique is recommended for the industrial-scale production of this mushroom, most of the other techniques are low-cost and appropriate for rural area development, especially when production is established at the community level.

Four examples of regional variations in cultivation conditions used to grow the straw mushroom are given below:

Mexico: *V. volvacea* culture is grown at 32-38°C on beds of fermented and pasteurized barley straw

TABLE 7. Approximate biological efficiency of edible mushrooms.

Species	Substrate	B.E. (%)
Lentinus edodes	Wood logs (outdoors,sometimes protected)	40
	Sterilized sawdust (polypropylene bags)	60-100
Auricularia spp.	Wood logs (outdoors, sometimes protected)	2-12
	Sterilized sawdust (polypropylene bags)	70-85
Tremella fuciformis	Wood logs (outdoors)	20
	Sterilized cotton-seed hull (polypropylene bags)	100-160
Pleurotus spp.	Pasteurized cereal straw (indoor)	80-100 or more
Agaricus bisporus	Composted cereal straw/animal manure mixtures	65-80
Volvariella volvacea	Straw (outdoor)	6-10
	Composted cotton waste	30-45
Flammulina velutipes	Sterilized sawdust (polypropylene bags)	70-100

Source: Smith *et al.*, (1988); Chang & Miles, (1989).

and cotton waste.

Thailand: *V. volvacea* is cultivated over a temperature range of 25-38°C in beds of pasteurized straw, cotton waste or kapok waste.

Indonesia: *V. volvacea* grows in the 30-35°C region. In 360 growing sheds on 3 locations. The mushroom company P.T. Dieng Djaya is producing approximately 40 tonnes per day on pasteurized straw and cotton waste as a growing medium (Vedder, 1991). In rural areas, the rice straw is still used as a traditional substrate for the cultivation of the mushroom. The main consideration is that the bed have an optimum height in order that sufficient heat, which is conductive to mycelial growth, can be developed.

China: *V. volvacea* has been traditionally cultivated in southern provinces (e.g., Guangdong, Guangxi and Fujian). In recent years, this mushroom has also been grown in other parts of the country during the summer months. In Nanhui (Shanghai), *V. volvacea* is cultivated in early summer. The major substrate is cotton waste, a byproduct of the textile factories, and the resulting B.E. was reported as 34-38%. In Qing Yuan County (Hebei Province), the cultivation technique using plastic shelter trenches (hut trenches) on a compost mixture consisting of spent corn cobs, wheat straw and cotton-seed hulls gave a B.E. of 28-29%. In Jiangxi, the pretreatment of paddy straw by water washing under pressure in a machine resulted in an increase of B.E. up to 20-30% from 6-10%.

5. UTILIZATION OF WASTE TEA-LEAVES IN THE PREPARATION OF SPAWN

Chang (1982) and Bisht and Harsh (1984) reported that waste tea-leaves, unavoidable waste materials after decoction of tea in both restaurants and households, can be used with good effect in straw mushroom spawn production when mixed with wheat grains or cotton waste. The selected cotton waste contains short fibre material as well as cotton-seed hulls. A mixture of cotton waste / wheat grain and used tea-leaves in equal ratio has been found suitable for the production of the spawn. The mushroom mycelium can grow both inside and outside the waste tea-leaves which also discourages the development of competing molds under the high temperature and humidity conditions in the mushroom beds. The spread of spawn (spawn running) on mushroom beds was also good and there was no difference in the yield by utilizing this spawn in comparison with chopped straw or grain spawns. A spawning rate of 2% (spawn to substrate, w/w) is sufficient.

6. GENERAL REMARKS

Volvariella volvacea is an edible mushroom of the tropics and subtropics. In traditional cultivation it grows on paddy straw in subtropical and tropical climates. In recent years, it has been cultivated on cotton wastes and cotton-seed hulls which contain a higher proportion of the cellulose component than does paddy straw, and it can be cultivated in temperate climates in summer months. Since *V. volvacea* lacks the capability of producing phenoloxidizing and lignin-transforming enzymes, it is difficult to obtain a reasonably high yield if the substrate contains a high lignin component, as is the case with wood chips and sawdust. On the other hand, if the compost contains a higher proportion of cellulose, as in cotton wastes, and growth is under optimal conditions, a reasonably high and stable yield can be achieved. It has been noted in a review of the literature that untreated paddy straw usually gives low biological conversion rates, in the range of 5-15%. It is recommended that cotton waste/cotton-seed hulls should be mixed with paddy straw or bagasse for

composting for growth of *V. volvacea*. Before spawning, the compost should be pasteurized, or the compost should be presoaked in 2% $CaCO_3$ overnight in order to inhibit or eliminate harmful molds. After spawning, the bed should be covered with plastic sheets for the first 3-4 days in order to create microclimatic conditions in the bed. This exercise can provide conditions of higher temperature, higher moisture and higher concentrations of CO_2 that promote mycelial running in the compost. After removal of the plastic sheets, the bed should be gently sprinkled with water in order to lower the temperature of the bed. In the meantime, the culture room or the surroundings of the bed should be provided with more fresh air and adequate light. It is suggested that these environmental factors can stimulate fructification. Furthermore, some reports suggest that the bed should be covered with a thin layer of casing soil.

Since *V. volvacea* has been regarded as a homothallic species, selection for desirable strains by single spore isolation may still be preferable. However, using protoplast fusion techniques, interspecific somatic hybrids, such as between *V. volvacea* and *V. esculenta* and between *V. volvacea* and *V. bombycina,* may be obtained. This approach to hybridization may open a new channel for breeding programmes. In practice, *V. volvacea* normally produces only one flush per crop (one compost duration). This is quite different from other mushrooms, e.g., *Agaricus bisporus* and *Pleurotus sajor-caju*, which can have more than 4 flushes in each crop. This is an important issue in *V. volvacea* cultivation. Researchers should consider both physiological and nutritive conditions as well as genetic and breeding aspects.

Lack of access to good quality spawn represents a major problem for mushroom growers in developing countries. Quality controls may be non-existent and there is no "in-house" testing of spawn by spawn producers to ensure the character and conditions of their product. Spawn cultures are normally obtained from outside the region concerned. The spawn may be well-suited to climatic conditions and the cultivation procedures adopted in the source regions, but little attention may be given to the particular requirements of local conditions. Furthermore, should the spawn prove defective or inappropriate, the grower is left to carry the burden of an unsuccessful harvest.

The absence of systematic government support for, and promotion of, mushroom growers appears to be responsible in part for the failure of some countries to sustain a viable mushroom cultivation programme. In countries with thriving mushroom cultivation industries, a coordinated government policy allots production quotas for each grower and provides price guarantees for those quotas, thereby reducing some of the financial uncertainly associated with the enterprise. In countries where no such coordinated government involvement exists potential growers are dissuaded from entering into the business.

REFERENCES

BAKER, J.A. (1934). Mushrooms growing in Wellesley and Penang Provinces. *Malaysian Agricultural Journal* **22**, 25-28.

BENEMERITO, A.N. (1936). Mushroom culture in Canton. *Philippine Agriculture* **24**, 624-34.

BISHT, N.S. & HARSH, N.S.K. (1984). Utilization of waste tea-leave - preparation of spawn of *Agaricus* and *Volvariella volvacea*. *Mushroom Journal* **139**, 231-233.

CAI, Y.J., BUSWELL, J.A. & CHANG, S.T. (1993). Effect of lignin-related phenolic monomers and tannin derivatives on the growth of the edible mushrooms *Lentinus edodes, Pleurotus sajor-caju* and *Volvariella volvacea*. *World Journal of Microbiology and Biotechnology* (in press).

CHAKRAVARTY, D.K. & MALLICK, A.K. (1979). Studies on the physiology of mushroom

fungi, *Volvariella displasia* and *V. esculenta*. *Indian Journal of Mycology and Plant Pathology* **9**, 9-12.

CHANG, S.T. (1974). Production of the straw mushroom (*Volvariella volvacea*) from cotton wastes. *Mushroom Journal* **21**, 348-53.

CHANG, S.T. (1977). The origin and early development of straw mushroom cultivation. *Economic Botany* **31**, 374-76.

CHANG, S.T. (1982). Mushroom spawn. In *Tropical Mushrooms - Biological Nature and Cultivation Methods*, pp.31-46. Edited by S.T. Chang, & T.H. Quimio. Hong Kong: The Chinese University Press.

CHANG, S.T. & MILES, P.G. (1989). *Edible Mushrooms and Their Cultivation*. Boca Raton, Florida: CRC Press, Inc.

CHANG, S.T. & YAU, C.K. (1971). *Volvariella volvacea* and its life history. *American Journal of Botany* **58**, 552-61.

CHANG-HO, Y., & HO, T.M. (1979). Effect of nitrogen amendment on the growth of *Volvariella volvacea*. *Mushroom Science* **10**, 619-28.

DALE, B.E. (1987). Lignocellulose conversion and future of fermentation biotechnology. *Trends in Biotechnology* **5**, 287-291.

DEACON, J.W. (1980). *Introduction to Modern Mycology,* pp.61-62. ELBS, UK.

KIRK, T.K. (1985). The discovery and promise of lignin-degrading enzymes. In *New Horizons for Biotechnological Utilization of theForest Resource*, pp.27-42. Marcus Wallenberg Foundation Symposium.

LI, Y., CHO, K.Y., WU, Y.Z. & NAIR, N.G. (1992). The effect of lipids and temperature on the physiology and growth of *Volvariella volvacea*. *World Journal of Microbiology and Biotechnology* **8**, 621-626.

OFOSU-ASIEDU, A., SCHMIDT, O. & LIESE, W. (1984). Growth studies of *Volvariella volvacea* for cultivation on wood waste. *Material and Organism* **19**, 241-251.

QUIMIO, T.H., CHANG, S.T. & ROYSE, D.J. (1990). *Technical Guidelines for Mushroom Growing in the Tropics.* FAO, Rome.

RAPER, J.R. & MILES, P.G. (1958). The genetics of *Schizophyllum commune*. *Genetics* **43**, 530-546.

SHAFFER, R.L. (1957). *Volvariella* in North America. *Mycologia* **49**, 545-79.

SMITH, J.F., FERMOR, T.R. & ZADRAZIL, F. (1988). Pretreatment of lignocellulosics for edible fungi. In *Treatment of Lignocellulosics with White Rot Fungi*, pp.3-13. Edited by F. Zadrazil & P. Reiniger. London: Elsevier Applied Science.

VEDDER, P.J.C. (1991). The biggest mushroom farm in the world. In *Science and Cultivation of Edible Fungi*, pp.813-819. Edited by M.J. Maher. A.A. Balkema/Rotterdam/Brookfield.

WONG, W.K.R., CURRY, C., PAREKH, R.S., WAYMAN, M., DAVIES, R.W., KILBURN, D.G. & SKIPPER, N. (1988). Wood hydrolysis by *Cellulomonas fini* endoglucanase and exoglucanase coexpressed as secreted enzymes in *Saccharomyces cerevisiae*. *Biotechnology* **6**, 713-718.

YU, M. & CHANG, S.T. (1989). Tolerance of tannin by the shiitake mushroom, *Lentinus edodes*. *World Journal of Microbiology and Biotechnology* **5**, 375-378.

CHAPTER 9

INTERACTIONS BETWEEN SUPPLEMENTATION, FRUCTIFICATION-SURFACE AND PRODUCTIVITY OF THE SUBSTRATE OF *PLEUROTUS* SPP.

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1. INTRODUCTION

The production of oyster mushrooms (*Pleurotus* spp.) rose rapidly in the last decade and reached second place behind the white mushroom (*Agaricus bisporus*). Their production and consumption became extremly popular in Asia, especially in China (Chang & Miles, 1991; Overstijns, 1992; Schmidt, 1993). However, production and consumption of oyster mushrooms in Europe and North America has not increased substantially over the last 15 years (Behr, 1992; Schmidt, 1989, 1993). Only a limited increase can be observed in some Middle European countries, where the production and export of mushrooms appears to be a desirable source of generating hard currency (Lelley & Janßen, 1993). However, the expectations of the researchers, growers and dealers involved, in relation to the possible role of the oyster mushroom for human consumption in the western hemisphere, have not been fulfilled.

We believe that the slow increase in production in the West is caused mainly by the generally low yields achieved by commercial growers. Taking into account the high costs for labour, investments, energy etc. , the mean fruitbody yield of 13-18 % (fresh mushrooms compared to the substrate wet weight, which corresponds to approx. 45-60 % biological efficiency) harvested by the growers is, in most cases, not high enough to reach the break-even point.

In order to change this situation and improve the economic efficiency, the average yield must be increased significantly. The other possible way, a decrease in production costs, does not appear realistic.

A significant increase in the oyster mushroom substrate productivity is realizable by supplementation, as in case of the white mushroom. Unfortunately, using the most popular bag cultivation system (after spawning, the basic material is immediately filled into perforated plastic bags and kept there until the end of the cropping period), the addition of supplements and their distribution in the substrate is only practical at spawning. Supplementation causes a significant increase in the nutrient content in the substrate. It is enriched by a large quantity of carbohydrates as

well as nitrogen. If this happens at spawning, the bacteria and competitive moulds living in the prepared substrate are also fed and a dramatic stimulation of their growth in the substrate can be unleashed. Due to the quick multiplication of the microflora, a fact well-known to *Agaricus* growers, a rapid increase of the substrate temperature can be observed. In the center of larger substrate units the temperature increases up to 40-60°C in less than 24 hours. Of course, at this temperature the oyster mushroom mycelium is killed. Several studies have been done in order to find useful solutions for the supplementation of the oyster mushroom substrate (Eder, 1990; Kalberer & Vogel, 1974; Royse & Bahler, 1988; Royse & Schisler, 1987 a, 1987 b; Royse & Zaki, 1991) but, unfortunately, a successful method for farm practice has still not been established.

Putting together the effects caused by supplementation, we think that the following problems should be solved:

* inhibit the growth of competitive moulds,
* avoid the rise in substrate temperature.

The use of delayed release supplements could be a possible way to manage these problems. The principle of such supplements is that their nutrients will more or less be released after the mycelium of the cultivated fungus has fully colonized the substrate. At this stage, the substrate microflora is already depressed and will no longer be able to utilize the nutrients. Unfortunately, the common delayed release additives, suitable for *Agaricus* cultivation, have still not been satisfactorily used in oyster mushroom farms. In most cases the supplementation was linked to a rapid increase in the substrate temperature because, due to the longer incubation period, the release of the nutrients obviously began before the oyster mushroom mycelium had completely colonized the substrate. Therefore, a new type of supplement should be tested.

However, by using the above mentioned bag cultivation system, in which the plastic material is not removed from the substrate surface before the end of the cropping period and the fruitbodies appear through the macroperforation, so called "fructification holes", an additional aspect, the interaction between the yield and the range of the fructification area, must also be clarified. The effect of successful supplementation could be limited by the range of fructification surface (Hettlich, 1989). Therefore, it might be necessary to enhance the number and size of the fructification holes, which might, however, be disadvantageous from the view of the cultivation technique. The following report presents the results of our relevant studies.

2. MATERIALS AND METHODS

Wheat straw was used as the basic material for substrate production. After delivery, the straw was chopped and compressed into briquetts of a diameter of approx. 5-7 cm. While being compressed, the temperature of the straw rose to 70-80°C for a few minutes. Straw treated in such a way can be free at least of pests (insects, nematodes). In fact, in several laboratory tests we did not find any living forms of pests in our samples. We added tapwater to the straw in order to adjust the substrate moisture to 73 %. In the water, 100 ppm Fermenta Forte (active substance 35 % Carbendazim) were added in order to protect the substrate against mould contamination. No substrate preparation (sterilisation, pasteurisation, aerobic or semianaerobic fermentation) took place, but immediately after moistening the basic material, it was inoculated and filled into macroperforated plastic bags. The spawn rate reached 2 %, compared to the substrate wet weight. The bags were adjusted to 6 kg each. We used a hybrid-strain (HK-35 and its subcultures, produced by different companies) of *Pleurotus* spp.

In the first trial we used 0.5 % urea (CRN), compared to the substrate wet weight, at spawning

as a controlled release supplement. The urea was encapsulated into pellets made from a special resin (product's common name: Osmocote-3900, made by Sierra Chemical, USA). Moisture in the form of vapor penetrates through the resinous case of the pellets and dissolves the urea. The water-dissolved nutrient is subsequently released. This process continues and guarantees, depending on the construction of the walls of the pellets, a constant nitrogen supply for a longer period of time.
We have also used at spawning 0.5 % encapsulated urea coated with a thin layer of manganese chloride (CRNM comb.).

As Niess and Grabbe (1990) showed in laboratory experiments, the manganese salts have a beneficial effect on mycelium development as well as on the fruitbody yield of the oyster mushroom. They also found that the provision of manganese salts significantly improved the ability of the oyster mushroom to decompose lignin. This phenomenon can be attributed to the activity of the manganese-peroxide enzyme which is much more effective than all other lignin decomposing enzymes. The use of manganese chloride in farm practice can be characterised as an indirect way of supplementation as only the oyster mushroom is able to utilize the straw extensively.

In the second trial only encapsulated urea and manganese chloride were used together. On the one hand, we tested the above described combined product, adding it at spawning. On the other hand, we used 0.5 % encapsulated urea at spawning and added 200 ppm manganese chloride to the tapwater. The fructification surface on the bags was varied between 0.76 %, 1.44 % and 2.12 %, compared to the total surface, by doubling and trebling the number of the fructification holes. The substrate bags were placed randomly on shelves in a cultivation room. Daily temperature measurements from each treatment were carried out. Colonization time was finished three weeks after spawning. A slightly reduced room temperature, more fresh-air and high humidity (above 85-90 %) were provided afterwards in order to induce primordia formation. Between two flushes the temperature was enhanced again and the fresh-air dose was significantly restricted. From the 21st day, twelve hours of fluorescent light was automatically provided each day. The harvesting period took 35 days. For yield determination only fruit bodies with a stem of one cm were counted. Six to eight repetitions were established for each treatment.

3. RESULTS

The daily temperature measurements showed that there was no significant increase in treated substrate temperature during the incubation period compared to the untreated control (Figure 1).

We were able to conclude that the use of controlled, delayed release urea and manganese chloride as a supplement did not cause a significant increase in the microbial activity in the substrate during spawn run.

Furthermore, we found that supplementation always significantly increased the substrate productivity (Figures 2 and 4). The first flush (1 and 2 weeks) was basically the highest in all treatments, including the control, in both trials, but the first crop of the supplemented variants were always significantly higher than those of the control. However, supplementation significantly increased not only the first but also the second flush (3 to 4 weeks). The enhancement of the second flush is especially detectable in both trials after using controlled release urea enriched with manganese chloride (Figures 2 and 4, CRNM comb. and CRN + M). The enhancement of the fructification surface does not increase the substrate productivity. The only effect observed in the first trial was a quicker appearance of the first flush on such substrate units of the control in which the fructification area was enhanced twice and three times (Figure 2). In the first trial no interactions were

found between the significantly higher fruitbody production (compared to the control) of the supplemented substrates - neither in the first nor in the second flush - and the range of the fructification surface (Figures 2 and 3).

It does not appear economical to continue the *Pleurotus* cropping period for longer than about 28 to 35 days. After using supplements nearly 70 % or more of the total yield can be harvested in the first flush (Figures 3 and 5). Especially in the second trial, the superiority of the first crop (1. and 2. week) was observed very clearly. In the second attempt about 20 % *Pleurotus*-biomass (prepared for selling), related to the fresh substrate weight, was harvested at the supplemented variants in the first flush (Figure 4). A similar relation, a strong first flush, was also detected in the control after enhancing the fructification surface of the substrate, but at a much lower level in its entirety.

In cases involving the use of controlled release urea enriched with manganese chloride (the combined product), the wait for the second crop, which appears in the 3. to 5. week, should be taken into consideration (Figure 2). In the second cultivation experiment a remarkable second flush, economical to wait for, was harvested only on the substrate that was separately supplemented by the controlled release urea and manganese chloride. Obviously, due to the use of manganese chloride, a stronger substrate transformation into fungal biomass, especially more effective lignin decomposition, also takes place in the later stage and remarkable fruitbody production, compared to the first crop, can be expected. A synergistic effect between nitrogen supplementation and the provision of manganese chloride might also be responsible for this relevant increase of the later substrate

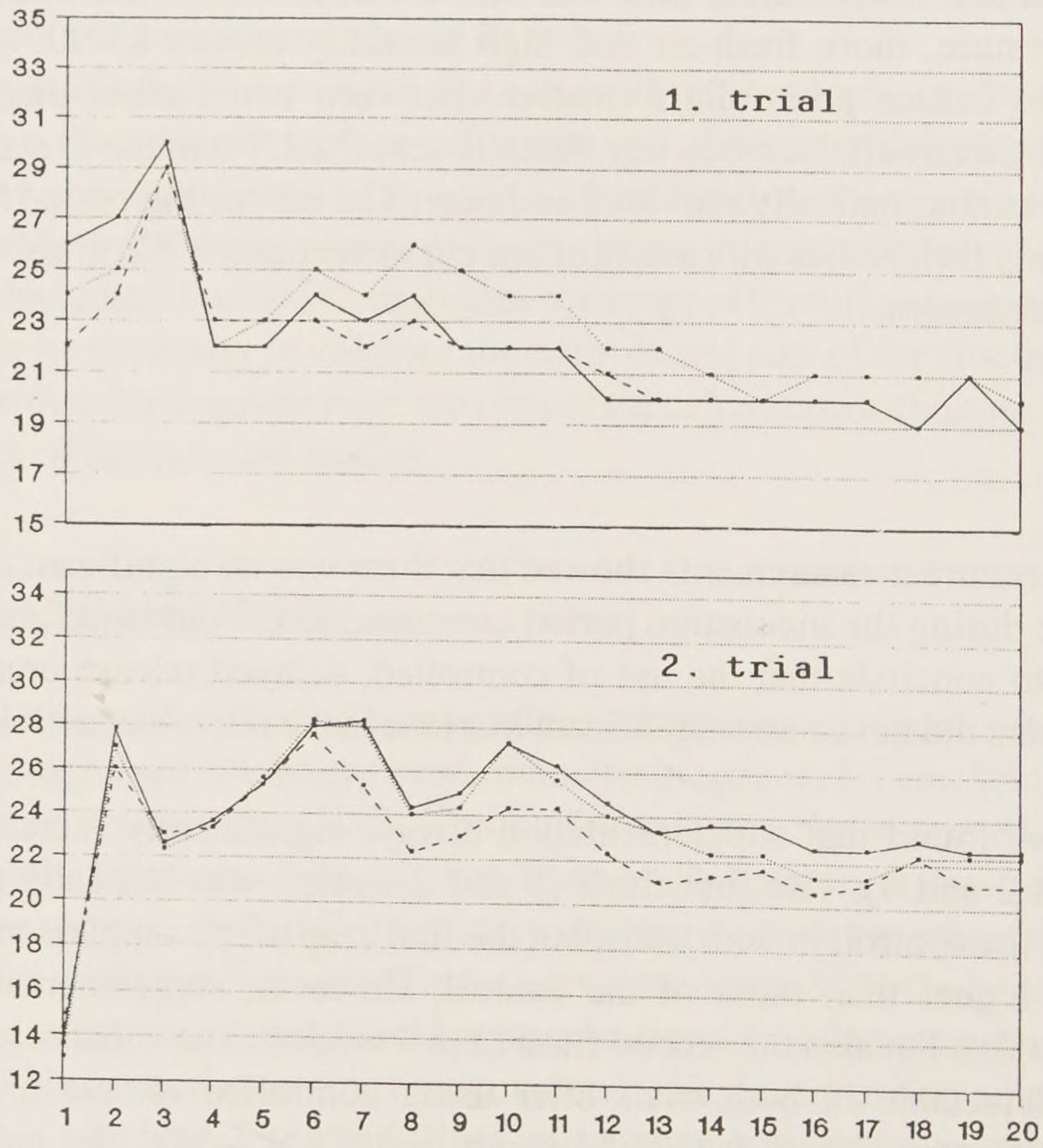
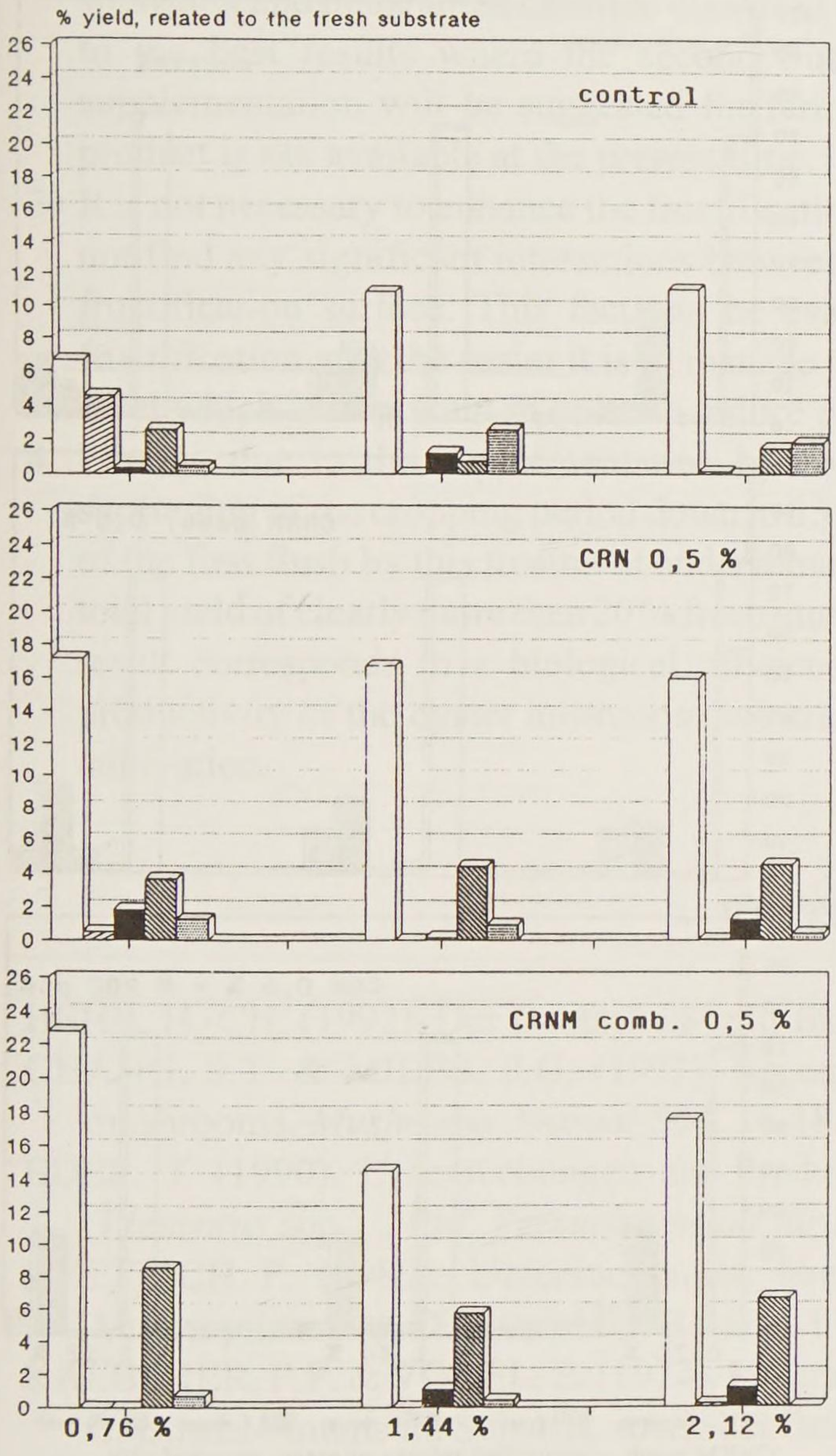


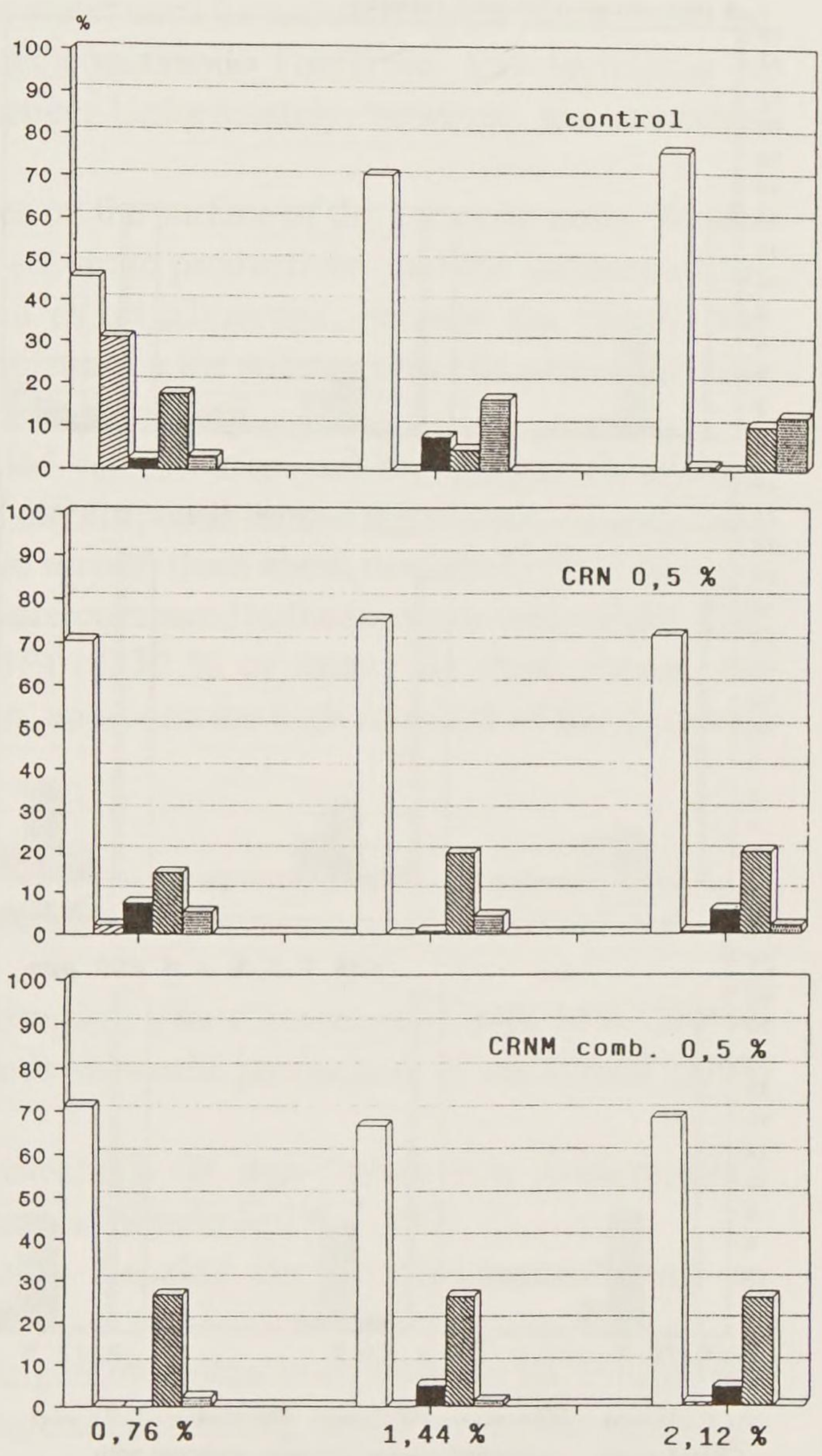
FIGURE 1. Average substrate temperature during the 20 days long incubation period in supplemented and non-supplemented units. ----- control; ——— controlled release nitrogen; ····· controlled release nitrogen + manganese chloride.

productivity, because only the use of the nitrogen-supplement always resulted in a considerably lower second flush (Figure 2, CRN and CRNM comb.). Moreover, the separate use of the manganese chloride (e.g. 200 ppm added to the water), which means a much higher concentration in the substrate than that only coated on the urea pellets, seems to be more benefical for the development of the second flush (Figure 4). In the second cultivation experiment in which untreated as well as controlled release urea and manganese chloride supplemented substrates were tested, the results of the first trial were mostly confirmed. But, contrary to the first attempt, a slight interaction between the enhancement of



CRN - controlled release nitrogen
CRNM comb. - controlled release nitrogen, enriched with manganese chloride (combined product)

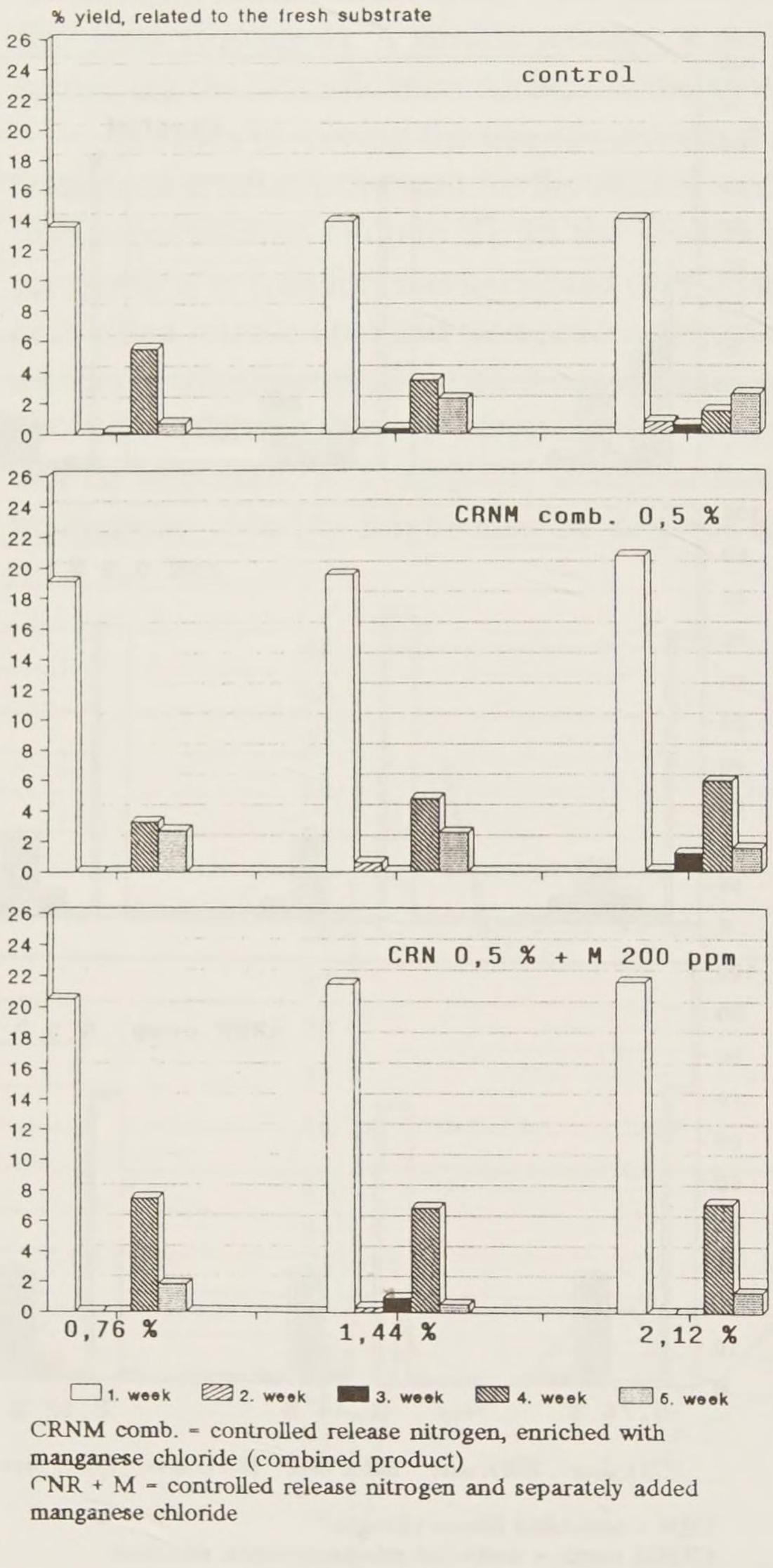
FIGURE 2. Interactions between supplementation, substrate productivity and fructification area (expressed as % of the total surface) at *Pleurotus* spp. Yield development during 5 weeks.



CRN - controlled release nitrogen
CRNM comb. - controlled release nitrogen, enriched with manganese chloride (combined product)

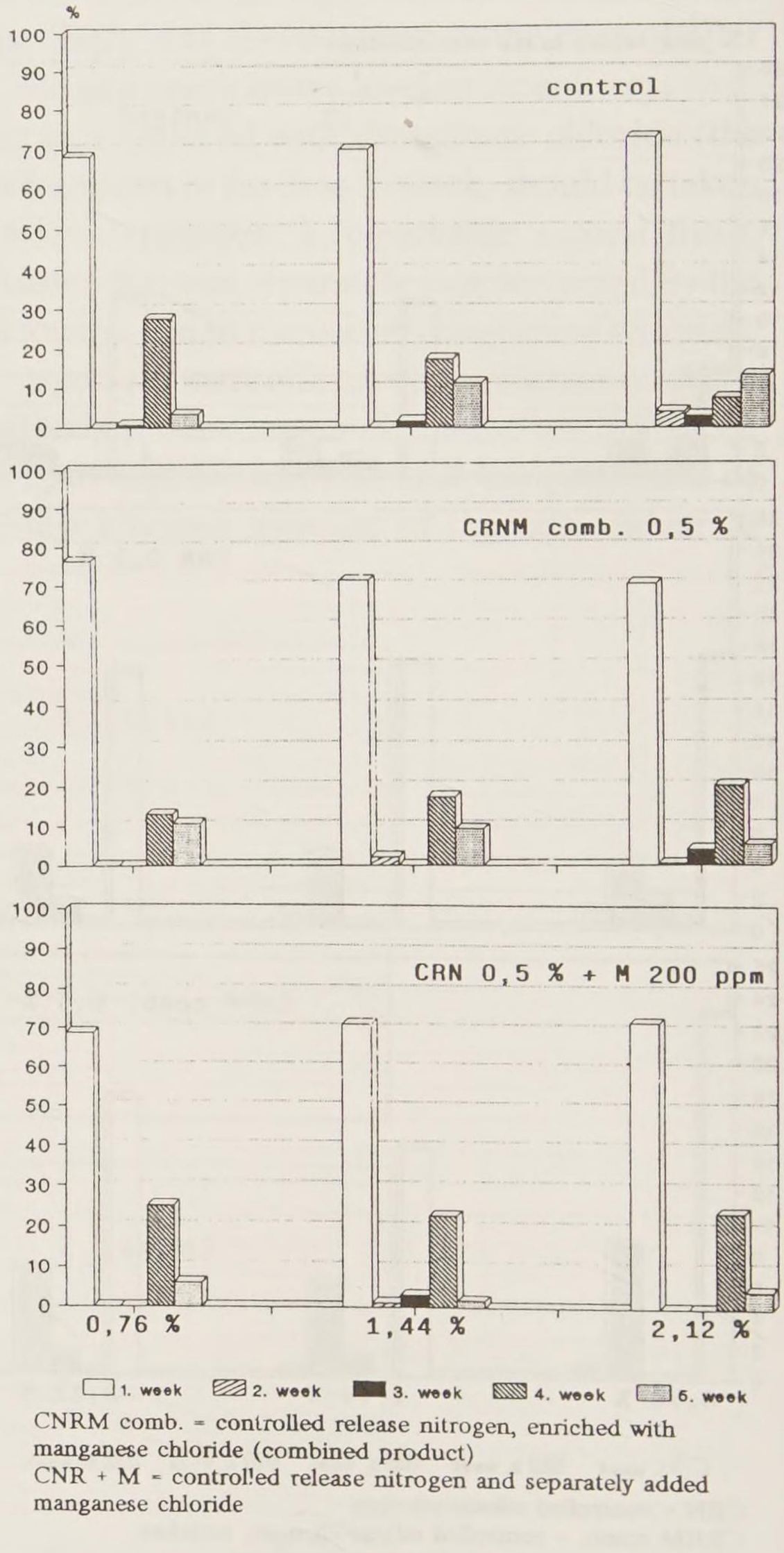
FIGURE 3. Interactions between supplementation, substrate productivity and fructification area (expressed as % of the total surface) at *Pleurotus* spp. Ratio of yield distribution during 5 weeks.

the fructification area and the substrate productivity was observed. In the first flush, we found a limited tendency to higher fruitbody yield if a larger fructification area was used. In the second flush, the same tendency was observed only when using the combined product (CRNM comb.). However, as mentioned, this interaction was only a slight one because the enhancement of the fructification area did not lead to significantly higher yields (Figure 4).



CRNM comb. = controlled release nitrogen, enriched with manganese chloride (combined product)
CNR + M = controlled release nitrogen and separately added manganese chloride

FIGURE 4. Interactions between supplementation, substrate productivity and fructification area (expressed as % of the total surface) at *Pleurotus* spp. Yield development during 5 weeks.



CNRM comb. = controlled release nitrogen, enriched with manganese chloride (combined product)
CNR + M = controlled release nitrogen and separately added manganese chloride

FIGURE 5. Interactions between supplementation, substrate productivity and fructification area (expressed as % of the total surface) at *Pleurotus* spp. Ratio of yield distribution during 5 weeks.

4. CONCLUSIONS

Summarizing the result achieved in both cultivation attempts, the following statements are possible:

1. The use of controlled release urea combined with manganese chloride for supplementation of the grain-straw-made-oyster mushroom substrate might be a successful way to significantly increase substrate productivity and improve the profitability of the cultivation.
2. The separate use of the encapsulated urea and the manganese chloride (the pellets given at spawning and manganese chloride dissolved in the water used for moistening the straw), also led to the best results where the second flush was concerned. Therefore, this technique of supplementation will be suggested for farm practice. Unfortunately, however, the combined product is not available at the present time.
3. It is not necessary to enhance the fructification area on the surface of the substrate units. We did not find any significant interactions between the substrate productivity and the increase of the fructification surface. This fact can be evaluated as an advantage, because the smaller the fructification area the easier it is to manage the moisture in the substrate and its carbon dioxide level which is important in order to induce primordia formation.
4. Finally, due to the supplementation by controlled release urea and manganese chloride, a shortening of the cropping period down to 35 to 28 days is possible. The significant enhancement of the first flush by this treatment and a remarkable second flush about two weeks later causes a total yield of clearly more than 20 % fresh mushrooms, compared to the substrate wet weight. This result corresponds to a biological efficiency (BE) of 70 % or more. By these means, the productivity of the oyster mushroom substrate can approach the high standard of the *Agaricus* cultivation.

REFERENCES

BEHR, H.-CH. (1992). Der Deutsche Markt für Speisepilze. *Der Champignon* **368**, 119-121.

CHANG, S.T. & MILES, P.G. (1991). Recent trends in world production of cultivated edible mushrooms. *Mushroom Journal* **504**, 14-18.

EDER, J. (1990). Untersuchungen zur Produktionstechnik für den Anbau von Austernpilzen (*Pleurotus* spp.). *Mittl. Versuchsanstalt für Pilzanbau. Sonderheft* **9**, 1-193.

HETTLICH P. (1989). Untersuchungen über einige Aspekte der Frucht-körperbildung im Austernpilzanbau. Diplomarbeit, Bonn. 1-113.

KALBERER, P.P. & VOGEL, E. (1974). Untersuchungen zur Kultur von *Pleurotus* III. Einfluß des Grasmehl-Anteils im Substrat, sowie der Brutmenge auf den Ertrag bei *Pleurotus ostreatus* Typ *florida*. *Schweiz. Z. Speisepilz-Anbau.* Nr. 1/1. 7-11.

LELLEY, J.I. & JANßEN, A. (1993). Productivity improvement of oyster mushroom substrate with a controlled release nutrient. *Mushroom News* **41**, 6-13.

NIESS, A. & GRABBE, K. (1990). Response of the oyster mushroom (*Pleurotus ostreatus*) to manganese supply. *Abstracts of the Fourth International Mycological Congress*. Regensburg, Germany. IIE-246/4.

OVERSTIJNS, A. (1992). De wereldproduktie van eetbare teeltpaddestoelen van 1986 tot 1989/90. *Champignonberichten* **92**, 2-7.

ROYSE, D.J. & BAHLER, B.D. (1988). The effect of alfalfa hay and delayed release nutrient on

biological efficiency of *Pleurotus sajor-caju*. *Mushroom Journal for the Tropics* **8**, 59-65.
ROYSE, D.J. & SCHISLER, L.C. (1987a). Yield and size of *Pleurotus ostreatus* and *Pleurotus sajor-caju* as effected by delayed-release nutrient. *Applied Microbiology and Biotechnology* **26**, 191-194.
ROYSE, D.J. & SCHISLER, L.C. (1987b). Influence of benomyl on yield response of *Pleurotus sajor-caju* to delayed release nutrient supplementation. *Horticulture Science* **22**, 60-62.
ROYSE, D.J. & ZAKI, S.A. (1991). Yield stimulation of *Pleurotus flabellatus* by dual nutrient supplementation of pasteurized wheat straw. *Mushroom Science XIII* **2**, 545-547.
SCHMIDT, E. (1989). Mushroom growing worldwide - production, marketing and consumption. *Mittl. Versuchsanstalt für Pilzanbau*. Nr. **11/12**, 85-137.
SCHMIDT, E. (1993). Pilzanbau weltweit - Produktion, Absatz und Verzehr. *Mittl. Versuchsanstalt für Pilzanbau*. Nr. **16**, 15-37.

CHAPTER 10

INDOOR COMPOSTING : GENERAL PRINCIPLES AND LARGE SCALE DEVELOPMENT IN ITALY

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1. HISTORICAL BACKGROUND

INDOOR COMPOSTING is a widely accepted expression in the mushroom industry used for defining most of the composting techniques carried out "indoor" under conditions of strictly controlled environment and process. It seems that Lambert was the first one to use that expression in a paper published in 1941. Mushroom compost is a unique support for the growth and fruiting of *Agaricus bisporus*. It must be altogether selective and productive (Ross & Harris, 1983 ; Stone & Laborde, 1991 ; Fermor & Macauley, 1991) ; i.e. favorable for the sole growth of the cultivated mushroom, and generating high yields of fruit bodies. In order to positively control composting, it is essential to have the best knowledge of the complex characteristics of a good compost, and to determine what specific steps should be taken to reproduce them. Historical background has been recently reviewed (Van Griensven, 1993).

Improving our understanding of composting has been, for the past sixty years, the permanent goal of all scientists involved in mushroom cultivation, starting from Waksman and colleagues in the thirties, continuing with Stoller, Lambert and Sinden in the late thirties, the forties and fifties, and many more from the sixties onwards.

Sinden & Hauser (1950, 1953) developed the Short Composting method, through which they demonstrated that complete composting could be successfully achieved in two short successive phases : Phase 1, outdoor, lasting 7 days, at relatively high temperatures, between 70 and 80°C, in long, narrow piles, rectangular in shape ; Phase 2, indoor, in trays or in shelves, under well controlled conditions, at medium uniform temperatures, between 45° and 58°C, lasting 5 to 7 days. Prevailing aerobic conditions were a prerequisite during the whole length of the process. Present composting techniques, referred to as classical or traditional, are mostly derived from this Short Composting scheme.

An important advance in the understanding of mushroom nutrition and substrates was brought

about by Till (1962) who succeeded in growing *Agaricus bisporus* under conditions maintained axenic up to fruiting, on sterilized complex substrates. Regular yields were then obtained under non-sterile conditions. Till's method was improved by colleagues, von Sengbusch (1965), and Lemke (1965), then modified by Huhnke & von Sengbusch (1968) and Huhnke (1971) : they replaced autoclaving by semi-sterilization at lower temperatures (80° to 100°C), then, by reinoculating with specific microorganisms, they were able to avoid spawn run under aseptic conditions. Maintaining sterile conditions was considered too technical and too expensive for large scale projects. Nevertheless their contribution, following the great step forward by Sinden and Hauser, demonstrated in the mid sixties that a high quality substrate for the cultivation of mushroom could be obtained from a wide range of raw materials, under well controlled conditions, and in a much shorter period than used to be the case at that time.

In the sixties, and early seventies, a profusion of scientific investigations on the microbiology of composting gave a series of publications by Bels-Köning *et al.* (1962), Fergus (1964), Hayes (1968), Laborde *et al.* (1968), Imbernon & Leplae (1971), Stanek (1971) and Olivier & Guillaumes (1978). The result was a clearer picture of the compost ecology which led to new interest into the development of "rapid composting techniques".

In France, Laborde & Delmas (1969, 1971) developed a process named P.E.S.(Express Preparation of Substrates), a fast, 3 to 6 days, indoor composting process in trays under strictly controlled environmental conditions. This was, in fact, a novel composting process carried out indoor, but also in trays rather than in bulk (not known at that time), thus disadvantaged by less efficient control. Today, P.E.S. is considered as a single phase medium temperature process, as opposed to the present double phase higher temperature process adopted by INRA and Italian specialists since 1984.

In England, Hayes (1968), Randle & Hayes (1971), Smith (1974), Smith & Spencer (1976), worked on the basics of composting, first in revolving drums, then later in trays and pasteurizing chambers. Revolving barrels or drums had been used previously by Stoller *et al.* (1939), Delmas (1953), Trocmé & Sarazin (1956), and on a large industrial scale in the U.S. by Campbell Soup Co (Murphy, 1972), a true early indoor composting process. They were also used later in Australia (Baker & Baker, 1981). Smith and colleagues kept working for many years on the elaboration of short duration composts (Smith, 1983).

In Germany, Huhnke and von Sengbusch (1968), and Huhnke (1971) modified the Till method by substituting sterilization of the substrate with a high temperature treatment followed by incorporation of specific microorganisms and a short Phase 2 at lower temperature. In fact, they were recommending, years earlier, a very short composting process close to the one proposed, after many more years of experimentation, by Laborde *et al.* in 1984. The scheme described by Huhnke appears today as a forerunner of current Indoor Composting techniques. It is not known why such a visionary work was discontinued : for lack of financing or interest in another research subject ?

In Holland, Bels Köning, as early as 1962, conceived a composting process involving the partial predigestion of lignocellulosic materials by strong acids or bases (liquid ammonia was on the list), followed by a transformation period by inoculated selected thermophilic microorganisms, mainly fungi, working at their optimum growth temperature range. Such a scheme was also visionary; the value of thermophilic fungi has been widely demonstrated in recent times, not only during the conditioning period of Phase 2, but also as a key element in the rapid preparation of indoor composts by the single phase medium temperature process, experimented since 1985 to a large extent in Australia, Holland, England and Belgium. Muller (1965), Gerrits and coworkers (1965, 1968, 1971, 1974) contributed very strongly to a better knowledge of the biochemical transformations occurring

within a compost either outdoor or indoor, and after spawning. Despite such wide interest in developing new composting techniques, our understanding of the basic chemical and biological reactions involved remained limited. Simple questions could be only partially answered such as :

- What are the desired characteristics of a nutritive and selective compost ?
- What substances, or families of substances, at what level, what balance, are we aiming at through composting : for example, do we need black substances ; what are they ; are they useful for the mushroom ?
- What specific steps should be taken to regularly produce such substances, under what conditions?

More basic approaches to composting was, and still is, needed to make real progress in this field. British scientists have been doing a tremendous amount of research, from the mid-seventies up to early nineties, to clarify the biological mechanisms involved both during composting and the mushroom growth and fruiting, work extensively reviewed recently by Fermor & Wood (1991), Fermor & Macauley (1990), Wood & Fermor (1985). Even more recently, more data have been made available by French scientists on the biological mechanisms occurring during Indoor Composting, including both the microbial activity and the availability of nutrients, all evaluated on a process very similar to the INRA Indoor Composting process (Chalaux *et al.*, 1991; Savoie *et al.,* 1992a; Savoie *et al.,* 1992b).

But, in the eighties, an empirical approach was sufficient to permit the explosive development of Indoor Composting on a large industrial scale in Italy, and to a smaller extent in Austria (Sohm, 1989), Switzerland (Kuhn, 1990 ; Leendertse, 1988)), Holland and Australia.

It was also in 1968 and the following years that two of the authors, Francescutti and Giordani, became responsible for introducing the presently worldwide used technique of Phase 2 or Pasteurization in bulk in tunnels. Conducting the composting process in bulk had been proposed by Stoller (1951) twenty years earlier, himself impressed by the existing similarity between composting for mushroom growing and malting for beer making. Franscescutti and Giordani, associated with Derks (1973), decided to first experiment with, then to adopt completely, Phase 2 in bulk. Although Stoller had presented his views in an extensive review of the scientific and practical problems of mushroom cultivation (Stoller, 1954), they remained almost unknown until the late sixties. Of course, the new types of tunnels were designed with characteristics widely different from Stoller's first version. But it is certain that such a development was not expected at the time to become the main worldwide technique adopted to run the Phase 2 stage of composting, and in more recent years, the whole process of Indoor Composting. Sarazin, in France, started experimenting with tunnels at about the same time (1969), and in 1972, pasteurization in tunnels became adopted by large Italian and French mushroom growers (d'Hardemare & Talon, 1974). Years later, other European growers followed.

Running Phase 2 in bulk in tunnels meant blowing air through the compost mass instead of around it. Formerly, Phase 2 was carried out with compost in trays or shelves. Phase 2 in bulk meant more uniform environmental conditions across the whole compost mass with a very precise control. The main difficulty which arose was that optimum conditions during Phase 2 were not well established and could only be set through more trial and error experimentation.

But the Italian developers were so enthusiastic about this new technique that they readily visualised extending its use, not only to Phase 2 of composting but to Phase 1 (formerly outdoor) as well, and "Phase 3" or spawn run after spawning. Their ideas were summarized by Derks (1973) under an elegant formula : the 3 Phase in 1 process : in fact a complete Indoor Process including Phases 1 and 2 of composting as well as spawn running, the three operations being carried out in bulk, in tunnels.

Phase 2 in bulk has presently been adopted by the majority of mushroom growers and compost

makers except in the USA where Phase 2 in trays or shelves is still predominant.

However, extending that technology to either Phase 3 or Phase 1 presented some difficulties i.e. economical reasons: it was cheaper to compost outside (Derks, 1992) ; technical difficulties : the best conditions to select and maintain throughout the process were not really known ; more research and experimentation have been required to adopt such a process either for Phase 3 or Phase 1.

As far as Phase 1 was concerned (Indoor Composting), two questions needed precise answers.

1. Is a short phase at higher temperatures (70 to 80°C) better than usual medium ones (50°C) to obtain both selectivity and nutrition ? And if such is the case, how to compensate for the loss of microorganisms at such very high temperatures ?
2. How to determine the temperature range and duration, and the oxygen levels, throughout the process, in order to end up with an optimum substrate ?

Answers to these questions are still debated, but the processes adopted to-day on a large scale are an indication that the options retained must be valid.

In Denmark, Bech (1978), developed a very rapid composting process, first on wood pallets acting as a double floor, then in a tunnel. Total duration of the process (Phase 1 plus Phase 2) varied from 5 to 8 days. As in standard composting, a wide range of high temperatures was obtained and maintained through Phase 1 which eliminated the need for reinoculation of the compost before Phase 2.

In France, Laborde *et al.* (1978, 1980) produced more data about the positive effects on indoor processes of high temperatures applied for a limited time, provided biological activators (i.e. microorganisms) could be introduced following the high heat treatment. Later, they presented, the INRA Indoor composting process, a double phase higher temperature process (Laborde *et al.*, 1984, 1986). Indoor Composting is successively carried out under two different temperature ranges : from 2 to 3 days at around 80°C, then from 5 to 7 days at around 50°C, except for a short peak heat at 58°C. Following the higher temperature period, the introduction and mixing of a limited quantity of pasteurized outdoor compost provided reinoculation of the partially sterilized indoor compost. Such a technique combined the positive effects on compost bulk density and nutritional quality of a higher temperature treatment, with the benefits provided by the development of thermophilic fungi and actinomycetes at the medium temperature range. Additional data have been regularly presented since 1984 : Laborde *et al.* (1986, 1987), Houdeau *et al.* (1991), Laborde (1990, 1991, 1992). Despite slight modifications, the principles of the technique remain the same.

Around 1985, research work was almost simultaneously started in England, Holland, Australia and Belgium along an entirely different line. The idea was to maintain optimal environmental conditions for the growth of thermophilic fungi, *Humicola* or *Scytalidium* (Straatsma & Samson, 1993), which are viewed as indicators of a good compost. The process was run at a medium temperature, the range 45°C to 50°C appearing as the most satisfactory. A good productive compost could then be obtained in about seven days. A very positive point of that technique was the large reduction in emissions of both malodorous and polluting gases. Many publications presented the progress realized in experimenting with such a new technology : Perrin & Gaze (1987), Gerrits (1987), Gerrits & van Griensven (1991), Derrikx *et al.* (1990 a & b, 1991), op den Camp *et al.* (1991), Nair & Price (1991), Miller (1992), Gulliver *et al.* (1991), Overstinjs (1992). Large scale development of that process was recently undertaken in Holland, Australia, New Zealand and Belgium.

It must be remembered that such a single phase medium temperature process was a logical development of the former P.E.S., but in bulk instead of trays, abandoned in the late seventies by Laborde *et al.* (1978) for the simple reasons that mushroom yields and quality were not consistent, and compost bulk density was below that of composts made at normal higher temperatures. It now

seems evident that such arguments were of value, since most new Indoor Composting development projects are managing a phase at higher temperature, previous to the longer period at lower temperature. From 1985 onwards, Francescutti on one hand, and Giordani on the other, started to develop their own conception of large scale Indoor Composting, followed closely by several Italian mushroom growers and compost makers. All these projects, developed in Italy in the past six years, have included a hot temperature phase lasting from two to three days, conducive to very consistent results. Some of these we are now going to present to you. Such is the choice being made in recent development projects, in Holland by Theewen and Pleunis (Janssen, 1992 ; Dicks, 1993), in Australia (Gulliver *et al.* 1992) and in Belgium (Janssen, 1992).

2. REQUIREMENTS FOR COMPOSTING INDOOR

A. Preparation stage: objectives to be attained before the temperature treatment.
(Many of the factors presented here were recently reviewed by Miller (1992) and Laborde (1992)).

2.1. Nutritionally Balanced Mix of Raw Ingredients

The raw materials employed, and their proportions, are the same as for a traditional outdoor compost. But the much shorter duration of the process means reduced losses of useful nutritive elements, i.e. a larger proportion of organic matter, more carbon, less ash, in the final compost. This means more filling capacity from a given initial volume of raw ingredients, and more organic matter per cropping area, or a larger potential yield (Harper *et al.* 1990).

It is a well admitted fact that higher yields are cropped on composts with a low C/N ratio, between 14 and 17. With short duration composts, C will remain high. Therefore N must be raised to a higher level. Higher N levels are generally obtained in straw plus poultry manure composts, up to 3% of D.M. Poultry manure is a rich source, not only of interesting organic substances, but also of important minerals like potassium, phosphorous, magnesium, calcium, sulphur and iron and, of course, nitrogen. With shorter composting, the general ratio of inorganic elements over organic ones will be much smaller, requiring an adjustment in both level and balance of these elements (Table 1). Therefore, in order to obtain a high nitrogen level in the finished compost, high level and high balance of P, K, Mg, Ca, Fe and S are needed, brought about by large, but not excessive, volumes of poultry manure (Laborde, 1991). The nitrogen level may be increased even more by the addition of an inorganic fertilizer such as ammonium sulphate. Although high levels of inorganic elements are correlated with maximum yields, they increase the risk of salinity, i.e. excessive osmotic pressure and toxicity (Treschow, 1944; Bohus, 1959) which can only be reduced by a higher water content of the compost. But beware of overwetting!

Specific organic additives may profit from being applied at this early stage: they are sugar, fat or protein rich activators, and enzyme complexes. They all contribute to a rapid build up of active

TABLE 1. Optimum Levels of Inorganic Elements (% D.M. of compost) (From Laborde, 1991).

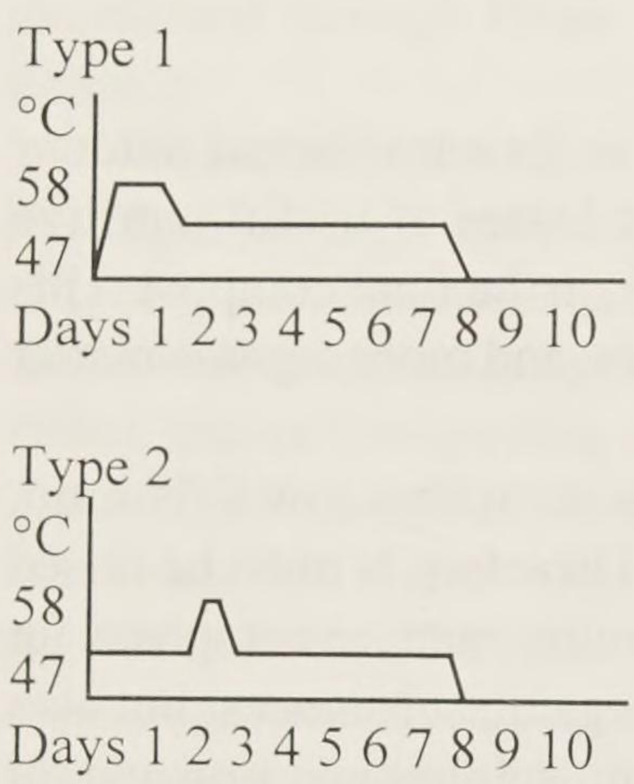
	N	P_2O_5	K_2O	CaO	MgO
Mixing	1.7-1.8	1.2-1.5	2.0-2.3	1.5-3.0	0.4-0.5
Spawning	2.3-2.4	1.6-2.0	2.7-3.2	2.0-4.0	0.5-0.7

microorganisms, which in turn will generate more heat.

2.2. Uniform Mix of Raw Ingredients

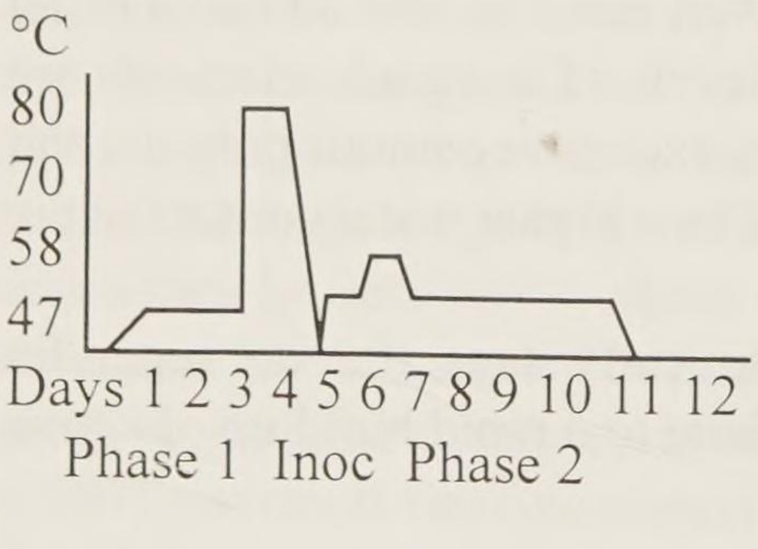
Premixing operations: To improve both the mix and the degradation process, it is a requirement that most raw materials should be shredded or milled. Mixing following milling disperses evenly compost nutrients and microorganisms (Miller, 1992) and permits a rapid and complete wetting of all the ingredients (water is readily absorbed by straw), and will promote vigorous thermogenesis through the action of thermophilic microbes. An important consequence of milling is that bulk density is immediately increased to the value which used to be obtained only at the end of composting. Better moisture content and distribution, smaller particles, and higher density all mean a more uniform and active compost.

Mixing equipment proposed nowadays is getting more efficient with auger type systems as used in feed wagons, and delivering a very uniform product in a shorter time. New blenders are so



SINGLE PHASE MEDIUM TEMPERATURE PROCESS
Filling tunnel
One Phase
Constant aerobiosis
Strong ventilation
Temperature rise: 1-2 days
Plateau 45-50°C : 5-7 days
Peak-Heat 58°C : 4-6 hours
Total duration : 6-9 days from filling to spawning.

FIGURE 1. Single phase medium temperature process.



DOUBLE PHASE HIGHER TEMPERATURE PROCESS
Filling tunnel for Phase 1
Aerobiosis + reduced ventilation
Range 45-50°C : 1-3 days
Up to and at 80°C: 1-2 days
End Phase 1; empty tunnel
Addition of microbial inoculum
Filling tunnel for Phase 2
Aerobiosis + strong ventilation
Plateau 45-50°C: 5-6 days
Peak-heat 58°C : 4-6 hours
Total duration : 8-12 days from 1st filling to spawning

FIGURE 2. Double phase higher temperature process.

efficient that all the water needed may be added and absorbed in a single pass.

B. The two thermal processes used to-day are shown in Fifures 1 and 2 (from Laborde, 1992).

2.3. Influence of the Temperature Level

Composting is an accelerated humification process at high temperature. In a compost pile, we find a full range of temperatures up to 80°C. Their effects are quite different at 50 to 60°C than at 70 to 80°C.

50-60°C: at this level, most mesophilic microorganisms are either destroyed or inactivated. Around 50°C, thermophilic microbes are booming and comprise fungi, actinomycetes and bacteria, while at 60°C, only bacteria remain active. *Scytalidium*, (*Humicola*), grows best between 45 and 50°C. Most predators and competitors are eliminated. The heat surge destroys many organisms, permitting the start of ammonification, or the release of free ammonia. Free ammonia acts as a sterilant, but is also a strong chemically active product which reacts with soluble sugars, other carbohydrates and phenols, to yield complex nutritive brown substances available only to the mushroom (Stone & Laborde, 1990). The higher the temperature, the stronger these reactions with ammonia. Thermophilic fungi, working at about 50°C, will rapidly use the ammonium ions and stop the production of free ammonia, known for its toxicity to the mushroom mycelium. So, when running a process continuously at around 50°C, the compost may be ready for spawning when no more free ammonia is detected. In fact, selectivity is also attained when the level of soluble sugars and phenols is very low. Dry matter losses at such temperatures are very high, between 3 and 4% per day, since they result from an intense biological activity (Table 2). The substrate is fully colonized by friendly microorganisms at the end of such a medium temperature treatment, mainly fungi and actinomycetes.

70°-80°C: above 70°C, a partial sterilization occurs, more pronounced at 80°C. Most microbial life is eliminated. Production of free ammonia is even greater, causing a larger complexing of products under self-sustained chemical reactions. Cellulose is also getting more pliable. Bulk density increases (Table 3). The compost is darker, almost black. Black products are complex substances useful to the mushroom (Eddy & Jacob, 1976, Goulas, 1987, Laborde, 1992). Dry matter losses are very low, 1 to 1.5% per day, since almost all biological activity has stopped, (Table 2). But the substrate is almost devoid of microorganisms, making it very susceptible to contamination by undesirable organisms. Following such a hot treatment, reinoculation with useful microorganisms is a must and their growth should be stimulated by maintaining optimum lower temperatures for a certain duration, generally several days.

TABLE 2. Losses during Indoor (2 phase) Composting Process (in % Fresh Weight or Dry Weight). From Laborde *et al.* (1984).

	Exp. 1		Exp. 2		Exp. 3	
	F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
Phase 1	8.4	6.8	14.8	5.3	9.9	3.5
Phase 2	18.9	13.1	15.0	14.6	17.8	14.4
TOTAL	24.8	18.6	25.9	18.6	25.4	16.9

TABLE 3. Bulk densities as measured in tunnels (not compacted). Indoor (2-Phase) Process (kg/m^3). From Laborde (1992).

	Exp. 1	Exp. 2	Exp. 3
End Phase 1	510	450	550
End Phase 2	430	420	450

2.4. Influence of Oxygen

The relationships between oxygen and compost have been thoroughly reviewed by Miller & Dahlberg (1990). Derikx *et al.* (1990b) found the highest consumption rate of oxygen at a temperature of 50°C, and lower consumption rates at all other temperatures.

Cellulose decomposing organisms may be found under aerobic or anaerobic conditions. But under lower oxygen levels, chemical products toxic to the mushroom may be formed such as short chain organic acids, amides, volatile sulphur compounds; and the residual microflora may not survive during spawn run under fully aerobic conditions, making room for competitors. So, even if some benefit may be obtained by maintaining anaerobic conditions for a certain length of time (Houdeau *et al.*, 1991), it seems preferable, either to entirely avoid them, or to limit them to very short periods of time if for no other reason than to avoid the production of offensive odors (Miller & Dahlberg, 1990). This is the reason why all Indoor Composting facilities, adopting the double phase higher temperature process, are trying to maintain, during the hot phase, a minimum oxygen level inside the compost mass of 10 to 12%. Such a level is interesting because it allows for limited introduction of colder fresh outside air, while maintaining sufficiently aerobic conditions. It may be regulated by small ventilation rates or alternate ventilation schedules, thus limiting the need for steam heating and its detrimental effect on compost aeration and moisture content through condensation. Let's not forget that fungi and actinomycetes require high oxygen levels, while *Chaetomium* (Olive green mold), will develop readily when high temperatures and low oxygen levels are combined.

2.5. Emission of Malodorous Gases and Ammonia

A recent review of odours and composting is available, (Miller & Macauley, 1988). It has been demonstrated (Op den Camp *et al.*, 1991; Derikx *et al.*, 1990a, 1990b, 1991) that very much reduced emissions of odorous sulphur compounds were produced with Indoor Composting conducted according to the single phase medium temperature process, under constant and very high aerobic conditions. Higher temperatures, between 70 and 80°C, do not generally result in increased emissions (Dicks, 1992) when truly aerobic conditions are maintained; much lower biological activity at these temperatures means a much reduced oxygen consumption, or very low fresh air intake, and low volumes of polluted air released in the atmosphere. Therefore, higher temperatures may not be associated with increased environmental problems.

Free ammonia, (gas), is beneficial to the composting process. Larger volumes may be expected to be released in the atmosphere under high fresh air admission, and exhaust, as customary with the single phase medium temperature process; under higher temperatures, we have just seen that very low volumes of air are rejected, i.e. less ammonia. In both cases, to remove any trace of ammonia, the exhaust air should be treated with sulphuric acid before being released outdoor.

2.6. Biological Activators

Activators commonly used in the composting process are generally bringing more nutrition to the microorganisms to generate maximum activity (Laborde, 1992) ; they are chemical activators, for example, sugars, oils and proteins.

Biological activators are designed to bring specific microorganisms to intervene at a preset stage of the process. For example, to replace destroyed microbial biomass immediately after the higher temperature treatment of the 2 Phase process. The objective is to introduce the right organisms doing the right work under the right conditions, which implies the following questions:

- What kinds of microorganisms and at what stage ?
- Under what form ; what volume of inoculum ?
- To carry out which reactions leading to which compounds ?
- Under what optimum conditions ?

The microflora of a compost is quite different at the beginning and at the end of composting. It is constituted predominantly of thermophilic microorganisms at the end, and of more mesophilic ones at the start (Wood & Smith, 1988 ; Fermor *et al.*, 1979) (Table 4).

Fungi like *Scytalidium* (formerly *Torula* and *Humicola*) appear massively at the end (Straastma & Samson, 1993). General evolution is presented in Table 5; we obtain a 1,000-fold increase in actinomycetes and fungi when incorporating a balanced biological activator at the end of Phase 1.

Pure cultures of thermophilic fungi have been introduced recently as biological activators in Indoor Composts by Straastma *et al.* (1988), and Houdeau *et al.* (1991), following earlier extensive studies by Gerrits *et al.* (1962) and Olivier & Guillaumes (1978). Other proprietary formulas may include thermophilic microorganisms. It seems evident that the chemical and physical conditions of a compost may not be suitable for the development of such micoorganisms at the early stages of composting (Gerrits *et al.*, 1962), as far as pH, concentration of ammonium ions, and temperature

TABLE 4. Major groups of microorganisms that can be isolated during substate preparations for *Agaricus* cultivation. From Wood & Smith, 1988.

Bacteria	Actinomycetes	Fungi
Mesophiles		
Flavobacterium	*Streptomyces* spp.	*Mucor* spp.
Pseudomonas spp.	*Nocardia* spp.	*Aspergillus* spp.
Serratia marcescens	*Micropolyspora* spp.	*Penicillium* spp.
Thermotolerants		
Pseudomonas spp.		*Aspergillus fumigatus*
Bacillus licheniformis		
Thermophiles		
Bacillus coagulans	*Thermoactinomyces* spp.	*Torula thermophile*
Bacillus stearothermophilus	*Thermomonospora* spp.	*Humicola insolens*
Bacillus subtilis		*Rhizomucor pusillus*
		Talaromyces lanuginosa

TABLE 5. Number of microorganisms isolated at several stages duting a 2 Phase Higher Temperature Indoor Composting Process (activator added at end of Phase 1). From Laborde *et al.*, 1986.

	Beginning Phase 1	End 50°C Stage	End Phase 1	End Phase 2
BACTERIA				
No activator	10^{10}	10^{8}	10^{6}	$>10^{9}$
+ activator				$>10^{9}$
ACTINOMYCETES				
No activator	10^{7}	10^{5}	10^{3}	$>10^{3}$
+ activator				$>10^{6}$
FUNGI				
No activator	10^{5}	10^{3}	10^{3}	$>10^{3}$
+ activator				$>10^{6}$

levels, among other factors, are concerned. Also, in order to increase their chances, pure cultures should preferably be introduced in a previously sterilized (Huhnke, 1974), or semi-sterilized medium (Laborde *et al.*, 1984). This would almost completely eliminate the risk of competition from other thermophiles or even mesophiles.

On these grounds, the single phase medium temperature process does not permit to reach temperatures high enough to eliminate most undesirable microbes. In fact, yields obtained so far from Indoor Composts inoculated with pure cultures of thermophilic fungi are lower than when incorporating a mixed culture of bacteria, actinomycetes and fungi (Houdeau *et al.*, 1991) (Table 6).

When using raw materials relatively deficient in active microflora, like wheat straw, adding a strong inoculum may be stimulatory. Otherwise, the expected chemical reactions may not proceed as expected and the optimum selectivity may not be attained (Savoie *et al.*, 1992b).

Addition of microorganisms at a given composting stage, for example following a semi-sterilization at the higher temperature, may also be made more effective by the previous or simultaneous use of specific enzyme complexes (Savoie & Libmond, 1993 ; Hayes, 1992).

Composition of the inoculum to be introduced is very critical, since it may bring along some undesirable competitors or antagonists after the hot phase ; for example yellow molds. Ways of checking the inoculum quality have to be developed. Adopting a short peak heat following introduction might be safer, but it may also reduce the effectiveness of the activator before it becomes strongly established in the compost.

Such problems demonstrate clearly that successful Indoor Mushroom Composting depends in a large part on the definition and the critical use of an effective biological activator (Oldham, 1992).

TABLE 6. Addition of pure cultures of thermophilic fungi at the end of Phase 1 in the 2-Phase Indoor. Composting Process. Effect on yields. From Houdeau *et al.*, 1991.

	Yields (kg/ton)	% of control with mixed culture
Torula	225.4	86.5 %
Humicola	216.8	88.0 %

3. INDOOR COMPOSTING FACILITIES

Indoor composting has been going through a booming development in Italy for the past five years, under the leadership of the authors of this publication. In 1992, total spawned compost production in Italy was probably in excess of 500,000 tons, representing a mushroom output of over 125,000 tons, uncut. More than 60,000 tons of compost were made Indoor, under stictly controlled conditions. The three Italian co-authors of this publication contributed to the production of over 45,000 tons of spawned Indoor Compost, i.e. 3/4th of the total output. This is why they feel ready to share their experiences to-day.

All four systems which will now be presented and evaluated have adopted the double-phase higher-temperature process, with some important adaptations, for the three Italian large-scale facilities. The French INRA system, already presented, remains the same. It must be pointed out that in France, public research has the charge to do most of the work for developing a fully controlled composting process. In Italy, this remit falls within the competence of the private mushroom industry, and their technicians, to improve on existing technologies and to design new processes based, of course, on scientific principles.

Each one of the three cooperating authors from Italy, E. Giordani, B. Francescutti, and G. Lanzi, has perfected his own method. The four systems may be regrouped into two categories, A and B, A for Giordani and Laborde, B for Francescutti and Lanzi. All have adopted the same type of Phase 2. For A, Phase 1 is conducted in closed tunnels with strictly controlled environmental conditions. Uniform and high temperatures between 75 and 80°C are reached, killing most of the microbial biomass. Reinoculation with a biological activator must be carried out following Phase 1.

For B, tunnels with open doors are used for Phase 1. Temperatures in the range 60°-80°C are reached in about 75 % of the compost mass, 25 % remaining below that level. No activator is needed before Phase 2, only a good mixing of warmer and cooler zones to get a uniform distribution of the remaining microorganisms.

There are strong differences between the two systems. In Group A, uniformly high temperatures during Phase 1 are inducing a semi-sterilization of the substrate. A specific, useful inoculum, has to be thoroughly mixed with the compost before the medium temperature Phase 2. The selection and the definition of such an inoculum are of primary importance for the subsequent success of the process : they rely to a great extent on scientific progress in that field. Biological inoculum must be able to carry out the most useful transformations of the raw lignocellulosic materials, but also to contribute to the defence of the mushroom by creating a state of selectivity. This is probably the best technique for the future, but experimental data are lacking to finalize it.

In Group B, 25 % of the microbial biomass are staying alive in almost one fourth of the compost mass during Phase 1. Less than 75 % of the compost mass undergoes a partial sterilization. Following Phase 1, the mixing of both zones distributes evenly the microorganisms throughout the compost. These may be useful ones, as well as resistant forms of less desirable ones. Here also, reinoculation is made out of an unknown microbial biomass, which may vary from one tunnel to the other, and in function under highly variable external environmental conditions. Process control appears less efficient than in Group A.

Despite such important disparities between both groups, both ways of preparing Indoor Compost are presently successful, except for some small periods, 2 to 3 weeks in a row, where detrimental conditions may be expressed by reduced yields.

In fact, dry matter losses of raw materials are considerably smaller than those observed with classic outdoor composting ; the savings may represent a 20 % bonus. But average yields obtained

with Indoor Compost are always at least equal to, and more often larger than, those from traditional compost : 25 to 30 % for the traditional as compared with 30 to 36 %, and sometimes 40 % for the Indoor Compost (of uncut mushrooms and as related to the weight of spawned compost). Such yields explain the rapid expansion of Indoor Composting processes in Italy : savings in raw materials, in occupied areas, in duration of the process, resulting finally in a lower production cost of the mushroom associated with larger yields. Also, quality of hybrids is given as exceptional when going through the double-phase higher-temperature process.

4. COMPARATIVE DESCRIPTION OF EACH PROCESS

For the past three years, the company Agrifung of B. Francescutti has been making over 30,000 tons of Indoor Compost a year, prepared in specific "Agrifung type" tunnels and partly spawn-runned in tunnels. The Company G.S. Pleurotus of E. Giordani is presently preparing around 10,000 tons of Indoor Compost a year, in very sophisticated tunnels. Both the above companies make custom compost. G. Lanzi prepares Indoor Compost in four Italian Mushroom farms, with a yearly output of over 6,000 tons of spawned compost. The different processes are compared in Table 7 and the thermal treatments shown in Figure 3.

Demand for Indoor Compost is increasing so fast in Italy that most other compost facilities are now experimenting with, or switching to, that type of product.

TABLE 7. Comparative characteristics of the four processes.

Outdoor preparation	A1 (INRA)	A2 (GIOR.)	B1 (FRANC.)	B2 (LANZ.)
Raw materials				
Straw	+	+	+	+
Horse manure	+	+	-	-
Poultry manure	+	+	+	+
Gypsum	+	+	+	+
Ammonium sulphate	+	+	+	+
Shredding of straw ; toring of horse manure ; grinding of mix of poultry manure plus gypsum and ammonium sulphate. Water addition and mixing in very efficient machines. Mix immediately filled in tunnels or left in large pile overnight.				
Immediate fill	+	+	-	-
Overnight pile	-	-	+	+
Duration in days	0.5	0.5	1	1
Heat treatment : 1/Phase 1	A1	A2	B1	B2
Fill in open tunnels	-	-	+	+
Fill in closed tunnels	+	+	-	-
One filling	+	-	-	+
Two fillings (ea. 2 days)	-	+	+	-
Constant air recycling	+	+	-	-

Constant admission fresh air	+	-	-	-
Alternate admission fresh air	-	+	+	+
Steam addition	+	+	-	-
Air recycled in M3./T/Hr.	200	50-80	-	-
Fresh air in M3./T/Hr.	20	var.	var.	25-50
Regulation from 02 levels	-	+	+	+
Regulation from CO2 levels	-	-	-	-
Perforated concrete floor	-	-	+	-
Perforated nets	-	-	-	+
Other floor types	+	+	-	-
Tons compost per tunnel	1.5	100	350	40
Higher temperature reached	80	76	80	80
Lower compost temperatures	76	72	45	50
Average duration in days	3-5	4	4	4
End Phase 1				
Addition of Inoculum	+	+	-	-
Kind of inoculum	Compost	Comp/Air		
Heat treatment : 2/ Phase 2	A1	A2	B1	B2
Average duration in days	6	3	5	5
Short Peak Heat at 58°C	+	-	+	+
Conditioning temperature	50	50	50	50
Total duration of process in days				
Outdoor preparation	0.5	0.5	1	1
Phase 1	3-5	4	4	4
Phase 2	6	3	5	5
TOTAL DURATION IN DAYS	9.5-11.5	7.5	10	10

Additional information about each process

INRA

Raw materials always shredded or milled
Mixing in an auger-equipped mixer of raw ingredients and water
Immediate filling in tunnel for Phase 1.
Following Phase 1, emptying, addition of biological activator + water.
Filling tunnel for Phase 2, including peak heat and conditioning.
Constant volumes of air recirculated in process: over 200 m^3/ton/hr.
Constant volumes of admitted fresh air: 20 to 30 m^3/ton/hr.
Temperature regulated through steam addition.
Temperature reached in Phase 1: 80°C.

GIORDANI

Raw materials either milled or coarsely shredded. Three methods have been experimented; a,b and c; b being consistently the best one.

a) Milled ingredients:
Mixing of raw products and all the water in an auger-equipped mixer.
Immediate filling in tunnel for Phase 1.
Constant high volumes of recirculated air: 200 m^3/ton/hr.
Constant small volumes of admitted fresh air: 5 m^3/ton/hr.
Monitoring of O_2 levels, by constant analysis. O_2 must exceed 10%.
Uniform temperatures in Phase 1, around 75°C.
Following Phase 1, addition of biological activator, and water..
Activator is 5% by weight of pasteurized compost.
Refilling and short Phase 2 with excess fresh air, no peak heat.

b) Coarsely shredded or non shredded straw.
Phase 1 is run with 2 fillings and 2 emptyings at 2-day intervals.
Prewatering of ingredients to get 60% moisture et 1st filling.
Much reduced air flows (recirculation and intake) at this 1st stage.
Temperature reached at 1st stage: 72°C.
Emptying, water addition to get 70% moisture, and filling.
Strong activity needs high air volumes, 200 m^3/ton/hr. recirculation.
Fresh air admitted function of oxygen level.
Temperature reached at 2nd stage: 76°C.
Emptying; addition of biological activator (5% pasteurized compost).
Addition of water to get 75% moisture.
Filling for standard short Phase 2 with no peak-heat.

c) Activator introduced by air-flow.
Same as a), with milled ingredients; but tunnel is filled only once.
Emptying only once at end of Phase 2.
Activator = 20000 m^3 air extracted in 4 hours from tunnel stage end Phase 2.
Activator injected in tunnel stage end Phase 1 = 50 m^3/ton/h in 4 hours.

FRANCESCUTTI

Raw materials shredded.
Mixing and water addition in auger-equipped mixers.
Large pile for overnight stay: water added during the night.
Filling of 350 tons tunnel for 1st stage of Phase 1: doors left opened.
Small volumes of fresh air blown underneath the compost.
No recirculation of air; no steam addition.
Fresh air admission regulated by O_2 measurement: above 8% at all times.
Maximum temperatures: 80°C; Minimum; 45°C bottom and sides compost pile.
2 days later, emptying, mixing, water addition and refilling of tunnel.
Same 2-day process with no air recirculation.
Emptying, 4 days after 1st fill, 2 days after 2nd fill.
Water addition and mixing before filling tunnel for shorter Phase 2.
No biological activator added before Phase 2; only mixing of zones.
5 day Phase 2 with short peak heat at 58°C.
High volumes of fresh and recirculated air during Phase 2.

LANZI

Process very similar to Francescutti's, except for:
One 4-day Phase 1 with no intermediate emptying and filling.
40 ton tunnels. 12-24 hrs with 50 m^3 fresh air per ton.
Later, reduced fresh air admission to maintain high enough temperatures.
No air recirculation during Phase 1. No steam used. No O_2 measurement.
Tunnel emptied, mixed and refilled, after water addition, for Phase 2.
Activator (non pasteurized) added when too high temperature reached at Phase 1.
Generally, no activator added.
As activator, non pasteurized compost is logically better than pasteurized one.
Standard 5-day Phase 2 with excess air and short peak-heat.

5. DISCUSSION

Indoor Composting has several advantages over traditional composting:

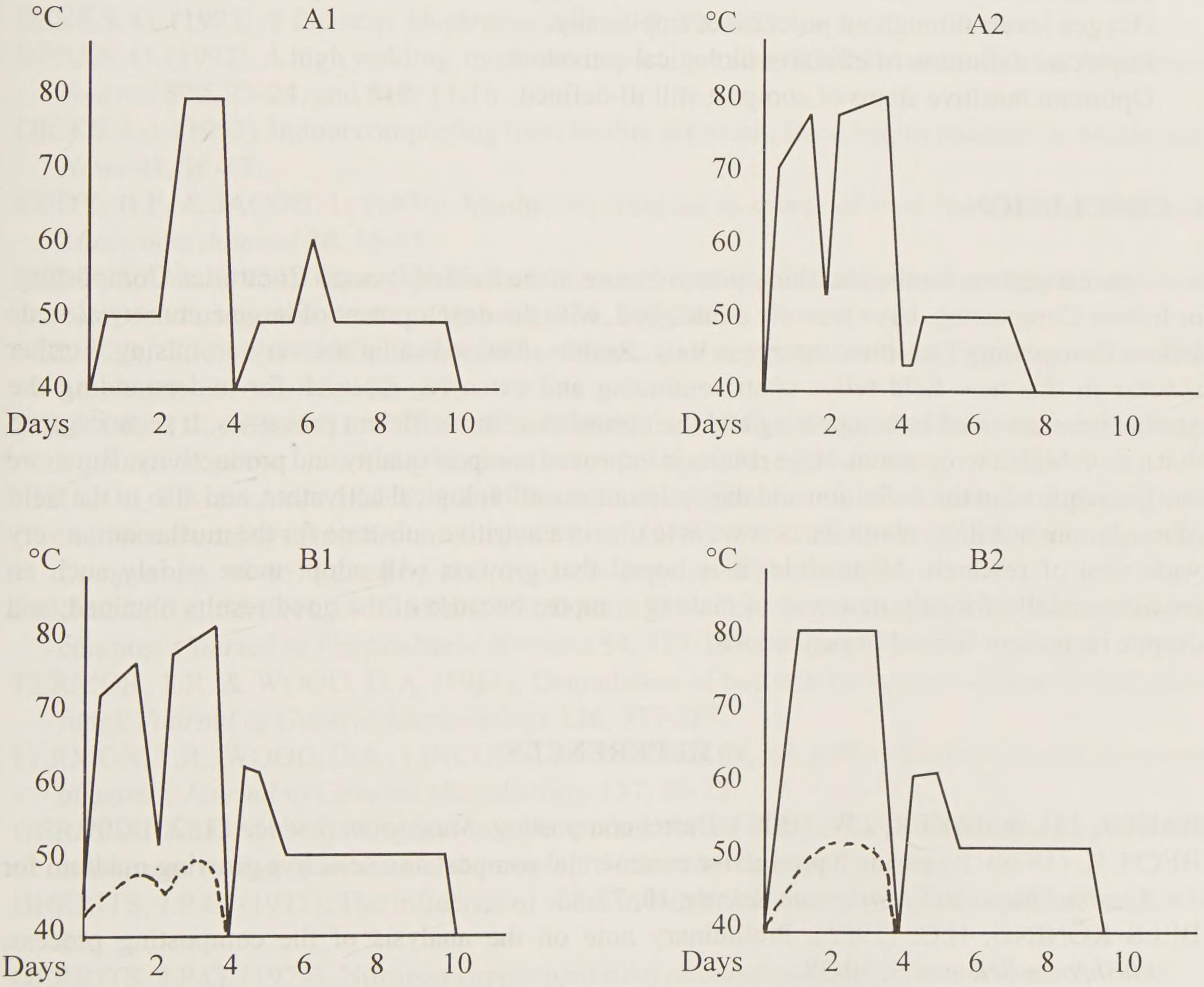


FIGURE 3. Thermal treatments in tunnels. —— Higher temperature; - - - lower temperature in compost.

1) Savings:
   savings in storing and working areas.
   savings in raw materials; more compost from starting material.
   savings in time and energy.
2) Controls:
   controlled substrate composition and physical condition.
   controlled environmental conditions.
   controlled process.
   controlled inoculation with optimum biological activator.
   controlled chemical and physical analysis.
   monitoring of CO_2, O_2, and NH_3.
   better sanitary control.
3) Yields and quality:
   in general, higher and better than for traditional composts. Good response of hybrids.

Problems:

Controlling means sophisticated facilities with computerized monitoring and regulation.
Heat treatment level, schedule and duration still experimental.
Oxygen levels throughout process set empirically.
Imprecise definition of effective biological activators.
Optimum nutritive status of compost still ill-defined.

6. CONCLUSION

Investigations for the past thirty years or more in the field of Process Controlled Composting, or Indoor Composting, have recently come good, with the development of large commercial scale Indoor Composting Facilities, mainly in Italy. Results obtained so far are very promising. Further success in this new field relies upon continuing and extensive research for understanding the mechanisms involved in composting and their translation into efficient processes. It seems agreed that a short higher temperature stage results in improved compost quality and productivity. But more work is required in the definition and the optimum use of biological activators, and also in the field of mushroom nutrition, about the best ways to obtain a nutritive substrate for the mushroom, a very wide area of research. Meanwhile, it is hoped that growers will adopt more widely such an environmentally-friendly new way of making compost because of the good results obtained, and despite its present limited imperfections.

REFERENCES

BAKER, J.D. & BAKER, J.W. (1981). Barrel composting. *Mushroom Science* **11**, 201-209.

BECH, K. (1978). Preparing a productive commercial compost as a selective growing medium for *Agaricus bisporus* . *Mushroom Science* **10**, 77-83.

BELS KÖNING, H.C. (1962). Preliminary note on the analysis of the composting process. *Mushroom Sciences* **5**, 30-38.

BELS-KÖNING, H.C., GERRITS, J.P.G., VAANDRAGER, M.H. (1962). Some fungi appearing towards the end of composting. *Mushroom Science* **5**, 165-169.

BOHUS, G. (1959). Investigations concerning the life processes of the cultivated mushroom. *Mushroom Science* **4**, 86-131.

CHALAUX, N., OLIVIER, J.M. & MINVIELLE, N. (1991). Bench scale composting and wheat straw biodegradability. *Mushroom Science* **13**, 207-214.

DELMAS, J. (1953). Orientation des recherches sur la culture du champignon de couche en France. *Mushroom Science* **2**, 118-123.

DERIKX, P.J.L., OP den CAMP, H.J.M., van der DRIFT, C. van GRIENSVEN, L.J.L.D. & VOGELS, G.D. (1990a). Odorous sulphur compounds emitted during production of compost used as a substrate in mushroom cultivation. *Applied and Environmental Microbiology* **56**, 176-180.

DERIKX, P.J.L., OP den CAMP, H.J.M., van der DRIFT, C. van GRIENSVEN, L.J.L.D. & VOGELS, G.D. (1990b). Biomass and biological activity during the production of compost used as a substrate in mushroom cultivation. *Applied Environmental Microbiology* **56**, 3029-3039.

DERIKX, P.J.L., SIMONS, F.H.M., OP den CAMP, H.J.M., van der DRIFT, C., van GRIENSVEN, L.J.L.D. & VOGELS, G.D. (1991). Evolution of volatile sulfur compounds during laboratory scale incubations and indoor preparation of compost used as a substrate in mushroom cultivation. *Applied and Environmental Microbiology* **57**, 563-567.

DERKS, G. (1973). 3 Phase 1. *Mushroom Journal* **9**, 396-403.

DERKS, G. (1992). A high yielding, modern compost, that simplifies the indoor system. *Mushroom Journal* **509**, 23-24, and **510**, 14-16.

DICKS, J.A. (1993). Indoor composting : new facility at Pleunis, Bree, begins production. *Mushroom News* **41**, 16-17.

EDDY, B.P. & JACOB, L. (1976). Mushroom compost as source of food for *Agaricus bisporus*. *Mushroom Journal* **38**, 56-67.

FERGUS, C.L. (1964). Thermophilic and thermotolerant molds and actinomycetes of mushroom compost during peak heating. *Mycologia* **56**, 267-284.

FERMOR, T.R. & WOOD, D.A. (1991). Mushroom compost microbial biomass : a review. *Mushroom Science* **13**, 191-199.

FERMOR, T.R., GRANT, W.D. (1985). Degradation of fungal and actinomycetes mycelia by *Agaricus bisporus*. *Journal of General Microbiology* **131**, 1729-1734.

FERMOR, T.R. & MACAULEY, B.J. (1990). Microbiological factors contributing to selectivity and nutritional quality of mushroom compost. *Proc. AMGA/ISMS International Seminar on Agaricus Compost* pp. 48-65. Edited by N.G. Nair. Sydney: Available AMGA.

FERMOR, T.R., SMITH, J.F. & SPENCER, D.M. (1979). The microflora of experimental mushroom compost. *Journal of Horticultural Sciences* **54**, 137-147.

FERMOR, T.R. & WOOD, D.A. (1981). Degradation of bacteria by *Agaricus bisporus* and other fungi. *Journal of General Microbiology* **126**, 377-387.

FERMOR, T.R., WOOD, D.A., LINCOLN, S.P. & FENLON, J.S. (1991). Bacteriolysis by *Agaricus bisporus*. *Journal of General Microbiology* **137**, 15-22.

GERRITS, J.P.G. (1968). Organic compost constituents and water utilized by the cultivated mushroom during spawn run and cropping. *Mushroom Science* **7**, 111-126.

GERRITS, J.P.G. (1971). The influence of water in mushroom compost. *Mushroom Science* **8**, 43-57.

GERRITS, J.P.G. (1974). Nitrogen supplementation of composts. *Mushroom Science* **9**, 77-98.

GERRITS, J.P.G. (1987). Bied composteren in tunnels perspectif. *De Champignoncultuur* **31**, 357-365.

GERRITS, J.P.G. (1988). Nutrition and compost. In *Cultivation of mushrooms*, pp. 29-72. Edited by L.J.L.D. van Griensven. Rustington - UK: Darlington Mushroom Laboratories.

GERRITS, J.P.G. & AMSING, J.G.M. (1991). Water relation in indoor compost. *Mushroom Science* **13**, 181-186.

GERRITS, J.P.G., BELS KÖNING, H.C. & MULLER, F.M. (1965). Changes in compost constituents during composting, pasteurization and cropping. *Mushroom Science* **6**, 225-243.

GERRITS, J.P.G., van GRIENSVEN, L.J.L.D., (1990). New developments in Indoor Composting (tunnel process). *Mushroom Journal* **205**, 21-29.

GERRITS, J.P.G. (1992). Trends in composting *Mushroom Journal* **508**, 46-51.

GOULAS, J.P. (1987). Production de peroxyde d'hydrogène par le mycélium du champignon de couche et sa contribution à la dégradation des constituants humiques du compost. *Agronomie* **7**, 853-858.

GULLIVER, A., MILLER, F.C., HARPER, E. & MACAULEY, B.J. (1991). Environmentally controlled composting on a commercial scale in Australia. *Mushroom Science* **13**, 155-164.

D'HARDEMARE, G. & TALON, J.L. (1974). Fermentation dirigée et controlée du compost en vrac. *Mushroom Science* **9**, 185-195.

HARPER, E., MILLER, F.C. & STUART, S. (1990). The energy balance of mushroom composting and production. *Proc. AMGA/ISMS International Seminar on Agaricus Compost*, pp. 68-79. Edited by N.G. Nair. Sydney.

HAYES, W.A. (1992). Absorbing the water and enzymes. *Mushroom Journal* **510**, 23.

HAYES, W.A. (1968). Microbiological changes in composting wheat straw/horse manure mixtures. *Mushroom Science* **7**, 173-186.

HOUDEAU, G., OLIVIER, J.M. & CHABBERT, B. (1991). Improvement of indoor short composting. *Mushroom Science* **13**, 215-220.

HUHNKE, W. & von SENGBUSCH, R. (1968). Mushroom cultivation on non composted nutritive substrates. *Mushroom Science* **7**, 405-419.

HUHNKE, W. (1971). Die Weiterentwicklung des Champignon-Anbauverfahrens auf nicht kompostiertem Nährsubstrat. *Mushroom Science.* **8**, 503-515.

JANSSEN, J., (1992). Selective composting ; peak heating ; indoor composting. *Australian M.G.A. Victorian Mushroom Laboratories,* pp. 1-26. Edited by SEAFORD. 3198 Victoria, Australia.

IMBERNON, M. & LEPLAE, M. (1971). Microbiologie des substrats destinés à la culture du champignon de couche : dénombrements, groupements fonctionnels. *Mushroom Science* **8**, 363-381.

KUHN, H. (1990). Why Kuhn Champignon in Switzerland had to build an indoor composting system and what it looks like. *Proc. of AMGA/ISMS International Seminar on Agaricus compost*. Edited by AMGA. Sydney.

LABORDE, J., DELMAS, J. & d'HARDEMARE, G. (1968). Preliminary note on some aspects of compost microbiology. *Mushroom Science* **7**, 187-203.

LABORDE, J. (1990). Moving towards a fully controlled mushroom substrate preparation : why a controlled preparation process? *Proc. AMGA/ISMS International Seminar on Agaricus Compost,* pp. 82:92. Edited by N.G. Nair. Sydney.

LABORDE, J. & DELMAS, J. (1969). La préparation express des substrats. *Bullatin FNSACC*: 2093-2109.

LABORDE, J., DELMAS, J., LAMAU, J.L. & BERTHAUD, J. (1971). La préparation express des substrats pour la culture du champignon de couche. *Mushroom Science* **8**, 675-706.

LABORDE, J., DELMAS, J. & DELPECH, P. (1978). Préparation rapide de substrats pour la culture

du champignon de couche : questions posées et tentative de réponse. *Mushroom Science* **10**, 85-103.
LABORDE, J. (1980). Rapid substrate making. *Mushroom Journal* 94: 349-361.
LABORDE, J., LOIRETTE BALDIT, N., DELPECH, P. & DELMAS, J. (1984). Rapid aerated compost for mushroom cultivation : preliminary trials. *ISMS International Symposium*, Budapest.
LABORDE, J., OLIVIER, J.M., HOUDEAU, G. & DELPECH, P. (1986). Indoor static composting for mushroom cultivation. In *Cultivating Edible Fungi*, pp.91-100. Edited by Wuest, Royse & Beelman. Elsevier.
LABORDE, J. (1992). Required qualities of indoor composting. *Mushroom World* **3**, 23-30.
LABORDE, J., HOUDEAU, G., BES, B., OLIVIER, J.M. & DELPECH, P. (1987). Compostage statique en salle : description du procédé ; analyses et principaux résultats. *Mushroom Science* **12**, 457-469.
LABORDE, J. (1991). Composting : current and future techniques in France and abroad. *Mushroom Information* **8**, 4-24.
LAMBERT, E.B. (1941). Indoor composting for mushroom culture. *U.S.D.A. Circular* 609.
LAMBERT, E.B., AYERS, T.T. (1952). An improved system of mushroom culture for better control of diseases. *U.S. Plant Disease Reporter* **36**, 261-268.
LAMBERT, E.B., AYERS, T.T. (1957). Thermal death times for some pests of cultivated mushroom. *U.S. Plant Disease Reporter* **41**, 348-353.
LEMKE, G. (1965). Kontrollmassnahmen beim Champignon-Kulturverfahren nach Till. *Mushroom Science* **6**, 393-402.
LEENDERTSE, I.K.D.K. (1988). Agrisystem Sohm Indoor Composting System. *Mushroom Journal* **181**, 427-431.
MILLER, F.C. (1992). Reducing the duration of traditional composting : consideration and consequences. *Mushroom World* **3**, 17-22.
MILLER, F.C. & MACAULEY, B.J. (1988). Odours arising from mushroom composting : a review. *Australian Journal of Experimental Agriculture* **28**, 553-560.
MILLER, F.C., DAHLBERG, K.R. (1990). Physical factors as determinants of composting activity and final compost quality. *Proc. AMGA/ISMS International Seminar on Agaricus Compost*, pp. 4-23. Edited by N.G. Nair. Sydney.
MULLER, F.M. (1965). Some thoughts about composting. *Mushroom Science* **6**, 213-223.
MURPHY, W.S. (1972). *Mushroom News* **20**, 4-22.
NAIR, N.G. & PRICE, G. (1991). A composting process to minimize odour pollution. *Mushroom Science* **13**, 205-206.
OLDHAM, S.L. (1992). Activate the microbes; are they pretting enough? *Mushroom Journal* **510**, 23-24.
OLIVIER, J.M., GUILLAUMES, J. (1978). Microbial evolution of composts during mycelium growth of *A. bisporus*. *Mushroom Science* **10**, 311-334.
OP den CAMP, H.J.M., DERIKX, P.J.L., van der DRIFT, C., VOGELS, G.D. & van GRIENSVEN, L.J.L.D. (1991). Odorous sulfur compounds emitted during conventional outdoor- and during indoor-composting. *Mushroom Science* **13**, 147-164.
PERRIN, P. & GAZE, R. (1987). Controlled environment composting in bulk chambers and in troughs. *Mushroom Science* **12**, 489-497.
POPE, S. & KNAUST, K. (1962). Production of compost by thermophilic fungi. *Mushroom Science* **5**, 123-126.
RANDLE, P.E. & HAYES, W.A. (1971). Progress in experimentation on the efficiency of

composting and compost. *Mushroom Science* **8**, 789-795.
RANDLE, P.E. & SMITH, J.F. (1986). Formulations for short duration mushroom composts. *Scientia Horticulturae* **28**, 37-45.
RENOUX-BLONDEAU, H. (1960). Etude de certaines actinomycètes se développant au cours de la pasteurisation du fumier. Leur action sur le développement ultérieur du champignon de couche. *Mushroom Science* **4**, 153-176.
ROSS, R.C. & HARRIS, P.J. (1983). An investigation into the selective nature of mushroom compost. *Scientia Horticulturae* **17**, 223-239.
SAVOIE, J.M., RICARD, I. & OLIVIER, J.M. (1992b). Microbial activities and transformations of substrates during composting, under controlled conditions, of straw plus corn steep liquor or poultry manure. *Mushroom Research* **1**, 103-114.
SAVOIE, J.M., CHALAUX, N. & OLIVIER, J.M. (1992a). Variability in straw quality and mushroom production : importance of fungicide schedules on chemical composting and potential degradability of wheat straw. *Bioresource Technology* **41**, 161-166.
SAVOIE, J.M. & LIBMOND, S. (1993). Stimulation of degradative activities by addition of polysaccharidases during environmentally controlled mushroom composting. *Applied Microbiology and Biotechnology*: submitted.
SENGBUSCH, R. von (1965). Das Till-Anbauverfahren für Champignons. *Mushroom Science* **6**, 389-391.
SINDEN, J.W. (1946). Synthetic compost for mushroom growing. *Pennsylvania Agricultural Experimental Station Bulletin* 482.
SINDEN, J.W. & HAUSER, E. (1950). The short method of composting. *Mushroom Science* **1**, 52-59.
SINDEN, J.W. & HAUSER, E. (1953). The nature of the composting process and its relation to short composting. *Mushroom Science* **2**, 123-131.
SMITH, J.F. (1974). Selective substrates and rapid methods of preparation. *Mushroom Journal* **23**, 423-426.
SMITH, J.F. & SPENCER D.M. (1976). Rapid preparation of composts in the production of the cultivated mushroom. *Scientia Horticulturae* **5**, 23-31.
SMITH, J.F. (1983). The formulation of mixtures suitable for economic, short duration mushroom composts. *Scientia Horticulturae* **19**, 65-78.
SOHM, D. (1989). The SOHM Indoor composting system. *Mushroom Science* **12**, 549-563.
STANEK, M. (1971). Microorganisms inhabiting mushroom compost during fermentation. *Mushroom Science* **8**, 797-811.
STOLLER, B.B., SMITH, F.B. & BROWN, P.E. (1937). A mechanical apparatus for the rapid, high temperature microbial decomposition of fibrous, cellulosic materials in the preparation of composts for mushroom culture. *Journal of the American Society of Agronomy* **29**, 717-723.
STOLLER, B.B. (1943). Preparation of synthetic compost for mushroom culture. *Plant Physiology* **18**, 296-414.
STOLLER, B.B. (1954). Principles and practice of mushroom culture. *Economic Botany* **8**, 48-95.
STOLLER, B.B. (1976). "No composting method". *Mushroom Journal* **42**, 185.
STOLLER, B.B. (1987). Quick composting. *Mushroom Journal* **180**, 379-383.
STONE, B. & LABORDE, J. (1990). Mushroom Nutrition and chemical aspects of composting. *Proc. AMGA/ISMS International Seminar on Agaricus Compost*, pp. 26-45. Edited by N.G. NAIR. Sydney.
STRAATSMA, G., GERRITS, J.P.G., AUGUSTIJN, M.P.A.M., OP den CAMP, H.J.M., VOGELS,

G.D. & van GRIENSVEN, L.J.L.D. (1989). Population dynamics of *Scytalidium thermophilum* in mushroom compost and stimulatory effects on growth rate and yield of *Agaricus bisporus*. *Journal of General Microbiology* **135**, 751-759.

STRAATSMA, G. & SAMSON R.A. (1993). Taxonomy of *Scytalidium thermophilum*, an important thermophilic fungus in mushroom compost. *Mycology Research* **97**, 321-328.

TILL, O. (1962). Cultivation of mushroom on sterile substrates and reutilisation of spent compost. *Mushroom Science* **5**, 127-133.

TROCME, S. & SARAZIN, A. (1956). Conditions d'utilisation d'un appareil mécanique dans l'étude de la préparation de fumiers pour champignons. *Mushroom Science* **3**, 165-171.

TRESCHOW, C. (1944). Nutrition of the cultivated mushroom. *Dansk Botanik Arkiv* **11**, 1-180.

TSCHIERPE, H. (1992). Are we headed in the right direction. *Mushroom World* **3**, 11-16.

Van GRIENSVEN L.J.L.D. (1993). The production of Indoor Cmpost; History and Prospects *Mushroom Information* **84**, 6-28.

WAKSMAN, S.A. & ALLEN, M.C. (1932). Comparative rate of decomposition of composted manure and spent mushroom compost. *Soil Science* **34**, 189-195.

WAKSMAN, S.A., CORDON, T.C. & HULPOI, N. (1939). Influence of temperature upon the microbial population and decomposing processes in composts of stable manure. *Soil Science* **47**, 83-114.

WAKSMAN, S.A., UMBREIT, W.W. & CORDON, T.C. (1939). Thermophilic actinomycètes and fungi in soils and in composts. *Soil. Science* **47**, 37-60.

WOOD, D.A. (1984). Microbial processes in mushroom cultivation : a large scale solid substrate fermentation. *Journal of Chemical Technology and Biotechnology* **34**, 232-240.

WOOD, D.A. & FERMOR, T.R. (1985). Nutrition of *Agaricus bisporus*. In *The Biology and Technology of the cultivated mushroom*. Chapter 4. John Wileys & Sons Ltd.,

WOOD D.A. & SMITH, J.L. (1988). The cultivation of mushrooms. *Mushroom Journal* **188**, 665-674.

CHAPTER 11

AN ABBREVIATED MUSHROOM COMPOSTING SYSTEM AIDED BY AN ACCELERATOR

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1. INTRODUCTION

Mushroom composting technology is currently facing a new problem of environmental pollution and several abbreviated composting procedures have been developed in response to this problem. Different terminologies such as express compost or PES (Laborde & Delmas, 1969), environmentally controlled compost or EC (Miller *et al.*, 1990), accelerated assisted compost or AAC (Nair & Price, 1990) etc, are currently being used to describe abbreviated composting processes. There have also been earlier attempts at developing rapid methods of composting (Till, 1962; Huhnke & Sengbush, 1968; Smith, 1974; Smith & Spencer, 1977) and today we have enough published documentation which indicate that the duration of composting can be reduced with little or no effect on mushroom production. The objective of an abbreviated composting system is to overcome the main disadvantages of the conventional process such as emission of objectionable odours, significant loss in dry matter and relatively low efficiency in process control. Limited or no outside composting and acceleration of the decomposition process are the main concepts that will enable us to achieve this objective.

This communication reports on some observations of the AAC process. We also point out that the mushroom industry will soon be subjected to environmental audit which will eventually lead to the greening of accountancy.

2. BACKGROUND TO THE AAC PROCESS

The AAC is characterised by the acceleration of biochemical and biological degradation of the basic compost materials. This has been brought about by the use of a specially formulated

TABLE 1. Compost formulation based on 1000 kg of wheat straw.

Raw material	Kg of fresh weight
Wheat straw	1,000
Poultry manure	883
Cottonseed hulls	444
Cottonseed meal	100
Gypsum	25
Lime	55

accelerator (proposed registered name: **PRITAN**). The accelerator encourages molecular changes in the basic materials and enables more-rapid decomposition. It also biochemically releases carbon for microbial activity.

The need for using a specially formulated accelerator stemmed from our preliminary trials on short composting. The raw ingredients (Table 1) were blended with the aid of a compost turner. Water was added to achieve a moisture content of 75% saturation. Steel bins with perforated basal plates were filled with the compost and mounted on a specially designed platform which enabled a sealed basal plenum to be created. The bins were placed in an enclosed chamber. The compost was then treated with aerated steam and composting carried out for 7 days. The pasteurisation was carried out at the end of 24 hours at 55°C (in the compost) for 3 hours and the conditioning temperature was 45 to 47°C for 5 days. The compost was cooled and spawned (with off-white hybrid strain) on the seventh day. We refer to this compost as short compost (SC). The spawned compost was filled into plastic bags, each bag containing 12 kg of compost. The bags were removed to environment-controlled experimental growing rooms and normal cropping procedures were carried out. The yield of mushrooms was recorded over a six-week cropping period.

The yield of mushrooms from the SC in two experimental trials is shown in Table 2 and it was relatively lower than that from the conventional compost. However, the analytical data of both the types of composts were similar in terms of total nitrogen and carbon, C:N ratio, pH and NH_4-N ammonium (Table 3).

The work of Holtz, Smith and Barkate (1975) pointed to the importance of lipid:protein ratio (L:P) as an index of compost quality. The reason for determining L:P ratio is based on the argument that the lipid content of the compost at spawning is the result of microbial activity. Therefore, one can assume that the majority of residual lipid at spawning is derived from microbial synthesis. The major sources of carbon for lipid synthesis are short chain or free carbohydrates. These compounds

TABLE 2. Yield of mushrooms from short and conventional composts.

Trial	Short Compost	Conventional Compost
a	9*	22
b	12	23

* Kg/sq.m
(after Nair and Price 1990)

TABLE 3. Analytical data of short compost and conventional compost at filling and at spawning.

	SC	CC	SC	CC
NH_4-N	0.18	0.18	0.07	0.01
Nitrogen(5)	1.52	1.87	2.01	2.58
Carbon (%)	47.80	37.70	37.00	36.10
Carbon:Nitrogen	24.80	20.16	17.61	13.99
Lipid:Protein	0.52	0.76	0.23	0.31
pH	7.50	7.60	7.00	7.10
Moisture (%) (approx.)	75.00	75.00	62.00	65.00

* SC = Short Compost; CC = Conventional Compost.
(after Nair and Price 1990)

are found in the compost as the hydrolytic products of cellulose. Based on the analysis of several compost samples, Holtz *et al.*, (1975) set the optimum level of L:P ratio at 0.8. If the ratio is > 0.8, it can be assumed that adequate carbon is available for microbial fat synthesis. However, if the value is <0.8 and, at the same time the C:N ratio is adequate, undercomposting (especially the breakdown of cellulose during composting) is indicated.

The L:P of the SC was < 0.8 and, since the C:N ratio of the compost was adequate, we concluded that the cellulose in the straw was not available for lipid synthesis by the resident microorganisms. Therefore, it was thought worthwhile to make the carbon available to the microorganisms by treating the straw chemically and biologically to hasten the release of straw cellulose.

In order to understand the need to use chemical, physical or biological agents to release straw cellulose, let us take a brief look at the structure of the straw. A lot of research has already been carried out by several workers on the anatomical and chemical characteristics of cereal straw. A characteristic of straw is that it mainly consists of highly lignified cell wall material which often constitutes up to 80% of the dry matter. These cell walls are mainly built up of structural polysaccharides and lignin. Plant cell walls generally contain three types of structural polysaccharides. In straw, the polysaccharide composition is rather simple with cellulose and xylans as the predominant components. Cellulose is one of the most abundant molecules in nature. Although it is apparently a simple molecule, being a linear polymer composed of up to 10,000 units (β-1, 4-linked glucopyranosyl), it is complicated by its three-dimensional structure. The conformation of cellulose favours the formation of bonds between the units in a chain and this bonding explains the mechanical strength of cellulose as well as its resistance to biological degradation.

The accessibility of cellulose to hydrolysis can be increased by treatment such as milling to increase the surface area, steaming or treatments with swelling chemicals to make the cellulose less crystalline and less hindered by associated compounds such as lignin or silica. Lignin is a bonding agent in the layers between cells and it contributes to further mechanical strength of cells.

There is, therefore, a need to treat straw in a suitable manner to release the tightly bonded cellulose for use by the microorganisms.

3. THE AAC PROCESS

The straw was chopped and blended with the other compost ingredients (Table 4) as uniformly

TABLE 4. Normal AAC formula.

		(kg)
Wheat straw	-	2000
Poultry manure	-	1500
Cottonseed hulls	-	800
Cottonseed meal	-	400
Gypsum	-	50
Lime	-	10
Crushed Sunflower seed	-	50
PRITAN accelerator 1.5 kg/1000 kg straw		
Moisture at fill = 72 to 75% saturation		

as possible. The mixture was then treated with the accelerator (PRITAN) and water added to bring the moisture content to 75% saturation. The compost mix was then subjected to the AAC process using steel bins as before and a commercial phase 2 tunnel. After filling the bins or the compost tunnel, the compost temperature was maintained at 42°C (range 42-47°C) for 72 hours followed by pasteurisation at 57°C (range 57-60°C) for 3 hours. The compost was then cooled to 25°C for spawning. The total duration of the composting was 5 days. Crops were grown in controlled environment commercial mushroom production rooms as well as in controlled environment experimental growing rooms.

The oxygen levels during the AAC process declined to 18.5% 6 hours after the start and then levelled at 20 to 20.5% for the remaining period. The concentration of NH_3 increased to 130 ppm 20 hours after fill and dropped sharply to 0 ppm during the following 45 hours. The NH_4-N decreased gradually from 375 ppm at the start to 150 ppm at the finish. The changes in the pattern of NH_4-N was reflected in the pH shift during the composting process. The level of total nitrogen increased steadily from 1.3 to 1.9 (average values). The ash content also increased from 14% to 23% during the AAC process. The compositional changes in AAC during processing showed a progressive breakdown and utilisation of carbon and nitrogen compounds (Table 5). The reduction in NH_4-N during the process points to microbial utilisation. The relatively high pH soon after the start of the

TABLE 5. Compositional changes in AAC during processing.

Day	Stage	NH_4-N (ppm)	N (%)	pH	Moisture (%)	Loss on ignition (%)	Lipids (%)
Friday	Fill	424	1.32	6.98	70.74	87.24	0.36
Saturday	-	536	1.40	8.31	68.97	85.21	0.43
Sunday	-	26	1.97	7.28	68.70	84.64	0.42
Monday	-	14	2.00	6.98	58.90	81.49	0.81
Tuesday	Spawning	12	2.00	7.00	65.00*	-	0.81

* Water added at spawning - (bag growing system).

TABLE 6. Comparisons of some physical characteristics of AAC and conventional compost.

Compost type	Replicate number	Wet bulk density (g cm^{-3})	Airfilled porosity (%)
AAC	1	1.00	76.2
	2	1.01	74.9
	3	0.99	73.6
	Mean	1.00[A]	74.9
Conventional	1	1.04	70.4
	2	1.01	69.0
	3	0.99	66.0
	Mean	1.04[B]	68.5

A = 1000 kgm^{-3}; B = 1040 kgm^{-3}

AAC process, generated by the high concentration of NH_3 at this stage, help in the disruption of covalent linkages between polysaccharides and lignin in the cell walls of the wheat straw (Iiyama *et al.,* 1990). This reaction, in conjunction with the cellulolytic activities of the enzymes and microorganisms in the accelerator PRITAN, help to breakdown the straw and allow microbial synthesis. The reduction in the C:N ratio from 26.8 at filling to 17.8 at spawning (Table 3) is indicative of microbial utilisation of carbon and nitrogen sources in the AAC. Such chemical changes lead to higher microbial biomass and aid in mushroom nutrition and compost productivity.

The wet bulk density of AAC appears to be similar to that of the conventional compost (Table 6). In fact, the density of AAC can be increased by increasing the manure: straw ratio (Table 7). Although increase in bulk density of conventional compost is correlated with increased mushroom production on a volume basis, composts of varying bulk densities produce similar crop yields based on the dry weight of compost (Randle & Flegg, 1985).

The yield of mushrooms from AAC ranged in the different trials from 120 kg/tonne of compost to 360 kg/tonne of compost. Data on the yield of mushrooms from 20 trials are presented in Table 8. All these trials were carried out in a commercial farm using the bag system of growing. The sudden drop in yield when the strain of spawn was changed from A357 to the S series (S35, S110 and S381) can be explained in part to inadequate crop management.

4. CONCLUSIONS

Observations made so far on the AAC process indicate that it can result in productive compost; however, consistent performance over a longer period of time is required before it can be considered a commercially viable system. The AAC process has overcome the common objections raised against abbreviated composting systems generally such as low yield of mushrooms, thermogenesis during spawn-run and casing, low bulk density, lack of nitrogen balance and non-selectivity. More importantly, the AAC process has overcome the odour and water pollution normally associated with the preparation of conventional compost. The fact that the mushroom industry will be subjected to

TABLE 7. Changes in the bulk density of AAC in relation to its formulation.

AAC mix		Weight of AAC per 1.67m² tray (kg)	Weight of conventional compost per 1.67m² tray	Decrease in compost/tray (%)
Wheat straw	27000[A]	115	160	28.1
Poultry manure	11000			
Gypsum	3500			
Lime	1000			
Wheat straw	2500[B]	154	160	3.7*
Poultry manure	10000			
Cottonseed meal	2000			
Gypsum1	2000			

* an increase of 24.4% of compost/tray.
A = N, 1.5%; C/N, 26.7
B = N, 1.9%; C/N, 20.7 (at fill)

TABLE 8. Yield of mushrooms from Accelerated Assisted Compost prepared in steel bins*.

Strain	Cropping days	Yield of mushrooms (kg) per bag	Yield of mushrooms (kg) per tonne of compost
A357	32	3.6	300
A357	24	4.3	350
A357	29	4.4	360
A357	41	3.7	310
A357	31	3.6	300
A357	32	2.4	200
A357	26	4.0	330
A357	27	3.9	320
A357	31	4.0	250
A357	31	3.2	200
A357	26	2.0	120
A357	27	2.2	130
A357	32	4.2	260
A357	29	3.7	230
S351	18	2.0	120
S110	24	2.6	160
S110	30	2.9	180
S381	30	2.3	140
A357	29	4.4	240
S381	30	4.7	260

* see text for description of bins (after Nair and Price 1990)

environmental audit is being increasingly realised. This means that a mushroom farm may be compelled to allocate funds for solving pollution problems on an annual basis. Anti-pollution expenditure would then be a normal component of company accounting procedure - the greening of accountancy. A farm would be judged as being well-managed on the basis of its commitment to antipollution strategy.

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REFERENCES

HUHNKE, W. & SENGBUSCH, R. VON (1968). Mushroom cultivation on non-compopsted nutritive substrates. *Mushroom Science* **7**, 405-409.

IIYAMA, K., LAM, T.B.T. & STONE, B.A. (1990). Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **29**, 733-737.

LABORDE, J. & DELMAS, J. (1969). *Bulletin de la federation nationale des syndicats agricoles des culticaterus de champignons* **184**, 2093-2109.

MILLER, F.C., HARPER, E.R., MACAULEY, B.J. & GULLIVERS, A. (1990). *Australian Journal of Experimental Agriculture* **36**, 287-296.

NAIR, N.G. & PRICE, G. (1990). Accelerated assisted compost - an abbreviated method of compost preparation. *Mushroom News*, November p.23-26.

RANDLE, P.E. & FLEGG, P.B. (1985). The effect of duration of composting on compost density and yield of mushrooms. *Scientia Horticulturae* **27**, 21-31.

SMITH, J.F. (1974). Selective substrates and rapid methods of preparation. *Mushroom Journal* **23**, 424-426.

SMITH, J.F. & SPENCER, D.M. (1976). Rapid preparation of compost suitable for the production of the cultivated mushroom. *Scientia Horticulturae* **7**, 197-205.

TILL, O. (1962). Cultivation of mushrooms on sterile substrates and reutilisation of spent compost. *Mushroom Science* **5**, 127-133.

CHAPTER 12

RECONSTRUCTION OF THE MUSHROOM INDUSTRY IN POLAND

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1. POLISH MUSHROOM INDUSTRY

The Champignon de Paris cultivation method was 'imported' from France at the end of the 19th century. The spawn was imported from France via Prussia. Before the Second World War, production was estimated at 100 tons a year. After the war, the stimuli for mushroom cultivation included the establishment of a spawn factory and the publishing of a handbook for mushroom growing. In 1960, the Horticulture Cooperative organized state controlled buying points where growers delivered their product. The farms themselves have always been private. Until 1985, practically all of the mushroom business was managed by the Horticulture Cooperative. This organization was responsible not only for the trading of mushrooms but also for organizing services, advice, endorsement of investment credit, granting of revolving credit and supporting the Mushroom Association in discussions with the Government, particularly with regard to taxation. That was very important because the mushroom growers were considered as small capitalists. In fact, good cooperation between the Cooperative and the Mushroom Association was very successful and the taxation level has never prevented the development of this branch. The export of fresh mushrooms began in 1961, first to Austria and then to former West Germany, and was supported by the Government since hard currency was highly welcome.

In 1970, Poland produced 2,000 tons of mushrooms. Ten years later this figure had risen to 22,000 tons including a 10% export level of fresh mushrooms.

In 1981, the Dutch processors discovered Poland to be a country with good quality and cheap mushrooms. The golden era for mushroom growers, Dutch processors, and particularly brokers, commenced. The export rate increased from 10% to 80% of total production. The price paid to the Polish blanching plants for the top quality-white button mushrooms was US$ 2.6 per kg and for lower quality between US$2.2 and US$1.8. The price paid to the growers was US$ 0.85.

In 1991, the harsh conditions imposed on Poland by the World Bank resulted in sharp increases in the price of energy and other commodities. The blanching business appeared to be unprofitable. Fortunately, the price of US$ 1.0-1.2 per kg, which was unacceptable for many blanching plants, has been accepted by the domestic market. The tradition of consumption and, more especially, the price

difference between meat products and mushrooms played important roles. The domestic market was considered interesting because the grower receives cash. Selling to blanching plants often required waiting for many weeks or sometimes months for payment. In a country with an inflation rate of almost 40%, this was not attractive (Swietlik, 1992).

The decreasing profitability in the last 2-3 years caused by increased production costs was evident from the drop in total production from 95,000 tons in 1990 to 65,000 tons in 1992. The reserves of cheap energy, cheap raw materials, etc. were completely exhausted. A number of growers suspended production, others used only part of their growing area (Pomiankowska, 1992).

2. STRATEGY PLAN

The Department of Edible Fungi at the Institute of Vegetable Crops in Skierniewice, in close cooperation with a group of growers, elaborated a strategy plan for the reconstruction of the Polish mushroom industry. The main features of this plan are as follows:

2.1. Strengths

+ a strong tradition in the production and consumption of mushrooms
+ abundant supplies of raw material for mushroom production
+ available energy sources (coal)
+ relatively low labour costs
+ existing growing houses with infrastructure
+ existing ' standard documentation ' for building a farm
+ a 30 year-old of tradition of exporting high quality, fresh, blanched and freshly canned mushrooms
+ close proximity to Germany, the largest mushroom importing country, and to other importing countries
+ an existing Mushroom Research Department
+ a handbook for growing mushroom under Polish conditions, and two mushroom bulletins
+ nowadays, the possibility to cooperate with western mushroom-supplying industries, and to attend international mushroom events.

2.2 Weaknesses

- relatively low yields per ton of compost
- lack of sufficient quantity of good quality compost
- seasonal production

2.3. Opportunities

* increasing domestic consumption
* an EEC import quota of 38,400 tons of mushroom per year
* increases in the yield per cycle, and in the number of cycles from 2 to 4 per year

Actually, the main reason for the poor profit was the relatively low and unstable yields, undoubtedly the result of incorrect pasteurization. However, in the simple mushroom houses, equipped with fans and a steam boiler, the growers are unable to properly fill the shelves with the

compost let alone control the whole complicated process. Nematodes are frequently found in samples of incorrectly pasteurized compost, and other important conditions are not optimized (Szudyga, 1991).

To overcome these difficulties, pasteurization needed to be done professionally. It was clear that pasteurization in tunnels was the correct way. The question now was, how to move the pasteurized compost to the small shelves on the farms without the necessity for heavy investment.

3. SOLUTION

As a solution, growing on compressed blocks was recommended (Vedder, 1978; Lelley, 1991). Since April 1992, at three locations in the the western, central and southern parts of Poland, the production of compressed blocks of spawned compost has been initiated.

4. PREPARATION OF COMPOST

Straw compost, based on rye straw and chicken manure, is used. The ingredients of the straw compost are :

1000 kg rye straw (15% moisture content)
1000 kg chicken manure litter (N-2.8%, 40% moisture)
75 kg gypsum
5000 litres water

The overall schedule for preparation is as follow:

Day minus 7 : wetting of the straw by pressing with a caterpillar in a laguna, mixing pretreated straw with chicken manure
Day minus 3 : mixing using front-loader and wetting
Day 0 : stacking the mixture in heaps 1.9 m wide and 2.2 m high
Day 3 : turning the stacks, addation of gypsum
Day 5 : turning the stacks
Day 7 : turning the stacks
Day 9 : turning the stacks and filling the tunnels

The above mixture produces, on average, 3,300 kg of compost with a moisture content of 72-74%, pH-8.2 and ammonium-nitrogen content, expressed as NH^{4+}, of 0.4% of dry matter.

The second phase of fermentation is carried out in tunnels (100 tons per tunnel). The process is computer controlled and comprises the following steps:

a) levelling the compost temperature
b) heating-up to pasteurization temperature
c) pasteurization at 57°C for 8 hours
d) cooling down to conditioning temperature
e) conditioning at 47°C
f) cooling down for spawning

It is important to emphasize that, during this process, the fresh air damper is regulated according to the optimum oxygen concentration in the circulated air. This allows for the 'breeding' of the required microflora without using steam.

Chemical analyses of the compost after pasteurization reveal the following:

(i) moisture content: 68-70%
(ii) pH: 7.5
(iii) NH^{4+}: 0.4 % based on dry matter
(iv) N-content: 2.2 % based on dry matter
(v) abundant presence of *Scytalidium thermophilum*

Pasteurized compost mixed with spawn (7 litres per ton) is pressed into a block, 0.5 x 0.4 x 0.14 m in size, and wrapped in plastic. Placed on pallets, the blocks are transported to the mushroom farms.

The size of the blocks are formed for a mushroom bed ranging from 1.2 to 1.6 m in width (recommended 1.4 m), so all existing shelves can be used without any correction and subsequent additional cost.

Immediately after collection of the required number of blocks, they are transported to the growers and there placed on the shelves as quickly as possible. The plastic on the surface of the blocks is removed and the surface then covered with paper. In autumn and winter, the compost is supplemented with Calprozyme at a concentration of 0.5%. Filling a room of 200 square metres requires 16 working hours. After 14 days of spawning, the compost is colonized and can be cased.

The casing soil is always prepared by using high peat and chalk to achieve a pH of 7.5. At present, the casing soil is prepared by the growers themselves but there is little doubt that, in the near future, professional casing soil makers will take over this role.

5. EXTENSION

Before the production of compressed blocks began in Poland, intensive training courses concerned with the technique of growing mushrooms on blocks of compost were organized. Quantities of compressed blocks were imported from Western Europe and demonstrations plots were adopted by good growers to collect information, particularly with regard to the productivity of the compost and the quality of mushroom (Saxon, 1992). The yields and the quality were surprisingly good and much better than previously achieved by the growers.

Simultaneously, training was provided on all cultivation procedures considered crucial for success including the preparation of casing soil, casing the beds, watering, ruffling, and air/compost temperature. In particular, the saturation of the casing soil and the type of ruffling required special emphasis to overcome some traditional habits of treating the mushroom beds.

Finally, it was important also to prepare the growers for the price of the blocks. It was necessary to discuss with the growers the calculation of the price because a simple comparison between the price paid previously (US$30 for one ton of green compost)] and the price of the blocks is not, of course, valid. The price of the blocks (US$77.5 per ton) includes the cost of the spawn, compost lost during the pasteurization (27 %), heating, labour costs for filling the green compost, spawning and tamping. Additionally, it was explained that if pasteurization ceased, the building would not be damaged.

Unfortunately, 27% of the price quoted above goes on banking costs. No aid is granted to the growers. It was only possible for private investors to bear the whole financial burden of the venture by significantly increasing yields. As a result of careful preparations made beforehand, the initial results have already been seen after only a few weeks of project implementation. The average yield has increased from 15 kg to 25 kg per square metre and the variable production cost per kg of mushroom decreased from US$ 0.71 to US$ 0.42 (calculated on the basis of the price of compressed compost). Since other costs (fixed assets, labour, general expenses) are not so important on a small

family farm, this decrease in variable production costs leads to increased profitability and provides a much needed boost to mushroom production.

This example illustrates the impact that investment can have when directed at a crucial link in the overall process. Investing US$700,000 in a mushroom producing region with existing infrastructure has created additional mushroom production valued at US$9,000,000.

REFERENCES

LELLEY, J. (1991). Pilzanbau: Biotechnology der Kulturspesepilze Stuttgart: Eugen Ulmer GmbII & Co.

POMIANKOWSKA, Z. (1992). Miedzynarodowy rynek grzybow. *Biuletyn Producenta Pieczarek* **3**, 32-34. (in Polish).

SAXON, N. (1992). Podstawowe problemy w uprawie pieczarek. *Biuletyn Producenta Pieczarek* **1**, 4-5. (in Polish).

SWIETLIK, J. (1992). Opodatkowanie produkeji grzybow w 1992 roku. *Biuletyn Producenta Pieczarek* **1**, 2-4. (in Polish).

SZUDYGA, K. (1987). Pieczarka. Edited by PWRiL, Warszawa.

SZUDYGA, K. (1991). Pieczarkarstwo na swiecie. *Biuletyn Producenta Pieczarek* **1**, 9-11. (in Polish).

VEDDER, P.J.C. (1978). Modern mushroom growing. Edited by Educabock, Culemborg.

CHAPTER 13

BIOLOGY OF ARTIFICIAL LOG CULTIVATION OF *AURICULARIA* MUSHROOMS

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1. INTRODUCTION

Auricularia mushrooms are a kind of jelly fungi, which are widely distributed in areas of the tropics and subtropics. *Auricularia*, the Jew's ear fungus, is a traditional food in China. Its nutritive and medicinal value has long been praised as "health food" (Yang, 1988). Annual production of fresh *Auricularia* species in Asia has been estimated to be 172 thousand tons with a value of about $255 million (Chang, 1985).

Auricularia species are reported to have been grown in China two thousand years ago. China has an ancient history in *Auricularia* mushroom utilization both as food and as a medicine. In recent years, artificial log cultivation for *Auricularia* mushrooms has undergone a great development in China.

2. CLASSIFICATION AND NATURAL DISTRIBUTION

Auricularia species are basidiomycetes. Their classification is based on the morphological characters and internal structure of the fruitbodies. It is reported that there are 15-20 species worldwide. Eight species, *A. auricula*, *A. polytricha*, *A. mesenterica*, *A. delicata*, *A. fuscosucoinea*, *A. peltata*, *A. cornea*, and *A. hispida*, have been identified in China. They are distributed in the tropical and subtropical areas or regions of China but the distribution of *A. auricula* is in the temperate zone regions and this species is found adventively in tropical areas.

Nowadays in China, *Auricularia auricula* and *A. polytricha* are widely cultivated by the artificial log method using diverse agricultural wastes.

3. MATING TYPE AND MONOKARYOTIC FRUITBODY

Both *A. auricula* and *A. polytricha* exhibit bipolar heterothallism. Their mating type is

controlled by one factor (Luo & Chen, 1989). Monokaryotic fruitbodies can be produced in the life cycle of *A. auricula*. However, this monokaryotic fruitbody is not ideal in terms of quality and quantity. Monokaryotic fruitbodies have not been observed in the life cycle of *A. polytricha*.

4. CONDITIONS FOR GROWTH AND DEVELOPMENT

4.1. Nutritive Conditions

The range of carbon source utilization for mycelial growth is wide. Monosaccharides, disaccharides and polysaccharides can be used as carbon sources. Monosaccharides (such as fructose, glucose, etc.), or maltose among the disaccharides, are the most suitable carbon sources for *A. auricula*.

Organic nitrogen sources are obviously superior to mineral nitrogen and only nitro-nitrogen used as nitrogen source is unsuitable for mycelial growth of *A. auricula*. Corn steep liquor, peptone and yeast extract is the most preferable nitrogen source.

The most suitable carbon-nitrogen ratio should be 35:1 and the amount of mycelium will decrease under conditions where the carbon-nitrogen ratio is higher or lower.

Different mineral elements and vitamins produce different effects on mycelial growth within a certain concentration range. Calcium and vitamin B_1 (0.5-1 ppm) facilitate mycelial growth of *A. auricula*. Cellobiose and refined peptone are the most suitable carbon and nitrogen sources, and vitamin B_6 has an obvious stimulatory effect on basidiospore germination.

4.2. Physical Conditions

Auricularia mushrooms are moderate temperature fungi. The temperature range for mycelial growth is 6-36°C with the most suitable temperature 22-30°C. Below 5°C and above 38°C, mycelial growth is restrained, but it has a strong tolerance to short-term cold and heat. The evolution and development of the fruitbody occurs between 15-27°C, but the best range is 20-24°C. The temperature range for basidiospore germination is 22-32°C.

At low temperatures, growth and development of *Auricularia* mushrooms is slow but the mycelium is strong and the fruitbody blacker and thicker, whereas at higher temperatures, the mycelium and fruitbody are weaker and thinner. At higher temperatures and air humidity, wood ear softening will appear.

Water plays an important role in the growth and development of *Auricularia* mushrooms. A water content of 60-65% in artificial logs is suitable for mycelial growth. Less water will affect the capacity of the mycelium to absorb and utilize nutritional substances, and more water will lead to a lack of oxygen and restrained mycelial growth. Air humidity of 90-95% is suitable for the formation and appearance of fruitbodies. Below 80% humidity, the fruitbody dries out and becomes shrivelled, and its growth and development stop.

Light plays an important role in fruitbody formation and the mushroom requires not only a quantity of diffused light but certain directly radiated light. The fruitbody thus formed is black and strong. A light intensity of 500 lux is preferable for fruitbody formation. Fruitbody formation does not occur in the dark. Mycelial growth is normal under dark conditions but diffused light has a facilitating action. *Auricularia* mushrooms are aerobic fungi. Air which is not fresh, or a lack of oxygen, will have an adverse effect on mycelial growth and fruitbody formation. Stagnant air causes

fruitbody deformities and soft rot, and the mushroom takes on the appearance of a toadstool.

4.3. pH

Vigorous mycelial growth occurs in substances where the pH ranges between 5.5 and 6.5. The growth rate of the mycelium markedly decreases at pH values below 5 and above 7.

5. CELLULASE AND LIGNINASE ACTIVITIES AND DEGRADATION OF THE CULTIVATION SUBSTRATE

Cellulose and lignin in the cultivation substrates are respectively lysed by extracellular enzymes and polyphenoloxidase produced by mycelia of *Auricularia* mushrooms.

Cellulases (C_1, C_x and β-glucosidase) and hemicellulase activities are closely related to the degradation rate of cultivation substrates and yields of fruitbodies. It is reported that a strain with the highest extracellular enzyme activity has the highest level of ligninase activity during the period of mycelial growth. However, the activity of ligninase is reduced during the period by primordia evolution. During this period, cellulase activity is higher than that of acid proteinase. By reason of the lignin in the substrates being degraded quickly, the amount of soluble carbohydrates produced not only supply the energy for mycelial growth but raise the carbon-nitrogen ratio. This produces an environment in which the mycelium reverts rapidly to generative growth. The increased cellulase activity during the generative period facilitates the formation of fruitbodies.

6. THE FIVE STAGES OF FRUITBODY DEVELOPMENT

The development process for fruitbodies in *Auricularia* mushrooms is divided into five stages, i.e. rice grain, coral, wood ear appearance, wood ear unfolding and maturation stage. The duration of each stage is 1.5, 1.5, 2, 1.5, 2 days respectively at 24°C and 95% air humidity.

7. ARTIFICIAL LOG CULTIVATION OF AURICULARIA MUSHROOMS

At present, artificial log cultivation of *Auricularia* mushrooms is very popular in China. Cotton seed shells, sawdusts, sugarcane, rice straw and corn residues are major cultivation materials. One of the major materials (90%) is mixed with 8% wheat and wheat bran, and 1% each of gypsum and sugar, and water is added. A water content of 60-65% is preferable for the mixed cultivation materials used for the artificial log. Rice straw must be softened in lime water at pH10 for 3 days if it is used as the major material.

The procedure for artificial log cultivation of *Auricularia* mushrooms is: preparation of artificial log - autoclave/sterilization - inoculation - incubation - control of fruitbody formation period - harvest. In general, the biological efficiency (i.e. ratio of fresh mushroom yield by 100kg dry substances), ranges from 70-80%.

REFERENCES

CHANG, S. T. & MILES, P. G. (1989). *Edible Mushrooms and Their Cultivation.* Boca Raton: CRC Press.

LUO, X. C. & CHENG, L. G. (1989). Sexuality and formation of monokaryotic fruitbody in *Auricularia auricula* and *A. polytricha. Mushroom Journal for the Tropics* **9**, 12-28.

LUO, X. C. & YANG, S. T. (1991). *Wood Ear Mushroom.* Chong-qin: Chong-qin University Press.

MA, W. Z. & LUO, X. C. (1992). Nutritional conditions of basidial spore germination and mycelial growth in *Auricularia auricula. Proceedings of Second China-Japan International Congress of Mycology*, p.107.

NIU, F. Z., YIN, G. I. & LIU, D. Z. (1989). Degradation of main chemical components and variation of enzyme activities in solid substrates during cultivation of *Auricularia. Proceedings of the International Symposium on Mushroom Biotechnology*, Nanjing, China. p.51.

CHAPTER 14

PROGRESS IN CULTIVATION TECHNIQUES FOR *AGARICUS BISPORUS* IN CHINA

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1. INTRODUCTION

The growing of *Agaricus bisporus* in China dates back about sixty years. Mushrooms produced at that time were marketed fresh as one kind of luxurious vegetable in only a few large cities (Huan,1987). From the beginning of the 1970's, in order to meet the requirements of the canning industry, the growing of mushrooms increased rapidly. However, most of the growers still operated on a small scale as a part-time job or a subsidiary family occupation. For years, mushroom growers attempted to increase the productivity of the crops, but in fact they gained little. In 1978, the reasons for the low yields were extensively studied and analysed as follows:

1. The composting process was primitive, no Phase II procedure was used.
2. The spawn used had not been well selected or bred, and the origins of spawn were confused.
3. The growing houses were not well constructed. There were neither cooling systems nor heating systems installed. The temperature and moisture could not be well controlled.

Research into these areas has been carried out in the main mushroom-growing provinces. In particular, the Fujian Mushroom Research and Development Station was established in the Fujian Research Institute of Light Industry, and is engaged in the problems mentioned above.

2. RESEARCH ON A NEW TECHNIQUE OF COMPOSTING (1978-1985)

Before 1978, the composting of straw and manure was only done outdoors for a duration of about one month with 5-6 turnings at an interval of 5-8 days each. After outdoor composting, the compost was filled on shelves and spawned. The average unit yield was only 3.6Kg/m^2. Under the instruction of Prof. S.T.Chang, The Chinese University of Hong Kong, the technique of Phase II composting was first introduced. Prof. Chang pointed out that the unit yield of mushroom was decided by whether or not growers adopt the technique of Phase II composting. In March 1979, on-the-spot Academic Exchange Meeting of Phase II composting was convened at the Fujian Putian experimental ground. Some simple type peak-heating systems which attemped to suit the circumstances

in the countryside were developed (Wang & Shou,1981). But, since a great deal of fuel would be consumed during the peak-heating stage due to the poor heat insulation of the growing house and the low efficiency of the heating systems, the popularizing of the technique of Phase II composting was restricted. In 1985, a modified mode of Phase II composting with low energy consumption was suggested after testing by the Fujian Research Institute of Light Industry. The main points were as follows.

1. Phase I composting was shortened to about 10 days.
2. The compost after Phase I composting was stacked in half bulk on the shelves (Figure 1) to increase the thickness of the compost and to impel the accumulation of heat.
3. The process of peak-heating or pasteurizing was arranged between two stages of conditioning (Figure 2).

The technique was characterized by the following: 1) The energy given out from the microbial fermentation could be fully utilized and much fuel would be saved. The first stage of conditioning spontaneously raised the temperature of the compost. Generally, no extra heat was required and the temperature of peak-heating would easily be reached by introducing just a small amount of steam. 2) Two conditioning stages would promote the growth of beneficial microbial populations and improve the quality of compost. 3) The total technique would be easy to learn and control, and convenient to popularize (Ke,1991). The technique led to a general increase in the unit yield of up to 15-30% and the unit yield was 4-5Kg/m^2. Fuel consumption was reduced by about 50%.

3. BREEDING OF STRAINS WITH HIGH PRODUCTIVITY AND OF GOOD QUALITY FOR SPECIAL PURPOSE CANNING (1983-1992)

Strains of *A. bisporus* were first introduced into China during the 1930's. Although the quality

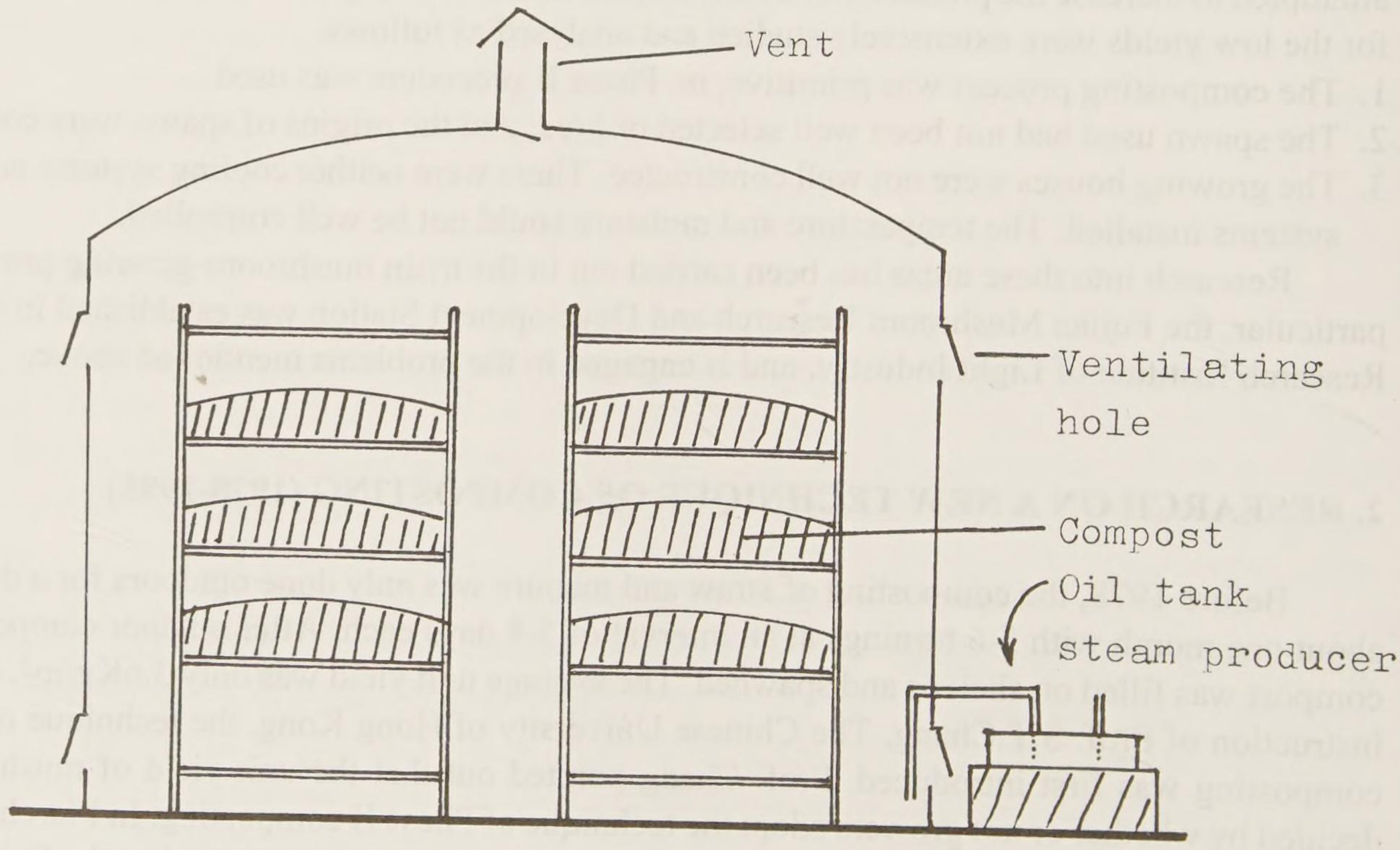

FIGURE 1. Sketch map of Phase II composting stacked in half bulk on the shelves.

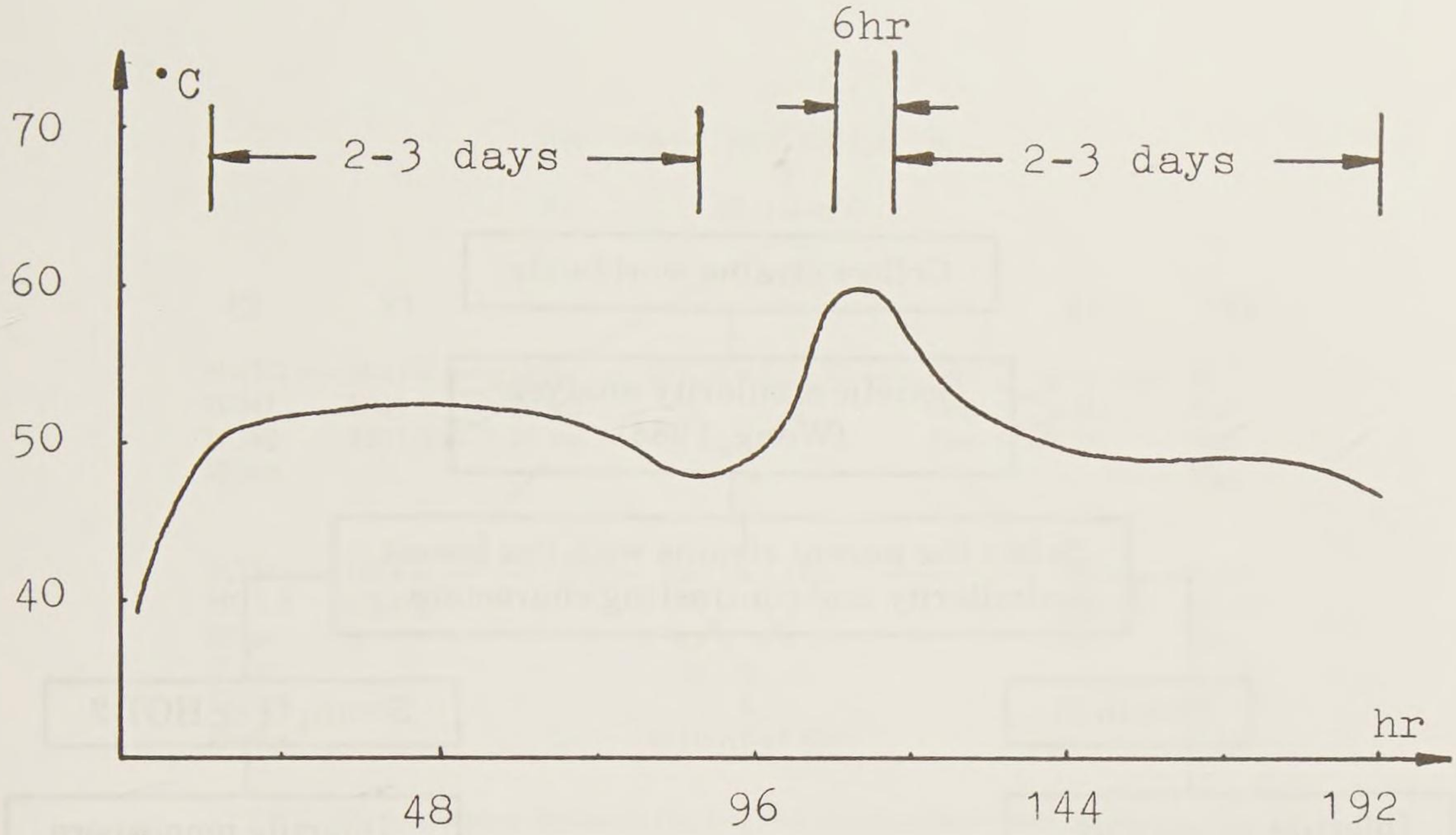

FIGURE 2. Diagrammatic variational curve of temperature in the compost and growing house during conditioning to culture first and pasteurizing afterwards. It shows the Modified Mode of Phase II composting.

of such strains was good, the unit yield was on the low side. From the end of the 1970's to the beginning of 1980's, some strains with better productivity were introduced, but the quality was unsatisfactory. In order to meet the need of the canning factories, new strains with high productivity and good quality suited for special purpose canning and also for Chinese cultivation conditions needed to be bred (Wang *et al*., 1983, 1989a, 1992). The Fujian Research Institute of Light Industry, Research Institute of Edible Fungi of Shanghai Academy of Agricultural Sciences, Zhejiang Agricultural University and the Institute of Food and Fermentation Industry of the Ministry of Light Industry, embarked on the necessary research. The Fujian Research Institute of Light Industry started the research on crossbreeding in 1983, and a series of cross-breeding techniques with practical value had been established after 6 years, including selecting hybrid parents, separating and purifying the tips of mycelia, identifying hybrids and predicting new strain characteristics (Wang, 1991; Wang, *et al*., 1989a,b, 1991a, 1992) (Figure 3). The main experimental work was concentrated on breeding new strains which combined certain good characteristics possessed by different types of parents, such as the combination of high productivity with good quality. Many types of hybrid strains, according to their isozyme patterns, were selected (Figures 4 and 5). For example, the esterase isozyme pattern of hybrid strain As1671 inclined to G type (Figure 6), and that of hybrid strain As2796 represented HG4 type (Figure 7). However, these hybrid strains have occupied 70-90% of the total spawn used in Fujian province and will soon completely replace old strains (Wang, 1992a). The characteristics of these strains may be generalized as follows: 1) Stronger resistance to natural climatic conditions; 2) High productivity combined with the good quality of the parent strains. As these hybrid strains have been adopted, the unit yield of mushrooms has increased by 20-50%, and the average unit yield in middle-sized testing grounds from 6-9Kg/m^2 to about 15Kg/m^2. They were well received by both growers and canning factories (Ke, 1991).

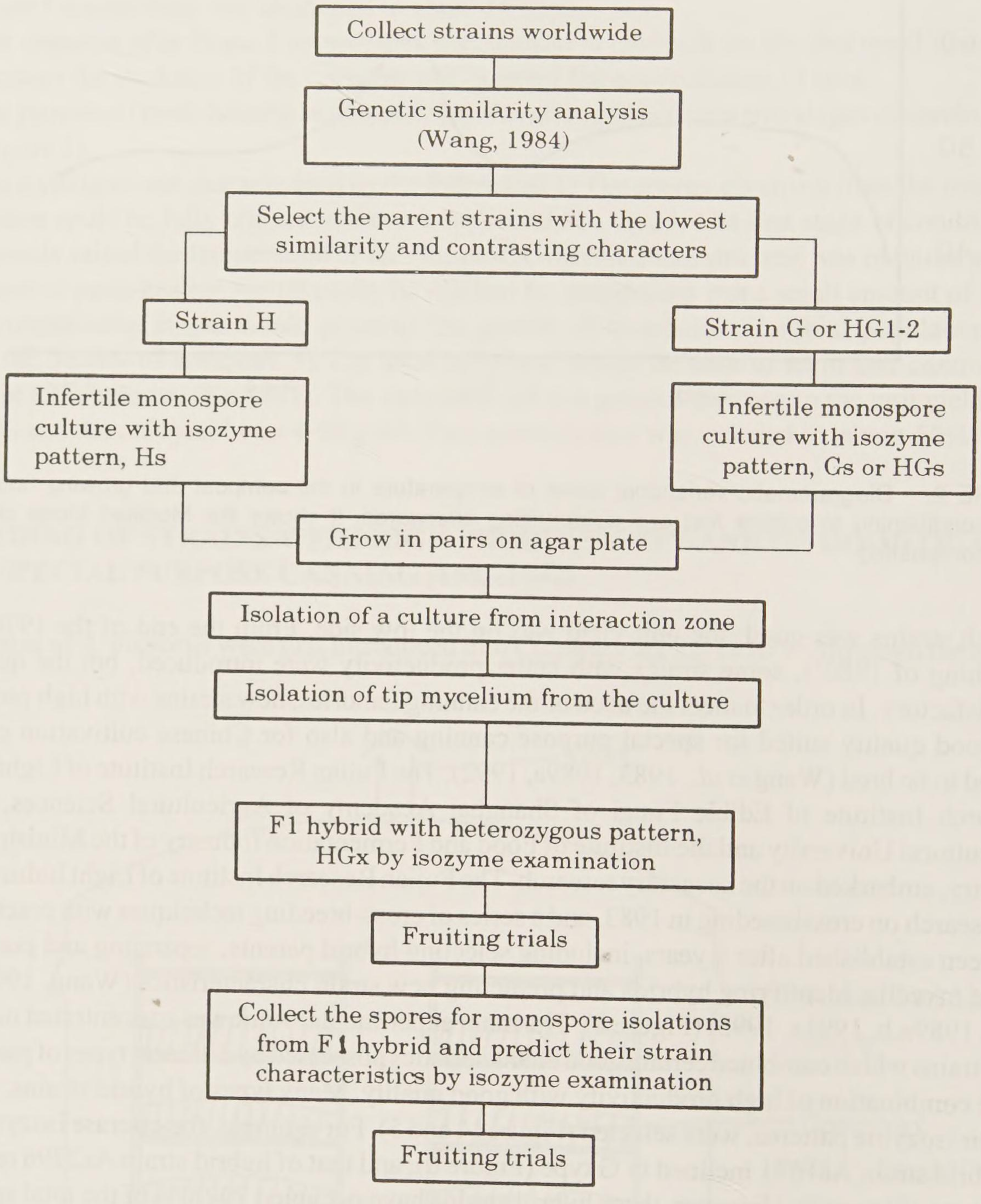

FIGURE 3. The crossbreeding program for hybrid formation in *Agaricus bisporus*.

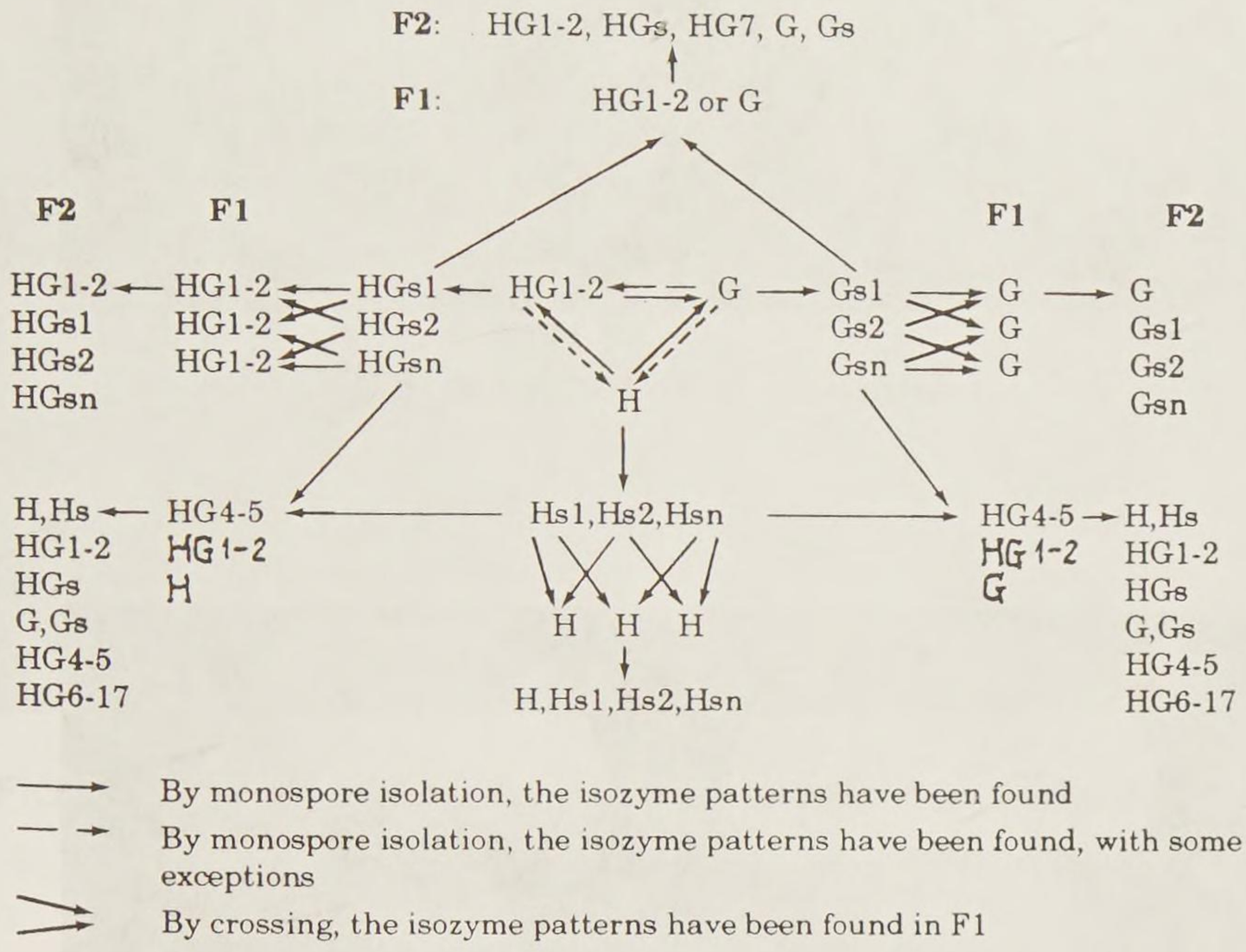

FIGURE 4. Analysis of genetic variations in traditional strains of *Agaricus bisporus* and their F1 and F2 hybrids by using esterase isozymes as biochemical markers.

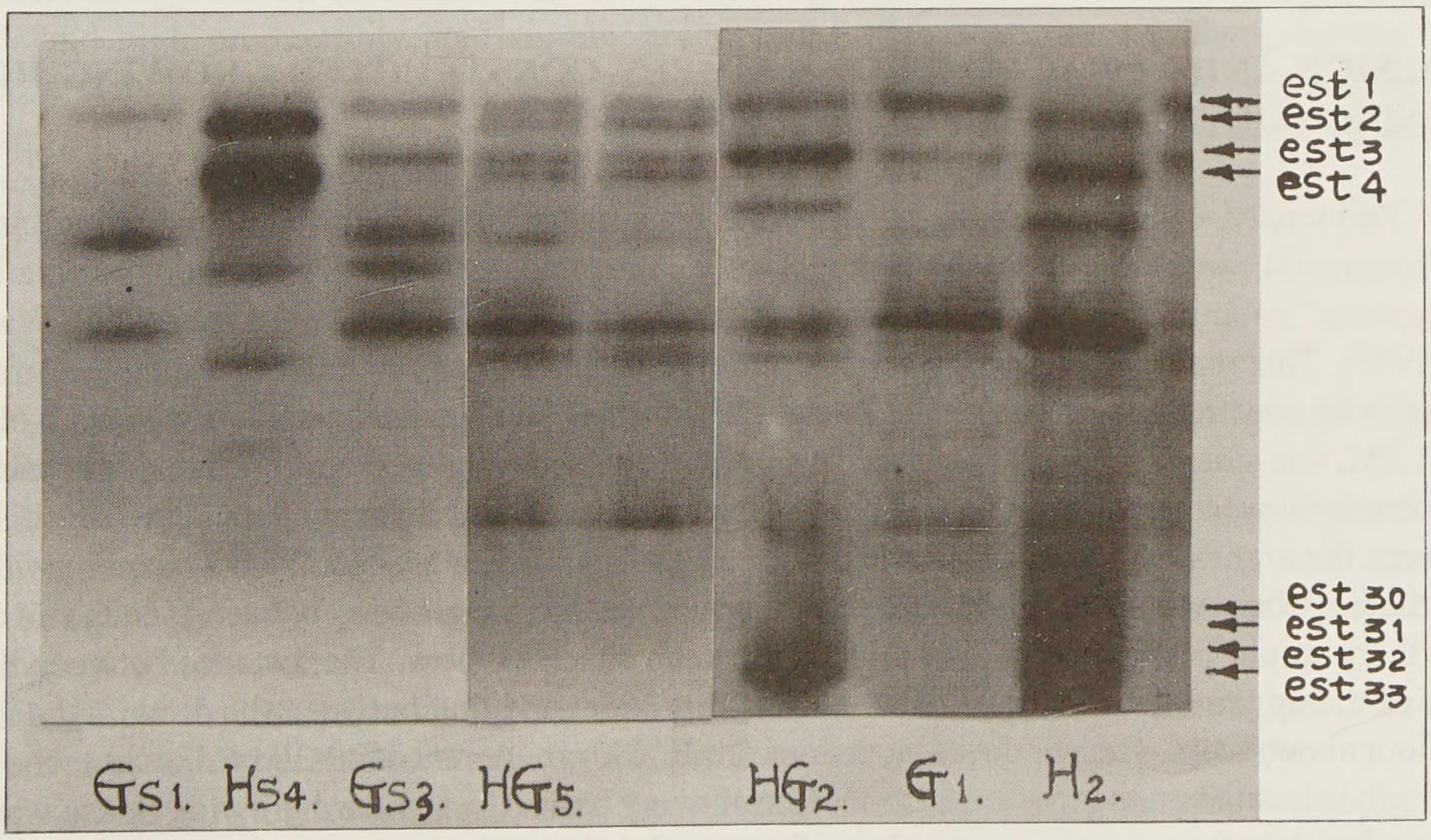

FIGURE 5. Esterase isozyme (EST) patterns of genetic variations in traditional strains, and their hybrid F1 and F2, of *A. bisporus*.

FIGURE 6. Hybrid strain As1671.

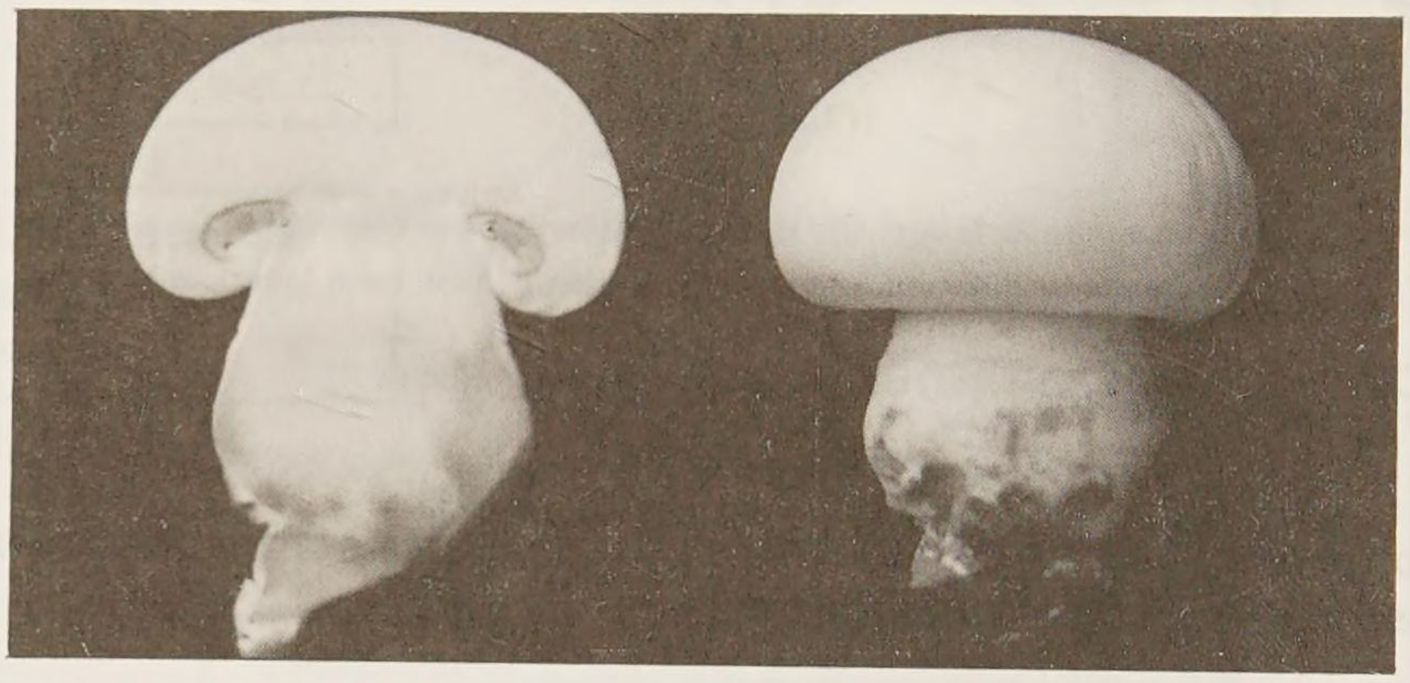

FIGURE 7. Hybrid strain As2796.

4. DESIGN AND APPLICATION OF A SIMPLY-CONSTRUCTED GROWING HOUSE USING POLYETHYLENE (P.E.) FILM (1988-1993)

Previously, Chinese growing houses were constructed mainly with clay and wood, and the environmental conditions could not be effectively controlled. In 1988, "The rules of cultivation of *A. bisporus*" submitted by the Fujian Research Institute of Light Industry were approved (Wang, *et al.*, 1990). This report advanced a kind of simple, economical, easy to build and manage, growing house to be constructed as follows: 1) Racks: the width of beds operated upon two sides would be 1.2-1.5M, one side, 0.8-1.0M. There are five beds in one rack arranged appropriately. The distance between the lowest bed and the floor should be 20cm, and each bed is about 60cm apart. The distance between the highest bed and ceiling should be at least 1m. The path would be 60-80cm in width. 2) Ventilation: there would be a buffer room at the entrance into the growing house. At each end of one path there would be a screen up window and a screen down window. The distance between the up-edge of the up window and eaves should be at least 30cm and that between the down window and the floor about 20cm. The window dimensions are 30X40 cm. A vent of 20-30cm diameter and 50cm in length is located on the roof over the middle of every path. The standard growing house was built using a bamboo framework in unused rice farming fields, tightly covered with polyethylene (P.E.) film and then with a rice straw curtain above. The area occupied by the growing house is about 50m^2,

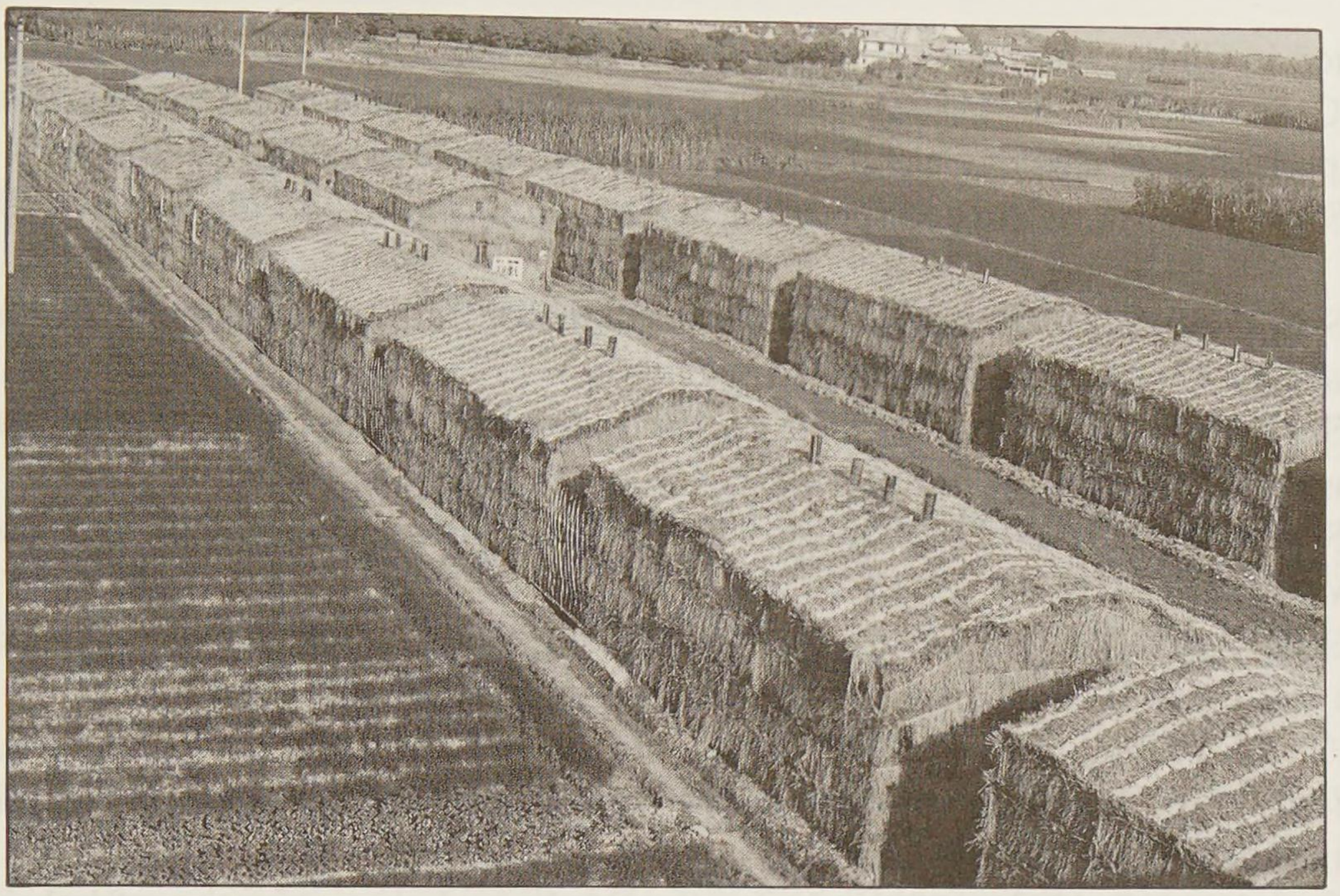

FIGURE 8. A specialized farm for standardized mushroom cultivation.

with a growing area of 130m^2.

During the growing season, from the winter 1992 to the spring 1993, there were about 8000 growing houses of this kind popularized in Fujian (Wang, 1992b). Many growers managed to use about 100 growing houses. For example, Mr.Y.H. Fang owned 135 growing houses of this kind in Putian (Figure 8) and has produced a total of 160 tonnes fresh mushrooms, average unit yield about 9Kg/m^2 and about 50% higher than that of a general growing house.

The energy saving modified Phase II composting technique, new hybrid strains with good quality and high productivity, and simple, practical P.E.film constructed growing houses have created a standard growing system for mushroom cultivation possessing Chinese features, and resulting not only in increases in yield of up to 50% (in some good cases, 150%), but also an obvious improvement in the quality of mushroom.

REFERENCE

HUAN, N.L. (1987). *Mushroom Science for self-studying*, Nanjing University Press, Nanjing (in Chinese). p.295.

KE, J.Y. (1991). Development on fermentation principle of mushroom compost and practice in China. *Experimental Report of Fujian Research Institute of Light Industry*.

KE, J.Y. (1991). A brief introduction of some new strains from Fujian Mushroom Research and Development Station. *Fujian Mushroom Journal* **1**, 141-148.

WANG, H.C. *et al*. (1983). Studies on breeding good strains of *A. bisporus* for canning and increasing the unit yield. *Experimental Report of Fujian Research Institute of Light Industry*.

WANG, Z.S. (1991). Genonomy analysis of the white *Agaricus bisporus* by isozyme electrophoresis. *Fujian Mushroom Journal* **1**, 19-25.

WANG, H.C. & SHOU, C.H. (1981). The development of Phase II composting for mushroom

cultivation in China. *Mushroom Science* **11**, 319-326.
WANG, H.C. & WANG, Z.S. (1989a). The prediction of strain characteristics of *Agaricus bisporus* by the application of isozyme electrophoresis. *Mushroom Science* **12**, 87-100.
WANG, Z.S. (1992). Progress of crossbreeding in *Agaricus bisporus*. *Edible Fungi of China* **11**, 10-12.
WANG, Z.S. (1992a). Popularization of the hybrid strains of *Agaricus bisporus* in Fujian province. *Mushroom Information of China* **7**, 10. (in Chinese).
WANG, Z.S. (1992b). Development of the standard growing system for mushroom cultivation in Fujian province. *Mushroom Information of China* **10**, 15. (in Chinese).
WANG, Z.S. & LIAO, J.H. (1990a). Study on the crossbreeding techniques of *Agaricus bisporus*. *Micol. Neotrop.Apl*. **3**, 1-12.
WANG, Z.S. & LIAO, J.H., LI, F.G. & WANG, H.C. (1991). Studies on genetic basis of esterase isozyme loci Est A,B and C in *Agaricus bisporus*. *Mushroom Science* **13**, 3-9.
WANG, Z.C. & WANG, H.C. (1989b). Study on the genetic variation of *Agaricus bisporus*. *Proceeding of the International Symposium on Mushroom Biotechnology*. Nanjing, China. Mushroom Biotechnology, pp. 329-338.
WANG, Z.S. & WANG, H.C. (1990b). Isozyme patterns and characteristics of hybrid strains of *Agaricus bisporus*. *Micol.Neotrop.Apl.* **3**, 19-29.
WANG, Z.C., WANG, H.C. & KE, J.Y. (1990). Standard syntheses of spawn and canning mushroom. Fujian local standard (recommended). p.14-20.

CHAPTER 15

FUNGAL- AND SUBSTRATE-ASSOCIATED FACTORS AFFECTING THE ABILITY OF INDIVIDUAL MUSHROOM SPECIES TO UTILIZE DIFFERENT LIGNOCELLULOSIC GROWTH SUBSTRATES

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1. INTRODUCTION

Vast quantities of organic wastes, particularly lignocellulosic materials, are generated annually through the activities of the agricultural, forest and food processing industries (Buswell, 1991). Currently, a large proportion of these wastes are either burnt, or shredded and composted and used for landfill or improving soil quality. However, with the application of appropriate bioconversion technology, these wastes represent a valuable resource especially for those regions with economies which are still largely agriculturally-based.

One of the most economically-viable processes for the bioconversion of many types of lignocellulosic wastes is represented by edible mushroom cultivation. Unlike chlorophyllous plants, mushrooms are unable to use solar energy for biosynthetic processes. Instead, they produce a wide range of extracellular enzymes that enable them to degrade complex organic substrates into soluble substances which can then be absorbed by the mushroom for nutrition (Wood & Fermor, 1982).

The ability of the different mushroom species to utilize various substrates will depend on both fungal- and substrate-associated factors. The major components of lignocellulosic wastes used for mushroom cultivation are cellulose, hemicellulose and lignin. Consequently, growth and fruiting of an individual mushroom species on a particular lignocellulosic substrate will depend largely upon the ability of the fungus to utilize the major components of that substrate as a nutritional source. This, in turn, will be determined by the mushroom's capacity to synthesize the hydrolytic and oxidative enzymes necessary to degrade the cellulose, hemicellulose and lignin components of the lignocellulose into low molecular weight compounds which can be readily assimilated. Substrate-associated factors include the presence of low molecular weight phenolic monomers which inhibit the growth of certain fungi. Lignocellulosic wastes of the type used for mushroom cultivation often contain phenolic monomers (Cherney *et al*. 1989), some of which are released during biological attack on the lignin component (Chen and Chang 1985). Several of these monomeric phenols are reported to inhibit

fungal growth (Akin and Rigsby 1985; Shea and Buswell 1992; Buswell and Eriksson, unpublished results) and the hydrolytic enzymes which catalyze the breakdown of the cellulolytic and hemicellulolytic constituents of plant cell walls (Borneman *et al.* 1986; Martin and Akin 1988).

In a recent study involving three mushroom species under commercial cultivation, *L. edodes, P. sajor-caju* and *V. volvacea*, both these major determinants have now been examined in an effort to explain the differing abilities of the three species to utilize different lignocellulosic wastes as a growth substrate.

2. LIGNOCELLULOLYTIC ENZYME PRODUCTION BY *L. EDODES*, *P. SAJOR-CAJU* AND *V. VOLVACEA*

2.1. Cellulases

L. edodes and P. sajor-caju both grew very poorly in agitated submerged culture on crystalline cellulose (Avicel). This poor growth is reflected by the absence of detectable cellulolytic activity (endoglucanase, exoglucanase and β-glucosidase) in culture supernatants. Moreover, no extracellular cellulolytic enzyme activity was detectable when these fungi were grown in a medium containing sawdust (*Castanopsis fossa*) and wheat bran. However, the two strains of *V. volvacea* included in this study (V14 and V34) grew well on crystalline cellulose, and endoglucanase, exoglucanase and β-glucosidase were readily detectable in culture supernatants. β-Glucosidase was also present in extracts of fungal mycelia. Similar patterns of enzyme production were observed when *V. volvacea*, strain V34, was grown in submerged culture on paddy straw in place of cellulose although enzymes levels were lower. Cellulolytic enzyme production by the three fungi is summarized in Table 1.

2.2. Xylanases

Birchwood xylan is a good growth substrate for *L. edodes* and the fungus produced relatively high levels of xylanase activity. No β-xylosidase was present in culture supernatants but was readily detectable in mycelial extracts. A similar pattern of enzyme production emerged when the mushroom was grown on a mixture of sawdust and wheat bran. *P. sajor-caju* also grew very well in agitated submerged culture with birchwood xylan, or a mixture of sawdust and wheat bran, as carbon source and produced appreciable levels of xylanase. Extracts of fungal mycelia grown under both sets of culture conditions also contained high levels of β-xylosidase. *V. volvacea* grew very poorly on extracted oat-spelt or birchwood xylan and no xylanase was detectable in the growth medium. However, when xylan was replaced by paddy straw, *V. volvacea* produced appreciable levels of extracellular xylanase and very high titres of intracellular β-xylosidase. Under these conditions, the mushroom also produced low but measurable levels of extracellular β-xylosidase. Xylanolytic enzyme production by the three mushroom species is summarized in Table 1.

2.3. Lignin-Transforming Enzymes

The activities of three recognized lignin-modifying extracellular oxido-reductases were examined in this study, namely lignin peroxidase (LiP) (Tien & Kirk, 1983; Glenn *et al.* 1983), manganese-dependent peroxidase (MnP) (Glenn & Gold, 1985) and laccase (Reinhammar, 1984). These enzymes may also play a role in the detoxification/removal of potentially inhibitory phenolic

TABLE 1. Lignocellulose-degrading enzyme profiles of *Volvariella volvacea* (V.v.), *Lentinus edodes* (L.e.) and *Pleurotus sajor-caju* (P.s.-c.).

Enzyme system	Enzyme	V.v.	L.e.	P.s.-c.
Cellulase complex	endoglucanase	+	ND	ND
	exoglucanase	+	ND	ND
	β-glucosidase	+	ND	ND
Hemicellulase complex	xylanase	+	+	+
	β-xylosidase	+	+	+
Lignin-degrading system	ligninase	ND	ND	+
	manganese-dependent peroxidase	ND	+	+
	laccase	ND	+	+

ND: Not detected.

compounds present in the substrate.

No LiP was detectable in cultures of *L. edodes* grown under various cultivation conditions. However, in stationary culture and under conditions of nutrient nitrogen limitation, this mushroom produces relatively high levels of MnP. Enzyme synthesis was suppressed by high concentrations of nitrogen in the culture medium. *L. edodes* also produced laccase; in this case, enzyme levels were unaffected by nutrient nitrogen availability (Table 1).

An H_2O_2-dependent veratryl alcohol oxidizing enzyme was present in culture supernatants of *P. sajor-caju* after 32 days growth in stationary culture. This mushroom also produced high levels of MnP and laccase (Table 1). MnP was detectable after only 4 days growth under both nitrogen-limiting and nitrogen-sufficient conditions although higher enzyme levels were recorded in low nitrogen cultures.

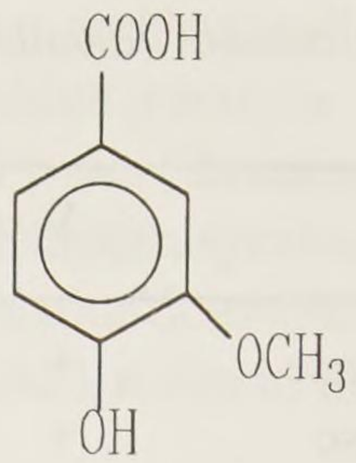
No ligninolytic enzyme activity has so far been detected in either of the two strains of *V. volvacea* used in this investigation (Table 1).

3. EFFECT OF LIGNIN-DERIVED PHENOLIC MONOMERS ON MUSHROOM GROWTH

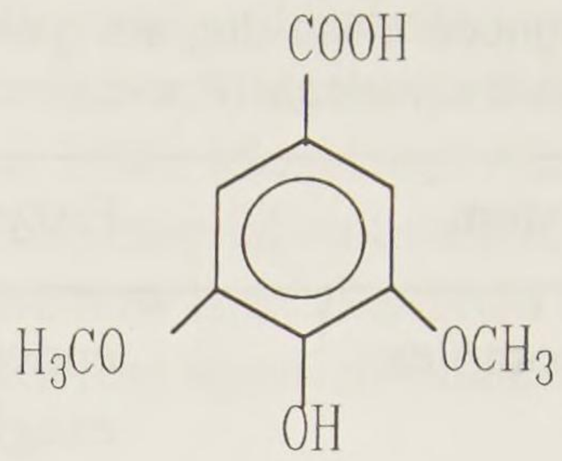
The chemical structures of the phenolic compounds used in this study are shown in Figure 1.

The effects of different concentrations of various phenolic monomers on the growth of *L. edodes*, *P. sajor-caju* and *V.volvacea* are shown in Figure 2 (Cai *et al.*, 1993). The most evident feature of the data is the generally higher tolerance to phenolics exhibited by *P. sajor-caju* compared to the other mushrooms. Of the benzoic acid derivatives tested, only syringic acid at concentrations above 1 mM was inhibitory. Supplementation of the culture medium with 1-10mM concentrations of vanillic, p-coumaric or caffeic acids, 1-5mM ferulic acid or 1mM syringate, actually resulted in

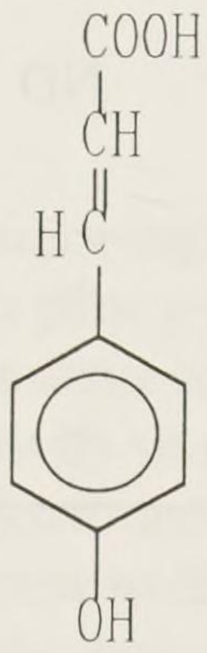
4–Hydroxybenzoic acid



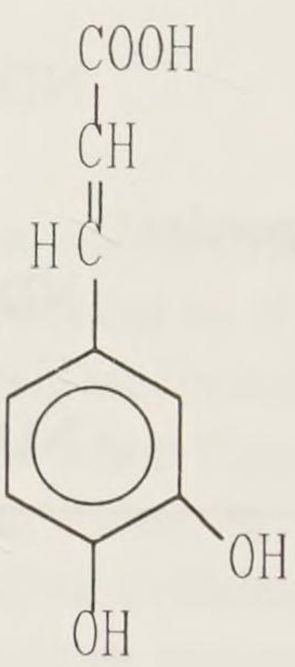
Vanillic acid



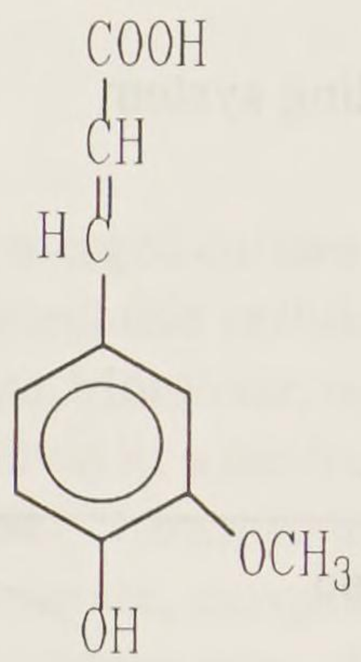
Syringic acid



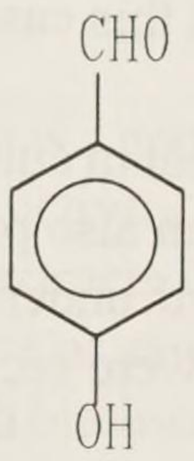
p – Coumaric acid



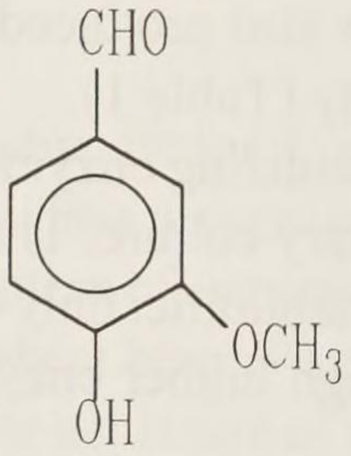
Caffeic acid



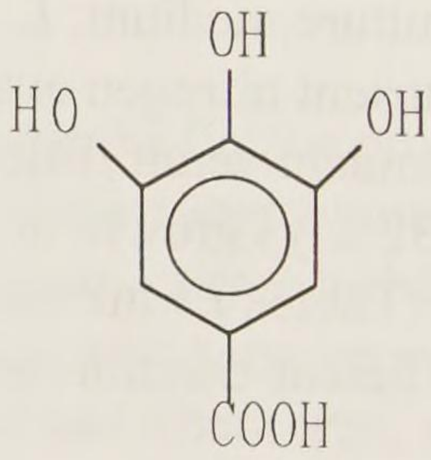
Ferulic acid



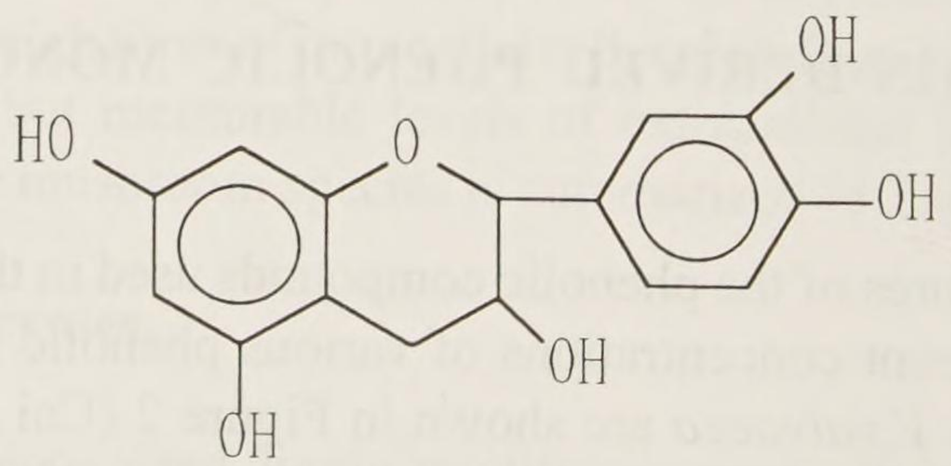
4–Hydroxybenzaldehyde



Vanillin



Gallic acid



Catechin

FIGURE 1. Chemical structure of lignin-related phenolic monomers and tannin compounds.

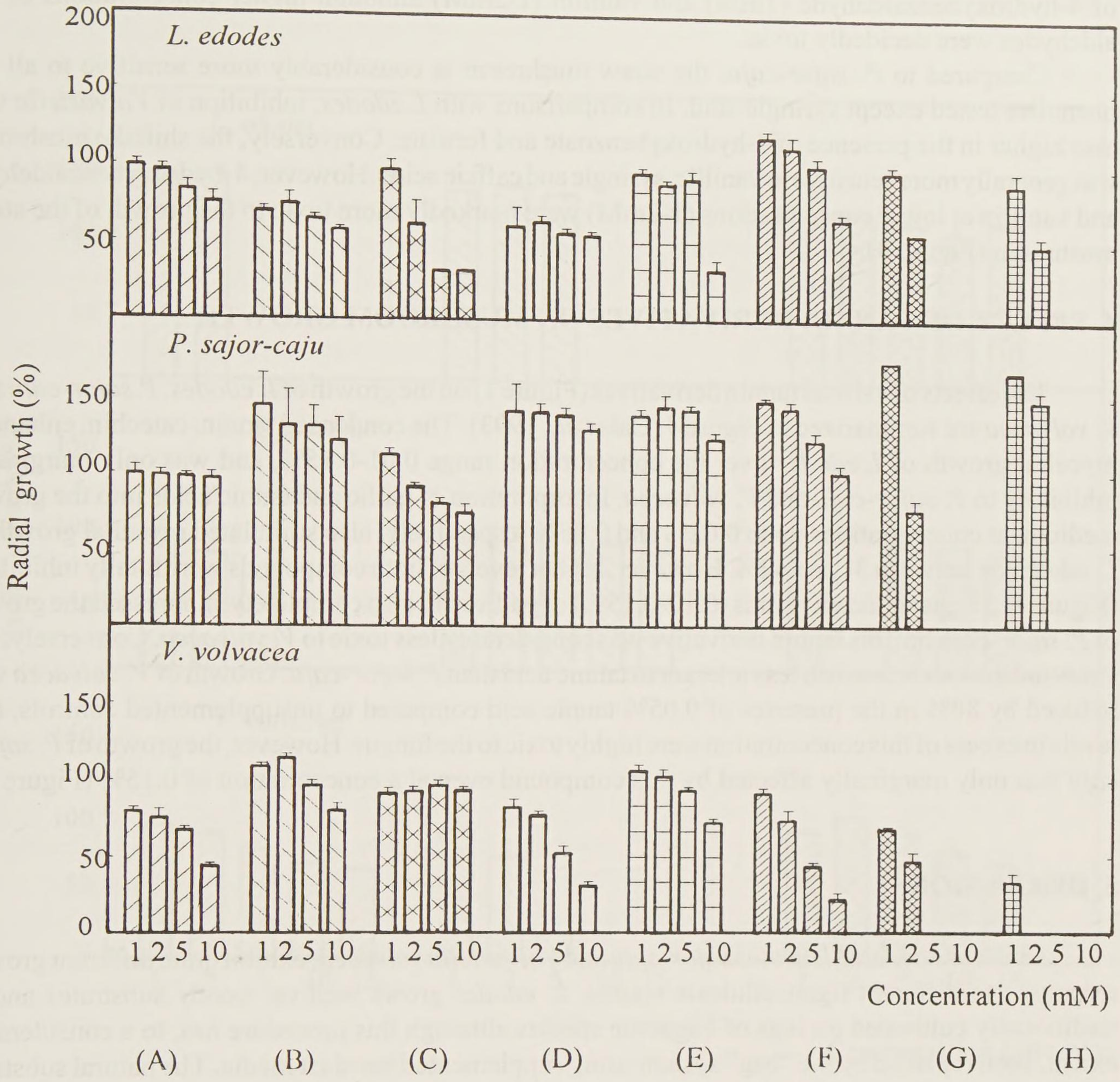


FIGURE 2. Effect of lignin-related phenolic monomers on the growth of *Lentinus edodes*, *Pleurotus sajor-caju* and *Volvariella volvacea*.

Values are expressed as a percentage of the growth recorded in the absence of aromatic compound and represent the mean of three replicates. The error bars represent the standard deviation. The growth rate of each fungus varied and the values presented are those recorded as follows: *L. edodes* (L.e), 252 hr; *P.sajor-caju* (P.s-c), 186hr; *V. volvacea* (V.v), 48hr. In all cases, fungal growth rates were linear with time when the values shown were taken. The aromatic compounds tested are:

(A) 4-hydroxybenzoic acid;
(B) 3-methoxy-4-hydroxybenzoic (vanillic) acid;
(C) 3,5-dimethoxy-4-hydroxybenzoic (syringic) acid;
(D) 4-hydroxycinnamic (p-coumaric) acid;
(E) 3,4-dihydroxycinnamic (caffeic) acid;
(F) 3-methoxy-4-hydroxycinnamic (ferulic) acid;
(G) 4-hydroxybenzaldehyde;
(H) 3-methoxy-4-hydroxybenzaldehyde.

a marked stimulation of fungal growth. Increased growth was also observed in the presence of 4-hydroxybenzaldehyde (1mM) and vanillin (1-2mM) although higher concentrations of the aldehydes were decidedly toxic.

Compared to *P. sajor-caju*, the straw mushroom is considerably more sensitive to all the phenolics tested except syringic acid. In comparisons with *L.edodes*, inhibition of *Volvariella* was also higher in the presence of 4-hydroxybenzoate and ferulate. Conversely, the shiitake mushroom was generally more sensitive to vanillic, syringic and caffeic acids. However, 4-hydroxybenzaldehyde and vanillin at lower concentrations (1-2mM) were markedly more toxic to the growth of the straw mushroom (Figure 3).

4. EFFECT OF TANNIN DERIVATIVES ON MUSHROOM GROWTH

The effects of various tannin derivatives (Figure 1) on the growth of *L.edodes*, *P.sajor-caju* and *V. volvacea* are summarized in Figure 4 (Cai *et al*., 1993). The condensed tannin, catechin, enhanced mycelial growth of *L.edodes* over the concentration range 0.01-0.15%, and was only marginally inhibitory to *P. sajor-caju* and *V. volvacea*. Incorporation of gallic and tannic acids into the growth medium, at concentrations up to 0.02% and 0.05% respectively, also stimulated mycelial growth of *L. edodes* by between 31 and 42%. However, higher levels of both compounds were totally inhibitory (Figure 4). Higher concentrations (0.10-0.15%) of gallic acid also completely suppressed the growth of *P. sajor-caju* but this tannin derivative was considerably less toxic to *V. volvacea*. Conversely, the straw mushroom was much less tolerant to tannic acid than *P. sajor-caju*. Growth of *V. volvacea* was reduced by 80% in the presence of 0.05% tannic acid compared to unsupplemented controls, and levels in excess of this concentration were highly toxic to the fungus. However, the growth of *P. sajor-caju* was only marginally affected by this compound even at a concentration of 0.15% (Figure 4).

5. DISCUSSION

Lentinus edodes, Pleurotus sajor-caju and *Volvariella volvacea*, exhibit quite different growth responses to different lignocellulosic wastes. *L. edodes* grows well on woody substrates and is traditionally cultivated on logs of Fagaceae species although this procedure has, to a considerable extent, been replaced by the "bag" system using supplemented sawdust media. The natural substrate for *V. volvacea* is paddy straw which has a relatively low lignin content (Dale, 1987). The preference of this mushroom for `less-lignified' substrates is exemplified by the increased production yields obtained when *V. volvacea* is cultivated on cotton wastes (Chang, 1991). *P. sajor-caju* is the most adaptable of the three species and can be grown on a wide variety of agricultural waste materials of differing composition in terms of polysaccharide/lignin ratio.

Although the cellulolytic and hemicellulolytic capacity of *V. volvacea* is clearly evident, the mushroom's aversion for highly lignified substrates is undoubtedly reflected in the apparent inability of the fungus to synthesize any of the recognized lignin-transforming enzymes. We have detected no laccase, lignin peroxidase or manganese peroxidase activity in the two strains of *V. volvacea* used here even though the fungus was grown under a range of culture conditions which support the production of these enzymes by other fungi (Cai, Buswell & Chang; unpublished results). The cellulose and hemicellulose components of the plant cell wall are intimately associated with the lignin moiety which presents a barrier to the hydrolytic enzymes catalyzing the degradation of the polysaccharides (Buswell, 1991). Thus, since *V. volvacea* lacks a ligninolytic or lignin transforming

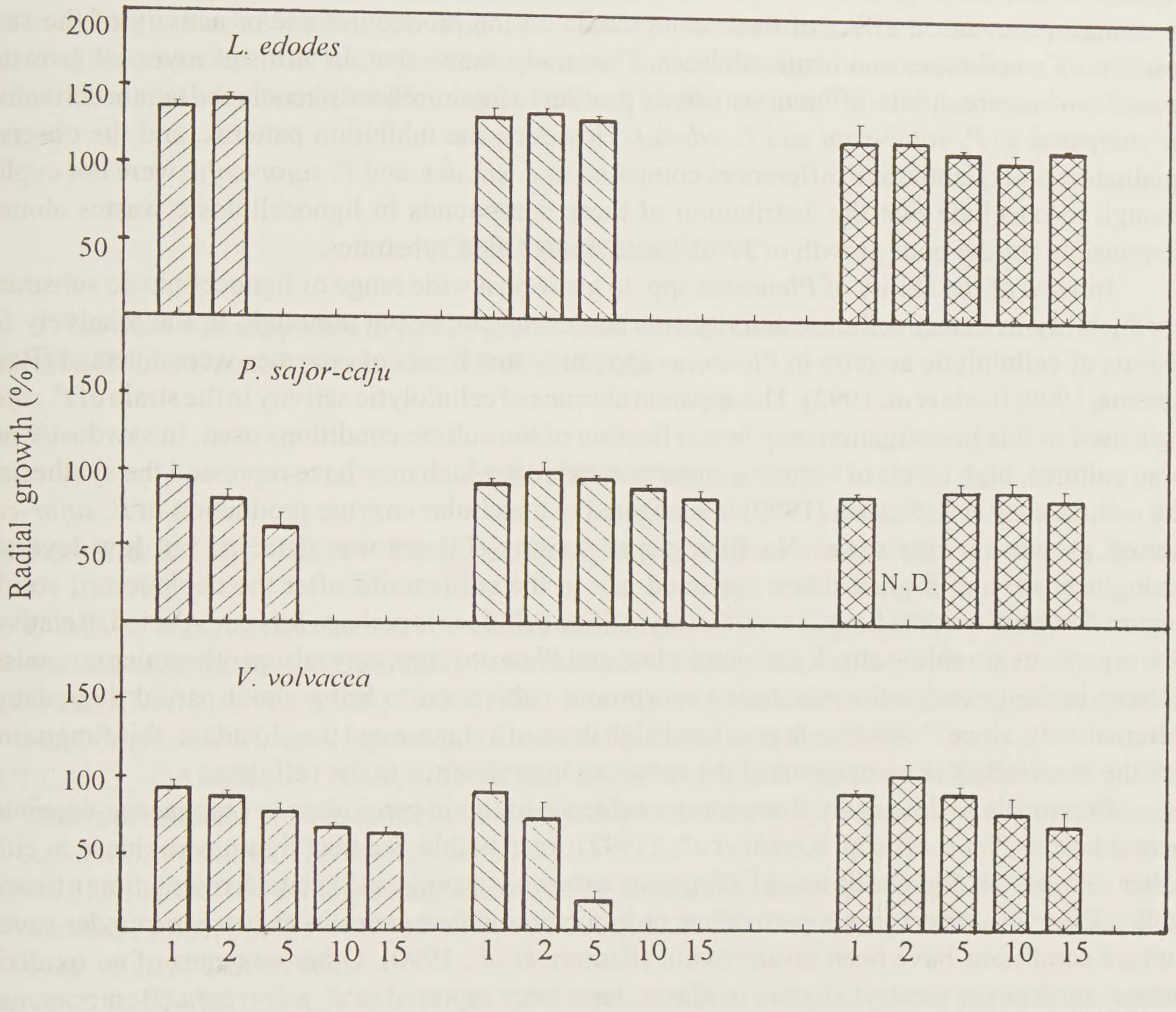


FIGURE 3. Effect of tannin derivatives on the growth of *Lentinus edodes*, *Pleurotus sajor-caju* and *Volvariella volvacea*.

Values are expressed as a percentage of the growth recorded in the absence of tannin compound and represent the mean of three replicates. The error bars represent the standard deviation. The growth rate of each fungus varied and the values presented are those recorded as follows: *L. edodes* (L.e), 264hr; *Pleurotus sajor-caju* (P.s-c), 168hr; *V.volvacea* (V.v), 42hr. In all cases, fungal growth rates were linear with time when the values shown were taken. The aromatic compounds tested are:

(GA) gallic acid;
(TA) tannic acid;
(CAT) catechin.

system, fungal access to the polysaccharide components will be restricted thereby markedly reducing the capacity of the fungus to grow and fruit on lignified substrates.

An alternative, or supplementary, explanation for the preference of *V. volvacea* for less-lignified substrates might also be found in a higher sensitivity to phenolic monomers and tannins, and in a more pronounced effect of these compounds on the production and/or activity of the straw mushroom's cellulases and hemicellulases. This study shows that, in terms of mycelial growth at least, *V. volvacea* exhibits different sensitivity profiles to lignin-related phenols and tannin derivatives as compared to *P. sajor-caju* and *L. edodes*. However, the inhibition patterns, and the observed qualitative and quantitative differences compared to *L. edodes* and *P. sajor-caju*, were not explicit enough to conclude that the distribution of these compounds in lignocellulosic wastes alone is responsible for the poor growth of *V. volvacea* on lignified substrates.

In view of the ability of *Pleurotus* spp. to grow on a wide range of lignocellulosic substrates, our inability to detect cellulase activity was somewhat surprising although, in the relatively few reports of cellulolytic activity in *Pleurotus* spp., only low levels of enzymes were detected (Rai & Saxena, 1990; Burla *et al*. 1992). The apparent absence of cellulolytic activity in the strain of *P. sajor-caju* used in this investigation may be a reflection of the culture conditions used. In sawdust/wheat bran cultures, high levels of reducing sugar was evident which may have repressed the synthesis of the cellulases. Rai & Saxena (1990) investigated extracellular enzyme production in *P. sajor-caju* during growth on rice straw. No filter paper activity (FPase) was detected but low levels of endoglucanase and β-glucosidase appeared late in the cultivations after the depletion of soluble sugars. The ability of the fungus to utilize crystalline cellulose is perhaps less unexpected. Relatively few organisms are able to attack native cellulose and *Pleurotus* spp. may rely on other microorganisms present in the growth substrate during mushroom cultivation to bring about partial degradation. Alternatively, since *P. sajor-caju* produced high titres of xylanase and β-xylosidase, this fungus may use the hemicellulose component of the substrate in preference to the cellulose.

Previously to this report, there was no evidence of lignin peroxidase or manganese-dependent peroxidase in *Pleurotus* spp. Kerem *et al*., (1992) were unable to detect lignin peroxidase in either water extracts or liquid cultures of *Pleurotus ostreatus* during solid state fermentation of cotton stalks. Efforts to detect lignin peroxidase or lignin peroxidase genes in *P. ostreatus* under several culture conditions have been unsuccessful (Kimura *et al*., 1990). Other enzymes of an oxidizing nature, such as the veratryl alcohol oxidases, have been reported in *P. sajor-caju* (Bourbonnais & Paice, 1988) and in *P. ostreatus* (Sannia *et al*., 1991), and may play a role in lignin degradation. Sannia *et al*.(1991) have suggested that a lignin metabolism different from that reported for other white-rot fungi may exist. *P. ostreatus* (Sannia *et al*., 1986) and *P. sajor-caju* produce copious amounts of laccase, a copper-containing phenol oxidase, which appears to play a broad role in lignin biodegradation.

The conversion of hardwood into edible shiitake mushroom is currently the only significant commercial bioconversion process utilizing wood (Mishra & Leatham, 1990). Our data support previous research findings indicating that *L. edodes* may lack LiP, and that MnP (Forrester *et al*., 1988) and laccase are the major ligninolytic enzymes produced by this fungus. On the basis of the enzyme activities detected in this study, *L. edodes* also seems to have a preference for the hemicellulose component of the wood substrate. No significant growth of *L. edodes* was observed when crystalline cellulose served as carbon source. Moreover, although the mushroom grew well on a sawdust-wheat bran mixture, we were unable to detect any cellulolytic activity. Mishra and Leatham (1990) have reported endoglucanase, exoglucanase and β-glucosidase activity in *L. edodes* grown in solid-state cultures on red oak wood (*Quercus rubra*). Clearly, the type of substrate and other culture parameters may be critical for cellulase biosynthesis.

Our results provide clear evidence for a direct relationship between the lignocellulolytic enzyme profiles, and sensitivity to lignin-related phenols and tannin substances, and the capacity of an individual mushroom species to grow and fruit on a particular lignocellulosic substrate. Manipulation of lignocellulolytic enzyme production to improve the efficiency of substrate utilization, and to detoxify/remove inhibitory substances present in the substrate, will lead to the increases in mycelial biomass and fruit body yields essential to successful mushroom cultivation. It will also extend the range of substrates which could be used for the cultivation of a particular mushroom species.

ACKNOWLEDGEMENT

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REFERENCES

AKIN, D.E. & RIGSBY, L.L. (1985). Influence of phenolic acids on rumen fungi. *Agronomy Journal* **77**, 180-182.

BORNMAN, W.S., AKIN, D.E. & VAN ESELTINE, W.P. (1986). Effect of phenolic monomers on ruminal bacteria. *Applied and Environmental Microbiology* **52**, 1331-1339.

BOURBONNAIS, R. & PAICE, M.G. (1988). Veratryl alcohol oxidase from the lignin-degrading basidiomycete *Pleurotus sajor-caju. Biochemical Journal* **255**, 445-450.

BURLA, G., GARZILLO, A,M., LUNA, M., CARDELLI, L.E. AND SCHIESSER, A. (1990). Effects of different growth conditions on enzyme production by *Pleurotus ostreatus* in submerged culture. *Bioresource Technology 42*, 89-94.

BUSWELL, J.A. (1991). Fungal degradation of lignin. In *Handbook of Applied Mycology. Vol. 1, Soil and Plants*, pp.425-480. Edited by A.K. Arora, B. Rai, G. Mukerji and G. Knudsen. New York: Marcel Dekker Inc.

CAI, Y.J., BUSWELL, J.A. AND CHANG, S.T. (1993). Effect of lignin-related phenolic monomers and tannin derivatives on the growth of the edible mushrooms *Lentinus edodes, Pleurotus sajor-caju* and *Volvariella volvacea.* World Journal of Microbiology and Biotechnology. (In press).

CHANG, S.T. (1991). Cultivated mushrooms. In *Handbook of Applied Mycology, Vol 3, Food and Feeds*, pp. 221-239. Edited by D. K. Arora, K.G. Mukerji and E.H. Marth. New York: Marcel Dekker Inc.

CHEN, C.-L. & CHANG, H.-M. (1985). Chemistry of lignin degradation. In *Biosynthesis and Biodegradation of Wood Components*, pp. 535-556. Edited by T. Higuchi. San Diego: Academic Press.

CHERNEY, J.H., ANLIKER, K.S., ALBRECHT, K.A. & WOOD, K.V. (1989). Soluble phenolic monomers in forage crops. *Journal of Agricultural and Food Chemistry* **37**, 345-350.

DALE, B.E. (1987). Lignocellulose conversion and future fermentation technology. *Trends in Biotechnology* **5**, 287-291.

FORRESTER, I.T., GRABSKI, C.G., BURGESS, R.R. & LEATHAM, G.F. (1988). Manganese, Mn-dependent peroxidases, and the biodegradation of lignin. *Biochemical Biophysical Research Communications* **157**, 922-929.

GLENN, J.K., MORGAN, M.A., MAYFIELD, M.B., KUWAHARA, M. AND GOLD. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochemical Biophysical Research Communications* **114**, 1077-1083.

GLENN, J.K. AND GOLD, M.H. (1985). Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **242**, 329-341.

KEREM, Z., FRIESEM, D. & HADAR, Y. (1992). Lignocellulose degradation during solid-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **58**, 1121-1127.

KIMURA, Y.Y., ASADA, Y. & KUWUHARA, M. (1990). Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. *Applied Microbiology and Biotechnology*. **32**, 436-442.

LEATHAM, G.F. 1985. Extracellular enzymes produced by the cultivated mushroom *Lentinus edodes* during degradation of a lignocellulosic medium. *Applied and Environmental Microbiology* **50**, 859-867.

MARTIN, S.A. & AKIN, D.E. (1988). Effect of phenolic monomers on the growth and β-glucosidase of *Bacteroides ruminocola* and on the carboxymethylcellulase, β-glucosidase and xylanase from *Bacteroides succinogenes*. *Applied and Environmental Microbiology* **54**, 3019-3022.

MISHRA, C. & LEATHAM, G.F. (1990). Recovery and fractionation of the extracellular degradative enzymes from *Lentinula edodes* cultures cultivated on a solid lignocellulosic substrate. *Journal of Fermentation and Engineering* **69**, 8-15.

RAI, R.D. & SAXENA, S. (1990). Extracellular enzymes and non-structural components during growth of *Pleurotus sajor-caju* on rice straw. *Mushroom Journal for the Tropics* **10**, 69-73.

REINHAMMAR, B. (1984). Laccase. In *Copper Proteins and Copper Enzymes, Vol 3*, pp. 1-36. Edited by L. Lontie. Boca Raton, Florida: CRC Press.

SANNIA, G., GIARDINA, P., LUNA, M., ROSSI, M. & BUONOCORE, V. (1986). Laccase from *Pleurotus ostreatus*. *Biotechnology Letters* **8**, 797-800.

SANNIA,G., LIMONGI. P., COCCA, E., BUONOCORE, F., NITTI, G. & GIARDINA, P. (1991). Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Biochimica Biophysica Acta* **1073**, 114-119.

SHEA, S.K. & BUSWELL, J.A. (1992). Effect of lignin-derived phenols and their methylated derivatives on the growth of *Lentinus* spp. *Letters in Applied Microbiology* **15**, 12-14.

TIEN, M. & KIRK, T.K. Lignin degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* **221**, 661-663.

WOOD, D.A. & FERMOR, T.R. (1982). Nutrition of *Agaricus bisporus* in compost. *The Mushroom Journal* **114**, 194-197.

CHAPTER 16

CONVERSION OF LIGNOCELLULOSICS INTO ANIMAL FEED WITH WHITE ROT FUNGI

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1. INTRODUCTION

1.1. Biological Upgrading of Lignocellulosics

The application of lignocellulosic agricultural wastes, particularly cereal straws, as animal feed has received considerable attention in past years. The high content of polysaccharides in lignocellulose is a potential source of dietary energy for ruminants. However, their nutritive value is limited because of the poor digestion of complex polysaccharide in the rumen. The restricted digestibility of these energy rich substances is due to the presence of a recalcitrant component, lignin, which acts as a physical barrier depriving the hydrolytic enzymes access to the polysaccharides.

In order to increase the digestibility of lignocellulose, biological methods of delignification can be used (Han, 1978). The principle of these methods is the splitting of the cellulose-lignin complex by extraction or decomposition of lignin. The main problem of biological upgrading of lignocelluloses into feed is to find suitable microorganisms, with metabolic patterns different from those of the rumen flora and fauna, and a cheap large scale process. "Ideal" microorganisms for upgrading lignocellulosics into animal feed should have a strong lignin metabolism with a low degradation of cellulose and hemicellulose. In this review, fungal activities on lignocelluloses are briefly summarized.

1.2. Fungal Delignification of Lignocellulose

Fungal delignification of wood has been observed in South Chile under natural conditions. The final product of delignification, known as "Palo podrido", is a white decomposed wood which is used as animal feed (Tangol, 1976). The wood degraded to Palo podrido is usually from the logs of *Drymis winteri* Forst, *Eucryphia cordifolia* Cav., *Laurelia philippiana* and *Nothofagus dombeyi* (Mirb.) and the fungi responsible for the degradation of the wood belong either to *Ganoderma applantum* or *Armillariella* spp. The *in vitro* digestibility of wood increased from a bare 3% to 77% in some cases. However, the process of degradation is very long and slow, and it is not possible to calculate how

much carbohydrate is lost before such a highly digestible product occurs in nature (Zadrazil *et al.*, 1982). Zadrazil and Brunnert (1981) confirmed that *G. applanatum* degrades lignin, in the case of wheat straw preferentially at low temperature, and helps to increase the digestibility of the fermented product. The cool weather of South Chile (2-18°C) seems to facilitate the growth of this fungus on wood.

Phanerochaete chrysosporium degrades hemicellulose, cellulose and lignin non-selectively, which adversely affects the digestion rate of cell walls in the rumen (Agosin *et al.*, 1986). In 15 days of fermentation, 32% of dry matter can be degraded, accompanied by only 11% increase in digestbility. Similar results have also been reported by Zadrazil and Brunnert (1982) and Zadrazil (1985). But Reade and McQueen (1983) reported that dry matter loss during fermentation of wood shavings was not as high as reported in the above studies. This might be due to the different physical structures of the two substrates used. Reid (1985) reported that aspen wood was degraded efficiently by *Merulius tremellosus* in solid state fermentation. The fungus degraded 52% of lignin accompanied by only 12% loss of dry matter and increased the cellulase digestibility of the fermented wood from 18 to 52%. More than 200 strains of white rot fungi have been screened by Zadrazil (1985) for substrate degradation, lignin decomposition and increase in digestibility of wheat straw at different temperatures (22, 25 and 30°C) and periods of fermentation.

2. ECOLOGICAL BACKGROUND OF CONVERSION OF LIGNOCELLULOSICS INTO ENRICHED ANIMAL FEED

2.1. Factors Determining Lignin Degradation and *in vitro* Digestibility

Figure 1 shows the factors determining the course of solid state fermentation of lignocelluloses. The number of possible variations between two, three or more factors is 256. However, during large-scale production the controlling key-factors of fermentation are limited.

2.2. Type of Fungal Strains

The influence of fungal species on the decomposition of wheat straw and the *in vitro*

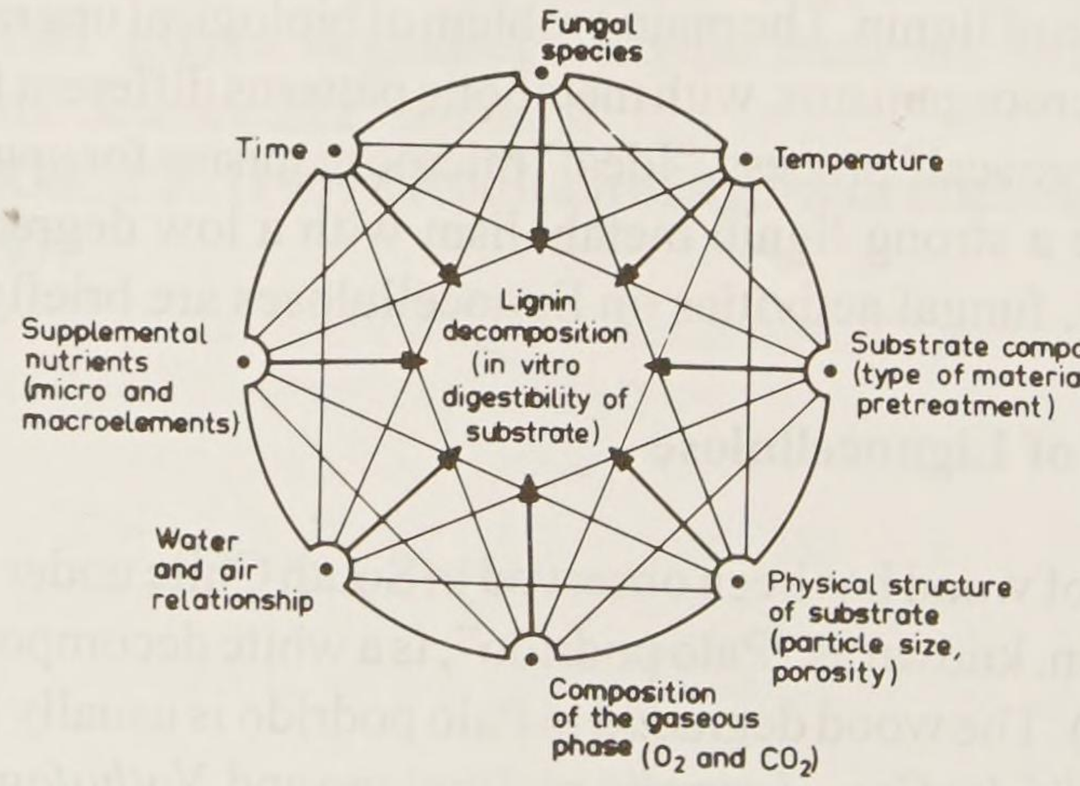

FIGURE 1. Interrelation between factors determining lignin decomposition and *in vitro* digestibility of lignocelluloses during solid state fermentation with white rot fungi.

digestibility and decomposition of lignin is comprehensively discussed by Zadrazil (1985). In Figure 2 differences between physiologically different fungi are shown.

Fungi in group (a) decompose the substrate without lignin degradation (brown rot fungi). *In vitro* digestibility is negative in comparison with untreated straw. Examples are *Agrocybe aegerita* and *Flammulina velutipes*. Similar results can be obtained by the cultivation of lower fungi, bacteria or yeasts on cereal straw.

2.3. Substrate Composition and Quality

Five different substrates, including beech sawdust, rape-, sunflower- and reed straw, rice husks, were tested with 4 different fungi for speed of decomposition of organic matter, lignin decomposition and *in vitro* digestibility. Each substrate was subjected to 60 days of solid state fermentation by each fungus. All these factors are strongly dependent on fungal species and the kind of plant waste substrate (Zadrazil, 1980). *Pleurotus* sp. *florida*, *Pleurotus cornucopiae* and *Stropharia rugosoannulata* showed good lignin decomposition and increase the *in vitro* digestibility of all substrates except the rice husks. *Agrocybe aegerita* decomposed lignin only to a small extent and decreased the *in vitro* digestibility (Zadrazil, 1980). This effect is probably caused by the high incrustation of rice husks with SiO_2.

2.4. Substrate Pretreatment

Interactions between indigenous substrate microorganisms and the consumption of liberated carbohydrates by the fungus influence the digestibility of substrate. Kamra and Zadrazil (1986) reported that pasteurization of wheat straw at 90°C or sterilization was required for a significant

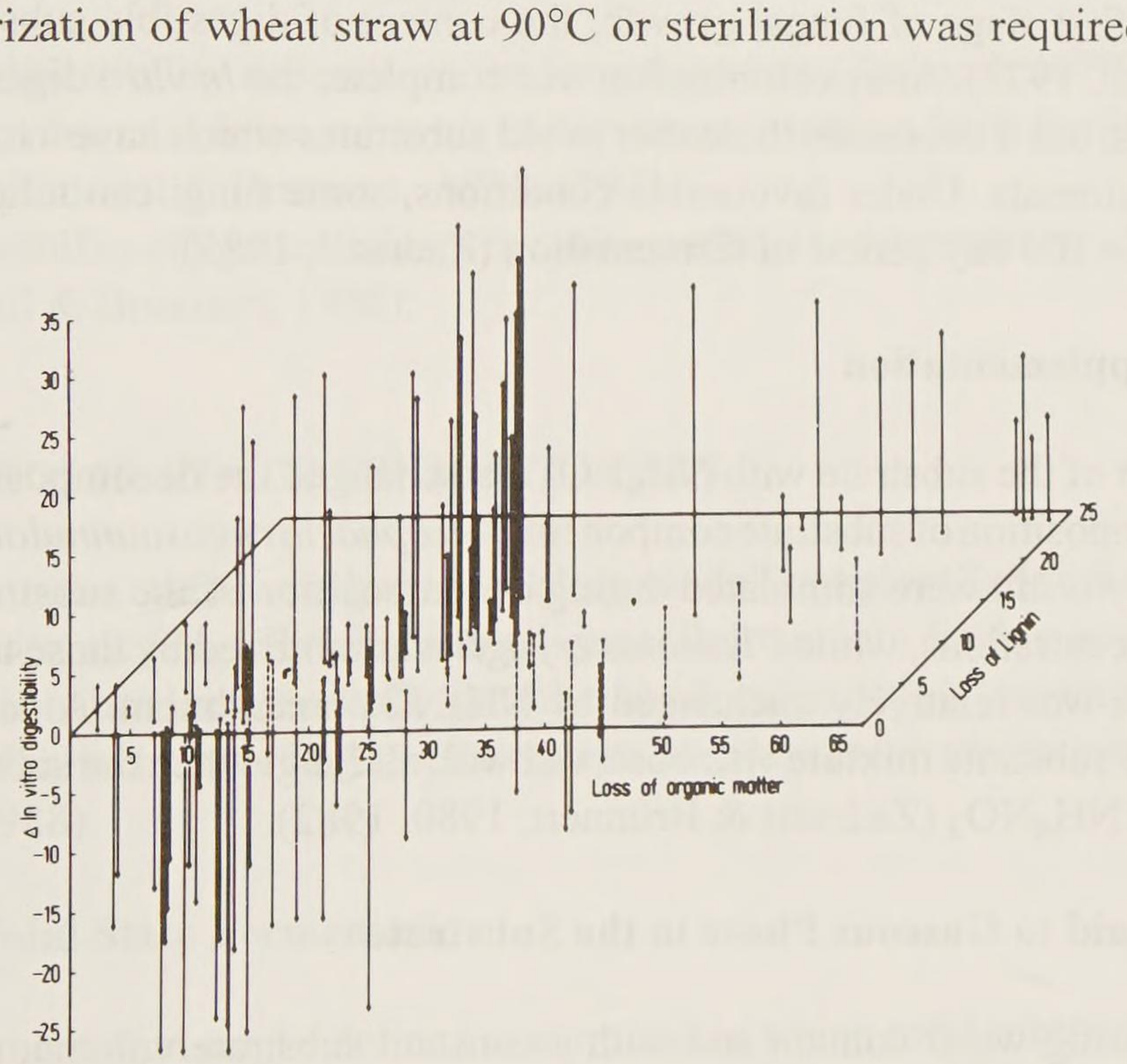

FIGURE 2. Relationship between the loss of organic matter, loss of lignin and differences in the *in vitro* digestibility of wheat straw after fungal treatment (60 days at22,25 and 30°C; *in vitro* digestibility of untreated straw = 0; increased digestibility +; decreased -). (a b c d - see text for explanation).

increase in *in vitro* digestibility following fermentation with *Pleurotus sajor-caju*. Zadrazil and Peerally (1986) reported that anaerobic fermentation of wheat straw at 50°C for 48 h before inoculation with the desired fungus is as effective as sterilization of substrate. It shows that to achieve good digestibility of lignocellulose, the natural microflora must be suppressed and the inoculum must be given the maximum opportunity to colonize the substrate quickly.

2.5. Temperature

The temperature of fermentation not only influenced the speed of decomposition of the organic matter, it also influenced the sequence of decomposition of the substrate components. With all fungi tested, increases in temperature between 22 and 30°C, increased the speed of decomposition of the organic matter (Zadrazil, 1977; 1985). Large differences in the rates of straw decomposition caused by temperature were observed for *Pleurotus cornucopiae* and *Stropharia rugosoannulata*. A positive correlation between increases in temperature and lignin decomposition or *in vitro* digestibility was only visible for *Stropharia rugosoannulata*.

2.6. Duration of Fermentation

In the fungal life cycle, the following stages are distinguished:

1. Colonization of the substrate
2. Maturation of fungus
3. Induction of fruiting bodies
4. Autolysis.

During the first stage of fungal growth, the content of digestible substances for ruminants decreases (Zadrazil, 1977). After colonization was complete, the *in vitro* digestibility of the fungal substrate increases, but it decreases thereafter in old substrates which have a relatively high content of accumulated minerals. Under favourable conditions, some fungi can totally mineralize cereal straw during a 80 - 100 day period of fermentation (Zadrazil, 1985).

2.7. Nitrogen Supplementation

Amendment of the substrate with NH_4NO_3 also changed the decomposition rate and also the sequence of decomposition of substrate components. *Stropharia rugosoannulata*, *Agrocybe aegerita* and *Pleurotus* sp. *florida* were stimulated during decomposition of the substrate by the addition of low NH_4NO_3 concentrations, while *Pleurotus eryngii* was inhibited by these treatments. The lignin decompositon rate was relatively unchanged by NH_4NO_3 amendment. A decrease in the *in vitro* digestibility of the substrate mixture was observed with all fungi when the substrate received higher concentrations of NH_4NO_3 (Zadrazil & Brunnert, 1980, 1982).

2.8. Ratio of Liquid to Gaseous Phase in the Substrate

With increasing water content and with a constant substrate volume, the air content of the substrate decreased. This resulted in increased water tension and an increased swelling of the substrate.

All fungi investigated showed good growth on substrates with varying water contents (from 25 to 150 ml water/25g straw). With both the lowest and the highest water contents, the decomposition rate of the total organic matter decreased as did decomposition of lignin and accumulation of

digestible substances. The fungi tested showed specific growth optima for various air and water contents of the substrate (Zadrazil & Brunnert, 1981, 1982).

2.9. Composition of Gaseous Phase

The losses in organic matter and lignin after fermentation of wheat straw with *Pleurotus sajor-caju* were found to be highest in a 100% oxygen atmosphere (Kamra & Zadrazil, 1986), followed by those in air in the case of *P. eryngii*. Carbon dioxide at 1-20% in the atmosphere influenced neither organic matter loss nor lignin degradation, but at 30% concentrations the organic matter loss was slightly increased and lignin degradation was considerably decreased. Lignin was degraded at a much lower rate with less than 20% oxygen in the atmosphere. The increase in *in vitro* digestibility was highest in pure oxygen, followed by that in an air atmosphere. Carbon dioxide at 1-10% positively influenced the increase in digestibility, but at higher CO_2 concentrations the digestibility was reduced. The process efficiency with respect to increase in digestibility of wheat straw was highest in the presence of oxygen (Kamra & Zadrazil, 1986).

Gaseous metabolites have a strong influence on the mineralization of organic matter, loss of lignin and *in vitro* digestibility following the fungal degradation of straw (Zadrazil *et al*., 1991). For large scale processes, the composition of gaseous phase is proposed to be the key-factor. The effects on the composition of the substrate of different gaseous treatments have been summarized by Buta *et al*. (1989) and by Chiavari *et al*. (1989). The above investigations can be summarized as follows:

1. *In vitro* digestibility of fungal substrates decreases at the beginning of colonization by white rot fungi and increases afterwards (Zadrazil, 1977; Zadrazil & Brunnert, 1982). During incubation, the content of soluble substances (partly sugars) increases (Zadrazil, 1976; Lindenfelser *et al*., 1979).
2. The increase in digestibility depends on the fungal species (Zadrazil, 1979), cultivation time, temperature, water/air ratio in the substrate and on the preparation, bulk density and composition of the substrate (Zadrazil & Brunnert, 1981, 1982).
3. The *in vitro* digestibility of lignocelluloses by white rot fungi is decreased by addition of inorganic nitrogen (Zadrazil & Brunnert, 1980).

3. SCALE UP OF SOLID STATE FERMENTATION

The above examples and the analysis of "Palo podrido" samples (Zadrazil *et al*., 1982) clearly show that use of white rot fungi for upgrading lignocelluloses into feed is possible, at least on a laboratory scale and in natural processes. On the other hand, only a little is known about the large scale process. Solid state fermentation of lignocelluloses in deep layers have been proposed (Schuchardt & Zadrazil, 1982, 1988).

3.1. Definition of Solid State Fermentation

Solid state fermentation can be defined as a process in which solid substrates are decomposed by known mono- or mixed cultures of microorganisms (mainly fungi, which can grow on and through this substrate) under controlled environmental conditions with the aim of producing a high quality standardized product (different to composting). The substrate (mixture of different particles) is characterized by a relatively low water content. Since much of the water is chemically or physically bound to the substrate, physical properties, e.g. porosity, density, are uniform. The substrate is not

mixed or moved during the process (Zadrazil *et al*., 1990a).

Prepared substrates can also be used in environmental control as biofilters (Hüttermann *et al*., 1988), or for decontamination of xenobiotics in soil and waste materials (Hüttermann *et al*., 1988, 1989; Martens & Zadrazil, 1992).

3.2. The Principle of the Process

Many factors influence the course of solid state fermentation, but only some factors such as temperature, humidity and composition of gas phase can be controlled and changed.

The pretreated substrate is filled into the reactor in 1.5-2.0 m deep layers and incubated by percolation of the gas phase through the substrate. The temperature in the substrate is indirectly controlled by conditioned, percolated gas. In the cultivation of *Agaricus* spp., fresh air is added in order to cool the substrate. In contrast, in the production of wood decaying fungi, high carbon dioxide and low oxygen concentrations are required during the period of colonization (Zadrazil, 1975).

3.3. Description of SSF- Reactor

The principal design of a small scale reactor and its control were described by Schuchardt and Zadrazil (1982, 1988). The reactor used in the Institute for Soil Biology, FAL, Braunschweig, Germany, was based on previous experience. The reactor is constructed of polyurethane foam sandwich panels which are covered on both sides with polyester board. The filling height is approximately 2.0 m, the internal width 2.3 m and the depth 2.0 m. This results in a net filling volume of 9.2 m^3 equivalent to 1.5 t of straw or 3.0 t wood chip substrate.

Two similarly insulated swing doors are situated at the front of the reactor, the width of the doors being the width of the reactor itself. Inside the reactor there is a raised slatted floor covered firstly with a gliding net and then with a drag net.

Two reactors of the same shape and construction are used (Fig 3). One reactor is used for substrate pretreatment and the second one for substrate colonisation (Zadrazil *et al*., 1990a, 1990b). On moving the substrate from one reactor to the other reactor, the substrate is inoculated.

3.4. Conditioning of Gas Phase

Fungal growth and heat exchange from the substrate is controlled by re-circulating the air within the reactor. The total quantity of circulated air can be varied by changing the speed of electronically controlled fans.

In order to humidify the air and raise the substrate temperature for pretreatment of substrate (e.g. pasteurisation), a steam injection pipe is installed under the slatted floor.

Fresh air, oxygen, or carbon dioxide can be added after gas analysis by computer-controlled valves (Fig. 4). Exhaust gases leave the fermentor through an over-pressure valve located above the substrate.

3.5. Control of Gas Humidity

The humidity of gaseous phase was controlled by hygrometers situated (Hygrotest 720) in different parts of the reactor. The air humidity fluctuates between 95 and 100%. Commercially available hygrometers are not sufficiently sensitive enough in this region to give more precise control.

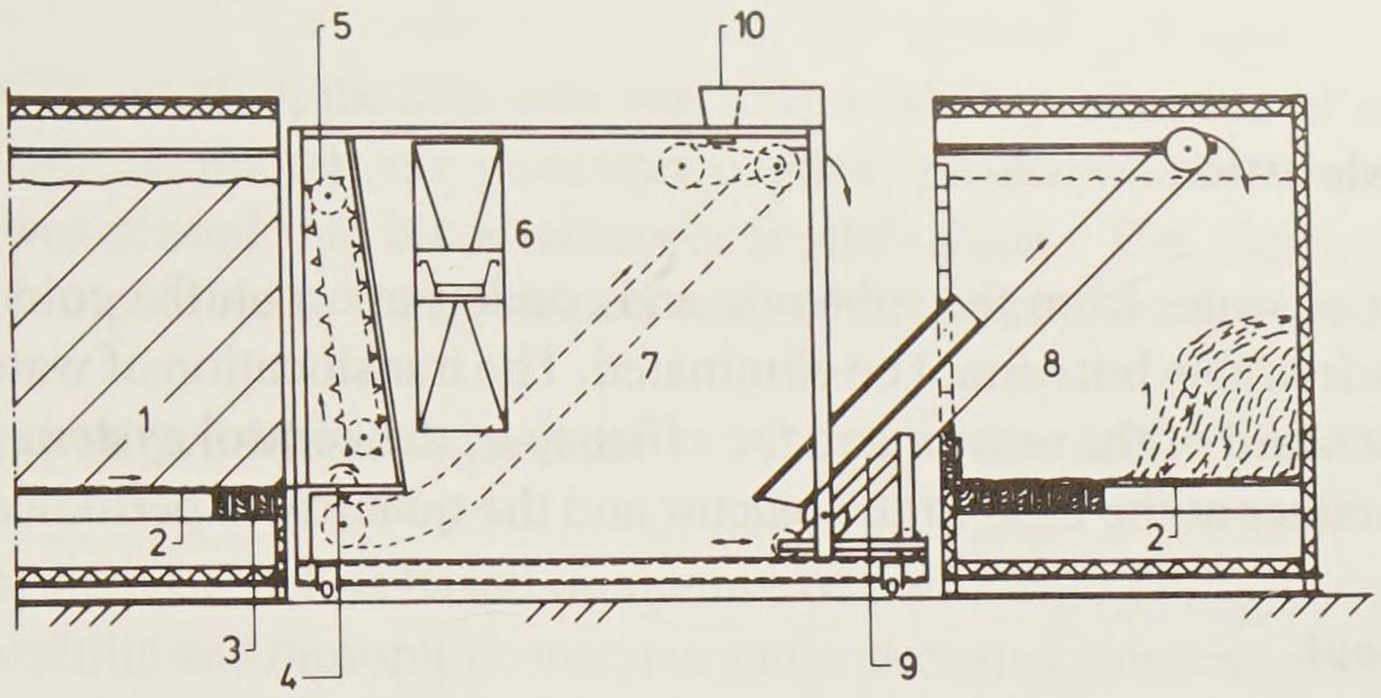

Figure 3. Horizontal cross -section of reactors for SSF and spawning-emptying machine. 1. SSF reactor; 2. Net for emptying the substrate; 3. Panels for aerating the substrate; 4. Equipment for for emptying the substrate; 5. Equipment for fragmenting the substrate; 6. Equipment for filling the substrate; 7. Conveyor to bring substrate to spawning machinery; 8. Conveyor for filling the reactor with substrate; 9. Carrier for moving the conveyor; 10. Spawning machine (Zadrazil *et al.*, 1990).

3.6. Control of Water Evaporation

Saturation deficit of water in the gaseous phase increases during the penetration of gas through the substrate. The gaseous phase has a lower temperature than the substrate and water evaporates. From the circulating gases, water condenses on the cooling equipment and within cold areas of substrate and reaches 100 % relative humidity again. Evaporation of water in circulating gases was

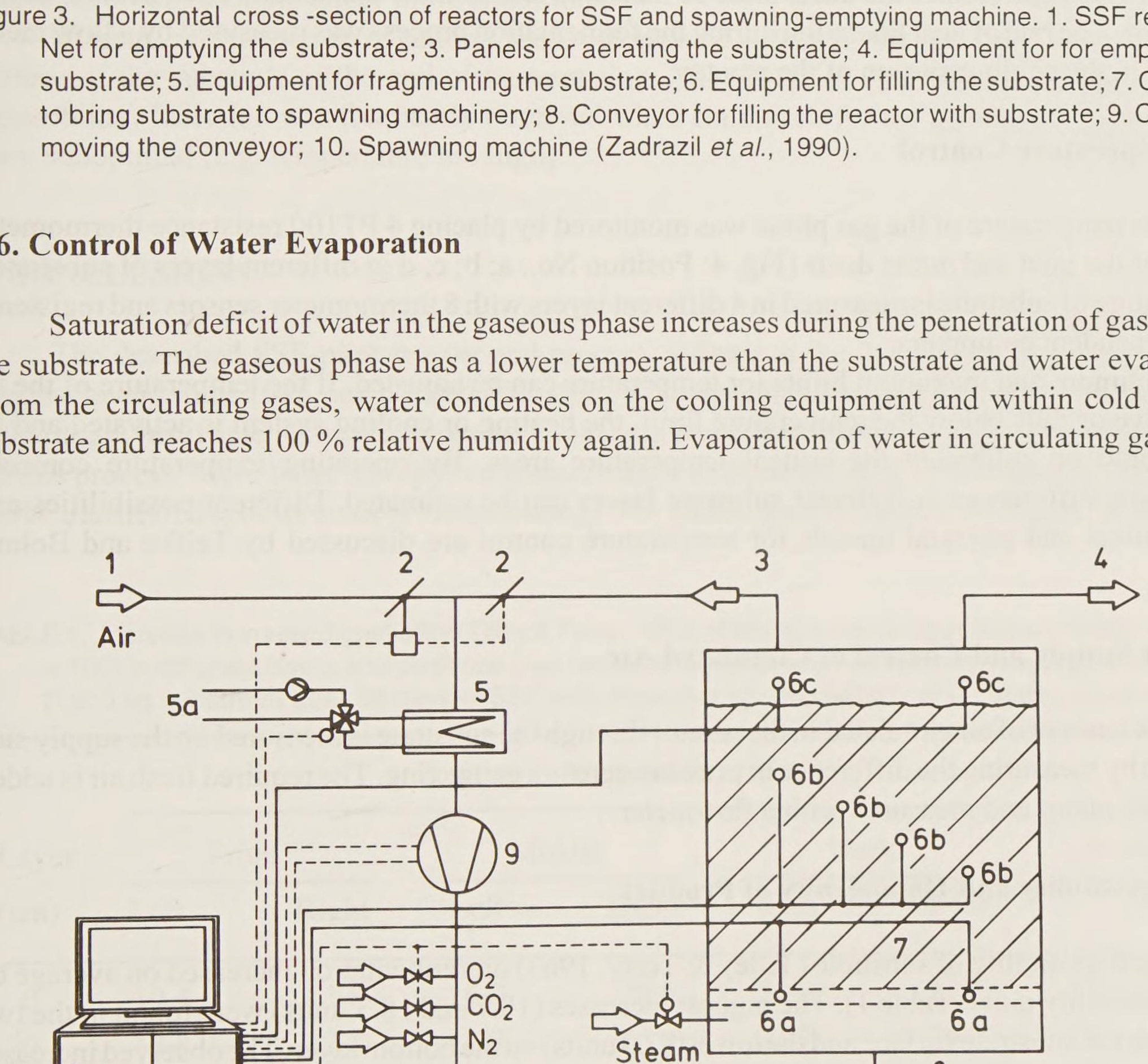

FIGURE 4. Scheme of control system of solid state reactor (Zadrazil *et al.*, 1990). 1. Fresh air supply; 2. Air damper; 3. Recirculation of gas phase; 4. Emptying of gas; 5. Gas-cooling system; a. Liquid-cooling system; 6. Sensors for temperature control; a. Gas-input b. Temperature of substrate c. Gas-output 7. Substrate; 8. Body of SSF reactor; 9. Fan.

measured by water loss from a 20 cm^2 ceramic disc (Czeratzki, 1968) placed in the space above the substrate.

3.7. Water Translocation

Evaporation of water from the substrate and condensation on the cold parts of reactors and cooling units is undesirable but cannot be eliminated. The translocation of water could be a measure of the technical standard of the reactor and the efficacy of the control system. All condensed water is led into the container at the base of the reactor and the quantity is periodically measured.

3.8. Metabolic Heat

For scientific studies the ducts must be insulated to eliminate the uncontrolled heat loss from the reactor. The rate of heat liberation during the fermentation process was measured by a flow meter installed in the cooling system of the reactor.

3.9. Temperature Control

The temperature of the gas phase was monitored by placing 4 PT100 resistance thermometer sensors at the inlet and outlet ducts (Fig. 4: Position No.: a; b; c; d in different layers of substrate). Temperature of substrate is measured in 4 different layers with 8 thermometer sensors and registered in 2 independent computers.

Minimum and maximum limits for temperature can be adjusted. If the temperature of the air rises above or falls below the temperature limit, the heating or cooling system is activated and an alarm could be calibrated for critical temperature areas. By operating temperature controls, temperature differences in different substrate layers can be estimated. Different possibilities and mathematical and physical models for temperature control are discussed by Teifke and Bohnet (1990).

3.10. Air Supply and Control of Circulated Air

The amount of air circulated in the reactor through the substrate is measured on the supply side of the fan by measuring the difference in pressure across a gauge ring. The required fresh air is added with an air pump and measured with a flowmeter.

3.11. Digestibility and Homogenity of Product

The digestibility of substrate (Tilley & Terry, 1963) on fermentation increased on average by 13.8 digestibility units (Table 1). The highest increases (18.7 and 18.3 units) were found in the two layers near the substrate surface and the lowest (7.0 units) on the bottom layer. The observed increases in the digestibility of cereal straw after fungal treatment on a large-scale were comparable with results obtained by sodium hydroxide or ammonia treatment (Sundstol & Owen, 1984). The final product (fungal substrate) differs in water content and digestibility. One may assume that differences between the water contents of different layers have an influence on the digestibility of substrate. We hope that this phenomenon can be eliminated by better control of the gas phase.

3.12. Utilisation of Substrate

After 14 days of incubation, the substrate was also used for production of edible fungi, or for other technologies (soil sanitation, air pollution control, production of chemical feedstocks). Colonized substrate was placed into a container for fructification . The yield of fruit bodies was satisfactory and was comparable to that obtained with other cultivation systems.

3.13. Growth of Competitive Microorganisms

The proposed system of SSF is based on the use of non-sterile culture conditions. Selective propagation of thermophilic and mesophilic microorganisms during substrate pretreatment supports the saprophytic colonization by the cultivated fungus. Infection of substrate was not observed during colonization of substrate under these conditions.

During the production of animal feed, colonies of *Trichoderma* sp. were observed on the surface of the substrate at the end of fermentation. This infection correlates with high substrate digestibility. Infection was frequently observed when conditions for the growth of *Pleurotus* spp. were suboptimal (e.g. temperature too high).

4. CONCLUSIONS

The described SSF-pilot reactor and process, utilized at the Institute of Soil Biology, FAL Braunschweig since 1985, can be used after some technical modifications as a model for large-scale technology. Insufficient knowledge exists about solid state fermentaion and about suitable reactors for this process. More basic and applied research must be done before this "mushroom" technology can be transferred to other areas of biotechnology. For each organism, special strategies for the design

TABLE 1. Increase in *in vitro* digestibility (Tilley & Terry, 1963) of wheat straw (indigestible = 0; totally digestible = 100) in different layers and positions (two replicate samples from each layer) in a large-scale reactor (1500 kg substrate) after 38 days of SSF with *Pleurotus* sp. (digestibility of untreated straw = 40).

Layer	Position in the reactor						
	Front		Middle		Back		Average
(cm)	Left	Right	Left	Right	Left	Right	
0	19.4	11.8	16.0	20.6	21.6	22.8	18.7 ± 4.12
20	18.8	12.8	17.9	16.0	23.7	20.2	18.3 ± 3.71
40	11.9	12.1	16.9	15.7	23.3	14.3	15.7 ± 4.22
60	14.8	15.0	5.7	16.6	14.4	18.5	14.1 ± 4.41
80	8.3	9.5	10.9	11.9	7.8	10.2	9.8 ± 1.57
100	0.0	7.1	7.0	10.2	10.3	7.5	7.0 ± 3.73
Av	12.2 ± 7.30	11.4 ± 2.73	10.7 ± 5.29	15.2 ± 3.70	16.8 ± 6.98	15.6 ± 5.95	13.8 ± 3.59 13.7 ± 3.49

of the reactor and for the control of the process must be developed. For application in developing countries, cheap and easily controlled reactors must therefore be developed.

Based on the studies reported here and elsewhere, one may conclude that there is a need for further research on several aspects of the SSF process. These include the following:

1. Development of new designs and constructions of SSF reactors.
   1.1 Requirements: Homogenous conditions during processing.
   1.2 Minimal differences between process parameters (growth conditions) in different layers of substrates.
2. Development of equipment and sensors for the control of the SSF process.
   2.1 Control of the air speed in different parts of reactors.
   2.2 Control of air humidity (95-100% relative humidity).
   2.3 Control of water evaporation from substrate.
   2.4 Control of water translocation.
3. Development of a strategy for process control.
4. Development of mathematical models of the SSF process.
5. Verification of results in laboratory and pilot-scale reactors.
6. Comparative economic studies with other lignocellulose-upgrading processes.

ACKNOWLEDGEMENTS

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REFERENCES

AGOSIN, E., TOLLIER, M.T., BRILLOUET, J.M., THIVEND, P. & ODIER, E. (1986). Fungal pretreatment of wheat straw: Effects on the biodegradibility of cell walls, structural polysaccharides, lignin and phenolic acids by rumen microorganisms. *Journal for Science in Food and Agriculture* **37**, 97-106.

BUTA, J.G., ZADRAZIL, F. & GALLETTI, G.C. (1989). FT-IR determination of lignin degradation in wheat straw by white rot fungus *Stropharia rugosoannulata* with different oxygen concentrations. *Journal of Agricultural and Food Chemistry* **37**, 1382-1384.

CHIAVARI, G., FRANCIOSO, O., GALLETTI, G.C., PICCAGLIA, R. & ZADRAZIL, F. (1989). Characterization by pyrolysis-gas chromatography of wheat straw fermented with white rot fungus *Stropharia rugosoannulata*. *Journal of Analytical and Applied Pyrolysis* **15**, 129-136.

CZERATZKI, W. (1968). Ein Verdunstungsmesser mit keramischer Scheibe. *Landbauforschung Völkenrode* **18**, 93-98.

HAN, Y.W. (1978). Microbial utilization of straw. In *Advances in Applied Microbiology*, Vol. 23, pp. 119-153. Edited by D. Perlman. New York: Academic Press.

HÜTTERMANN, A., LOSKE, D., BRAUN-LÜLLEMANN, A. & MAJCHERCZYK, A. (1988). Der Einsatz von Weißfäulepilzen bei der Sanierung kontaminierter Böden und als Biofilter. *Bio-Engineering* **4**, 156-160.

HÜTTERMANN, A., LOSKE, A., MAJCHERCZYK, A. & ZADRAZIL, F. (1989). Einsatz von Weißfäulepilzen bei der Sanierung kontaminierter Böden. In Fortschrittliche Anwendungen der Biotechnologie, *Der Bundesminister für Forschung und Technologie* 115-121.

KAMRA, D.N. & ZADRAZIL, F. (1986). Influence of gaseous phase, light and substrate pretreatment on fruit body formation, lignin degradation and *in-vitro* digestibility of wheat straw fermented with *Pleurotus* spp. *Agricultural Wastes* **18**, 1-17.

LINDENFELSER, L.A., DETROY, R.W., RAMSTACK, J.M. & WORDEN, K.A. (1979). Biological modification of the lignin and cellulose components of wheat straw by *Pleurotus ostreatus*. *Developments in Industrial Microbiology* **20**, 541-551.

MARTENS, R. & ZADRAZIL, F. (1992). Screening of white rot fungi for their ability to meneralize polycyclic aromatic hydrocarbons in soil. In *Soil Decontamination Using Biological Processes*, pp. 505-510. Karlsruhe: Dechema.

READE, A.E., & MACQUEEN, R.E. (1983). Investigation of white rot fungi for the conversion of poplar into a potential feedstuff for ruminants. *Canadian Journal of Microbiology* **29**, 457-463.

REID, I.D. (1985). Biological delignification of aspen wood by solid state fermentation with white rot fungi *Merulius tremellosus*. *Applied and Environmental Microbiology* **50**, 133-139.

SCHUCHARDT, F. & ZADRAZIL, F. (1982). Aufschluß von Lignozellulose durch höhere Pilze - Entwicklung eines Feststoff-Fermenters. In *5. Symp. Techn. Mikrobiologie "Energie durch Biotechnologie"*, pp. 421-428. Edited by H. Dellweg. Berlin.

SCHUCHARDT, F. & ZADRAZIL, F. (1988). A 352 L fermenter for solid state fermentation of straw by white rot fungi. In *Treatment of Lignocellulosics with White Rot Fungi*, pp. 77-89. Edited by F. Zadrazil & P. Reiniger. Elsevier Applied Science.

SUNDSTOL, F. & OWEN, E. (1984). "Straw and other fibrous by-products as feed", Elsevier Science Publishers.

TANGOL, R. (1976). Diccionario etimologico Chiloe, Chiloe, Chile.

TEIFKE, J. & BOHNET, M. (1990). Modelling of the physical process parameters of technical lignin degradation by *Pleurotus* spp. In *Advances in Biological Treatment of Lignocellulosic Materials*, pp. 71-83. Edited by M.P. Coughlan & M.T. Amaral Collaco. Elsevier Applied Science.

TILLEY, J.M.A. & TERRY, R.A. (1963). A two stage technique for *in-vitro* digestion of forage crops. *Journal of British Grassland Society* **18**, 104-111.

ZADRAZIL, F. (1975). Influence of CO_2 concentration on the mycelium growth of three *Pleurotus* species. *European Journal of Applied Microbiology* **1**, 327-335.

ZADRAZIL, F. (1976). Freisetzung wasserlöslicher Verbindungen während der Strohzersetzung durch Basidiomyceten als Grundlage für eine biologische Strohaufwertung. *Zeitschrift für Acker- und Pflanzenbau* **142**, 44-53.

ZADRAZIL, F. (1977). The conversion of straw into feed by basidiomycetes. *European Journal of Applied Microbiology* **4**, 273-281.

ZADRAZIL, F. (1979). Umwandlung von Pflanzenabfall in Tierfutter durch höhere Pilze. *Mushroom Science* **10**, 231-241, Bordeaux.

ZADRAZIL, F. (1980). Conversion of different plant wastes into feed by basidiomycetes. *European Journal of Applied Microbiology and Biotechnology* **9**, 243-248.

ZADRAZIL, F. (1985). Screening of fungi for lignin decomposition and conversion of straw into feed. *Angewandte Botanik* **59**, 433-452.

ZADRAZIL, F. & BRUNNERT, H. (1980). The influence of ammonium nitrate supplementation on degradation and *in vitro* digestibility of straw colonized by higher fungi. *European Journal of Applied Microbiology and Biotechnology* **9**, 37-44.

ZADRAZIL, F. & BRUNNERT, H. (1981). Investigation of physical parameters important for the solid state fermentation of straw by white rot fungi. *European Journal of Applied Microbiology and Biotechnology* **11**, 183-188.

ZADRAZIL, F. & BRUNNERT, H. (1982). Solid state fermentation of lignocellulose containing plant residues with *Sporotrichum pulverulentum* Nov. and *Dichomitus squalens* (Karst.) Reid. *European Journal of Applied Microbiology and Biotechnology* **16**, 45-51.

ZADRAZIL, F., DIEDRICHS, M., JANSSEN, H., SCHUCHARDT, F. & PARK, J.S. (1990a). Large scale solid state fermentation of cereal straw with *Pleurotus* spp. In *Advances in Biological Treatment of Lignocellulosic Materials*, pp. 43-58. Edited by M.P. Coughlan & M.T. Amaral Collaco. Elsevier Applied Science.

ZADRAZIL, F., GALLETTI, G.C., PICCAGLIA, R., CHIAVARI, G. & FRANCIOSO, O. (1991). Influence of oxygen and carbon dioxide on cell wall degradation by white rot fungi. *Animal Feed Science and Technology* **32**, 137-142.

ZADRAZIL, F., GRINBERGS, J. & GONZALES, A. (1982). Palo Podrido - Decomposed wood used as feed. *European Journal of Applied Microbiology and Biotechnology* **15**, 167-171.

ZADRAZIL, F., JANSSEN, H., DIEDRICHS, M. & SCHUCHARDT, F. (1990b). Pilot-scale reactor for solid-state fermentation of lignocellulosics with higher fungi: Production of feed, chemical feedstocks and substrates suitable for biofilters. In *Advances in Biological Treatment of Lignocellulosic Materials*, pp. 31-41. Edited by M.P. Coughlan & M.T. Amaral Collaco. Elsevier Applied Science.

ZADRAZIL, F. & PEERALLY, A. (1986). Effects of heat and microbiological pretreatments of wheat straw substrate on the initial phase of solid state fermentation by white rot fungi. *Biotechnology Letters* **8**, 663-666.

CHAPTER 17

PHYSIOLOGY AND BIOCHEMISTRY OF LIGNOCELLULOSE UTILIZATION BY *PHOLIOTA NAMEKO*

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1. INTRODUCTION

Pholiota nemako, an edible fungus, is delicious and nutritious. It has been cultivated for 70 years in Japan and also in three provinces in Northeast China. This mushroom was cultivated with sawdust as substrate. A systemic study of the physiology and biochemistry of lignocellulose utilization by this fungus has so far been lacking. In this paper, the degradation pattern for lignin, hemicellulose and cellulose by *Pholiota nameko* have been studied.

2. MATERIALS AND METHODS

2.1. Cultivation

Pholiota nameko strain no. 2, which originated from Japan, was cultivated using the following substrate in a 500ml bottle: sawdust, 70gm; wheat bran, 17gm; $CaCO_3$, 1gm; KH_2PO_4, 0.5gm; $MgSO_4.7H_2O$, 0.5gm; dextrose, 0.5gm, water, 160ml. The substrate was autoclaved at 1kg/cm^2 for 1.5hr. After inoculation, cultures were incubated at 23-25°C, 16-20°C, 20-28°C, 14-20°C, 14-17°C and 11-14°C for 0-20, 20-72, 72-150, 150-175, 175-190 and 190-230 days, respectively.

2.2. Analysis of Components

The reduction in the amount of dry matter in the substrate, and the lignin, hemicellulose and cellulose contents were analysed according to Wang and Xu (1986).

2.3. Analysis of Enzyme Activities

Analyses of CMCase, FPase and hemicellulase were carried out according to Wang and Xu (1986). 1 unit of CMCase is equivalent to 1mg dextrose/30min/1gm dry culture; 1 unit of FPase =

1mg dextrose/60min/1gm dry culture; and 1 unit of hemicellulase = 1 mg xylose/30min/1gm dry culture.

3. RESULTS AND DISCUSSION

3.1. Quantitative Changes in Substrate Components

3.1.1. Reduction of substrate and consumption of respiration. When *Pholiota nameko* was incubated for 230 days, the dry matter in the substrate was reduced by 73.36% (Table 1). Within this figure, 60.93% was consumed by respiration of mycelium and another 12.43% was converted into fruitbody biomass (absolute biological efficiency). The production index (dry weight of fruitbody/ reduction of substrate x 100) is 16.94%. That is to say, the effective bioconversion of the substrate by *Pholiota nameko* is very low. The reason is that the fungus has a long period of vegetative growth and the nutrients in the substrate are consumed by mycelial respiration. The analysis above which is based on dry weight is less variable than when the fresh weight of fruitbody is used as is done conventionally.

It is shown also in Table 1 that the rate of lignin and hemicellulose degradation is more rapid than for cellulose, during the mycelium growing stage (0-199 days), but during the fruitbody developing stage (199-230 days) the rate of cellulose degradation is far more rapid than that of lignin and hemicellulose. This increase in the amount of cellulose degraded during the fruitbody development stage was attributed to: (1). During the fruitbody development stage, the amount of cellulose in the substrate is 3-4 times greater than the hemicellulose or lignin. (2). Cellulase activity increases markedly at the fruitbody stage. (3). When the primordia appeared (199 days later), the lignin and hemicellulose that inhibit cellulase had decreased by about 80% in the substrate (Table 1). This is beneficial for the degradation of cellulose (Rajarathnam, 1979; Dekker, 1983). (4). The activity peaks of cellulase are responsible for fruitbody development. In this period, the absorption rate of nutrients from the substrate by mycelium, and the bioconversion rate, were

TABLE 1. Reduction of several main components of the substrate during the cultivation period.

stage of growth (days)*	substrate reduction (%)***	consumption- via respiration (%)***	cellulose		hemicellulose		lignin	
			content (%)	reduction (%)	content (%)	reduction (%)	content (%)	reduction (%)
0			32.26		25.62		20.09	
18	8.41	8.41	33.00	6.32	22.18	18.64	15.65	28.49
125	42.46	42.46	37.77	32.64	10.81	75.72	8.82	74.75
199	54.39	54.39	30.33	57.13	11.25	80.00	9.22	81.35
230	73.76	60.93	16.94	86.01	6.75	92.98	9.89	86.88

* 0-18 days, rapid growth stage; 18-199 d, mycelium ripening stage; 199-230 d, fruitbody development stage.

** reduction of dry substrate (%)=(A-B)/A x100

*** consumption via respiration(%) =(A-B-C)/A x100

A: dry weight of substrate at the beginning;

B: dry weight of substrate at the different stage of growth;

C: dry weight of fruitbody.

promoted. Feedback inhibition of enzyme production was lifted so that the exocellulases of the mycelium exhibited maximum hydrolytic activity. This is one of the important reasons why the cellulose degradation rate increased during fruitbody developing. (5). The pH of the substrate, 3.8-4.1, is similar to the optimum pH (4.0-4.1) of cellulase.

3.1.2. Degradation of lignocellulosics. When the cultivation ended, the cellulose, hemicellulose and lignin in the substrate were reduced by 86.01%, 92.98% and 86.88%, respectively (Table 1). This showed that *Pholiota nameko* is a wood-rotting fungus that has a very strong ability to degrade sawdust.

Through analysis of the rates listed in Table 2, the nutritional characteristics of *P. nameko* at different growth stages are as follows: (1). In the stage of rapid mycelial growth (0-18 days), the amount of lignocellulose degraded was more than the reduction of substrate. At the same time, the amount of reducing sugar in the substrate also decreased rapidly and about 32% of degraded lignocellulose was converted into mycelial biomass. (2). In the stage of mycelial ripening, the amount of lignocellulose degraded was slightly lower than the reduction in the weight of the substrate. In this stage (18-199 days), the total amount of non-lignocellulosics in the substrate did not increase. Non-lignocellulosics were consumed to a small extent, equivalent to 6.96% of the reduced weight of substrate. (3). The nutrients consumed by fruitbody (used for respiration and constructing the fruitbody) originate from the degraded cellulose (49.13%), hemicellulose (17.54%), lignin (5.85%), and mycelium biomass or bioconverted product (27.48%), respectively. That is to say, cellulose is the main carbon source for fruitbody development, and the amount of cellulose degraded at this stage markedly affected the yield of the fruitbody. It is of great physiological significance that the lignin and hemicellulose are degraded largely during the stage of vegetative growth. This is beneficial to the degradation of cellulose in the substrate at the stage of fruitbody development. (4). In the stage of fruitbody development, the amount of hemicellulose degraded is not enough. This phenomenon does not mean that the hemicellulase activity was weak. In fact, degradation due to hemicellulase was reduced markedly before the stage of fruitbody development. It is necessary to study the regulation of the physiology of mycelial ripening (it could be delayed by high temperatures or promoted by low temperatures). This regulation affects the fruitbody development and the yield of fruitbody.

3.2. Cellulase Activity of *Pholiota nameko*

As shown in Figure 1, peaks of CMCase, FPase and hemicellulase activity appeared during the

TABLE 2. Relationship between the amount of degraded lignocellulose and the reduction of substrate at different stages of growth.

stages of growth	LR(g)	HR(g)	CR(g)	LHCR(g)	SR(g)	LR/SR x100	HR/SR x100	CR/SR x100	LHCR/SR x100
0-18	4.49	3.71	1.60	9.80	6.60	68.03	56.21	24.24	148.48
18-199	8.33	12.37	12.86	33.56	36.07	23.09	34.29	35.65	93.04
199-230	0.87	2.61	7.31	10.79	14.88	5.85	17.54	49.13	72.51

"LR"-reduction in lignin; "HR"-reduction in hemicellulose; "CR"-reduction in cellulose.
"LHCR"-reduction in lignocellulosics=LR+HR+CR; "SR"-reduction in substrate.

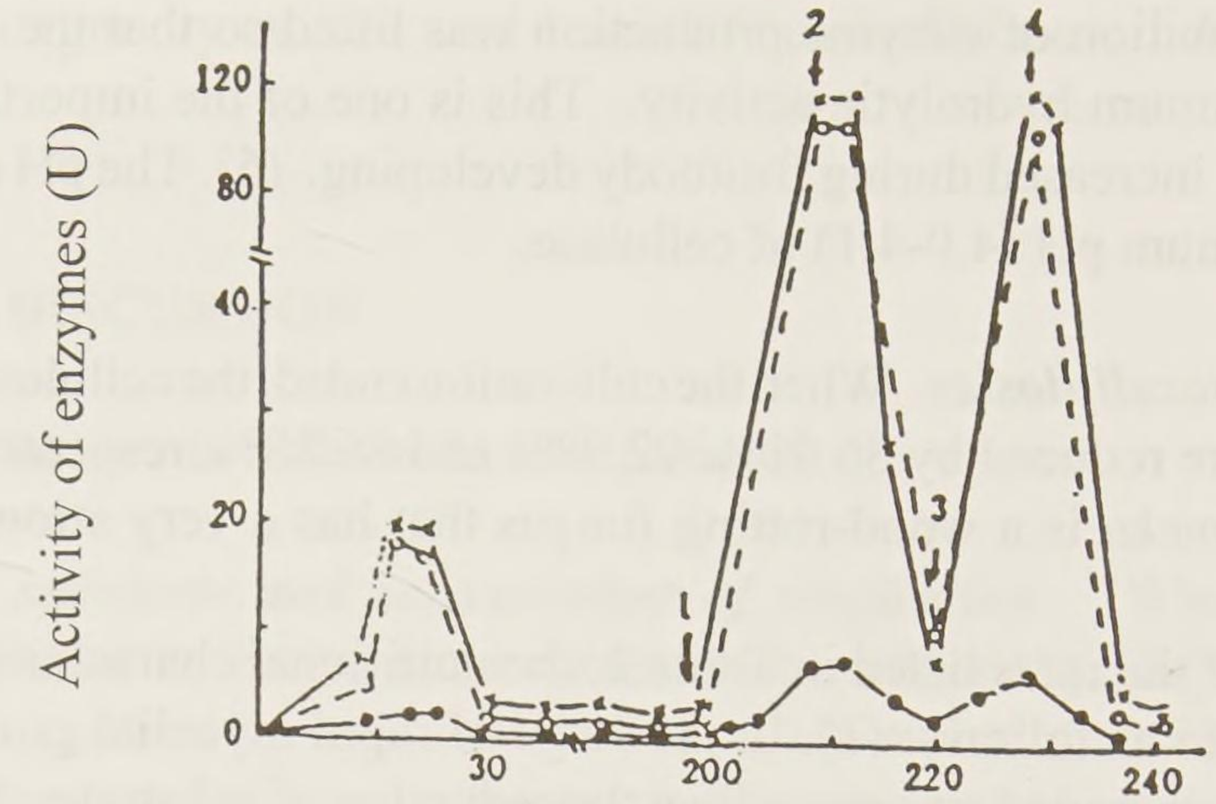

FIGURE 1. Activity of lignocellulases of *Pholiota nameko* in substrate. o—o CMCase cellulase; ●—● FP cellulase; x—— x hemicellulase; 1, 2, 3,4 show the primordia stage and the ripening stage of the first and second flushes.

stage of mycelium growth and the stage of fruitbody development. However, the highest peaks corresponded to the later stage. This is very beneficial to fruitbody development. The increase in enzyme activity was related to fruitbody development because the enzyme activity increased rapidly during the first and second flush stages and decreased rapidly between the two flushes (212-220 days). Additional experimental results are shown in Figs. 2, 3, 4 and 5. When cultivated at 25°C, or if the primordia were cut off to stop the fruitbody development, the peak of cellulase disappeared (only 10-18 units). This fact indicates that the low temperature is an essential condition for induction of fruitbody development.

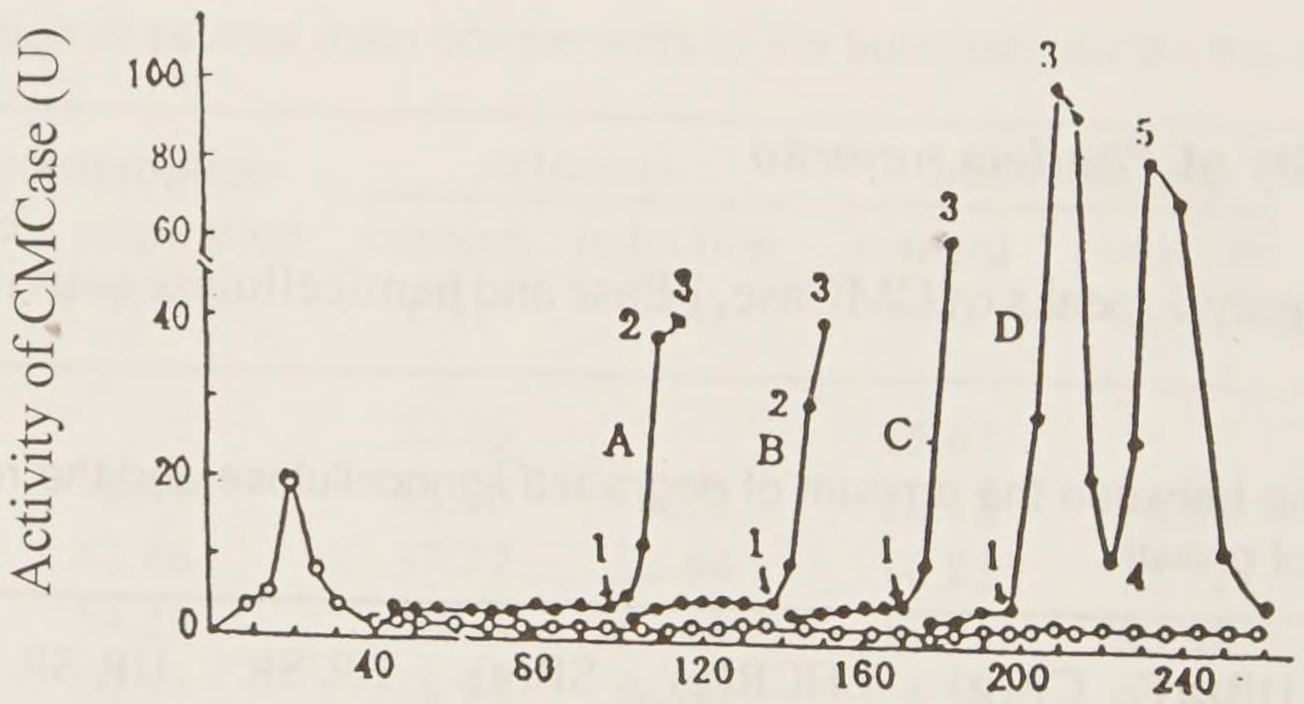

FIGURE 2. Effects of incubation temperature on *Pholiota nameko* fruitbody formation and activity of CMCase in the substrate. o—o incubated at 23-25°C for 0-260 days; ●—● incubated at 9-11°C; A, B, C, D incubated at 9-11°C after 40, 102, 142 and 178 days, respectively; 1, 2, 3 stages at primordia formation, pileus opening and spore dissemination of first flush; 4, 5 stages at primordia formation and spore dissemination of second flush, respectively.

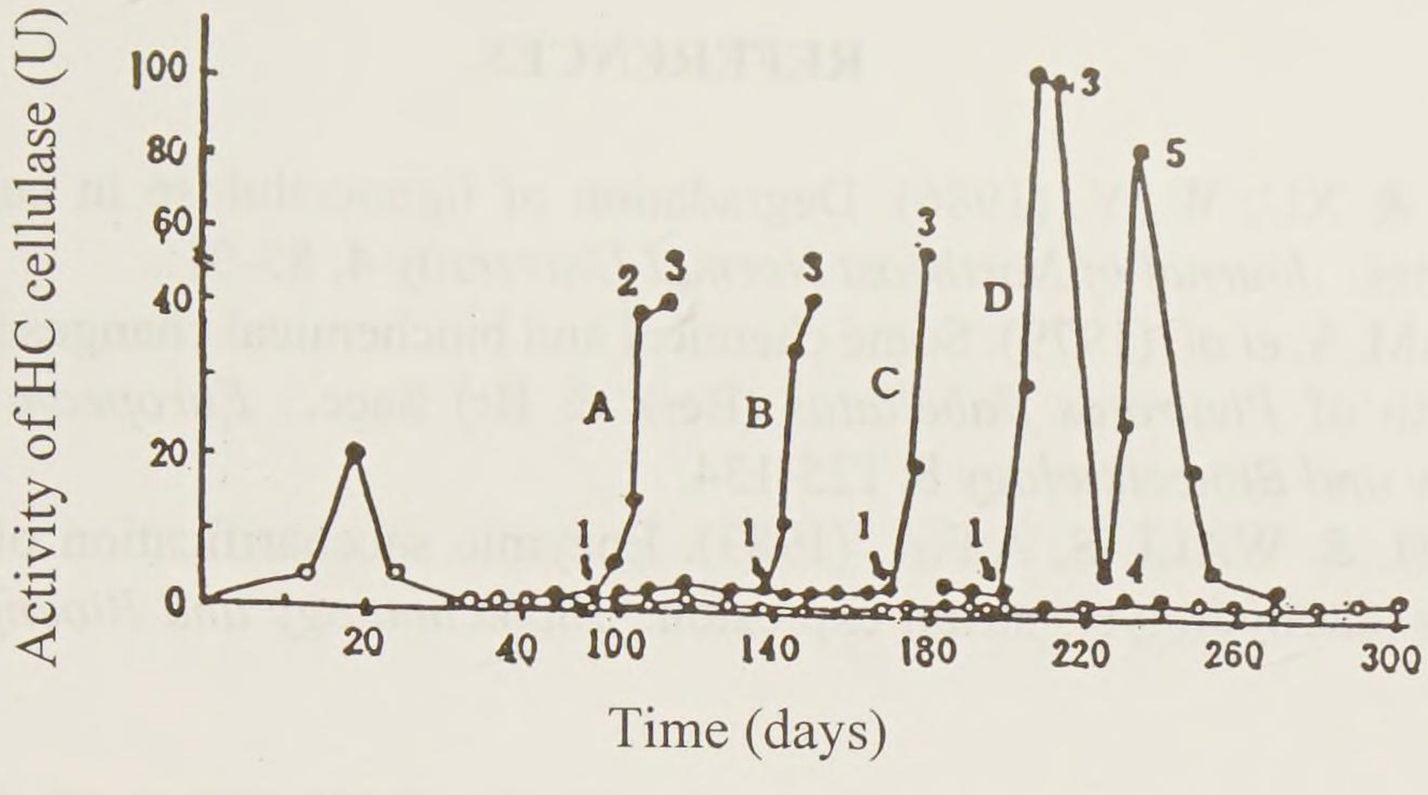

FIGURE 3. Effects of incubation temperature on *Pholiota nameko* fruitbody formation and activity of hemicellulase in the substrate (Legend as for FIGURE 2).

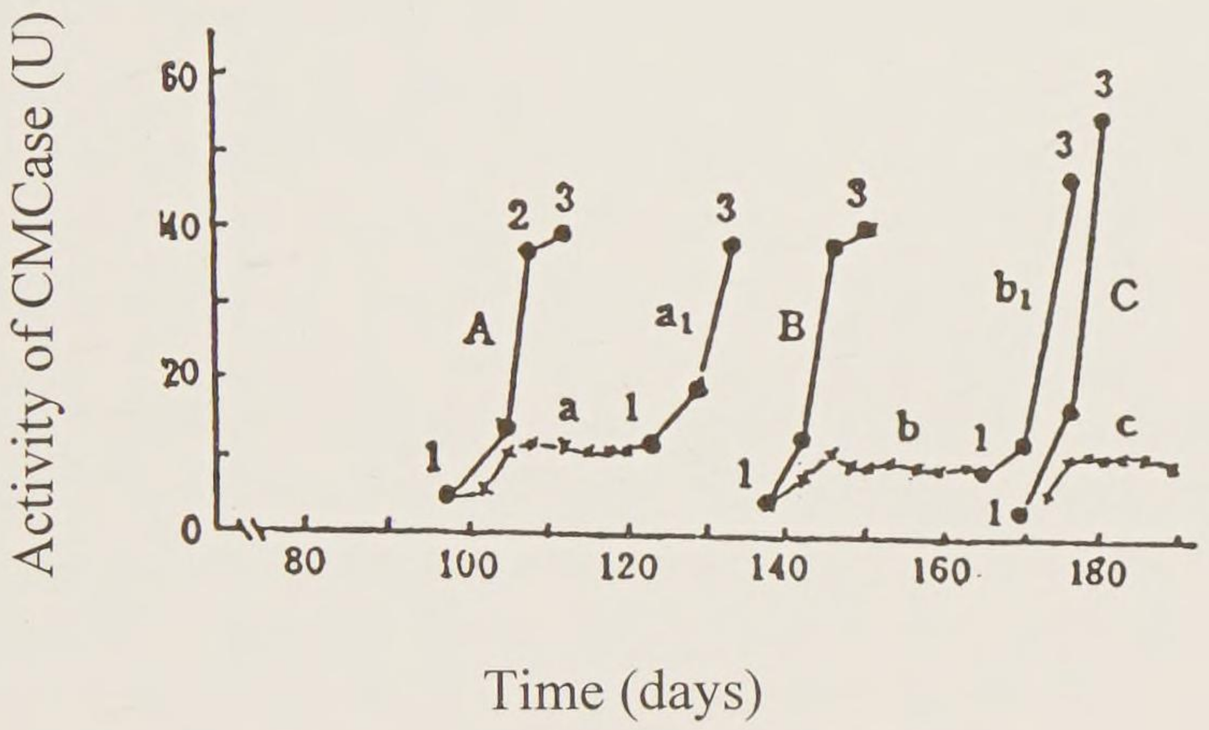

FIGURE 4. Cutting off of primordia to inhibit the activity of CMCase. o —— o enzyme activity when fruiting; A, B, C treatment at 9-10°C after different incubation times; x—— x enzyme activity after cutting off primordia; a, b, c treatment at 9-10°C after different incubation times; 1, 2, 3 different stages of fruitbody development; a1, b1 when fruiting again, the enzyme peak appears in substrate.

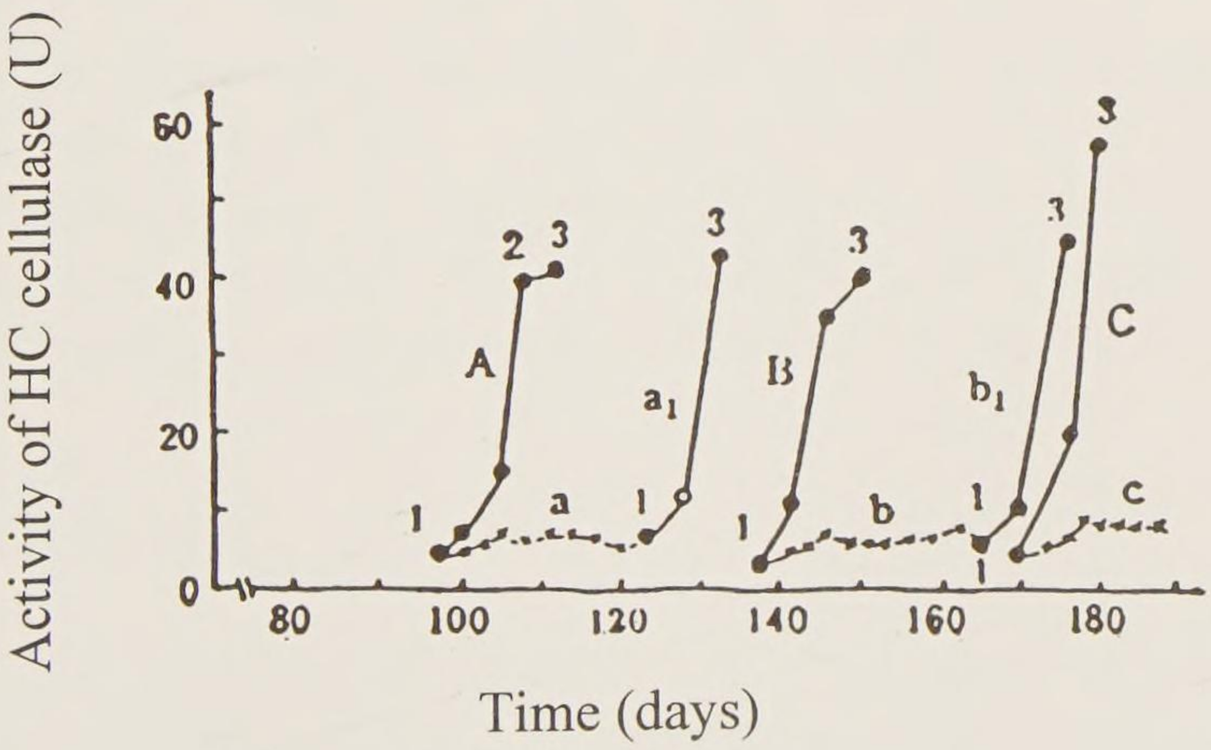

FIGURE 5. Cutting off of primordia to inhibit the activity of hemicellulase (Legend as for FIGURE 4).

REFERENCES

WANG, Y. W. & XU, W. Y. (1986). Degradation of lignocellulose in sugarcane bagasse by basidiomycetes. *Journal of Northeast Normal University* **4**, 85-91.

RAJARATHNAM, S. *et al*. (1979). Some chemical and biochemical changes in straw constituents during growth of *Pleurotus flabellatus* (Berk & Br) Sacc. *European Journal of Applied Microbiology and Biotechnology* **8**, 125-134.

DEKKER, R.F.H. & WALLIS, A.F.A. (1983). Enzymic saccharification of sugarcane bagasse pretreated by autohydrolysis-steam explosion. *Biotechnology and Bioengineering* **25**, 3027-3048.

Part III

POST-HARVEST PROCESSING AND QUALITY CONTROL

CHAPTER 18

MUSHROOM SENESCENCE: ITS MECHANISM AND CONTROL

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1. INTRODUCTION

The mushroom is a high value food product with a short storage life. To ensure that mushrooms are acceptable and nutritious to the consumer at the time of purchase, it is necessary to delay or prevent senescence. Technologies such as cooling and modified atmosphere packaging can be used to delay the rate of senescence, while preservative technologies such as canning, drying, pickling, freezing and γ-irradiation arrest biological function to prevent senescence. In the U.K., 91% of mushrooms grown are purchased fresh (Anon, 1993). In other European countries and in North America, an increasing proportion of mushrooms are bought fresh as part of the trend for eating healthy food. Consequently, research into quality and storage-life of *Agaricus* spp. has concentrated on non-preservative technologies to delay senescence and the regulation of mushroom senescence. This paper describes research into postharvest biology of *Agaricus* mushrooms and the mechanism of action of modified atmosphere packaging.

Sporophore growth prior to harvest is sustained by nutrients and water from the compost, and casing material. When the sporophore is harvested and the nutrient and water supply are cut off, its metabolism adapts to sustain gill growth and spore production. There have been relatively few areas of investigation into mushroom postharvest physiology, two of which, carbon and nitrogen metabolism are concerned with nutrients and their redistribution.

2. CARBON CATABOLISM

Non-structural carbohydrate can account for 30-50% of the sporophore dry weight. This is mainly in the form of mannitol (which is considered to be the major osmoticum for cell expansion), trehalose and glycogen (Hammond & Nichols, 1975; Hammond, 1979). During 4 days storage at 18°C mannitol levels in intact sporophores fall from 24.5 to 4% and glycogen levels fall from 11.8 to 4.5% (Hammond & Nichols, 1975; Hammond, 1979). These compositional changes are not uniform throughout the sporophore. While the mannitol level in the pileus and cap falls during

postharvest storage, a peak in the mannitol concentration occurs in the stipe after 2 days followed by a decline. Levels of trehalose and glucose within the stipe of stored mushrooms also peak at the same time as mannitol (Hammond & Nichols, 1975).

Mannitol is catabolised by the enzyme NADP-dependent mannitol dehydrogenase producing fructose and NADPH. Mushrooms growing in contact with their mycelium oxidise a relatively large proportion of their hexoses via the pentose phosphate pathway. While the flux through both the pentose phosphate pathway and glycolysis declines after harvest, a greater decline was reported to occur in the pentose phosphate pathway (Hammond 1978). This change facilitates mannitol oxidation by reducing NADPH production. An increase in NADPH oxidase activity also occurred after harvest (Hammond, 1978). Glucosephosphate isomerase activity was shown to increase for the first two days of storage at 18°C followed by a rapid decline. Hammond (1978) speculated that this increased activity was to facilitate hexose transfer from the breakdown of trehalose and glycogen to glycolysis.

3. NITROGEN METABOLISM

Protein is the main source of nitrogen for redistribution after harvest. The level of soluble protein in sporophore tissues decreases during postharvest storage of *A. bisporus* and *A. bitorquis* (Burton, 1988a; Burton *et al*., 1993a). After 5 days storage at 18°C, the soluble protein level falls to 30-70% of that in freshly harvested sporophores. Levels of chitin, urea and cell wall associated protein were shown to increase after harvest (Hammond, 1979). Urea, which is 46.6% nitrogen, increased 9-fold over 4 days storage which is equivalent to approximately 2.4 mg protein g^{-1} (dry weight) (Hammond, 1979). The function of urea is probably as a replacement osmoticum for mannitol. Moore (1984) has shown that urea is the major osmoticum in the developing fruitbodies of *Coprinus cinereus*.

Hammond (1979) speculated that nucleic acid breakdown could provide another nitrogen source. However, both DNA and RNA levels in whole sporophores have been shown to increase postharvest (Minamide & Iwata, 1986). Care should be taken in interpreting this result, as major tissue differences in the nucleic acid levels of pre-harvest developing mushrooms have been observed (Minamide & Hammond, 1985). Nucleic acid levels in the gill tissues have been shown to increase during development while those in the stipe and pileus tissues generally fall.

Proteinase (or protease) activity increases during sporophore senescence (Murr and Morris, 1975a) and this increase has been shown to be a harvest-induced event (Burton, 1988a). The protein level starts to fall prior to the induction of proteinase activity. This is probably due to a distorted protein turnover system, i.e. synthesis is reduced by nutrient limitation but degradation rates remain unchanged. Inhibitor studies have revealed that the proteinases from senescent sporophores of *A. bisporus* and *A. bitorquis* are predominantly metallo- and serine-proteinases (Burton *et al*., 1993a).

A serine-proteinase from senescent sporophores of *A. bisporus* has recently been purified and characterized (Burton *et al.*, 1993b). The enzyme has a broad pH optimum, 6.5-11.5 and a narrow substrate specificity requiring both a hydrophobic amino acid on the amino-terminal side of the scissile bond and a minimum peptide chain length. The molecular mass of the proteinase is 27 kDa (when determined by SDS-PAGE) and its isoelectric point is 9.0. The N-terminal amino sequence of the serine proteinase has 60% identity with that of proteinase K from the fungus, *Tritirachium album*. The serine proteinase of *A. bisporus* accounts for approximately 3% of the soluble protein in the senescent mushroom stipe at the 5 day postharvest stage. For complete degradation of proteins

to amino acids, additional activities of as yet uncharacterised proteinases and peptidases may be required because the serine proteinase has such a narrow substrate specificity.

4. TISSUE BROWNING

Mushroom sporophores are normally white when harvested and develop brown discolouration during storage. The browning reaction is a normal feature of postharvest ageing, but can be increased by mechanical damage or bacterial infection by *Pseudomonas tolaasii*. The enzyme which initiates browning is tyrosinase, which catalyses the oxidation of phenols. This is followed by a series of chemical reactions which convert the oxidation products to the brown polymer, melanin. Tyrosinase is also responsible for melanogenesis during spore maturation (Hegnauer *et al.,* 1985) but in non-reproductive tissues, the most plausible function is as a defensive mechanism against invading micro-organisms. This is supported by the higher tyrosinase activity and phenol concentration found in skin of the pileus (*pilei pellis*) than in the underlying pileus flesh (*pileus trama*) of both *A. bisporus* and *A. bitorquis* (Burton *et al*., 1993a).

Tyrosinase is a copper-containing oxidase and has been described as a tetramer consisting of two heavy (43 kDa) and two light (13 kDa) subunits (Strothkamp *et al*., 1976). Tyrosinase antibodies have been used to immunoprecipitate *in vitro* translation products of total purified m-RNA. The two proteins so identified have molecular weights of 41 kDa and 15 kDa (Podila & Flurkey, 1986). Two research groups using PCR techniques have recently identified putative sequences containing the *Agaricus* tyrosinase gene (Khush *et al*., 1991; Wichers *et al*., 1993). A stated aim of several groups world-wide is to remove or reduce tyrosinase activity by molecular genetical techniques and so enhance the colour characteristics of the mushroom. If this aim is achieved, it will be interesting to observe not only changes in any quality characteristics but also possible changes in resistance to bacterial attack.

Tyrosinase from *A. bisporus* has been shown to be present in two forms, active and latent (Yamaguchi *et al*., 1970). The enzyme can be activated *in vitro* by the detergent sodium dodecyl sulphate, SDS (Yamaguchi *et al*., 1970) or by proteolytic action (Burton, 1988b). During postharvest storage of sporophores, when cap browning develops in a linear manner (Smith *et al*., 1993), no increases have been observed in the tyrosinase activity or phenol level of the sporophore skin in both *A. bisporus* and *A. bitorquis* (Burton *et al*., 1993). However, the *in vitro* activated tyrosinase activity (by SDS or trypsin) declines with increasing storage duration possibly indicating *in vivo* activation during storage. The whiteness of different strains **at the time of harvest** is consistent with their low phenol levels and tyrosinase activities but no correlation between these factors and **postharvest** discolouration could be observed (Burton *et al*., 1993a). It has been hypothesized that tyrosinase and phenols may be confined to separate compartments in the cell and during postharvest ageing the intracellular membranes may break down (Burton, 1986).

5. CELL STRUCTURE AND TISSUE PARTITIONING

The membrane components have been examined during postharvest storage (Braaksma & Schaap, 1991; Braaksma *et al.*, 1993). Phosphatidic acid was reported to increase in the stipe and cap during ageing, which may be indicative of increased activity of phospholipase D. Membrane protein content showed a marked decrease during storage. The overall conclusion of these reports,

however, is that membrane integrity is not lost during storage.

Expansion of the pileus by growth of gills and elongation of the stipe postharvest is supported by increased cell wall chitin and protein (Hammond, 1979). Chitin synthase can be activated by proteinases (Hänsler *et al.*, 1983). Recently, a compound 10-oxo-*trans*-8-decanoic acid (ODA) has been isolated from mushrooms and shown to stimulate mycelial growth and stipe elongation (Mau *et al.*, 1992). ODA is produced when the fruitbody tissues are damaged (e.g. harvested). Hammond and Nichols (1975) have shown that during ageing, there is a major re-distribution of dry weight between tissues with gill dry weight increasing during storage while that of the pileus and stipe diminishing. The rate of cap opening depends on the stipe length (the longer the stipe, the greater the expansion), indicating that the stipe is acting as a major nutrient source for the expanding gill tissue (Ajlouni *et al.*, 1992).

6. MODIFIED ATMOSPHERE PACKAGING

Modified atmosphere packaging (MAP) has been shown experimentally to delay senescence by delaying cap browning, retarding development and microbial spoilage (Burton, 1991 and references therein). However, this technology is currently not widely used largely due to economic factors. Under MAP mushroom sporophores are subjected to a raised carbon dioxide level, a reduced oxygen level and a higher relative humidity. Tyrosinase has a Km for oxygen equivalent to water saturated in 12% oxygen (Duckworth & Coleman, 1970) with catechol as a co-substrate. Therefore, a reduction in oxygen concentration as found in MAP would be expected to reduce tyrosinase activity and tissue discolouration. However, Briones *et al.* (1992) found that carbon dioxide rather than oxygen has the major influence on cap discolouration. Murr and Morris (1975b) showed that oxygen levels reduced to 2% or carbon dioxide levels raised to 5% had no effect on the induction of postharvest proteases. Modified atmosphere inhibits respiration and mannitol loss but the sporophore can adapt by increasing its rate of respiration (measured in air) particularly in response to increased carbon dioxide concentrations (Briones *et al.*, 1992). Carbon dioxide was also shown by Briones *et al.* (1992) to be the major factor influencing texture and cap opening. This is consistent with the widely described role of carbon dioxide as a regulator for mycelial growth and mushroom morphogenesis (Hammond & Wood, 1985).

REFERENCES

AJLOUNI, S.O., BEELMAN, R.B., THOMPSON, D.B. & MAU, J-L. (1992). Stipe trimming at harvest increases shelf-life of fresh mushrooms (*Agaricus bisporus*). *Journal of Food Science* **57**, 1361-1363.

ANON (1993). Mushroom statistics 1992. *The Mushroom Journal* **520**, 31-33.

BRAAKSMA, A. & SCHAAP, D.J. (1991). Senescence in mushrooms. In *Plant Lipid, Biochemistry Structure and Utilization*, pp. 421-423. Edited by P.J. Quinn & J.L. Harwood. Colchester: Porthead Press.

BRAAKSMA, A., SCHAAP, D.J. DE VRIJE, T., JONGEN, W.M.F., WOLTERING, E.J. & WICHERS, H.T. (1993). Ageing of the mushroom (*Agaricus bisporus*) under postharvest conditions. *Conference Proceedings from First International Conference on Mushroom Biology and Mushroom Products*, Hong Kong, 23-26 August 1993.

BRIONES, G.L., VAROQUAUX, P., CHAMBROY, V., BOUQUANT, J., BUREAU, G. & PASCAT, B. (1992). Storage of common mushroom under controlled atmospheres. *International Journal of Food Science and Technology* **27**, 493-505.

BURTON, K.S. (1986). Quality - Investigations into mushroom browning. *The Mushroom Journal* **158**, 68-70.

BURTON, K.S. (1988*a*). The effects of storage and development on *Agaricus bisporus* proteases. *Journal of Horticultural Science* **63**, 103-108.

BURTON, K.S. (1988*b*). The effects of pre- and post-harvest development on mushroom tyrosinase. *Journal of Horticultural Science* **63**, 255-260.

BURTON, K.S. (1991). Modified atmosphere packaging of mushrooms - Review and recent developments. *Mushroom Science* **13**, *Science and Cultivation of Edible Fungi* (Maher, M.J. Ed.), A.A. Balkema, Rotterdam.

BURTON, K.S., LOVE, M.E. & SMITH, J.F. (1993a). Biochemical changes associated with mushroom quality in *Agaricus* spp. *Enzyme and Microbial Technology* **15** (in press).

BURTON, K.S., WOOD, D.A., THURSTON, C.F. & BARKER, P.J. (1993b). Purification and characterization of a serine proteinase from senescent sporophores of the commercial mushroom *Agaricus bisporus*. *Journal of General Microbiology* **139**, (in press).

DUCKWORTH, H.W. & COLEMAN, J.E. (1970). Physiochemical and kinetic properties of mushroom tyrosinase. *Journal of Biological Chemistry* **245**, 1613-1625.

HAMMOND, J.B.W. (1978). Carbohydrate catabolism in harvested mushrooms. *Phytochemistry* **17**, 1717-1719.

HAMMOND, J.B.W. (1979). Changes in the composition of the harvested mushroom (*Agaricus bisporus*). *Phytochemistry* **18**, 415-418.

HAMMOND, J.B.W. & NICHOLS, R. (1975). Changes in respiration and soluble carbohydrates during the post-harvest storage of mushrooms (*Agaricus bisporus*). *Journal of Science of Food and Agriculture* **26** 835-842.

HAMMOND, J.B.W. & WOOD, D.A. (1985). Metabolism, biochemistry and physiology. In *The Biology and Technology of the Cultivated Mushroom*, pp. 63-80. Edited by P.B. Flegg, D.M. Spencer & D.A. Wood. Chichester, UK: Wiley.

HANSELER, E., NYLEN, L.E. & RAST, D.M. (1983). Isolation and properties of chitin synthase from *Agaricus bisporus* mycelium. *Experimental Mycology* **7**, 17-30.

HEGNAUER, H., NYLEN, L.E. & RAST, D.M. (1985). Ultrastructure of native and synthetic *Agaricus bisporus* melanins - Implications as to compartmentation of melanogenesis in fungi. *Experimental Mycology* **9**, 221-229.

KHUSH, R.S., MORGAN, L., BECKER, E. & WACH, M. (1991). Use of the polymerase chain reaction (PCR) in *Agaricus bisporus* breeding programs. In *Genetics and Breeding of Agaricus, Proceedings of the First International Seminar on Mushroom Science*. Edited by L.J.L.V. van Griensven. Wageningen, The Netherlands: Pudoc Press.

MAU, J-L., BEELMAN, R.B. & ZIEGLER, G.R. (1992). Effect of 10-oxo-*trans*-8-decanoic acid on growth of *Agaricus bisporus*. *Phytochemistry* **31**, 4059-4064.

MINAMIDE, T. & HAMMOND, J.B.W. (1985). The influence of the periodic fruiting (flushing) cycle on the biochemical development of *Agaricus bisporus* sporophores. *New Phytologist* **100**, 571-578.

MINAMIDE, T. & IWATA, T. (1986). Studies on physiological and chemical properties and postharvest keeping quality of mushrooms. XI. Effect of storage temperature on the taste compounds and on protease and nucleic acid degrading enzyme activities of mushrooms

(*Agaricus bisporus*). *Bulletin of the University of Osaka Prefecture (Series B)* **28**, 1-11.
MOORE, D. (1984). Developmental biology of the *Coprinus cinereus* carpophore: metabolic regulation in relation to cap morphogenesis. *Experimental Mycology* **8**, 283-297.
MURR, D.P. & MORRIS, L.L. (1975a). Effect of storage temeprature on post-harvest changes in mushrooms. *Journal of the American Society of Horticultural Science* **100**, 16-19.
MURR, D.P. & MORRIS, L.L. (1975b). Effect of storage atmosphere on postharvest growth of mushrooms. *Journal of the American Society Horticulture* **100**, 298-301.
PODILA, G.K. & FLURKEY, W.H. (1986). *In vitro* translation of mushroom tyrosinase. *Biochemical and Biophysical Research Communications* **141**, 697-703.
SMITH, J.F., LOVE, M.E. & BURTON, K.S. (1993). Comparative studies of the quality of fresh and stored mushrooms of *Agaricus bisporus* with two tropical *Agaricus bitorquis* strains. *Annals of Applied Biology* **123** (in press).
STROTHKAMP, K.G., JOLLEY, R.L. & MASON, H.S. (1976). Quaternary structure of mushroom tyrosinase. *Biochemical and Biophysical Research Communications* **70**, 519-524.
WICHERS, H.J., GERRITSEN, Y.A.M., VAN DUIJNHOVEN, G.C.F. & RECOURT, K. (1993). Molecular characteristics of *Agaricus bisporus* tyrosinase. *Conference Proceedings from First International Conference on Mushroom Biology and Mushroom Products*, Hong Kong, 23-26 August 1993.
YAMAGUCHI, M., HWANG, P.M. & CAMPBELL, J.D. (1970). Latent o-diphenol oxidase in mushrooms (*Agaricus bisporus*). *Canadian Journal of Biochemistry* **48**, 198-202.

CHAPTER 19

SELECTED CULTURAL AND HARVEST PRACTICES TO IMPROVE QUALITY AND SHELF LIFE OF *AGARICUS* MUSHROOMS

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1. INTRODUCTION

Cultivated mushrooms, especially the commonly grown strains of *Agaricus bisporus* are currently grown under intense conditions which can often result in compromised quality. Also, they are highly perishable and tend to rapidly lose the quality attributes that make them appealing to consumers, thereby reducing their value and/or market demand. Most consumers appear to favor tight buttons, light or white in color with smooth, unspotted surface appearance and a crisp, tender texture. Unfortunately, mushrooms may be off-white in color with scaly surface at harvest. At harvest, mushrooms may rapidly begin to darken, the caps open and expose the darkening gills as spores develop, the stems lengthen, and all of the tissues toughen and become chewy. These changes occur as a result of normal senescence phenomena and/or microbial activity. This paper will focus on research conducted at Penn State University related to identifying selected cultural and harvesting practices that can improve quality and shelf life.

2. TREATMENT OF IRRIGATION WATER WITH CALCIUM CHLORIDE

Extensive experimentation over the past 7 years has demonstrated that appropriate addition of calcium chloride to irrigation water reduces bacterial blotch, improves initial quality and postharvest shelf life without reducing crop yield (Beelman *et al.*, 1987; Barden *et al.*, 1990; Bartley *et al.*, 1991; Solomon *et al.*, 1991; Beelman *et al.*, 1992; Mau *et al.*, 1993a). Optimum results to date were achieved by addition of 0.3 per cent $CaCl_2$ (Dow Flake, Process R Grade; Dow Chemical Co, Midland, MI) to all water applied to the casing layer after pinset through the end of the crop.

Two recent crops were grown under semi-commercial scale conditions at the Mushroom Test Demonstration Facility (MTDF) on our campus. One crop (MTDF #1907 was treated with 0.3% calcium chloride and the other (MTDF #1906) served as an untreated (control) crop. The harvested

mushrooms were weighed to determine yield. The quality of mushrooms was determined by evaluating color using a Minolta Chromameter CR200. Color and maturity index (rate of cap opening) were determined during postharvest storage in PVC-overwrapped packages using methods employed routinely in our laboratory (Ajlouni, 1991).

The effects of calcium chloride added to irrigation water on yield and initial color of mushrooms at harvest are presented in Figure 1. The effect on postharvest shelf life as evidenced by postharvest browning is presented in Figure 2. Yield curves (Fig. 1A) illustrate that calcium chloride treatment gave a more even yield especially in the first and second flush. However, calcium chloride did not significantly reduce overall yield. Yields were 24.4 vs. 25.7 Kg/m^2, and biological efficiencies were 87.0% vs. 86.4% for $CaCl_2$ treatment and control, respectively. The data in Figure 1B clearly demonstrate that $CaCl_2$ added to irrigation water improved the colour (higher L value indicates whiter) of mushrooms from harvest throughout the crop cycle (Fig. 1B) despite the general decline in whiteness that occurred in later flushes. Also, mushrooms grown with added calcium chloride showed a reduced rate of postharvest browning compared to non-treated mushrooms (Fig. 2). Since calcium chloride is an inexpensive, food grade chemical commonly used to improve quality of fruits and vegetables, it appears that this technique has considerable potential to improve quality of fresh market mushrooms that can be easily employed by growers without any need for regulatory approval.

3. INFLUENCE OF MATURITY AT HARVEST

An experiment involving two crops using the same off-white hybrid spawn (Amycel U-1 #20) grown at the MTDF (Crops #1711 and #1715) employed the same cultural conditions except harvesting. Crop (#1715) was harvested normally (veils intact but slightly stretched) while the other (#1711) was harvested at a more immature stage (veils intact and tight). With crop #1711, pickers were instructed to harvest immature mushrooms by selecting those they would normally wait to pick the following day.

Surprisingly, harvesting more immature mushrooms resulted in slightly higher yield (33.4 vs. 30.0 kg/m^2 in five flushes) than the crop harvested normally (Fig. 3). Mushrooms harvested at a more immature state had better shelf life as evidenced by both a slower rate of browning (Fig. 4A) and reduced rate of cap opening (Fig. 4B).

These results indicate that harvesting mushrooms at an earlier stage of maturity can improve quality and shelf life without sacrificing yield. This could be an easy and inexpensive way for growers to improve the quality of their product. However, influence on mushroom size was not determined which should be done before this practice can be fully assessed for its potential. Also, this kind of experiment needs to be repeated to determine the consistency of the results that can be realized.

4. INFLUENCE OF STIPE TRIMMING AT HARVEST

Stipe trimming is the process of cutting off a portion of the lower stipe and the attached mycelium and casing material at harvest. Some mushroom growers instruct pickers to leave as much lower stipe as possible on the fruiting bodies (up to about 35mm) in order to maximize yield and consequent economic return. However, one noticeable feature of harvested mushrooms during storage is the elongation of the stipe; hence, mushrooms with short stipes result in proportionally less

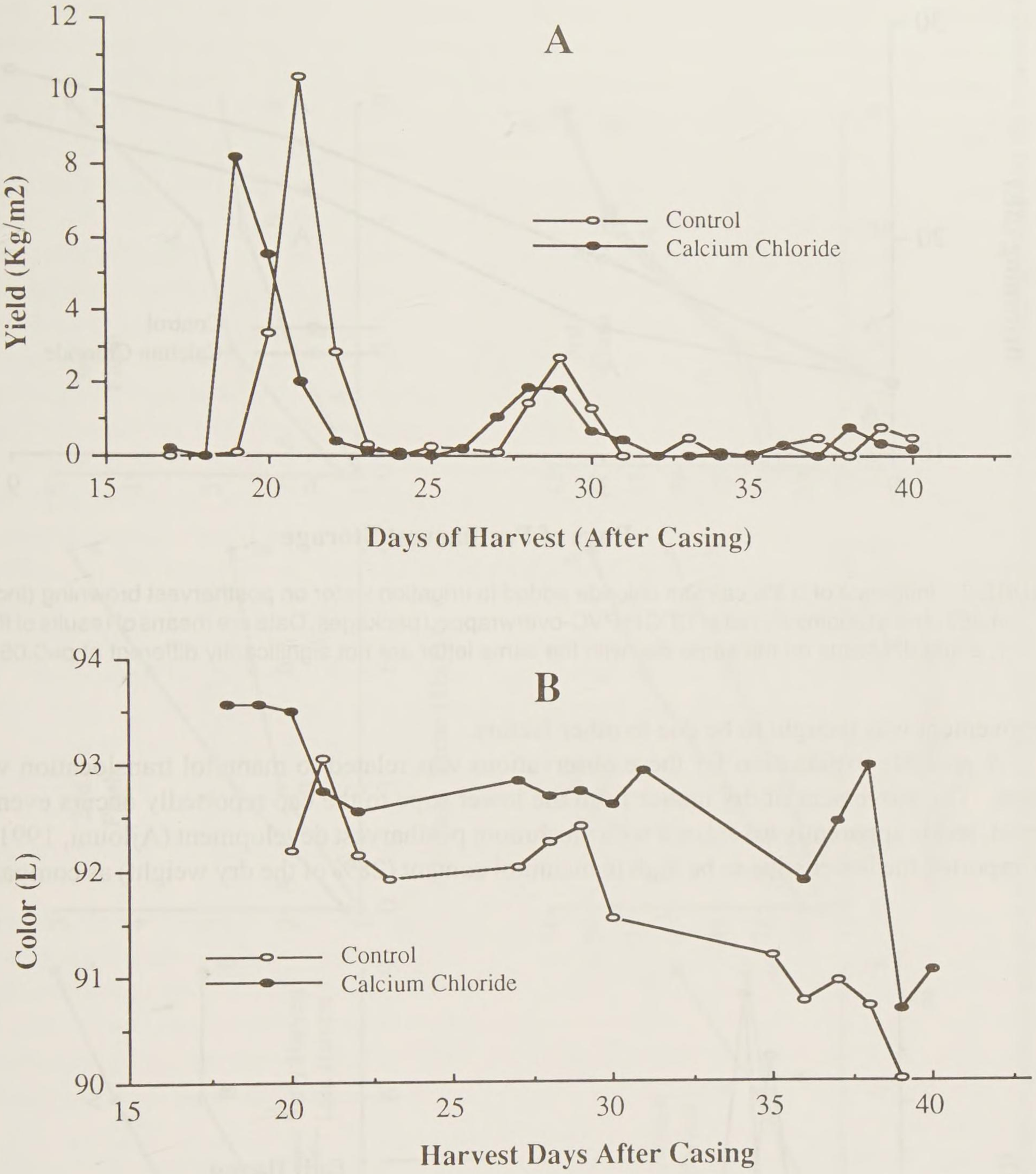


FIGURE 1. Influence of 0.3% calcium chloride added to irrigation water on (A) yield and (B) initial color (L value) of off-white hybrid mushrooms grown at the MTDF.

stipe after storage when compared to mushrooms with initial long stipes. Therefore, some growers trim stipes closer to the cap to minimize change in appearance after storage.

Recently, experiments in our laboratory have demonstrated that trimming stipes closer to the cap at harvest can also improve postharvest shelf life (Ajlouni *et al.*, 1993). They indicated that trimming the stipe of mushrooms, from 35mm to 5mm from the cap immediately after harvest resulted in improved shelf life as indicated by reduced browning and slower cap opening. This effect was evident after 3 days storage at 12°C and became more pronounced after 6 days. Trimming the stipes had no significant effect on postharvest respiration rate or bacterial growth. Thus, the shelf life

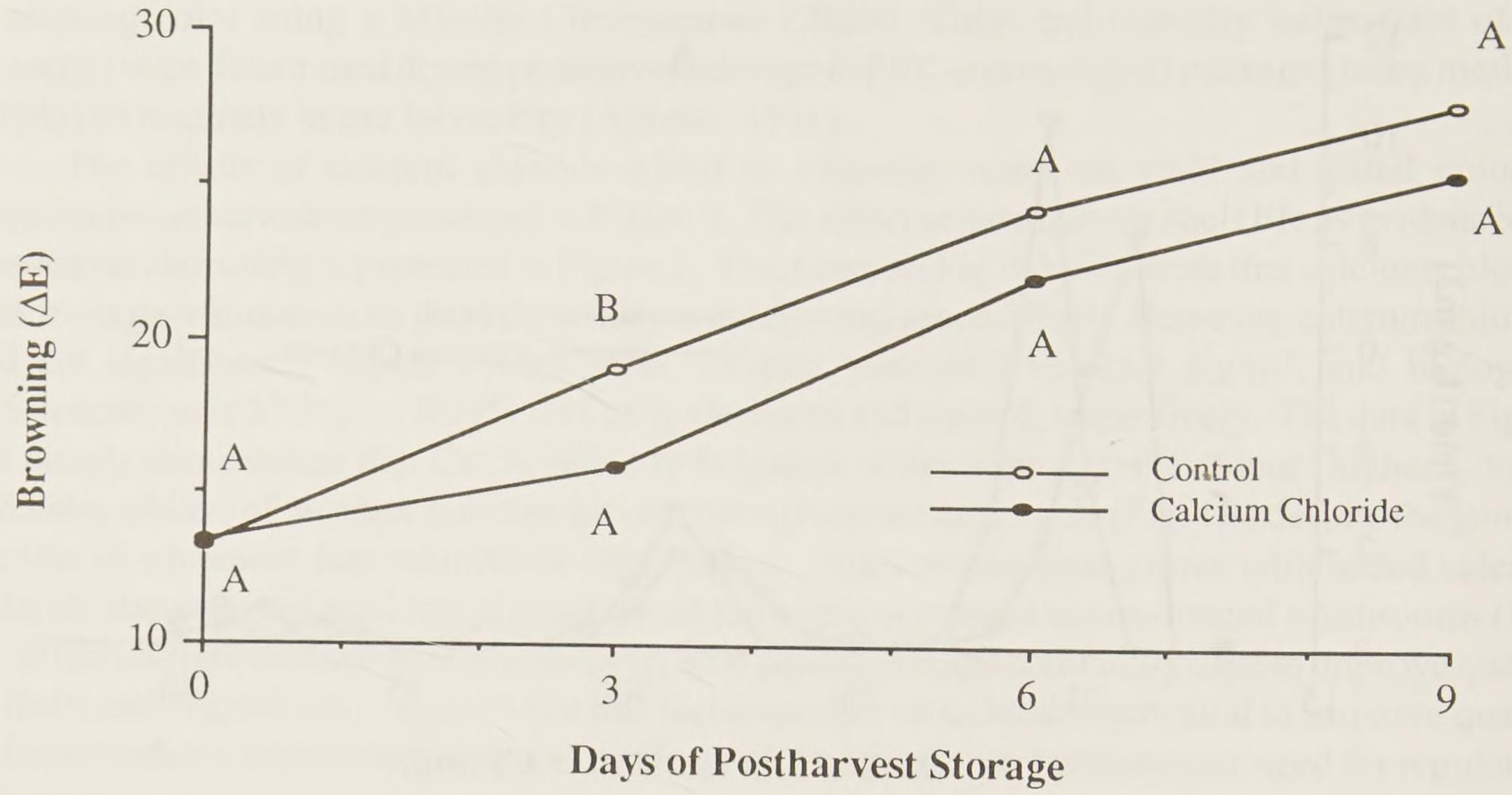


FIGURE 2. Influence of 0.3% calcium chloride added to irrigation water on postharvest browning (increase in ΔE) of mushrooms stored at 13°C in PVC-overwrapped packages. Data are means of results of flushes 1, 2 and 3. Means on the same day with the same letter are not significantly different at p=0.05.

improvement was thought to be due to other factors.

A possible explanation for these observations was related to mannitol translocation within tissues. The movement of dry matter from the lower stipe to the cap reportedly occurs even after harvest, and is apparently associated with mushroom postharvest development (Ajlouni, 1991). He also reported the lower stipe to be high in mannitol content (28% of the dry weight) as compared to

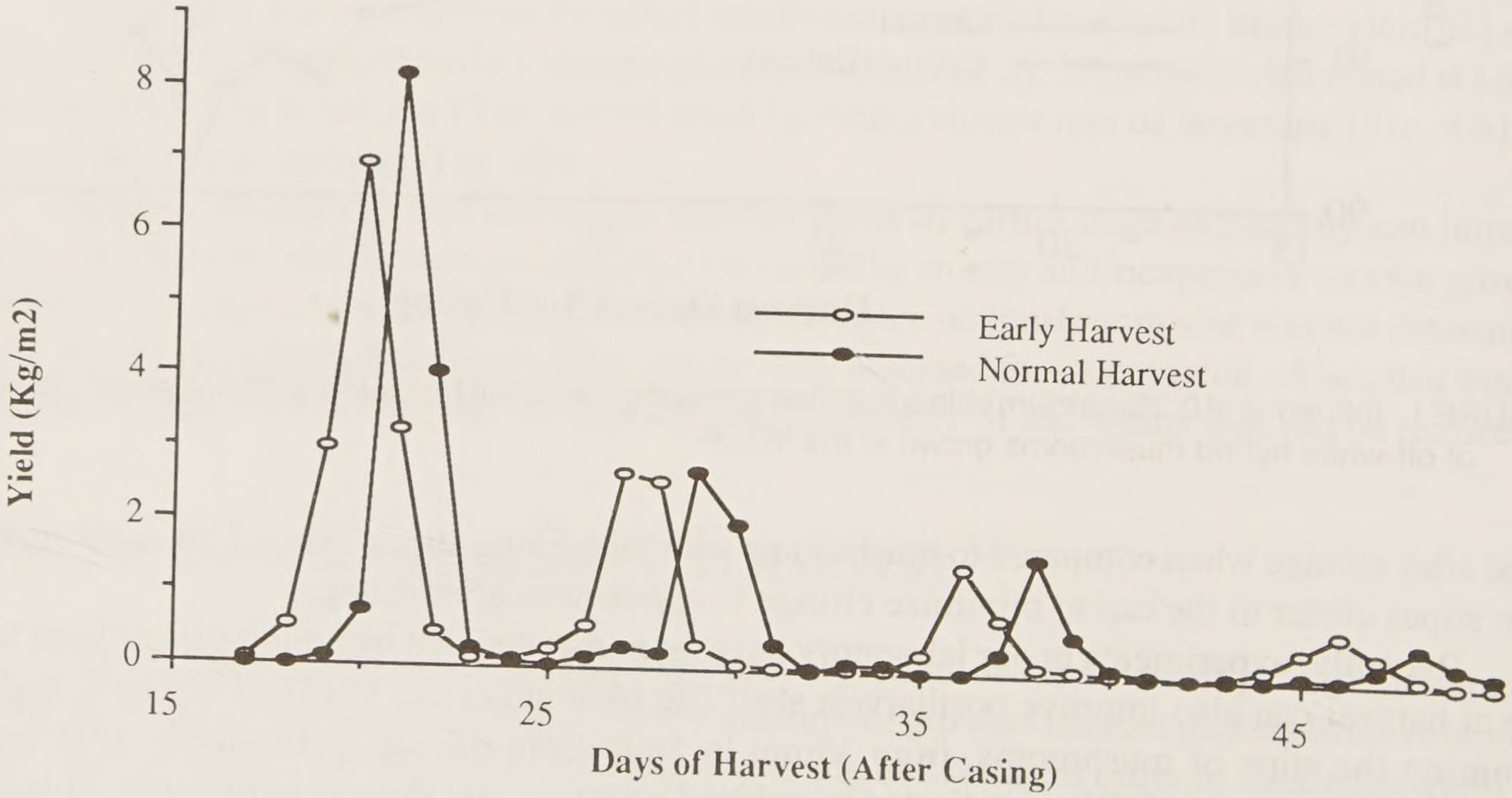


FIGURE 3. Influence of maturity at harvest on yield of mushrooms.

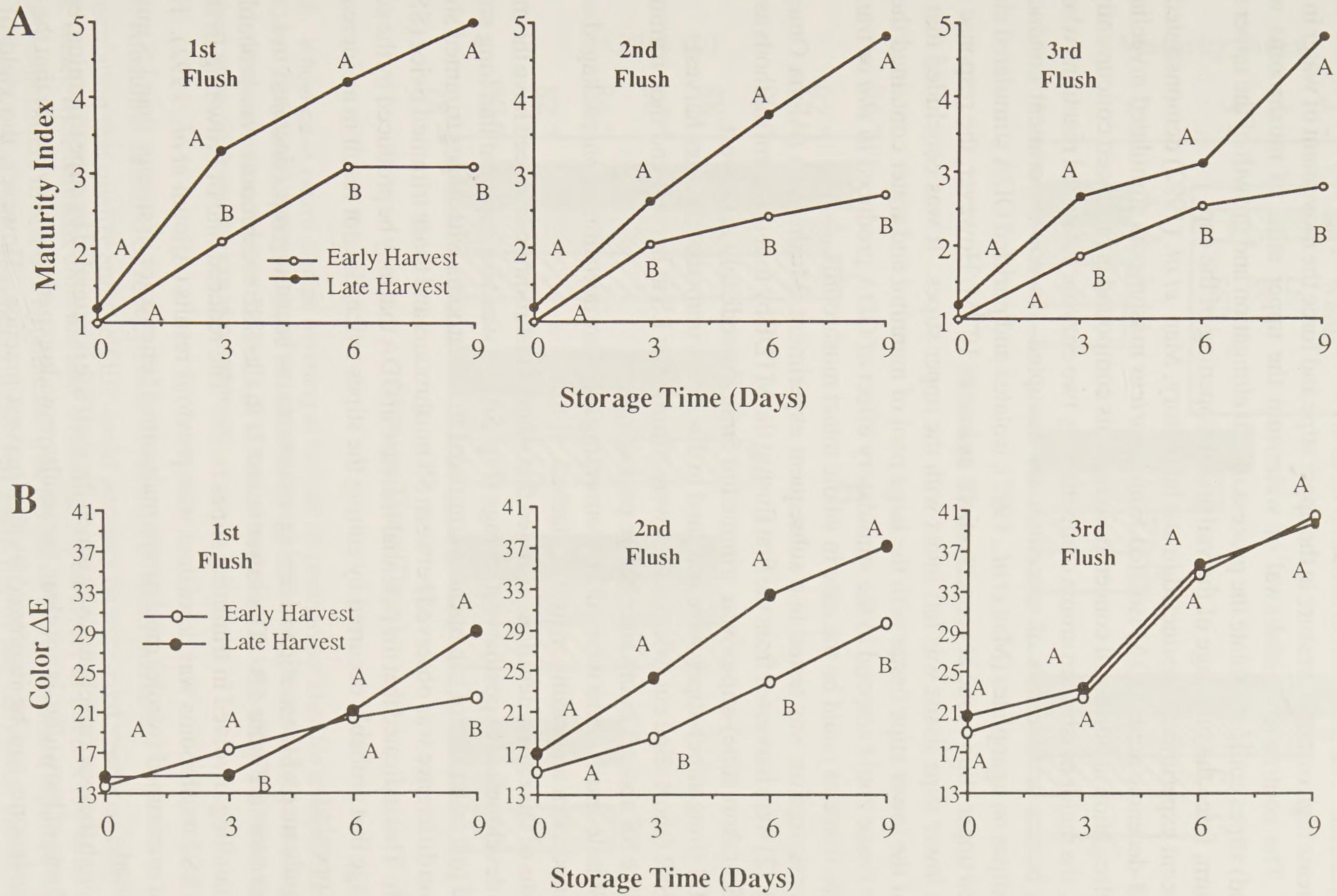


FIGURE 4. Influence of maturity at harvest on postharvest shelf life as indicated by (A) cap development (increase in maturity index) and (B) browning rate (increase in △E) in storage at 13°C. Means followed by the same letter on the same day are not significantly different at p=0.05.

the gills (10%) and the upper stipe (19%), and demonstrated apparent translocation of mannitol from the lower to the upper stipes during postharvest storage. Because the process of upper stipe cell elongation is involved in cap opening, normal maturation would be slowed if substrate for elongation were not available. Translocation of mannitol from the lower to the upper stipes of mushrooms could possibly create high osmotic pressure in the upper stipe and force the movement of water in the same direction. The continuous withdrawal of water into the upper stipe of mushrooms with long (untrimmed) stipes could stimulate the process of cell elongation and growth of the upper stipe that could, in turn, force the breakage of the veil and the opening of the cap.

In recent experiments conducted in our laboratory, Mau *et al.* (1992) demonstrated that 10-oxo-trans-8-decenoic acid (ODA) isolated from *Agaricus* mushrooms stimulated mycelial growth and stipe elongation at hormonal concentrations. This compound is produced concurrently with 1-octen-3-ol, the major mushroom aroma compound, by two enzyme catalyzed reactions when tissues of fruiting bodies or filaments of mycelium are disrupted. In one experiment conducted with separated stipes on agar plates (Mau *et al.*, 1992), isolated and purified ODA stimulated elongation of separated upper stipe tissue at concentrations as low as 10^{-8}M. However, the response occurred only when lower stipe tissue was left intact with the upper stipes. It was concluded that with the presence of the lower stipe tissue with the large pool of mannitol and water contained therein, the upper stipe tissue could respond to the stimulatory effect of ODA produced *in situ* or translocated from the gill tissue as could be the case in whole intact mushrooms.

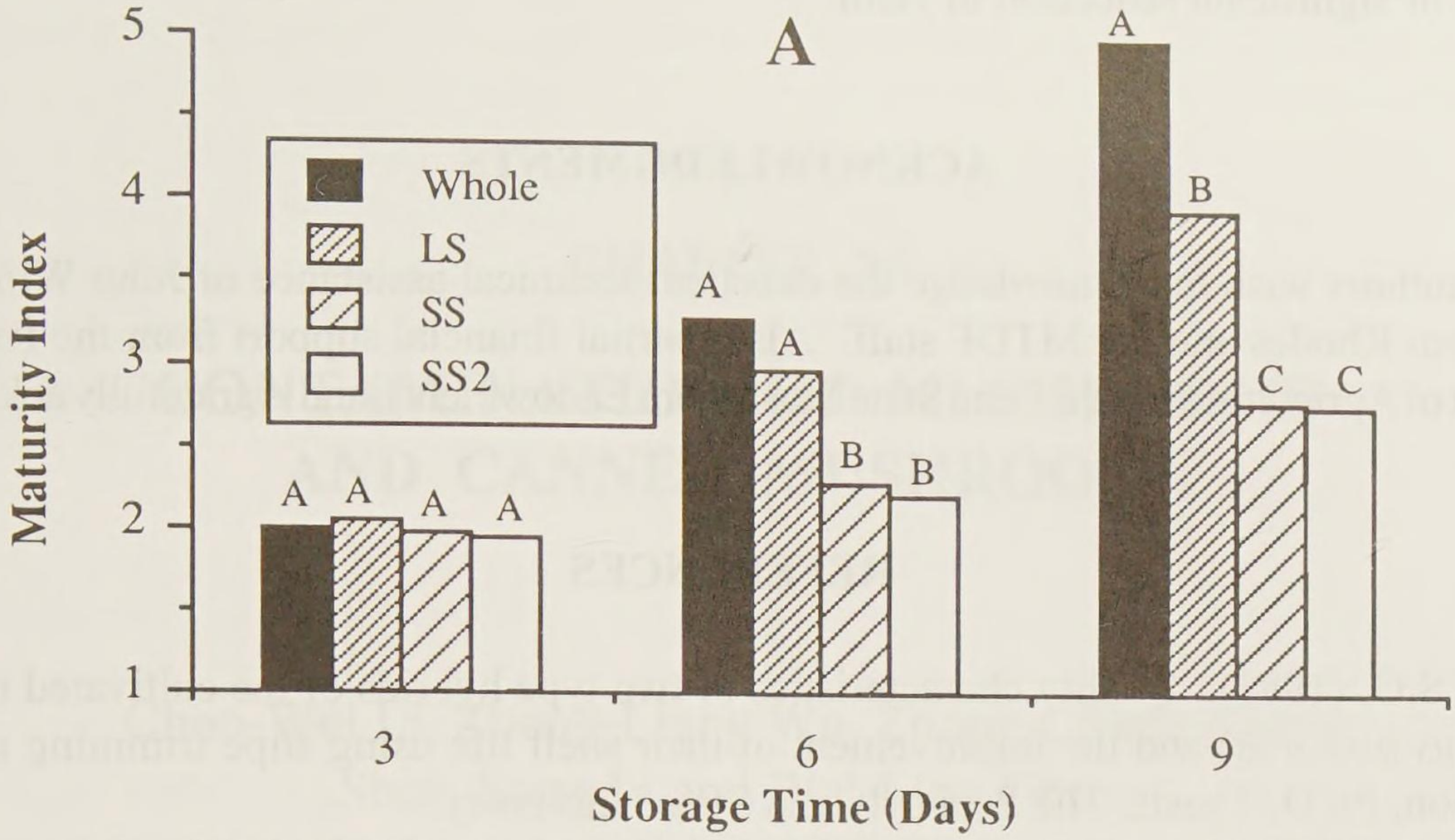
This assumption was tested in a subsequent experiment. Mushrooms (Alpha Omega white hybrid #20-22) were harvested from the first flush at the MTDF by four different methods as follows:

1. SS (short stipe)-stipes were trimmed to 5mm immediately at harvest.
2. LS (long stipe)-stipes were trimmed to 30-35mm immediately after harvest.
3. SS2 (short stipe cut twice)-stipes were trimmed as LS at harvest and then trimmed again like SS about 3 hours later before packaging.
4. Whole-mushrooms were pulled from casing and were not trimmed but packaged with some mycelium and casing material attached.

Results of this experiment demonstrated that whole mushrooms did indeed have the most rapid rate of cap development in postharvest storage (Fig. 5A). It was also apparent that long stipes (LS) contributed to a faster rate of cap opening compared to mushrooms with stipes trimmed short (SS). However, no difference was observed between SS mushrooms and those trimmed twice (SS2) to the same length. This indicated that the potential increase in ODA that may be produced by the additional tissue damage that probably occurred by cutting the stipes twice, did not result in an increase in the rate of cap opening.

The influence of these stipe trimming treatments on browning rate (decrease in L value) in storage is shown in Figure 5B. The lower initial L in the SS2 mushrooms was probably due to increased handling involved in trimming stipes twice. The reduced rate of browning in storage in the SS and SS2 mushrooms was consistent with previous results (Ajlouni *et al.*, 1992). However, the fact that untrimmed (whole) mushrooms maintained better color in storage than LS mushrooms is unexplained.

Although the exact mechanism by which the shelf life is extended by stipe trimming, especially the reduced rate of browning, is not clear, the results provide experimental evidence that the shelf life of fresh mushrooms can be improved by this harvest practice. However, the yield of saleable mushrooms may be decreased by this practice (Ajlouni, 1991). This reduction in yield would decrease potential revenue for growers unless they receive a differential price for their product based on improvement in initial appearance due to shorter stipes or improved postharvest shelf life resulting



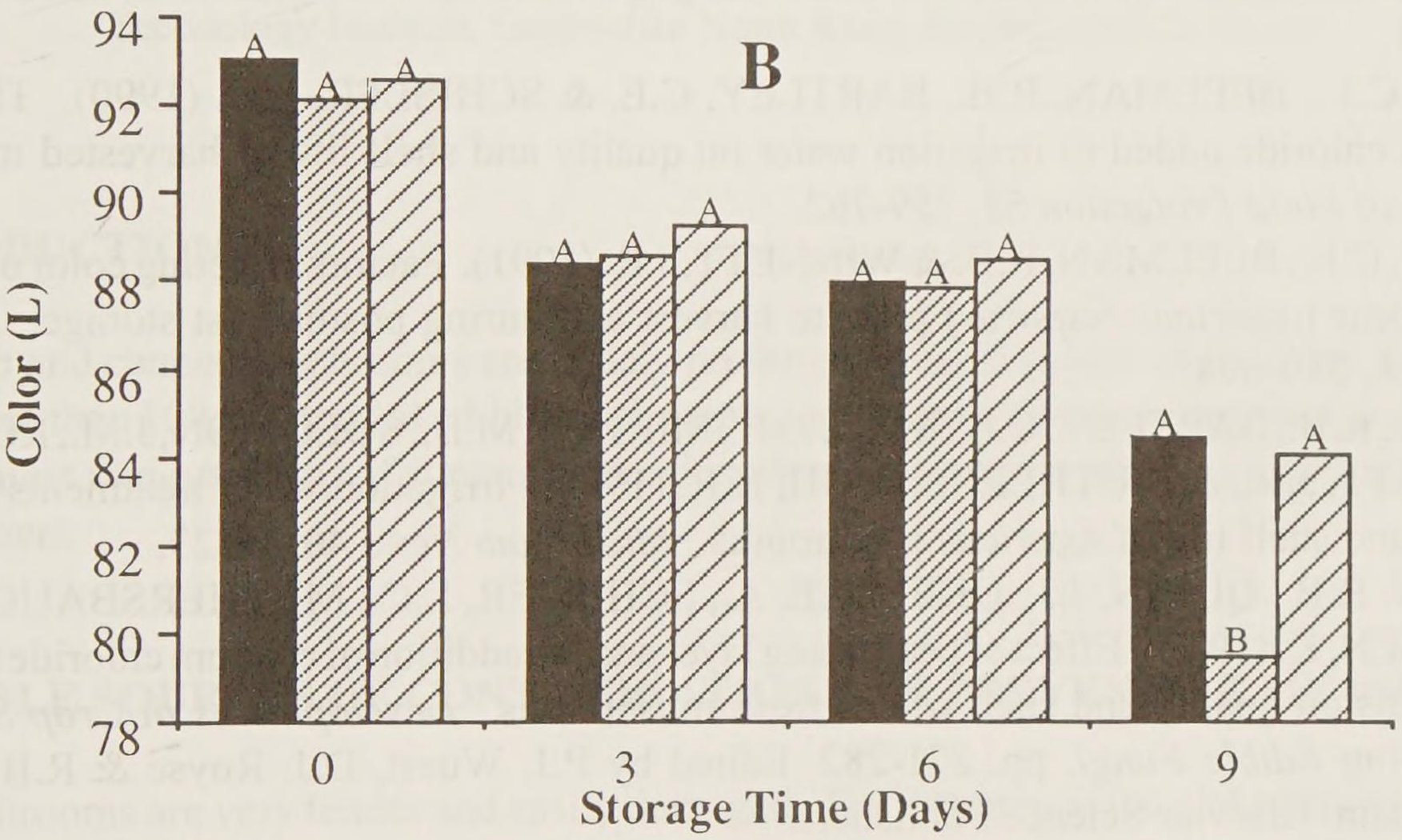


FIGURE 5. Influence of extent of stipe trimming at harvest on postharvest shelf life of mushrooms stored at 13°C in PVC overwrapped packages as indicated by (A) cap development and (B) browning (decrease in L). Whole: whole mushroom picked; LS: trimmed mushroom to long stipe; SS: trimmed mushroom to short stipe; SS2: mushroom trimmed twice.

in greater consumer demand or reduced loss of product in retail markets.

Speculation that quality and shelf life could possibly be improved synergistically by treating irrigation water with calcium chloride and trimming stipes closer at harvest led us to conduct an appropriate experiment to test this hypothesis. The results of this experiment reported by Mau *et al.* (1993b) indicated that both treatments improved shelf life of the harvested mushrooms, and a synergism was evident when both treatments were employed together. Shelf life was dramatically improved by the combination treatment. These results were very encouraging and demonstrate that quality and shelf life of fresh *Agaricus* mushrooms can be improved without expensive or elaborate

procedures or significant reduction in yield.

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REFERENCES

AJLOUNI, S.O. (1991). Quality characteristics of two type hybrids of the cultivated mushrooms (*Agaricus bisporus*) and the improvement of their shelf life using stipe trimming and gamma irradiation. Ph.D. Thesis. The Pennsylvania State University.

AJLOUNI, S., BEELMAN, R.B., THOMPSON, D.B. & J.-L. MAU. (1992). Stipe trimming at harvest increases shelf life of fresh mushrooms (*Agaricus bisporus*). *Journal of Food Science* **57**, 1361-63.

BARDEN, C.L., BEELMAN, R.B., BARTLEY, C.E. & SCHISLER, L.C. (1990). The effect of calcium chloride added to irrigation water on quality and shelf life of harvested mushrooms. *Journal of Food Protection* **53**, 759-762.

BARTLEY, C.E., BEELMAN, R.B. & WINNETT, J.R. (1991). Factors affecting color of cultivated mushrooms (*Agaricus bisporus*) prior to harvest and during postharvest storage. *Mushroom Science* **8**, 689-694.

BEELMAN, R.B., BARTLEY, C.E., MAU, J.-L., MIKLUS, M.B., SOLOMON, J.M., ROYSE, D.J., WINNETT, J.R., & MUTHERSBAUGH, H.R. (1992). Irrigation water treatments to improve quality and shelf life of *Agaricus* mushrooms. *Mushroom News* **40**, 18-23.

BEELMAN, R.B., QUINN, J.J., OKEREKE, A., SCHISLER, L.C., MUTHERSBAUGH, H.R. & EVENSEN, K. (1987). Effect of peat casing layer and the addition of calcium chloride to watering treatments on quality and shelf life of fresh mushrooms. *Developments in Crop Science 10, Cultivating Edible Fungi,* pp. 271-282. Edited by P.J. Wuest, D.J. Royse & R.B. Beelman. Amsterdam: Elsevier Science Publishers.

MAU, J.-L., BEELMAN, R.B. & ZIEGLER, G.R. (1992). Effect of 10-oxo-trans-8-decenoic acid on growth of *Agaricus bisporus*. *Phytochemistry* **31**, 4059-4064.

MAU, J.-L., BEELMAN, R.B. & ZIEGLER, G.R. (1993a). Factors affecting 1-octen-3-ol in mushrooms at harvest and during postharvest storage. *Journal of Food Science* **58**, 331-334.

MAU, J.-L., MIKLUS, M.B. & BEELMAN, R.B. (1993b). The shelf life of *Agaricus* mushrooms. In *The Shelf Life of Foods and Beverages*. Edited by G. Charalambous. Amsterdam: Elsevier Science Publishers.

SOLOMON, J.M., BEELMAN, R.B. & BARTLEY, C.E. (1991). Addition of calcium chloride and stabilized chlorine dioxide to irrigation water to improve quality and shelf life of *Agaricus bisporus*. *Mushroom Science* **13**, 695-701.

CHAPTER 20

CONTAMINATION OF MUSHROOMS AND CANNED MUSHROOMS

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1. INTRODUCTION

Fresh and canned mushrooms are widely preferred by most people as nutritive foods. China exports more than 100,000 tons of edible mushrooms each year so it is very important to have quality control against various forms of contamination in order to protect the people's health and to benefit the consumers.

2. POSSIBLE SOURCES OF CONTAMINATION AND PREVENTIVE MEASURES

Mushrooms are very tender and easily damaged. For instance, if the picked fresh mushrooms are not refrigerated and processed in the proper time, air-borne microorganisms will grow on them, and endogenous oxidation will take place, causing a change in colour. In this way, the quality of mushrooms will deteriorate and the mushrooms are spoiled and become unacceptable for eating or processing. Contamination of canned mushrooms comes from two main sources. One is filth contamination. In this case, unacceptable contaminated mushrooms have been used or the mushrooms are not thoroughly washed, or sanitary measures are not practised during processing, so the contamination ends up in the finished products. Sometimes, this contamination may be very dangerous for the consumer. Another form is microbial contamination that arise for various reasons, e.g. the fresh mushrooms are transported within unclean containers; raw materials are laid up for long peroids and not processed in time; practices which did not meet the sanitary requirements for the environment and processing; can defects which are caused by machinery or poor practices; retort equipment and temperature recorders are not operated properly and/or the cooling water did not contain enough chlorine, and so on. In this way, air-borne microorganisms are introduced and allowed to flourish, deteriorating the mushroom products.

In order to guarantee the quality of mushrooms and canned mushrooms, the related departments and canneries have undertaken many measures to strengthen quality control. Specific regulations and rules have been made for the mushroom growing, collection, transportation, processing and storage. Training classes have been held to introduce some advanced equipment and to improve the testing methods. The "Good Manufacture Practice" (GMP) and "Hazard Analysis Critical Control Point" (HACCP), which are the scientific systems for maintaining quality control for food safety, have been set up. Simultaneously, the quality of Chinese canned mushrooms has improved.

3. NON-SPECIFIC REACTION OF THE TECRA KIT

When speaking about the contamination of canned mushrooms, people often think about the contamination by staphylococcal enterotoxins (SET). In 1989, the US Food and Drug Adminstration (FDA) declared SET contamination found in Chinese canned mushrooms, and they issued an order to automatically detain all canned mushrooms processed in China. At that time, the Chinese Government and the related departments responded promptly. The response was not only to take several efficient measures to keep the quality control for the canned mushrooms, but also to organize a scientific research group to study the reasons for SET contamination of canned mushrooms and to develop preventive measures.

At the beginning, we adopted the TECRA Kit which is used and recommended by the FDA to detect SET. We expected that SET would be found in the canned mushroom products. However, we often found that the results initially appeared positive but, after incubation for a while, turned negative. Sometimes, when using the TECRA Kit to examine mushrooms or material from the growing mushrooms, a very strong positive reaction could be changed to negative after treating the extracts with normal animal (goat, rabbit) serum. Another important observation was that one bacterium, *Serratia marcescens,* which does not produce SET, was isolated and gave a strong positive reaction with the TECRA Kit. So we doubt the specificity of the TECRA Kit. Through different experiments, especially comparisons of the specificity among four commercial screening kits, the nonspecific reaction of the TECRA Kit used for the detection of SET was confirmed (Wu *et al.*, 1992).

4. SPECIFICITY OF FOUR COMMERCIAL SCREENING KITS

The four test kits for detection of SET are as follows:

i. TECRA Kit (Bioenterprises Pty. Co., Australia), (Anon., 1990),
ii. SET-PRLA Kit (Oxoid Co., U.K.), (Shingaki *et al.*, 1990),
iii. TRANSIA Detection of SET Kit (TRANSIA Co., France), (Drouet, 1991),
iv. SET-EIA Kit (Brommelia Bern, Switzerland for Toxin Technology Inc., U.S.A.). (Bommell, 1990).

Two bacterial strains, *Serratia marcescens* and *Staphylococcus aureus*, were cultured in nutrient broth at 37°C for 48hr. The culture was then centrifuged and the supernatant divided into two portions: one was diluted with an equal volume of normal rabbit serum and stored at 37°C for 1 hr while the other portion was diluted with Tris buffer and stored at 37°C for 1 hr. Afterwards, the two portions were added to the four SET test kits, respectively. The results are showed in Table 1. All of the four SET test kits gave a positive result for the *S. aureus* culture and the results were not affected by animal serum. Only the TECRA Kit gave a positive result for *S. marcescens* and the

Table 1. Specificity of the Commercial Kits.

Bacteria	TECRA-KIT		SET-RPLA		TRANSIA		SET-EIA	
	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1
S. aureus	+	+	+	+	+	+	+	+
S. marcescens	+	-	-	-	-	-	-	-

positive result turned negative after treatment with serum. This indicates that the cross-reaction of TECRA Kit with *S. marcescens* exists even though *S. marcescens* does not produce SET.

5. DETECTION OF SET IN MUSHROOM PRODUCTS USING TEST KITS

Cultivated material from three different mushroom growing areas were diluted with Tris buffer (1:4), homogenized and centrifuged. After centrifugation, the supernatant was filtered and divided into two portions. Subsequent work-up procedures were the same as described in Part 4. The results are shown in Table 2.

All three samples gave a positive reaction with the TECRA Kit. However, after treatment with serum, the samples gave negative results. Meanwhile, all three samples gave negative results with the other SET test kits. Based on these results, it is concluded that the substances in the cultivated materials which caused the positive reaction with the TECRA Kit were not SET. Cultivated mushroom material from Canada also gave a positive result with the TECRA Kit as did the two microbial species *Pseudomonas maltophilia* and a *Kluyveromyces* spp.isolated from the Canadian mushroom products. Therefore, it is suggested that substances which cause cross-reactions with the TECRA Kit also exist in cultivated mushroom material both from within and outside China.

Extracts from the same canned mushrooms which tested positive in the first detection by the TECRA Kit were re-examined. All the results were negative. However, if mushroom broth inoculated with staphylococcal enterotoxin A (2ng/ml) was stored under the same conditions and examined after a 4-week interval, it consistently gave a positive result. On examination of extracts from canned mushrooms using the TECRA Kit, we often found that the results were initially positive but turned negative when a second test was carried out within a 24 hr period. These results suggest that the positive TECRA reaction of true staphylococcal enterotoxin is stable but that of the cross-reacting

Table 2. Detection of SET in Mushrooms.

Location	TECRA-KIT		SET-RPLA		TRANSIA		SET-EIA	
	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1	Buffer 1:1	Serum 1:1
Shanghai	+	-	-	-	-	-	-	-
Fuzhou	+	-	-	-	-	-	-	-
Sichuan	+	-	-	-	-	-	-	-

substances is not.

The non-specific reaction of the TECRA Kit was confirmed not only in China, but also in other countries (Park *et al.*, 1992). So far, there are at least seven micro-organisms other than *S. aureus* that have been isolated from swollen cans or some other materials related to mushrooms or mushroom products, and which show positive results with the TECRA Kit. Therefore, the data described above cast doubt on the validity of decisions to ban the import of canned mushrooms from China based solely on results obtained using the TECRA Kit.

6. AN INVESTIGATION OF POTENTIAL SOURCES OF SET CONTAMINATION IN CHINA

In order to establish the possibility of SET contamination of canned mushrooms produced under the present Production Technology Procedure in China, samples were collected and examined from different processing steps such as: growing mushrooms, freshly picked mushrooms (before blanching), half-processed products (after blanching and before retortion), and finished products (after retortion).

S. aureus producing SET was intentionally inoculated into samples which showed a negative result with the TECRA Kit. Then, the population of *S. aureus* and the production of SET was followed.

6.1. Materials and Procedures

Materials:

a) SET Testing Kit; TECRA Kit (Bioenterprise Pty Co., Australia)
b) Organon Micro ELISA system
c) *S. aureus* strain producing Enterotoxin A (provided by the Food Research Institute, University of Wisconsin)
Serratia marcescens, isolated by the Fujian Import and Export Commodity Inspection Bureau and identified by the China Identification Institute for Medical and Biochemical Products.
Escherichia coli and a *Proteus* sp., provided by the Fujian Health and Quarantine Station.
d) Cultivated mushroom samples: fresh, half-processed and canned.

Methods:

1. Deliberate contamination was carried out by adding SET-positive *S. aureus* to cultivated mushroom material which showed a negative SET result with the TECRA Kit. Also, a TECRA-positive SET solution was added to the roots of growing mushrooms. After the mushrooms had been grown under normal laboratory cultivation conditions, the fruit bodies were picked, washed and tested for SET using the TECRA Kit.
2. The growth and decline of *S. aureus*, and SET production, in cultivated mushroom material inoculated with 2.7×10^6 cells/g and maintained at 25°C was examined at 24h intervals.
3. The growth and decline of *S. aureus*, and SET production, was also examined in freshly picked mushrooms. Fresh mushrooms were inoculated with 1.75×10^7

cells/g, tied off in plastic bags, incubated at 25°C and the parameters measured at 6 h intervals.

4. Half-processed mushrooms, after blanching and before retortion, were inoculated with *S. aureus* (10^3 cells/g), incubated at 28°C and examined for SET at 4 h intervals.
5. Contamination of finished canned mushrooms: 10 cans were deliberately damaged by dropping from a height of 1 metre, 5 of the cans were soaked in a suspension of *S. aureus* and the remainder in tap water for a period of 4-6 h. The cans were then incubated at 37°C for 10 days and the contents examined for SET using the TECRA Kit.

Canned mushrooms were also contaminated with a mixture of microorganisms by drilling a small hole in the top of the can and inoculating separately with *S. aureus* and *S. marcescens.* The holes were sealed immediately and the cans incubated at 35°C for 10 days. Other cans were inoculated with a mixture of *E. coli* and a *Proteus* sp., both of which gave negative results with the TECRA Kit, together with either *S. aureus* or *S. marcescens,* in a 1:1:1 ratio. The cans were sealed immediately, incubated at 35°C for 10 days and examined for SET using the TECRA Kit.

6.2. Results and Discussion

Mushrooms which had been deliberately contaminated with SET-positive *S. aureus* or SET during cultivation were divided into two groups, one with cut and the other with uncut roots. All the samples examined tested negative with the TECRA Kit showing that SET was not absorbed or enriched even if the mushrooms were grown on SET contaminated substrates.

The levels of *S. aureus* and SET in cultivated mushroom material during an 11-day period following inoculation are shown in Figure 1. Cell counts declined sharply after the first day and no viable bacteria were detectable after day 9. Tests for SET using the TECRA Kit were negative at the end of the 11th day. The strain of *S. aureus* grown in broth for 12 h gave a positive result with the TECRA Kit showing that the experimental strain was reliable and working. These data suggest that the conditions in the cultivated material are not suitable for the growth of *S. aureus* and even less suitable for SET production.

The survival of *S. aureus* and SET production in freshly picked mushrooms is shown in Figure 2. Cell counts declined from 1.7×10^7 cells/g to 3.0×10^5 cells/g during the first 12 hours and then began to slowly increase, while the tests for SET using the TECRA Kit were negative after 42 h. Since the 42 h experimental period is way beyond the processing specifications, which stipulate that the time for mushroom collection and transportation to the canneries will not exceed 6-8 h, it is unreasonable to believe that the freshly picked mushrooms will become contaminated with SET under the present processing procedures adopted by mushroom canneries in China.

In the case of half-processed (i.e. after blanching, before retortion) mushroom products, Table 3 shows that all tests for the detection of SET, for a period of 32 h from the time of inoculation, were negative. Processing specifications state that the time from bleaching to can filling and seaming must not exceed 2.5 hr, the work benches must be clean, and workers must wash their hands at 2 hr intervals. Therefore, even if *S. aureus* contamination occurred occasionally, the bacterium does not have enough time to multiply and produce SET. The half-processed products would not be contaminated with SET under the present processing procedures adopted by mushroom canneries in China.

All SET tests conducted on damaged cans soaked in a suspension of SET-producing *S. aureus* were negative. This result suggests that the bacterium does not readily survive and produce SET in

TABLE 3. Test results for SET in contaminated half-processed products.

Time (hours)	0	4	8	12	16	20	24	28	32
SET Test	-	-	-	-	-	-	-	-	-
OD Values	0.075	0.076	0.066	0.074	0.072	0.076	0.074	0.075	0.074

Negative: OD value <0.2; positive: OD value >0.2.

canned mushrooms as a result of can defects. In actual situations, can defects may well give rise to microbial contamination although post-process contamination solely by *S. aureus* seems unlikely (English & York, 1990). Therefore, multi-species contamination was introduced in order to imitate more closely the actual situation (Table 4). When pure cultures of *S. aureus* or *S. marcescens* were introduced into cans of mushrooms through small holes drilled into the cans, and the cans incubated at 35°C for 10 days, tests for SET carried out with the TECRA Kit were positive. When *S. aureus* was inoculated together with *E. coli* and a *Proteus* sp., the can was swollen but the SET test was negative. However, when *S. marcescens* was inoculated along with *E. coli* and a *Proteus* sp., the can was again swollen and the SET test proved positive.

Although it is theoretically possible for *S. aureus* to produce SET in canned mushrooms, in reality, any can defects will lead to contamination by more than one microorganism. Under these conditions, *S. aureus* may not compete very well with the other contaminants and, under such circumstances, may not readily produce SET (English & York, 1990). Therefore, it is unlikely that the canned mushrooms will be contaminated with SET.

It should be noted that, even in the absence of *S. aureus*, other organisms in the swollen, contaminated can may cross-react with the TECRA Kit and give a 'positive' SET result.

7. CONCLUSIONS

1. Two major types of contamination of mushrooms and canned mushrooms are filth and microbial contamination. Adoption of "Good Manufacture Practice" (GMP), and "Hazard Analysis Critical Control Point" (HACCP) represent efficient measures to prevent these forms of contamination.
2. Errors can arise when the TECRA Kit is used to detect SET in Chinese canned mushrooms due to non-specific reactions shown by this kit.

Table 4. Detection of SET in canned mushrooms.

Strain	Condition of Can	TECRA Kit
Staphylococcus aureus	Flat	+
Serratia marcescens	Swollen	+
S.a + E + P	Swollen	-
S.m + E + P	Swollen	+

S.a: *Staphylococcus aureus*; S.m, *Serratia marcescens*; E: *Escherichia coli*; P: *Proteus*.

3. Experimental evidence indicates that there is no SET contamination of canned mushrooms based on the present Production Technology Procedure in China.
4. From now on, it should not be necessary to conduct SET testing of fresh and canned mushrooms as a conventional testing item.

REFERENCES

ANONYMOUS (1990). Staphylococcal enterotoxin visual immunoassay for the detection of SET A, B, C_1, C_2, C_a, D, E in food ingredients. *TECRA, Australia, Instruction booklet.*

BOMMELL (1990). SET-EIA Kit for staphylococcal enterotoxin detection. *Toxin Technology, American, Instruction booklet.*

DROUET (1991). Detection of staphylococcal enterotoxins. *TRANSIA, France, Instruction booklet.*

ENGLISH, P.H. & YORK, G. (1990). Staphylococcal food poisoning outbreaks caused by canned mushrooms from China. *Food Technology* Dec.,74-76.

PARK, CE., AKHTAR, M. & RAYMAN, M.K. (1992). Nonspecific reactions of a commercial enzyme-linked immunosorbent assay kit (TECRA) for detection of staphylococcal enterotoxins in foods. *Applied and Environmental Microbiology* **58**, 2509-2512.

SHINGAKI *et al.* (1990). SET-RPLA. A kit for the detection of staphylococcal enterotoxin A, B, C, in food samples or culture filtrates by reversed passive latex agglutination. *Oxoid, England, Instruction booklet.*

WU, Z.L., ZHANG, Z.C. & LI, C.W. (1992). Study on the nonspecific reaction problems in the procedure of using TECRA Kit (Bioenterprise Pty. Co. Australia) for detection of staphylococcal enterotoxins. *Journal of the Association for the Development of Rapid Methods and Automation in Microbiology* **4**, 105-109.

CHAPTER 21

BEHAVIOR AND SEROLOGICAL IDENTIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN IN THERMALLY PROCESSED MUSHROOMS

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1. INTRODUCTION

Enterotoxin produced by some strains of *Staphylococcus* spp. is a relatively common cause of bacterial foodborne illness. This preformed microbial toxin is produced in a variety of foods over a wide range of conditions. Bacterial populations proliferate when food is time-temperature abused and the contaminating organism, if enterotoxigenic, subsequently produces the enterotoxin. When this abused food is consumed, foodborne illness may occur. Although most outbreaks are due to the contaminated food that has not received severe heat treatment, evidence shows that foods receiving medium to high heat treatment also may cause staphylococcal intoxication. Examples of such foods include boiled goat's milk (Drysdale, 1950), spray dried milk (Anderson & Stone, 1955; Armijo *et al.*, 1957), cooked sausage (Tatini, 1976), canned lobster bisque (CDC, 1976) and processed infant formula (Bennett, 1982). In 1989, canned mushrooms were purported to be responsible for a number of outbreaks in the United States (CDC, 1989), and staphylococcal toxin was found in product that was untreated or exposed to 6.0-8.0 M urea before serological testing (Bennett, 1991, 1992).

Since the mid-1960's, a number of studies on the thermal stability and behavior of the staphylococcal enterotoxins have been conducted in actual food substrates or in extracts of foods (Read *et al.*, 1965; Denny *et al.*, 1971; Humber *et al.*, 1975; Soo *et al.*, 1973; Tatini, 1976; Lee *et al.*, 1977; Bennett & Berry, 1987). Although it was generally concluded that the heat process normally used in canning destroys any enterotoxin in a food substrate (Denny *et al.*, 1966; Denny *et al.*, 1971; Humber *et al.*, 1974; Bergdoll, 1989), more recent serological evidence with canned mushrooms (Bennett, 1991, 1992) indicates otherwise. Previous studies showed a retention of biological toxin activity in kittens fed heated, serologically inactive enterotoxins A and D in selected food substrates (Bennett & Berry, 1987). In addition, it was suggested that the clinical symptoms of enterotoxemia

were enhanced in humans who ingested enterotoxin type A in milk heated at 100°C for 25.4 min. (Dangerfield, 1973). Monkeys fed heated toxin of serologically measured 5 ng activity appeared to be more severely ill than those fed the corresponding unheated toxin (Tatini, 1976). The serological renaturation of enterotoxin A has been successfully demonstrated in pasteurized milk (Tatini, 1976) and canned mushrooms (Bennett, 1991, 1992) when these foods were treated with 6.0-8.0 M urea.

Similarly, serological reactivation of staphylococcal enterotoxin by high pH treatment has been reported for a variety of foods and other menstrua heated at 80-100°C for 10 min (Schwabe *et al.*, 1990). This paper addresses the thermal inactivation kinetics of enterotoxin A, the denaturation and renaturation behavior of enterotoxins after treatment with urea and the toxin's attendant degradation, or spontaneous changes in toxin serology after thermal and chemical treatment.

2. RECOVERY AND SEROLOGICAL IDENTIFICATION OF ENTEROTOXIN IN CANNED MUSHROOMS

Naturally contaminated mushrooms that purportedly received normal thermal time-temperature processing were used to study the serological fate of enterotoxin and its response to chemical renaturation (serological reactivation).

To simulate the canning process, fresh mushrooms were sliced into "pieces and stems," and staphylococcal enterotoxin type A (SEA) was added at levels of 5, 10, and 20 ng toxin/g of product. The product (4 oz drained wt) was then placed in appropriate cans, sealed, and retorted at $F_0 = 7.5$ min, monitored with thermocouples.

After retorting of the product to which toxin had been added, the cans were chilled in cold water and given an initial toxin assay approximately 4 days after the thermal processing event. Other studies used various temperature/time conditions for mushrooms to which SEA was added in various concentrations before heating at 121.1°C for 15-30 min.

General steps in identifying toxin in thermally processed mushrooms are (1) extraction of toxin from food components; (2) urea treatment of extract to renature toxin that may have been preformed (i.e., formed before thermal processing); and (3) serological identification of enterotoxin by an enzyme-linked immunosorbent assay (ELISA) or other acceptable serological tool to determine whether suspect toxin was in its native or heat-altered form.

2.1. Extraction of Thermally Processed Mushrooms

A 200-ml volume of 0.25 M Tris, pH 8.0, was added to each 200 g of product and homogenized at high speed for 3 min in a Waring blender. The mushroom slurries were transferred to centrifuge bottles (stainless steel or equivalent), and then centrifuged at 27,300 x g for 20 min at 5°C. The resulting extracts were decanted into suitable containers. A 100-ml amount of untreated extract was removed and dialyzed in polyethylene glycol (PEG) as described under "Urea Treatment," below. From each extract, 100 ml was reserved for urea treatment. After dialysis of the 100 ml amounts of each untreated extract, the dialyzed volume was kept at 5.0 ml and clarified by centrifugation. Each dialyzed extract was filtered through a cotton-plugged disposable syringe (the cotton-plugged syringe was prepared by inserting a piece of cotton about 0.5 cm thick into a syringe; it was wetted by passing distilled water through the syringe to ensure tight packing of the cotton). For filtration, the plunger was removed and the extract was placed in the syringe; the plunger was then reinserted, and the extract was carefully pumped through the cotton plug into a plastic vial (preferably

polypropylene or equivalent).

The pH of the 5.0-ml cotton-filtered extracts was adjusted, when necessary, to between pH 7.0 and 8.0 with NaOH or HCl. Then, 50 µl of "sample additive" contained in the ELISA kit was added and the preparation was thoroughly mixed. A 200-µl volume of each extract was tested, as required by the kit's instructions.

2.2. Urea Treatment to Renature Thermally Denatured Toxin

The extracts (100 ml) reserved for urea treatment were placed in 250-ml beakers to which 36.0 g urea (M.W. 60.06) was added to achieve a 6.0 M solution. Where appropriate, other concentrations (5.0, 7.0, and 8 M) of urea were used for treatment of the heat-altered toxins.

The mixtures were stirred on a magnetic stirrer for 4-5 h at 25°C. After urea treatment, the mixtures were placed in dialysis tubing (cellulose casing of 2-3 cm flat width with 12,000 to 14,000 M.W. exclusion). Before the mixture was placed in the dialysis tubing, the tubing was checked for leaks by filling it with distilled water. Dialysis tubing containing the urea-treated extracts was placed in polyethylene glycol flakes (PEG, 20,000 M.W.) and allowed to remain until a volume of approximately 5 ml or less was reached; this is normally an overnight process. After dialysis, the tubing's exterior was washed thoroughly with tap water to remove residual PEG. The tubing was then soaked in 0.25 M Tris, pH 8.0, but only for as long as its total volume did not exceed 5 ml of the urea-treated extract. Extracts were removed from the dialysis tubing as previously described, clarified by centrifugation, and filtered through absorbent cotton. When necessary, the pH of the 5.0 ml extracts was adjusted to between 7.0 and 8.0 with NaOH or HCl; then 50 µl of "sample additive" was mixed with each extract.

3. SEROLOGICAL IDENTIFICATION OF STAPHYLOCOCCAL ENTEROTOXINS

An ELISA using polyvalent antisera (antibody serotypes A, B, C, D, and E) in a double polyclonal antibody "sandwich" configuration was used to screen test portions for the presence of staphylococcal enterotoxin in the untreated and urea-treated mushroom extracts (Bennett *et al.*, 1990).

The commercially available ELISA used was the TECRA, Staphylococcal Enterotoxin Visual Immunoassay (manufactured by TECRA Diagnostics, 28 Barcoo St., Roseville, NSW 2069, Australia and distributed in the USA by International Bioproducts, Redmond, WA, USA). This method was used to screen mushrooms for staphylococcal enterotoxin because of its previous success with a wide variety of foods contaminated with these toxins (Bennett & Atrache, 1989; Bennett *et al.*, 1989).

The protocol consisted of adding 200-µl aliquots of the 5.0-ml additive-treated extracts (pH adjusted to 7.0-8.0) to prewashed (10 min at 25°C) anti-staphylococcal enterotoxin (serotypes A-E) coated microtiter wells, incubated for 2.0 h at 35°C. Wells were washed (3X), treated with 200 µl of polyvalent antisera (A-E) enzyme conjugate, and incubated for 1 h at 25°C; wells were again washed (5X) and substrates (200 µl) were treated for 30-45 min at 25°C, when 20 µl stop solution was added. A positive test was indicated by enzyme action (green) on the colorless substrate. For visual determinations, the tray was placed on a white background and individual test wells were compared to a color standard (color comparator) supplied in the kit. Absorbance (A) of the test solutions was determined with a microtiter tray reader (V Max Kinetics Microplate Reader,

Molecular Devices, Menlo Park, CA 94025-9994, USA) or comparable, using dual wavelengths [405 and 490 (or 492) nm]. Test solutions that gave an absorbance of >0.200 were considered positive for enterotoxin. Those that gave an absorbance of ≤0.200 were considered negative for enterotoxin.

If confirmation by the AOAC method was necessary, the 5-ml (200 µl) urea-treated preparation was extracted with $CHCl_3$, diluted with 40 volumes of 0.005 M phosphate buffer, pH 5.7, chromatographed, and tested by the microslide gel double diffusion test (AOAC, 1990). When necessary, untreated mushroom extracts were processed by the AOAC method (Bennett *et al.*, 1988).

4. HEAT AND CHEMICAL DENATURATION OF TOXIN

The action of heat on globular proteins such as the enterotoxins leads to changes in molecular structure (denaturation) and/or particle size (Jaenicke, 1967). Such changes in the conformation of the protein, as a result of heat stress, may occur in a series of steps, from the native form through a number or intermediate, partially denatured forms to a fully denatured type which lacks a conformational likeness to the structure of the native protein.

Many studies on the heat inactivation of staphylococcal enterotoxins have indicated the thermal stability of the toxins. Nevertheless, high thermal exposure appears to destroy the serological activity of the enterotoxins while retaining some of their biological activity (Bennett & Berry, 1987).

If mushrooms are contaminated with enterotoxigenic *Staphylococcus* spp., followed by time and temperature abuse of the product, various amounts of enterotoxin may be produced. When the product is retorted, the canning conditions ultimately determine whether enterotoxins will be completely or partially inactivated. Studies have shown that the degree of serological inactivation differs with the effects of heat (F_0 = 7.5 min) on 50 and 100 ng SEA/g of product (Fig. 1). The preparation containing 50 ng SEA/g was totally inactivated and, when serologically analyzed by ELISA, produced an absorbance ≤0.200 (0.180). Although the serological inactivation pattern was similar in the preparation containing 100 ng SEA/g of product, total inactivation by heat did not occur and produced an absorbance > 0.200 (0.270).

Urea can also act as a chemical denaturant on certain proteins and has been shown to have a denaturing effect on staphylococcal enterotoxin (Warren *et al.*, 1974; Bennett, 1991, 1992). Figure 2 shows the effect of urea treatment time on the denaturation of 5.0, 10.0, and 20.0 ng staphylococcal enterotoxin/g in its native form in mushroom extracts. While 5 ng SEA/g was denatured in 6 h, producing negative ELISA results, denaturing of the toxin in the preparation containing 10 ng SEA/g required 12 hr. Table 1 presents data on the identification of enterotoxin in its native form in naturally contaminated canned mushrooms and the effect of urea treatment on rendering the toxin serologically inactive.

5. UREA RENATURATION OF TOXIN

The effects of urea and other chemicals as denaturants have been shown in pasteurized milk (Tatini, 1976) and, more recently, in mushrooms that received even more severe heat treatment (Bennett, 1991, 1992). Figure 3 compares the effect of urea concentration and treatment times of 4, 5, and 6 h on the serological renaturation of staphylococcal enterotoxin from its heat-altered form in naturally contaminated canned mushrooms. The data suggest that treatment time is very important, although the urea concentration is also a factor in the protein renaturation process. In this study, 6

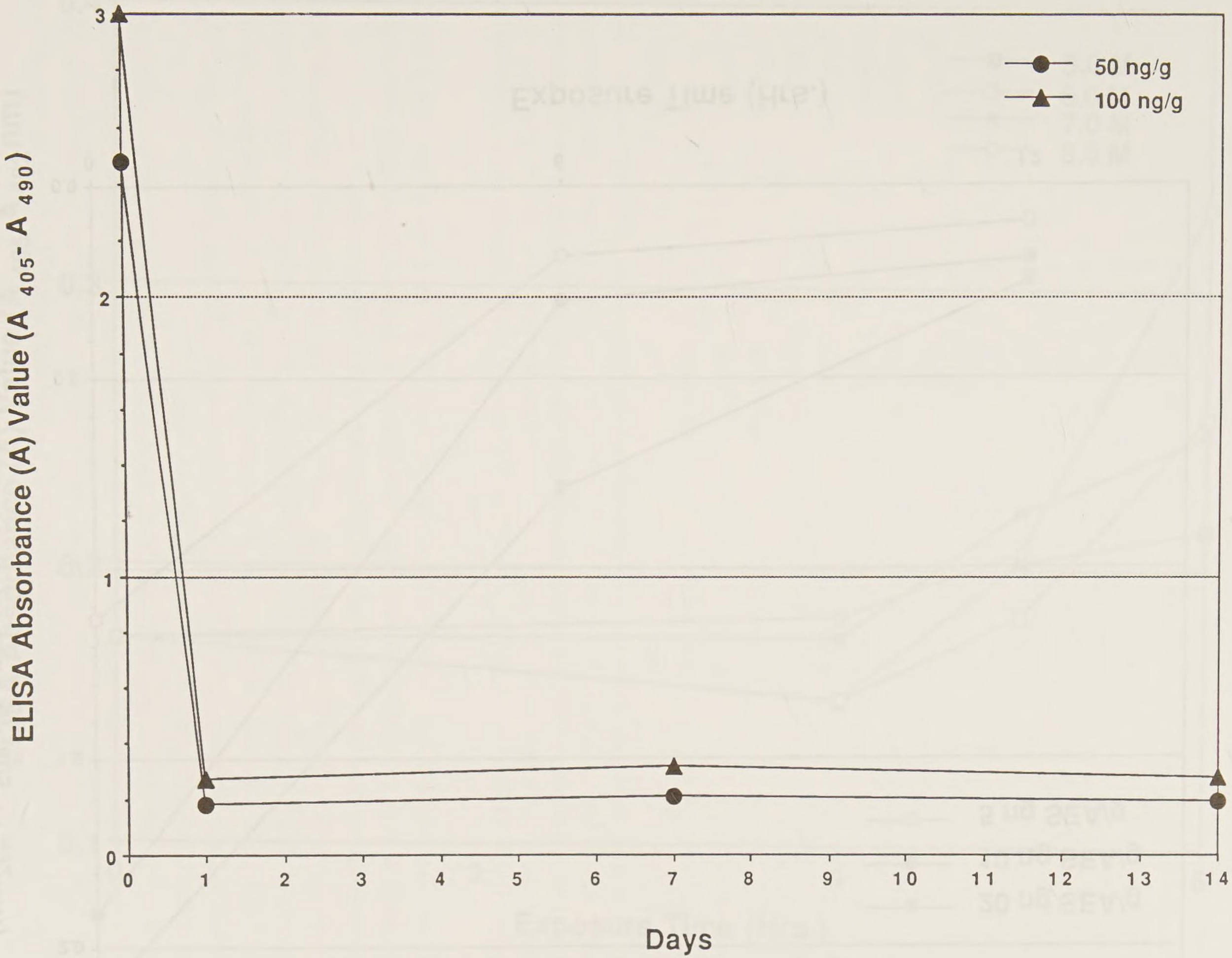

FIGURE 1. Comparative serological inactivation of various concentrations (50 and 100 ng SEA/g product) of staphylococcal enterotoxin in canned mushrooms (F_0 = 7.5 min).

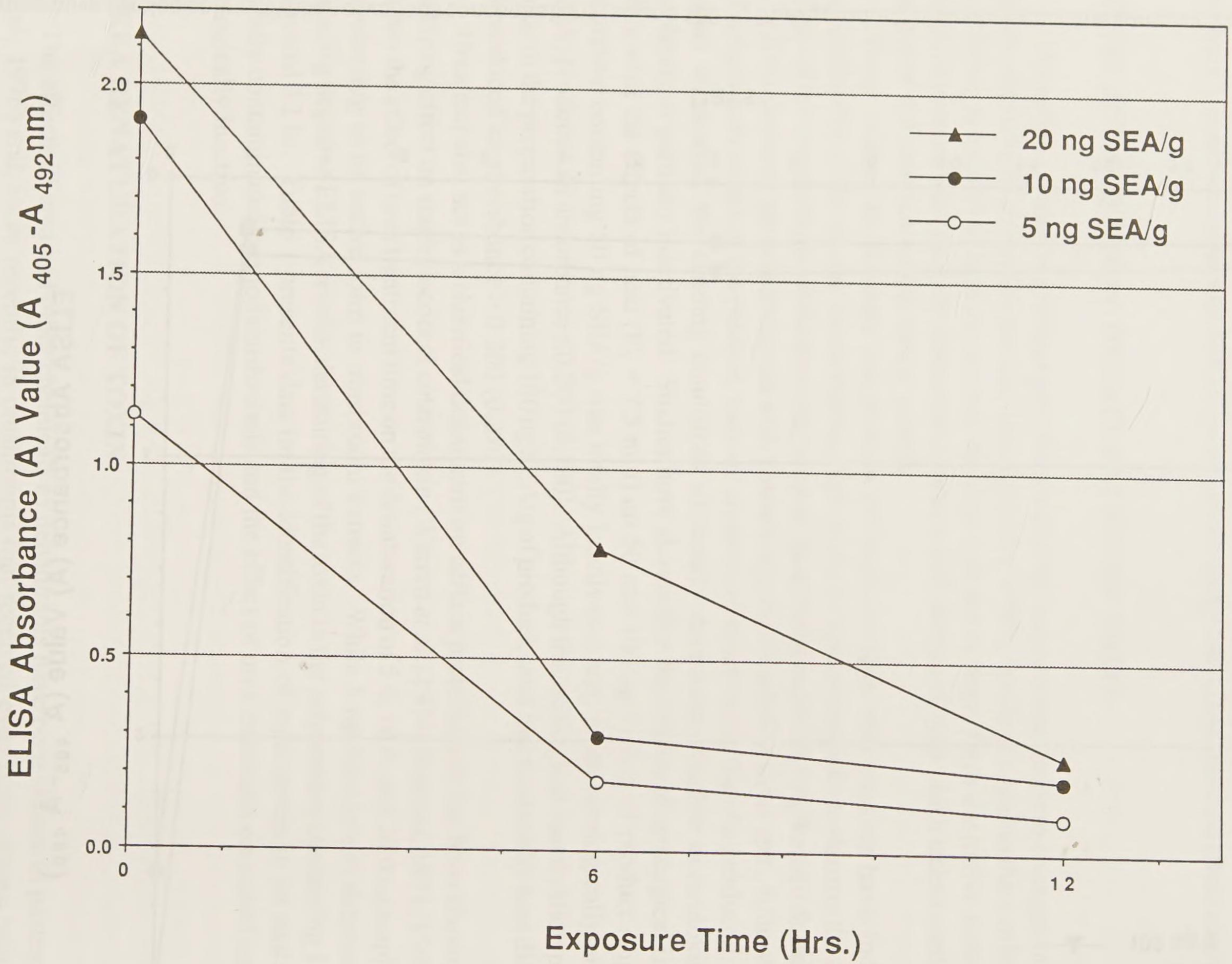

FIGURE 2. Effect of urea (6.0 M) exposure time on the denaturation of various concentrations (5.0, 10.0, and 20.0 ng/g) of staphylococcal enterotoxin in the native form in mushroom extracts. Absorbance 0.200 indicates serological absence of toxin.

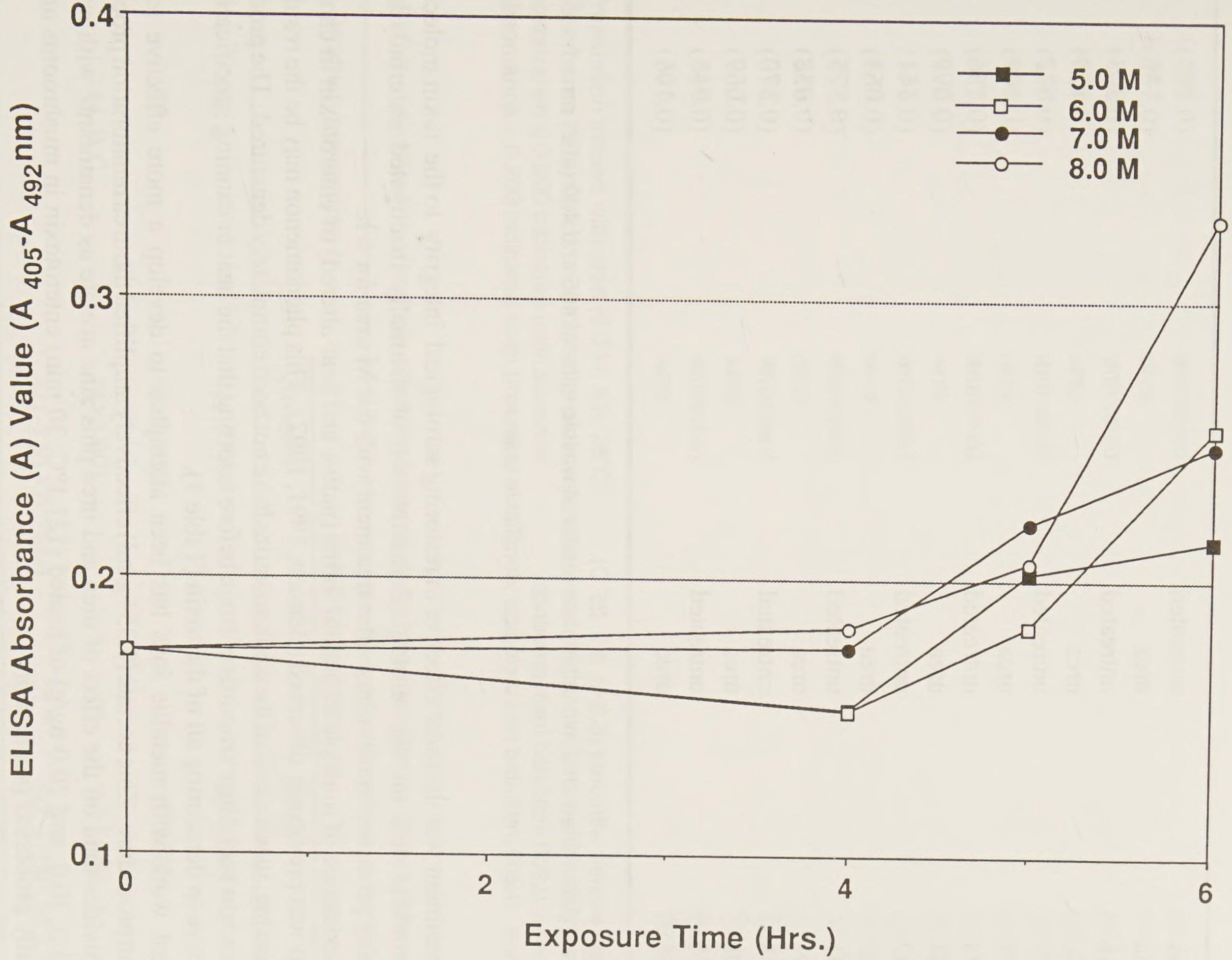

FIGURE 3. Comparative effect of urea concentration (5.0, 6.0, 7.0, and 8.0 M) and exposure time (4, 5, and 6 h) on the serological renaturation of staphylococcal enterotoxin in naturally contaminated canned mushrooms. Absorbance ≤0.200 indicates nonidentifiable toxin; absorbance > 0.200 indicates serological identification of toxin.

TABLE 1. Identification of staphylococcal enterotoxin in the native form and the effect of urea[1] on denaturation (serological inactivation) of the toxin

Product designation	Extract treatment	ELISA response	
		visual	(absorbance)[2]
26a	untreated	+	(0.223)[3]
26a	urea	-	(0.130)[4]
51a	untreated	+	(0.343)
51a	urea	-	(0.139)
779	untreated	+	(0.612)
779	urea	-	(0.149)
784	untreated	+	(0.756)
784	urea	-	(0.099)
182	untreated	+	(0.341)
182	urea	-	(0.063)
191	untreated	+	(0.575)
191	urea	-	(0.058)
198	untreated	+	(0.370)
198	urea	-	(0.069)
656	untreated	+	(0.945)
656	urea	-	(0.106)

[1] Preparation treated with urea (6.0 M, 6 h, 25°C).
[2] Absorbance determined on a microtiter plate reader at wavelengths of 405 and 490 (492) nm.
[3] Absorbance > 0.200 indicated toxin presence.
[4] Absorbance 0.200 indicated no serological identifiable toxin.

h of urea treatment was the most effective in restoring serological integrity to the toxin molecule. Table 2 provides data on the serological reactivation of thermally inactivated enterotoxin in commercially processed mushrooms after treatment with 6.0 M urea for 6 h.

The existence of multiple structural forms (native and heat-altered) of enterotoxin in canned mushrooms was previously observed (Bennett, 1991, 1992). This phenomenon may be the result of underprocessing, in which all of the toxin moieties have not been completely denatured. The product may also contain such large amounts of toxin before retorting that the heat or canning specifications are ineffective in denaturing all of the toxin (Table 3).

Recent work with metallic ions has been attempted to develop a more effective toxin renaturation procedure. Zinc acetate (0.354 g/ml) effectively amplified the urea renaturation process. Table 4 provides data on the effect of urea and urea plus zinc acetate as denaturants with small amounts (5.0, 10.0, and 20.0 ng/g) of heated (121.1°C, 30 min) enterotoxin in mushrooms and a commercially processed preparation.

6. SEROLOGICAL DEGRADATION OF HEAT-ALTERED ENTEROTOXIN

Naturally contaminated mushrooms that purportedly received the normal thermal processing time and temperature were tested to determine the serological stability of the enterotoxin under

TABLE 2. Serological reactivation (renaturation) of thermally inactivated (denatured) staphylococcal entertoxin in canned mushrooms after urea treatment[1].

Product designation	Extract treatment	ELISA response	
		visual	(absorbance)[2]
847	untreated	-	(0.176)[3]
847	urea	+	(0.234)[4]
Eden	untreated	-	(0.189)
Eden	urea	+	(0.227)
103	untreated	-	(0.123)
103	urea	+	(0.257)
175	untreated	-	(0.078)
175	urea	+	(0.291)
538	untreated	-	(0.109)
538	urea	+	(0.212)
278	untreated	-	(0.060)
278	urea	+	(0.217)
337	untreated	-	(0.061)
337	urea	+	(0.225)
816	untreated	-	(0.115)
816	urea	+	(0.236)

1 Preparation treated with urea (6.0 M, 6 h, 25˚C).
2 Absorbance determined on a microtiter plate reader at wavelengths of 405 and 490 (492) nm.
3 Absorbance ≤ 0.200 indicated toxin absence.
4 Absorbance > 0.200 indicated toxin presence.

TABLE 3. Identification of multiple structural forms of staphylococcal enterotoxin in canned mushrooms

Product designation	Extract treatment	
	No urea	Urea
448	+(1.727)[1]	+(>3.00)
377	+(>3.00)[2]	+(0.618)
344-B.C.	+(>3.00)	+(>3.00)
	+(1:320,[3] 0.234[4])	+(1:1,280,[3]0.220[4])

Source:Modified from Bennett, 1992.
1 Absorbance measurement on microtiter plate reader at wavelengths of 405 and 490 nm.
2 Absorbance limit, 3.00.
3 Dilution of extract in positive (> 0.200) absorbance range.
4 Absorbance reading at specified extract dilution; absorbance reading ≤ 0.200 negative for toxin. Absorbance reading > 0.200 indicated toxin presence.

TABLE 4. Effect of urea[1] and Zn^{++}[2] amplifier on the renaturation of heat-altered staphylococcal enterotoxin A in mushrooms.

Product status	Toxin ng/g	Treatment		ELISA-Serology (absorbance)[3] Renaturation Process, 6h	
		unheated	heated	Urea	Urea + Zn
Toxin added	5	+(0.747)[4]	-(0.195)[5]	-(0.186)	+(0.221)
Toxin added	10	+(1.436)	-(0.136)	-(0.127)	+(0.226)
Toxin added	20	+(1.907)	-(0.142)	-(0.131)	+(0.249)
Natural	TPF[6]	NA[7]	-(0.170)	-(0.184)	+(0.426)

1 Treatment with urea (6.0 M, 6 h, 25°C).
2 Zinc acetate (0.354 g/ml).
3 Absorbance on microtiter plate reader at wavelengths of 405 and 492 nm.
4 Absorbance > 0.200 indicated toxin presence.
5 Absorbance ≤ 0.200 indicated no serologically identifiable toxin.
6 Toxin preformed (TPF).
7 NA = not available; product commercially retorted.

selected storage conditions.

Figure 4 shows serological patterns that occurred in thermal processing simulation studies in which 5, 10.0 and 20.0 ng toxin/g was added to mushrooms before retorting that rendered the toxin serologically inactive. However, treatment of the toxin- containing preparations with urea resulted in the serological reactivation of the toxin. Immediately after exposure of the heat-altered toxin to urea, toxin was spontaneously reactivated for approximately 5 days; then subsequent serological degradation began regardless of the concentration of SEA.

Serological degradation of staphylococcal enterotoxin in naturally contaminated canned mushrooms held under conditions of abuse during transport is summarized in Table 5. Initial analysis of the product indicated the presence of toxin; however, after transport under abuse conditions, during a 4-day period, only one of seven of the mushroom extracts remained positive for toxin. The one positive urea sample (367a) in this series contained the greatest amount of toxin and was not completely degraded between the initial and later assays. However, the degree of serological degradation was generally equal to that of the other degraded preparations. These findings prompted further investigation of the effect of storage temperature on the serological behavior of staphylococcal enterotoxin in canned mushrooms. Figure 5 compares the effect of temperature (5 and 25°C) on untreated canned mushrooms. Both preparations showed drastic serological degradation within the first week of storage regardless of storage temperature, although degradation of both preparations was greatest when they were held at 25°C. Generally, degradation of serologically identifiable toxin continued, but at a slower rate, during the remaining weeks of monitoring. Interestingly, one preparation (Q-1-2,/5°C) showed degradation the first week, with intermittent spontaneous serological reactivation, and then showed rather drastic serological degradation. Figure 6 compares the effect of storage temperature on the serological behavior of untreated and urea treated mushroom preparations. The similar serological degradation patterns of these preparations suggest that this phenomenon is not inhibited by urea treatment of the heat-altered toxin.

These studies indicate that assays to determine the presence of toxin should be performed

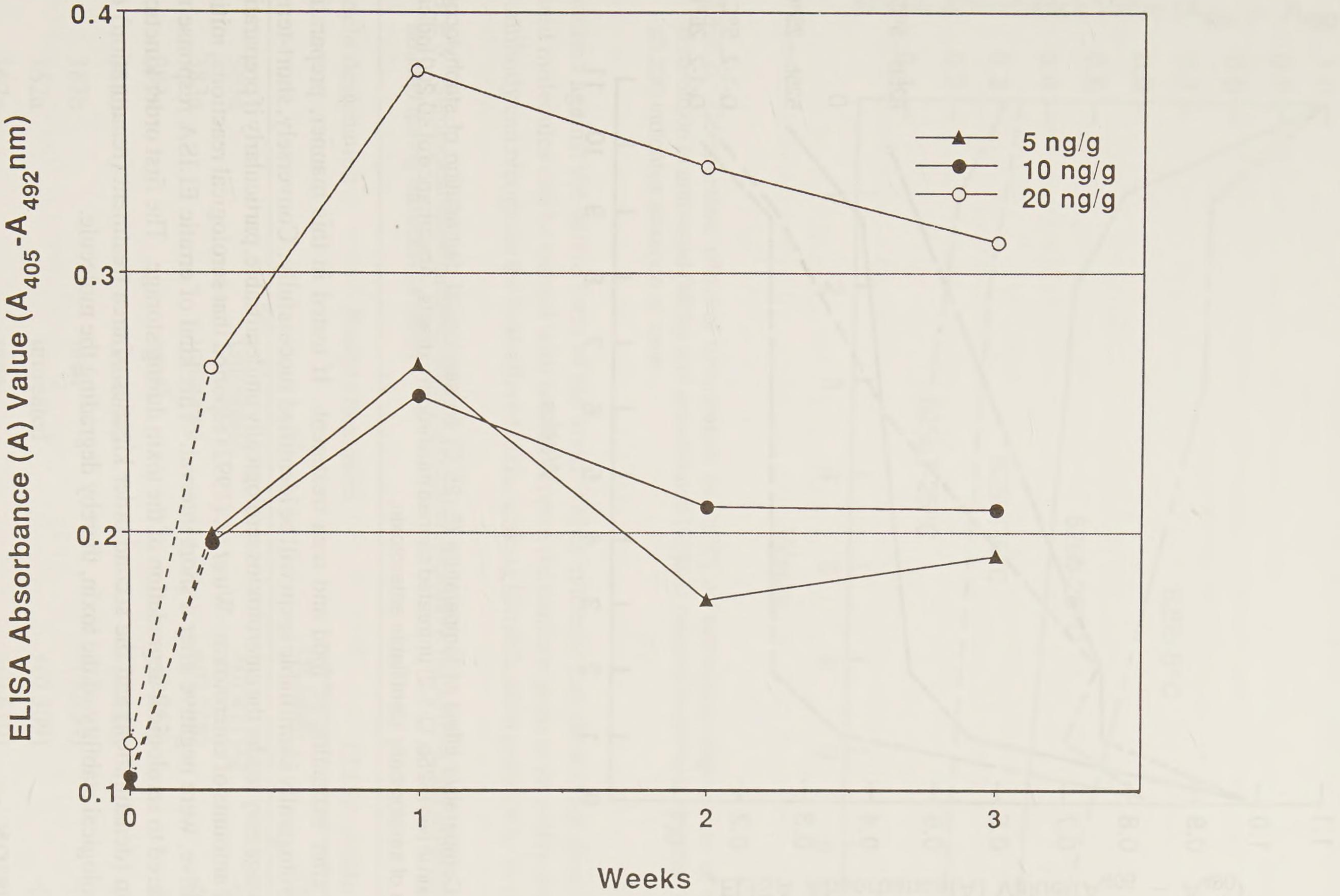

FIGURE 4. Comparative effect of renaturation (----, 6.0 M urea) and storage (5°C, 3 wk) on various concentrations (5.0, 10.0, 20.0 ng/g) of heat-altered (F_0 = 7.5 min) staphylococcal enterotoxin type A (SEA) in canned mushrooms and their spontaneous and/or degradative serological behavior (—). Absorbance > 0.200 indicates serological presence of SEA; absorbance 0.200 indicates absence of serologically identifiable SEA.

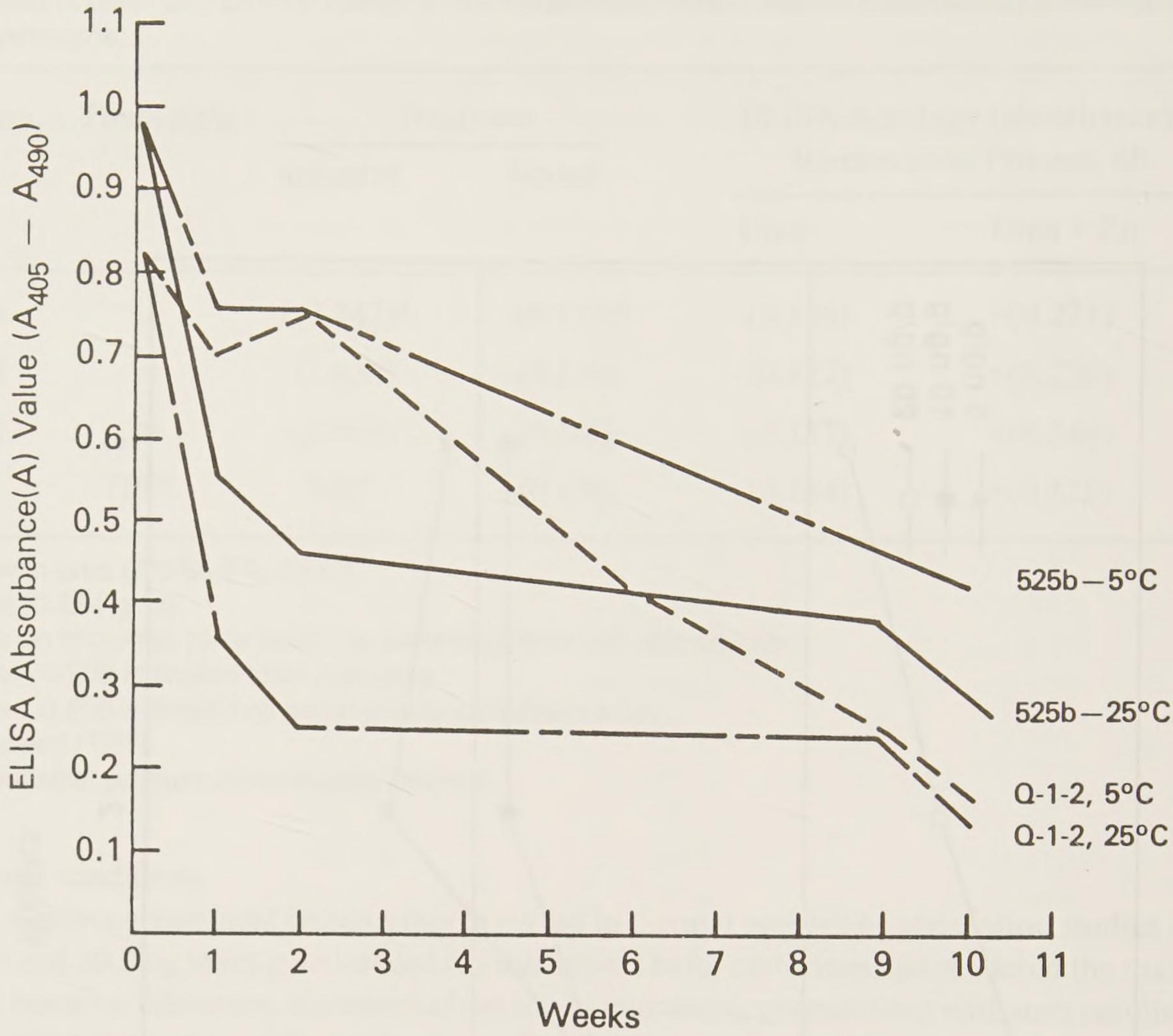

FIGURE 5. Comparative effect of temperature (5, 25°C) on serological degradation of staphylococcal enterotoxin in two (525b, Q-1-2) untreated canned mushroom extracts. Absorbance of ≤0.200 indicates absence of serologically identifiable enterotoxin.

immediately after extraction of food and urea treatment. If tested in this manner, preparations containing serologically identifiable toxin will be identified successfully. Conversely, short-term or prolonged storage may render the enterotoxins serologically unidentifiable, particularly if preparations contain small amounts of enterotoxin. Wu *et al.* (1992) reported that serological reactions, initially reported positive, were negative after a short interval. This kind of erratic ELISA response may perhaps be traced to serological degradation of the toxin during storage. The first order kinetics of heat alteration (denaturation) and the second order kinetics of urea treatment (renaturation) may affect the serological stability of the toxin, thereby degrading the molecule.

7. CONCLUSION

This paper has addressed the thermal inactivation/reactivation kinetics of SEA in contaminated mushrooms which have been simulated in the laboratory. Evidence shows that preformed staphylococcal enterotoxin can be altered serologically in foods and that this alteration, probably because of the conformation of the molecule, prevents recognition of the specific antibody that was

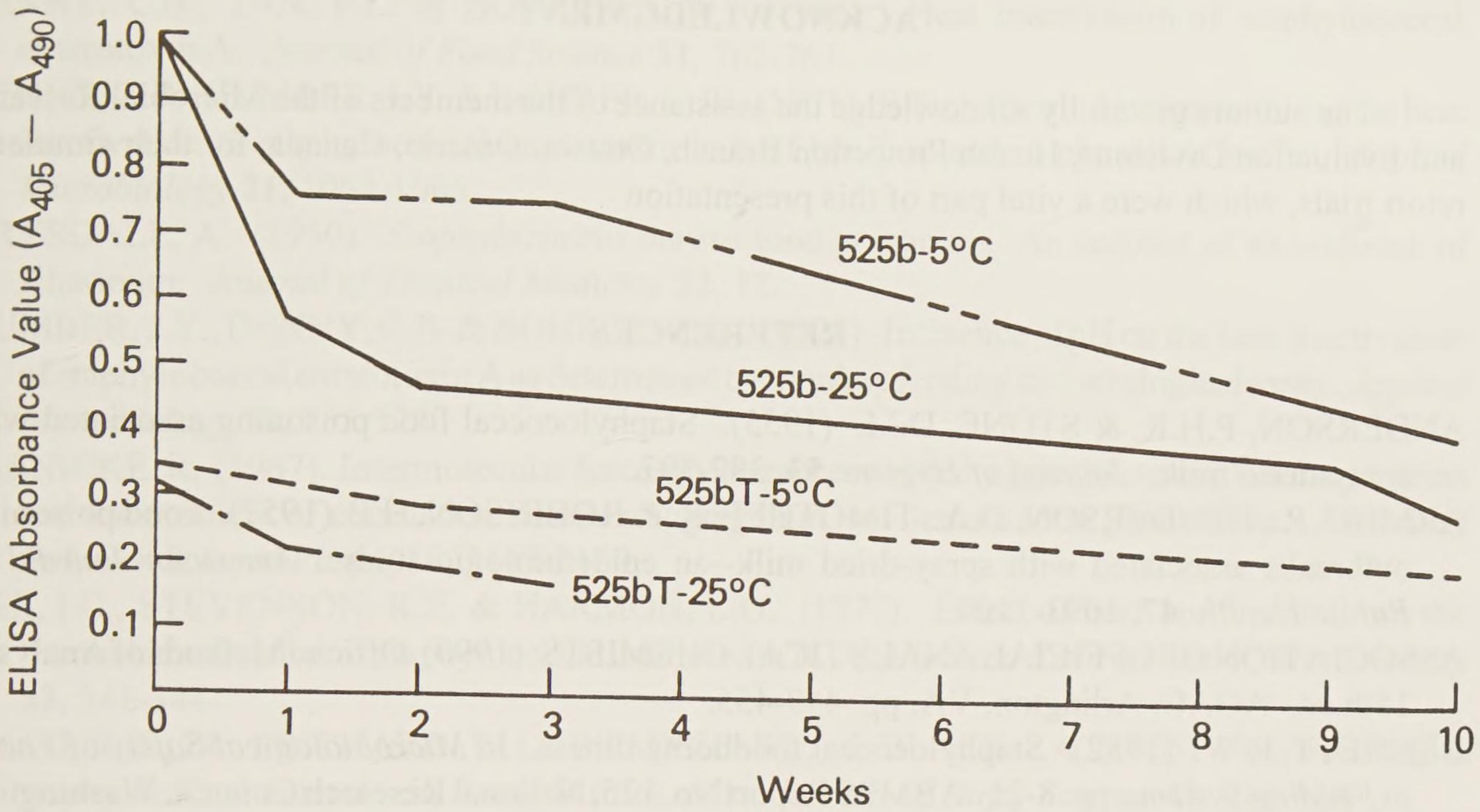

FIGURE 6. Comparative effect of temperature (5, 25˚C) on serological degradation of staphylococcal enterotoxin in untreated (525b) and urea-treated (525bT) canned mushroom extracts. Absorbance of ≤0.200 indicates absence of toxin.

generated against the native form of the toxin. Other evidence has shown that these serologically altered molecules can be treated with urea to reverse the conformation to its native position. Thus, the antibody can recognize the heat-altered toxin, making identification possible by *in vitro* serology.

TABLE 5. Serological degradation of staphylococcal enterotoxin A (SEA) from canned mushroom extracts abused during transport (4 days).

Sample designation	Sample treatment	ELISA results	
		1st assay	2nd assay
367a	untreated[1]	+(0.431)[2,3]	-(0.183)[4]
383a	untreated	+(0.228)	-(0.163)
383a	urea[5]	+(0.263)	-(0.174)
383b	untreated	+(0.486)	-(0.153)
383b	urea	+(0.534)	-(0.185)
362a	untreated	+(0.309)	-(0.185)
367a	urea	+(0.610)	+(0.229)

1 No urea treatment.
2 Absorbance reading on microtiter plate reader (A_{405}-A_{490}nm).
3 Readings > 0.200 indicated serologically identifiable SEA.
4 Absorbance ≤ 0.200 indicated absence of serologically identifiable SEA.
5 Treatment of the extract with 6.0 M urea for 5 h at 25˚C.

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REFERENCES

ANDERSON, P.H.R. & STONE, D.M. (1955). Staphylococcal food poisoning associated with spray-dried milk. *Journal of Hygiene* **53**, 387-397.

ARMIJO, R., HENDERSON, D.A., TIMOTHEE, R. & ROBINSON, H.B. (1957). Food poisoning outbreaks associated with spray-dried milk--an epidemiologic study. *American Journal of Public Health* **47**, 1093-1100.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. (1990). Official Methods of Analysis, 15th ed. AOAC, Arlington, VA, pp. 449-455.

BENNETT, R.W. (1982). Staphylococcal foodborne illness. In *Microbiological Safety of Foods in Feeding Systems*, pp. 8-21. ABMPS Report No. 125, National Research Council, Washington, DC: National Academy Press.

BENNETT, R.W. & BERRY, M.R., JR. (1987). Serological reactivity and *in vivo* toxicity of *Staphylococcus aureus* enterotoxins A and D in selected canned foods. *Journal of Food Science* **52**, 416-418.

BENNETT, R.W., ARCHER, D.L. & LANCETTE, G. (1988). Modified procedure to eliminate elution of food proteins during seroassay for staphylococcal enterotoxins. *Journal of Food Safety* **9**, 135-143.

BENNETT, R.W. & ATRACHE, V. (1989). Applicability of visualimmunoassay for simultaneous indication to staphylococcal enterotoxin serotype presence in foods. *Abstracts of the 89th Annual Meeting of American Society for Microbiology* 1989, 28.

BENNETT, R.W., ASH, M. & ATRACHE, V. (1989). Visual screening with enzyme immunoassay for staphylococcal enterotoxin in foods: an interlaboratory study. *AOAC Abstracts* 1989, 277.

BENNETT, R.W., ASH, M. & ATRACHE, V. (1990). Serotype differentiation of staphylococcal enterotoxin with an ELISA polyvalent antibody. *Abstracts of the 90th Annual Meeting of American Society for Microbiology* 1990, 31.

BENNETT, R.W. (1991). The effects of thermal processing on *S. aureus enterotoxin*. In *Proceedings of the 10th International Congress on Canned Foods*, pp. 441-459, Paris, France.

BENNETT, R.W. (1992). The bimolecular temperament of staphylococcal enterotoxin in thermally processed foods. *JAOAC International* **75**, 6-12.

BERGDOLL, M.S. (1989). *Staphylococcus aureus*. In *Foodborne Bacterial Pathogens*, pp. 464-522. Edited by M. Doyle. New York: Marcel Dekker.

CENTERS FOR DISEASE CONTROL. (1976). *Foodborne and Waterborne Disease Outbreaks--Annual Summary, 1975*. CDC, Atlanta, GA.

CENTERS FOR DISEASE CONTROL. (1989). Multiple outbreaks of staphylococcal food poisoning caused by canned mushrooms. *Morbidity and Mortality Weekly Reports* **38**, 17-418.

DANGERFIELD, H.G. (1973). Effects of enterotoxin after ingestion by humans. *Abstracts of the 73rd Annual Meeting of the American Society for Microbiology*, May 6-11, 1973, Miami Beach, FL.

DENNY, C.B., TAN, P.L. & BOHRER, C.W. (1966). Heat inactivation of staphylococcal enterotoxin A. *Journal of Food Science* **31**, 762-767.

DENNY, C.B., HUMBER, J.Y. & BOHRER, C.W. (1971). Effect of toxin concentration on the heat inactivation of staphylococcal enterotoxin in beef bouillon and in phosphate buffer. *Applied Microbiology* **21**, 1064-1066.

DRYSDALE, A. (1950). *Staphylococcus aureus* food poisoning. An account of an outbreak of Khartoum. *Journal of Tropical Medicine* **53**, 12.

HUMBER, J.Y., DENNY, C.B. & BOHRER, C.W. (1975). Influence of pH on the heat inactivation of staphylococcal enterotoxin A as determined by monkey feeding and serological assay. *Applied Microbiology* **30**, 755-578.

JAENICKE, R. (1967). Intermolecular forces in the process of heat aggregation of globular proteins and the problem of correlation between aggregation and denaturation phenomena. *Journal of Polymer Science Part C* **16**, 2143-2160.

LEE, I.C., STEVENSON, K.E. & HARMON, L.G. (1977). Effect of beef broth protein on the thermal inactivation of staphylococcal enterotoxin B. *Applied and Environmental Microbiology* **33**, 341-344.

READ, R.B., JR., PRICHARD, W.L., BRADSHAW, J. & BLACK, I.A. (1965). *In vitro* assay of staphylococcal enterotoxin A and B from milk. *Journal of Dairy Science* **48**, 411-419.

SCHWABE M., NOTERMANS, S., BOAT, R., TATINI, S.R., & KRÄMER, J.(1990). Inactivation of staphylococcal enterotoxins by heat and reactivation by high pH treatment. *International Journal of Food Microbiology* **10**, 33-42.

SOO, H.M., TATINI, S.R. & BENNETT, R.W. (1973). Thermal inactivation of staphylococcal enterotoxins A and D. *Abstracts of the 73rd Annual Meeting of American Society for Microbiology* 1973:1.

TATINI, S.R. (1976). Thermal stability of enterotoxins in food. *Journal of Milk and Food Technology* **39**, 432.

WARREN, J.R., SPERO, L. & METZGER, J.F. (1974). Isothermal denaturation of aqueous staphylococcal enterotoxin B by guanidine hydrochloride, urea and acid pH. *Biochemistry* **13**, 1678-1683.

WU, Z.-L, ZHANG, Z.-C., LI, C.-W., LI, S.-S., XI, Z.-Y., ZHOU Y.-N. & YIN, C.-R. (1992). Study on the nonspecific reaction problems in the procedure of using TECRA Kit (Bioenterprises Pty. Co., Australia) for detection of staphylococcal enterotoxins. *Journal of the Association of Rapid Methods and Automation in Microbiology* **4**, 105-109.

CHAPTER 22

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN IN MUSHROOMS

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1. INTRODUCTION

The detection of the staphylococcal enterotoxins (SE) in foods is a subject that has attracted wide attention and has resulted in research in a number of countries. There are two main reasons for this interest: 1) to determine whether a particular food was involved in food poisoning; and 2) whether a processed food suspected of possible contamination is marketable. Detection of enterotoxin in a food implicated in food poisoning is normally easier than establishing the safety of a food because in most instances the amount of enterotoxin present in the foods involved in food poisoning outbreaks is much more than the minimum amount necessary to establish the safety of a food.

2. DEVELOPMENT OF DETECTION METHODS

It was not until the first enterotoxin was purified (Bergdoll *et al.*, 1959b) and specific antibodies to it were prepared (Bergdoll *et al.*, 1959a) that it was possible to develop laboratory methods for its detection. The fact that the enterotoxin was a protein made impossible the development of chemical methods for its detection as such methods were not specific for any given protein. As it turned out, only methods that involved the reaction of the specific antibody with the enterotoxin were useful in its detection. During the course of purifying the first enterotoxin, gel diffusion methods based on antigen-antibody reactions to produce a precipitate were being developed. Such methods as the single gel diffusion (Oudin, 1952) and double gel diffusion tube methods (Oakley & Fulthorpe, 1953), the plate methods (Ouchterlony, 1948), and the microslide (Crowle, 1961) were among those presented. The Ouchterlony plate method was the method used initially to check culture supernatant fluids for enterotoxin because of its simplicity (Robbins *et al.*, 1974). However, its sensitivity was not such that it could be used to detect enterotoxin in food extracts from suspect foods.

Dr. Ezra Casman at the United States Food and Drug Association (FDA) also was working on the staphylococcal food poisoning problem. The method that he and Mr. Reginald Bennett (1965) developed required the concentration of the food extract from 100 g of food to 0.1 or 0.2 ml with the

final analysis by microslide. The microslide is the most sensitive of the gel diffusion methods, but not an easy method to use. The first use of this method was in the testing of cheese that had been implicated in food poisoning (Zehren & Zehren, 1968). At that time, the limit of sensitivity appeared to be about 1 μg/ml but, with later improvements, some individuals were able to achieve a sensitivity of 0.1 μg/ml (Casman *et al.*, 1969). However, most users of this method are unable to detect less than 0.5 μg/ml. The maximum sensitivity reportedly achieved is 30-50 ng/ml, but this would be a rare occurrence. It is doubtful that any individual using the extraction-concentration microslide method would be able to detect less than 1 ng of enterotoxin/ml and for most individuals, 1 ng/ml would be impossible. One of the problems with this method is that the percentage of enterotoxin recoverable, particularly at the very low levels, is probably not more than 10% and may be much less. Although this method is referred to as the "Official Method" for detection of enterotoxin in foods (Bennett & McClure, 1980), the fact that most individuals cannot achieve the maximum sensitivity claimed for it discredits the method. Thus, this method is not one to be recommended for use in the detection of enterotoxin in foods, especially in the establishment of the safety of foods.

3. SENSITIVE DETECTION METHODS

The quantity of enterotoxin required to cause illness in sensitive individuals was estimated to be less than 1 μg from analysis of foods involved in food poisoning (Bergdoll, 1979). It was only an estimate because enterotoxin is not uniformly distributed in foods nor is the amount of food consumed by any one individual known. It was not until an outbreak among school children who had drunk a known quantity of chocolate milk could a more accurate estimate be made. The amount present in the amount of milk drunk was 144 ng, or 0.4 to 0.75 ng/ml of milk, which was barely detectable by enzyme-linked immunosorbent assay (ELISA) without some concentration of the milk (Evenson *et al.*, 1988). It should be pointed out that the FDA laboratories were unable to detect enterotoxin in the milk served on the day of the outbreak (Schwab, personal communication). In most food poisoning outbreaks, much more enterotoxin is present and is easily detectable. However, it is essential that methods be available to detect less than 0.5 ng/g of food, to declare a suspect food consumable.

Fortunately, sensitive methods for detection of enterotoxin are now available, such as radioimmunoassay (RIA) (Miller *et al.*, 1976), ELISA (Freed *et al.*, 1982), and reversed passive latex agglutination (Igarashi *et al.*, 1985), These require no concentration of the food extract to detect ≤0.5 ng enterotoxin/g food although, if necessary, the extract can be concentrated several fold without interfering with the analysis. Although these sensitive methods have been available for a number of years and even though personnel in the FDA laboratories developed satisfactory procedures for both RIA (Kaufman, personal communication) and ELISA (Kaufman, 1980), FDA still insisted on using the cumbersome and outdated extraction-concentration microslide method. The most disturbing aspect of this was that, as a result of a collaborative study on the use of this method, it was declared the "Official Method" for the detection of enterotoxin in foods (Bennett & McClure, 1980). This was done without the involvement of the Bergdoll laboratory, the leading group in the development of methods for the detection of enterotoxin in foods. It was only after the paper had been published that Bergdoll was aware of the collaborative study.

As a result of the publication, Bergdoll wrote to FDA about the acceptance of the collaborative study as adequate for it being made the "Official Method". One paragraph from his letter read as follows: "The method presented in the article does not agree with the one presented in the 'Compendium of Methods for the Microbiological Examination of Foods' (Bergdoll & Bennett,

1984). The amount of food used in the collaborative study was 20 g instead of 100 g and the amount of toxin added was 0.1 µg to 0.2 µg per gram of food which is much higher than is recommended in the Compendium (0.1 to 0.25 µg/100 g of food, 2nd Edition, p 388). It is recognized that the extraction-concentration-microslide method is not an easy method and many people have difficulty obtaining the sensitivity possible, but this does not justify changing the sensitivity required and making the extraction easier to get good results in a collaborative study. Even so, only 73% were able to obtain positive results with the samples to which toxin was added. The fact that 99% were able to identify negative samples is of less importance." It is questionable whether there should be an official method for the detection of enterotoxin in foods. The basic reaction is immunological instead of chemical and is not easily quantifiable. Some individuals will find one procedure better for them than others, and they should be allowed to use that method. The only stipulation should be that a minimal amount be detectable. Certainly, when better methods are developed, they should replace the older methods. It is difficult to understand why FDA insists on using a long outdated method in favor of methods which are more sensitive and easier to use.

4. SCREENING KITS

Kits are now available commercially for the detection of enterotoxin in foods. The first one available was the ELISA ball kit produced in Bern, Switzerland (Fey & Pfister, 1983). In this kit, the specific antibodies are adsorbed to the surface of polystyrene balls, each color coded for a particular enterotoxin. One ball for each SE (SEA, SEB, SEC, SED) and two control balls coated with normal rabbit sera are added to 20 ml of the food extract and shaken over night to allow the enterotoxin to attach to the specific antibodies. The value of using this volume of extract results in increased sensitivity. The conjugate is alkaline phosphatase attached to the specific antibodies. The substrate is nitrophosphate which is converted to a yellow compound by the phosphatase. The sensitivity of the method can be as high as 0.1 ng/ml. In tests to date, it appears to be the method of choice (Wieneke & Gilbert, 1987; Wieneke, 1991).

The RPLA kit was developed in Japan and employs latex particles coated with the specific antibodies for SEA-SED (Igarashi *et al.*, 1986; Fujikawa & Igarashi, 1988). The treatment of the coated particles with SE results in agglutination of the latex particles. There are at least two problems with this method: 1) it is necessary in many instances to filter the food extract through a non-protein adsorbing filter to clarify the extract, and 2) an occasional food extract will give a false agglutination (Wieneke & Gilbert, 1987). The sensitivity of the method appears to be somewhat less than the ELISA ball kit (Wieneke & Gilbert, 1987).

More recently, screening kits have been developed which contain all of the specific antibodies adsorbed to the same surface such as wells in a microtiter plate. The first one marketed was the TECHRA kit developed in Australia and promoted by FDA. One problem with screening kits is obtaining an equal quantity of specific antibodies for each SE to each surface. Apparently this is very difficult since, in the TECRA kit, the analysis for SEB is twice as sensitive as that for the other SEs. Tests on at least one kit in Japan showed very poor results with SED (Igarashi, personal communication). Nonspecific reactions have been encountered (Park *et al.*, 1992).

A second screening kit has been produced, that by TRANSIA in Lyon, France. In this kit the inside bottom of small tubes is coated with the specific antibodies. The sensitivity is equal to 0.02 ng of SE; a mixture of 0.004 ng of each of the five SE gives a beautiful blue color. A collaborative study with this kit has been done and a paper was submitted to the AOAC journal

(De Solan *et al.*, 1992)

The screening kit idea is a good one providing all SEs can be detected equally. The advantage is that if more than one SE is present, and present at below the individual detectable levels, it is possible that together they could be detected. This is important because it is known that the biological reaction of the SEs is accumulative (unpublished data).

A procedure included in the TECRA kit that has caused a lot of concern is the use of urea to supposedly renature heat-treated SEs. The exact effect of heat on proteins is not certain but, from experience, the structure is so altered that they are no longer active, for example, enzymes. The idea that heated SE can be regenerated is fascinating, but unproved. It is known that urea can partially unfold the SE molecule and treatment with guanidine hydrochloride results in complete unfolding (unpublished results). However, removal of the denaturing agent allows the molecule to refold to its original structure and retain its biological activity. Denaturation by heat is quite different and it is very doubtful that heated proteins can be renatured by urea. Even so, it is known that the heat-denatured SEs are not biologically active when taken intragastrically. This has been demonstrated by boiling the SE over a 30 minute heating period at 100°C. It is true that the results reported by Dangerfield with heated SEA appear to show that the activity is enhanced by the heating procedure (Dangerfield, 1973). This was never confirmed and remains questionable, especially in the light of the experiments carried out at the National Canners Association (Denny *et al.*, 1966, 1971; Humber *et al.*, 1975). The time required to inactivate SEA by heating in beef boullion decreased exponentially with the increase in temperature. The activity was followed by intragastric injection into monkeys and intrapertioneal injection in cats. The SE could be detected serologically even though it was no longer active in monkeys. This is not unexpected because the action in monkeys is not very sensitive. The important point is that SEA was definitely inactivated with time.

There is one curious aspect to the procedure employed in the treatment with urea. The food extract treated with urea is concentrated 20-fold, and the result compared to the unconcentrated extract. It would be presumed that one should be able to detect any SE that was present in too small amount to be detected in the unconcentrated extract.

If more SE were detectable after the urea treatment, it could be due to one or two other phenomena. It is known that, on relative mild heating, the SE can associate with food proteins and not be detectable when the usual extraction is done (Satterlee & Kraft, 1969). In this case, any SE adsorbed would be disassociated with urea and could be detectable. The other phenomenon is that the SE may dimerize and could be dedimerized with urea, thus increasing the amount of SE detectable. In either case, the SE would not be denatured and would still be biologically active. In the case of the mushrooms, it would be expected that the retorting would result in denaturation of the SE, and it would be biologically inactive.

The 20-fold concentration step raises the question of how sensitive do the methods need to be. If such a step is desirable, it would complicate the SE analysis because it would make it more difficult as a means of concentration would be needed. The sensitive methods now in use made it possible to eliminate the cumbersome concentration procedures. It is hoped it would not be necessary to revert to concentration procedures.

5. CASE STUDY

The mushroom problem arose because of several supposedly staphylococcal food poisoning outbreaks in the United States from mushrooms imported from Hong Kong that supposedly were

canned in China. A complete description of the outbreaks and the mushrooms involved was never published. I was told by an authority at FDA that the mushrooms came from Hong Kong and were thought to be blackmarket mushrooms as the lot numbers were incomplete in some cases and different lot numbers were present in the same case. In some outbreaks, the can from which the mushrooms were taken was not available for examination and the mushrooms were not available for testing. Subsequently, a listing of canned mushrooms from several canneries in China that reportedly were positive for enterotoxin appeared in Food Industry Report (Anonymous, 1990). In my opinion, this appears highly improbable as others have examined hundreds of cans of mushrooms from China without ever finding a positive reaction, both with the ELISA ball kit and the RPLA method. Even those who used the TECRA kit, recommended by FDA, did not find any positive reactions. The major possibility of entertoxin being present in canned foods is through cans that are leakers, and I was informed that leakers were found occasionally. It is difficult to understand how enterotoxin could be present in the mushrooms initially or, even if they were, how they could survive the processing. There was a report that, if staphylococci were present in the mushrooms at harvest, these organisms could grow and produce enterotoxin during transportation in sealed plastic bags (Hardt-English *et al.*, 1990). Others were unable to confirm this (Wu, personal communication; Brunner & Wong, 1992). For this to take place, the mushrooms would have to be in transportation for several hours. There is no evidence that staphylococci will grow on raw mushrooms, much less produce enterotoxin. Even if enterotoxin was present on the mushrooms at the time of processing, certainly most of it would be removed during the washing and preparing the mushrooms for retorting. It has been shown that enterotoxin does not survive retorting (Denny, personal communication). It is still a mystery to me how FDA could find so many cans to contain enterotoxin.

This raises a question about the methods used by FDA for enterotoxin detection and the ones they asked the Chinese to use in testing their mushrooms. It was impossible for them to use the "Official Method" because they could do no better than 500 ng of SE with the microslide which is the best many users can achieve. They did purchase 100 of the TECRA kits to be used in China, but this method is not a proven method. They and others obtained false positive reactions with the method. Why FDA recommended this method before it had been adequately tested by other investigators, I do not know. Even FDA was unable to identify blind samples containing enterotoxin using this method (Park, personal communication).

6. INTERNATIONAL COLLABORATION FOR DEVELOPMENT

The commercial kits are quite expensive. Hence, the Chinese desired to develop their own kits for use in China. I was asked to give them an assist in the development of an ELISA kit. I aided them in the purification of the enterotoxins, but they prepared their own enterotoxin antibodies and ELISA kit. I helped them in testing the kit against the ELISA ball kit and the RPLA kit. Their kit was equal in sensitivity to the ELISA ball kit and somewhat more sensitive than the RPLA kit. I congratulate them on their success as I started working with them in October 1990 and helped them test the kit in April 1992. In my opinion it is more reliable that either of the methods required by FDA.

REFERENCES

ANONYMOUS (1990). Big problem with mushrooms. *Food Industry Report.* July 23, pp 2, 6-7.

BENNETT, R. W. & MCCLURE, F. (1980). Extraction and separation of staphylococcal enterotoxin in foods: collaborative study. *Journal of the Association of Official Analytical Chemists* **63,** 1205-1210.

BERGDOLL, M. S. (1979). Staphylococcal intoxications. In: *Foodborne Infections and Intoxications*, 2nd Ed. pp. 443-494. Edited by H. Reimann & F. L. Bryan. New York: Academic Press.

BERGDOLL, M. S. & BENNETT, R. W. (1984). Staphylococcal enterotoxins. In *Compendium of Methods for the Microbiological Examination of Foods,* pp. 428-457. Edited by M. L. Speck. Washington, DC: American Public Health Association.

BERGDOLL, M. S., SUARGALLA, M. J. & DACK, G. M. (1959a). Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin-neutralizing property. *Journal of Immunology* **83**, 334-338.

BERGDOLL, M. S., SUGIYAMA, H. & DACK, G. M. (1959b). Staphylococcal enterotoxin. I. Purification. *Archives of Biochemistry and Biophysics* **85**, 62-69.

BRUNNER, K. G. & WONG, C. L. (1992). *Staphylococcus aureus* growth and enterotoxin production in mushrooms. *Journal of Food Science* **57**, 700-703.

CASMAN, E. P. & BENNETT, R. W. (1965). Detection of staphylococcal enterotoxin in food. *Applied Microbiology* **13,** 181-189.

CASMAN, E. P., BENNETT, R. W., DORSEY, A. E. & STONE, J. E. (1969). The microslide gel double gel diffusion test for the detection and assay of staphylococcal enterotoxins. *Health Laboratory Science* **6,** 185-198.

CROWLE, A. J. (Ed.). (1961). *Immunodiffusion*. New York: Academic Press.

DANGERFIELD, H. D. (1973). Effects of enterotoxin after ingestion by humans. *Abstracts of the 73rd Annual Meeting of the American Society for Microbiology.*

DENNY, C. B., HUMBER, J. Y. & BORRER, W. (1971). Effect of toxin concentration on the heat inactivation of staphylococcal enterotoxin A in beef boullion and in phosphate buffer. *Applied Microbiology* **21**, 1046-1066.

DENNY, C. B., TAN, P. L. & BORRER, C. W. (1966). Heat inactivation of staphylococcal enterotoxin A. *Journal of Food Science* **31**, 762-767.

DE SOLAN, M. N., LAPEURE, C. & DROUET, X. (1992). Global immunoenzymatic detection of staphylococcal enterotoxins: collaborative study. *Journal of the Association of Official Analytical Chemists* Submitted.

EVENSON, M. L., HINDS, W. M., BERNSTEIN, R. S. & BERGDOLL, M. S. (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of food poisoning involving chocolate milk. *International Journal of Food Microbiology* **7,** 311-316.

FEY, H. & PFISTER, H. (1983). A diagnostic kit for the detection of staphylococcal enterotoxins (SET) A, B, C and D (SEA, SEB, SEC, SED), In *Immunoenzymatic techniques*, pp. 345-348. Edited by S. Avrameas *et al.* Amsterdam: Elsevier/North-Holland Publishing Co.

FREED, R. C., EVENSON, M. L., REISER, R. F. & BERGDOLL, M. S. (1982). Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxin in food. *Applied and Environmental Microbiology* **44,** 1349-1355.

FUJIKAWA, H. & IGARASHI, H. (1988). Rapid latex agglutination test for detection of staphylococcal enterotoxins A and E that uses high-density latex particles. *Applied and Environmental Microbiology* **54**, 2345-2348.

HARDT-ENGLISH, P., YORK, G., STIER, R. & COCOTAS, P. (1990). Staphylococcal food poisoning outbreaks caused by canned mushrooms from China. *Food Technology* **44**, 74, 76-77.

HUMBER, J. Y., DENNY, C. B. & BORRER, C. W. (1975). Influence of pH on the heat inactivation

of staphylococcal enterotoxin A as determined by monkey feeding and serological assay. *Applied Microbiology* **30**, 755-758.

IGARASHI, H., FUJIKAWA, SHINGAKI, M. & BERGDOLL, M. S. (1986). Latex agglutination test for staphylococcal toxic shock syndrome toxin 1. *Journal of Clinical Microbiology* **23**, 509-512.

IGARASHI, H., SHINGAKA, M., FUJIKAWA, H., USHIODA, H. & TERAYAMA, T. (1985). Detection of staphylococcal enterotoxins in food poisoning outbreaks by reversed passive latex agglutination. *Zentralblatt für Bakteriologie Supple*ment **14**, 255-257.

KAUFMAN, P. E. (1980). Enzyme immunoassay for staphylococcal enterotoxin A. *Journal of the Association of Official Analytical Chemists* **63**, 1138-1143.

MILLER, R. A., REISER, R. F. & BERGDOLL, M. S. (1976). The detection of staphylococcal enterotoxin A, B, C, D, and E in foods by radioimmunoassay, using staphylococcal cells containing protein A as immunosorbent. *Applied and Environmental Microbiology* **36,** 421-426.

OAKLEY, C. L. & FULTHORPE, A. J. (1953). Antigenic analysis by diffusion. *Journal of Pathology and Bacteriology* **65,** 49-60.

OUCHTERLONY, O. (1948). Antigen-antibody reactions in gels. *Archiv fürKemi, Mineralogi, och Geologi* **26B,** 1-9.

OUDIN, J. (1952). III. Techniques and analysis of the quantitative precipitin reaction. B. Specific precipitation in gels and its application to immunochemical analysis. *Methods in Medical Research* **5,** 335-378.

PARK, C. E., AKHTAR, M. & RAYMAN, M. K. (1992). Nonspecific reactions of a commercial enzyme-linked immunosorbent assay kit (TECRA) for detection of staphylococcal enterotoxins in foods. *Applied of Environmental and Microbiology* **58**, 2509-2512.

ROBBINS, R., GOULD, S. & BERGDOLL, M. S. (1974). Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Applied Microbiol*ogy **28,** 946-950.

SATTERLEE, L. D. & KRAFT, A. A. (1969). Effect of meat and isolated meat proteins on the thermal inactivation of staphylococcal enterotoxin B. *Applied Microbiology* **17**, 906-909.

WIENEKE, A. (1991). Comparison of four kits for the detection of staphylococcal enterotoxin in foods from outbreaks of food poisoning. *International Journal of Food Microbiology* **4**, 135-143.

WIENEKE, A. A. & GILBERT, R. J. (1987). Comparison of four methods for the detection of staphylococcal enterotoxin in foods from outbreaks of food poisoning. *International Journal of Food Microbiol*ogy **4**, 135-143.

ZEHREN, V. L. & ZEHREN, V. F. (1968). Relation of acid development during cheese making to development of staphylococcal enterotoxin A. *Journal of Dairy Science* **51,** 645-649.

Part IV

NUTRITIONAL AND MEDICINAL ATTRIBUTES OF MUSHROOMS

CHAPTER 23

ANALYSIS, DIGESTIBILITY AND THE NUTRITIONAL VALUE OF MUSHROOMS

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1. INTRODUCTION

In many parts of the world people have recently become much more aware of nutrition and its importance. Several years ago, the U.S. Congress passed new nutritional labeling laws requiring that virtually all processed food and much of the fresh produce, including mushrooms, must show their nutritional content on the label. Those laws take effect this year. In Europe, the EEC also has a new nutritional labeling law. Such laws depend solely on chemical analysis.

Chemical analysis tells what is present, but it does not tell how well it is used. Like any experimental data, it is subject to varied interpretations or misinterpretation.

For many years, we have known that the primary structural carbohydrate of mushrooms is chitin. Chitin is poly-N-acetylglucosamine. The nitrogen of the chitin will be included in a Kjeldahl analysis. While the chitin is non-protein, there seem to be no definitive experiments that allow us to conclude anything about the nutritional value of mushroom chitin. All living tissue also contains nucleic acids which will account for some of the nitrogen.

Many people routinely report crude protein of most materials as 6.25 X Kjeldahl nitrogen (A.O.A.C., 1990; FAO, 1970; Kurtzman, 1975; Watts & Merill, 1963). The nucleic acid, chitin and other miscellaneous nitrogen are normally included, so it should be clear that the reported protein will often be too high. Nonprotein structural nitrogen may be relatively uncommon. It does occur in all mushrooms, yeasts and arthropods. Many plants contain nitrogenous glycosides. All living tissue, but especially actively growing tissue will contain nucleic acid nitrogen. Thus, poultry eggs and seeds contain large amounts of storage protein, with few (eggs, one) living cells. Milk is a secretion which has only a few sloughed cells and contaminating microorganisms.

Official Methods of the Association of Official Analytical Chemists (A.O.A.C., 1990) call for several different factors to be used for various commodities. Those factors range from 5.18 for almonds (amygdalin) to 6.38 for dairy products. Most commodities are lumped together and most often 6.25 is recommended. It is surprising that they specifically recommend 6.25 for yeast. Yeast would be more like mushrooms than anything else that is specifically mentioned, but yeast would

usually have a higher nucleic acid content. Watt and Merrill (1963) proposed 6.25 X 2/3 or 4.17 for *Agaricus bisporus*. Doesburg and Meijer (1965) assayed the amino acids of *Agaricus bisporus* and recovered 5.162 grams of amino acid per gram of nitrogen. Assuming that the amino acids weigh 6% more than the protein they are derived from, due to water of hydrolysis, a factor of 4.78 is a minimum conversion factor for *Agaricus bisporus*. Even 5.03 would be reasonable, if Doesburg and Meijer accounted for 95% of the protein nitrogen. Other mushroom species probably are similar.

2. LABELING LAWS

Nutritional labeling laws introduce new problems into our nutritional understanding. It is good to let the public know what nutritional benefits they should expect from fresh produce. Often regulations may require that the analysis be done in such a manner as to assure that the data applies to all packages of the produce. That too sounds good, but it means that old data, considered to be carefully done, is no longer acceptable. The growers are not prepared to do such analysis and must find a contractor to do it for them.

I was privileged to see the complete contractor's report which is the basis for the official U.S. nutritional label for mushrooms (Table 1) (Contractors Report to Produce Marketing Association, 1982). Several of their data appear to be out of the expected range, based on the analysis of others.

They used samples taken from retail supermarkets, shipped to them from all of the 48 states, but they reported moisture contents of 90.2% to 93.5%. In my own experience, I find that mushrooms picked less than one hour before the initial weighting will usually be less than 92% water when dried at 80°C. However, they ranged from 88.6 to 93.2% (Table 1). There will be some apparent caramelization, that is, some of the water lost will be chemically part of the sugar. I also find that after 20 hours storage in a refrigerator, the same mushrooms have lost water and are about 88% water. The values in the literature vary from the mid eighty-percents to the mid ninety-percents.

TABLE 1. Proximate composition of mushrooms, gm per 100 gm fresh weight. Minerals mg per 100 gm fresh weight.

Species and Ref.	Moisture	Ash	Nitrogen	Protein N X 5.0	Fat	Crude Fiber	Na	K	Ca	Fe	P
Agaricus bisporus[a]	89.7	0.82	0.78	3.90	0.20	0.38					
A. bisporus[b]	89.5	1.26	0.61	3.07	0.19	1.09					
A. bisporus[c]	90.4	0.9	0.64	3.24	0.30	0.80	14.98	414.0	6.05	.797	116.0
A. bisporus[d]	88.7	0.9	0.62	3.08	0.90	0.80	11.98	322.0	8.02	.938	103.1
A. bisporus[e] High	93.0	1.40									
A. bisporus[e] low	87.2	0.87									
A. bisporus[f] High	93.5	0.9*	0.64	3.20	0.5*	1.00	5.40	389	5.03	1.20	124.4
A. bisporus[f] Low	90.5	0.6	0.43	2.16	0.2	0.500*	3.90	329	1.48	0.420	92.6
A. bisporus[f] Label				2.23	2.23	0.558	4.46	357			
Pleurotus ostreatus[g]	92.5		0.34	1.72							
P. ostreatus "florida"[h]	91.5	0.79	0.25	1.25	0.14	1.01					
Volvariella volvacea[a]	88.4	1.46	0.80	3.99	0.74	1.38					

* Exact value in three or more composite samples.

References: [a]Chang (1992); [b]Esselen and Fellers(1946); [c]Watts and Merrill(1963); [d]F.A.O.(1972); [e]Kurtzman, unpublished; [f]Contractor'sReport (1982); [g]Kalberer and Kunsch(1974); [h]Bano, *et al.* (1981).

The mushrooms shipped to the contractor from the greatest distance and from the driest climate had the highest water content. That is also the part of the country where the soil is most saline. If we believe the official analysis we must reject experiments that show that increased salt in cultivation yields mushrooms of higher solid content, although those from the hot-dry-saline area also contained the least ash.

There were other interesting variations in the ash, and calcium was 3.4 times greater from one area of the country than from another (Table 1). As far as I know, all U.S. growers use large quantities of gypsum in their compost and limestone in their casing. Thus, it is not reasonable that calcium should vary by locale. The magnesium content of soils does vary from region to region and from one source of limestone to the next, but the report showed a variation of only 9.6 to 12.0 mg magnesium per 100 g mushrooms. Magnesium and calcium are antagonistic in many biochemical reactions and although most growers avoid magnesium, the largest portion of magnesium is in the limestone. So, although some correlation between magnesium and calcium content might be expected, none was apparent.

While others (Crisan & Sands, 1978; FAO, 1970; Kurtzman, 1975; Watts & Merrill, 1963) have found 0.65 to 0.78% nitrogen, the contractor found 0.52%. It is interesting that 0.52% is exactly 2/3 of the 0.78%. Watt and Merrill (1963) used 2/3 in their calculation of protein from nitrogen. While others found 0.2 to 0.3% fat (Crisan & Sands, 1978; Kurtzman, 1975), the official label says 2%.

The sampling protocol was such that it assured that the sampling was not random, but even the questionable sampling would not appear to account for the unexpected results. It seems equally unlikely that it was due to varietal differences.

3. ANTI-NUTRITIONAL FACTORS

In 1972, Presant and Kornfeld (1972) isolated a hemagglutinin from *Agaricus bisporus*. The hemagglutinin was similar to, but distinct from, the hemagglutinin of bean, *Phaseolus vulgaris*. Apparently, that work went unnoticed by people interested in the nutritional value of mushroom until very recently. Ortiz *et al.* (1992), used the purified hemagglutinin and found that it caused intestinal malabsorption in rats. Malabsorption was determined by weight gain, fecal nitrogen and nitrogen digestibility. Histological lesions were found in the small intestine. They found no histological lesions in rats that were fed material that had been heated at 100°C for 1 h. Weight gain, fecal nitrogen and nitrogen digestibility were not determined for the rats fed the heated material.

Hemagglutinins have also been reported in *Pleurotus ostreatus* and *Pleurotus spodoleucus* (Kogure, 1975). Until some mushroom is specifically shown not to contain hemagglutinins, we should expect that all mushrooms have them.

Bean hemagglutinin is well known to cause intestinal malabsorption (Liener, 1975), so the results of Ortiz *et al.* (1992) were not surprising. It appears that a thorough study is needed to determine the minimum degree of cooking or other processing that will be required to inactivate the hemagglutinin. Other mushrooms should be examined for hemagglutinins. As long as we are aware of the problem, it need not decrease the nutritional value of mushrooms, but we need to know more than we have learned so far.

4. VITAMINS

Fungi have long been used as a source of vitamins. Yeast has been sold as a vitamin source. Years ago it was sold, along with an iron supplement as vitamin pills in the U.S.A. In Australia, New Zealand and the United Kingdom, yeast extract is sold as a spread for breakfast toast. The beneficial value of fungi as yeast was appreciated, at least indirectly, by western civilizations long before yeast was clearly understood. Governor William Bradford, the primary civil leader of the American Pilgrims of the Plymouth Colony, wrote that the colonists had suffered greatly from want of food and from pestilence (Bradford, 1648). He indicated that it was surprising to him that things had not been worse because they did not have beer to drink to give them health; the colonists were reduced to drinking water! If you ever think that mushroom or nutritional science is lagging behind other intellectual endeavors, consider the social sciences in the U.S.A., who tell us that Governor Bradford and his associates, who considered beer a health drink, were somehow responsible for our alcohol prohibition 250 years later. A lesson that we should learn from their error is to read older literature. People a decade or a century ago did have good brains. It is important to build on both past errors and past knowledge.

Most vitamin analyses have shown mushrooms to be very similar to yeast, except that thiamine is usually found to be low in mushrooms. Thiamine is required for decarboxylation, a reaction required by all living tissue. Why then is it low in mushrooms? According to Wakita (1976) most mushroom species have thiaminases, enzymes that destroy thiamine. There are two thiaminases: I, base transferase (Wittliff & Airth, 1970a) and II, hydrolase (Wittliff & Airth, 1970b). Apparently, mushrooms have the hydrolase and a few also have the transferase. Both enzymes destroy the vitamin. If thiaminase is the reason for low thiamine analysis, it may be released during normal food preparation, but destroyed by cooking procedures. It is possible that with proper care, the thiamine in mushrooms could be greatly increased.

If mushrooms contain at least two anti-nutritional factors, a few might discard them as worthless food. That would not be wise, since most foods have some negative factors and both factors known in mushrooms occur in other, commonly consumed foods. Beans and other legumes contain hemagglutinins (Liener, 1975), and fresh-water fish have thiamine base transferase (Wittliff & Airth, 1970a).

5. FEEDING EXPERIMENTS

Another appealing answer is to make all determinations by feeding experiments. Feeding experiments are expensive and rats or other species are generally used. Rodent digestive systems are clearly different than the human digestive system. Specific differences can easily become of great importance. Chitin might easily pass down the short gut of a rodent without being digested. That might keep the digestive enzymes from reaching the cell contents of unbroken cells. Both the chitin and all cell contents might be digested in the complex gut of a ruminant, neither experiment would tell us about humans.

Human feeding experiments can tell much about nutritional value. It might seem that human feeding experiments would give conclusive answers, but it would be nearly impossible to feed only mushrooms to people. What influence can we expect from the remaining diet? If the basal diet and the control were adequate in thiamine, there might be no affect from the thiaminase, but it might be great if the basal diet contained any less than the minimum requirement. It should be apparent that the degree of cooking could make a considerable difference.

Table 2. Protein Efficiency Ratio and digestibility of tunnel-dried mushrooms in rats.

Expt.	Dietary Protein	Final Body	Total Feed	PER[5]			% Digestibility[6]		
No.[1]	10% of diet[2]	Wt., gm[3]	Consumed[3]	Actual[4]	Adjusted	5.0/6.25	Diet	N	5.0/6.25
1	Casein (ANRC)	201 ±9	438 ±36	3.35 ± .13	2.50			93	91
1	*A. bisporus*	141 ±7	286 ±19	3.01 ± .10	2.25	2.81	88	65	81
2	Casein (ANRC)	183 ±11	372 ±24	3.42 ± .09	2.50			94	92
2	*A. bisporus*	116 ±4	278 ±12	2.18 ± .05	1.59	1.99	82	59	74

[1]See Table 3 for the diets used in experiment No.1 and No. 2.
[2]The 10% protein is based on N X 6.25.
[3]Mean ± S.E. Duncan's Multiple Range Test. All values within each experiment are significantly different: P<.01.
[4]Mean ± S.E. Duncan's Multiple Range Test. All values within experiments are significantly different: Experiment No. 1 P<.05. Experiment No. 2 P<.01.
[5]PER (Protein Efficiency Ratio) = weight gain/protein intake. "Adjusted" equivalent with casein reduced to standard 2.5. "5.0/6.25" = "Adjusted"/ 5.0/6.25.
[6]Digestibility: Diet = (feed intake - fecal weight)/feed intake X 100. N = (N intake - fecal N)/ N intake X 100. "5.0/6.25" = (N intake - fecal N)/ N intake X 5/6.25 X 100. Pooled data, day 7 through day 14.
Male, Sprague-Dawley rats, initial age 21 days, initial weight 55 grams.

Some of the problems with feeding experiments are more subtile. Years ago I had some Protein Efficiency Ratios (PER) run. While conferring with the pharmacologist who was in charge of running the experiments, he explained that the results would depend largely on the basal diet even though it would be used as the control.

The results of those experiments are shown in Table 2. The experimental rats never ate as much as the controls and more nitrogen was found in their feces. If we assume that the chitin nitrogen should not be counted, and try to recalculate on the basis of 5.0 rather than 6.25 as the nitrogen to protein conversion factor, the PERs look better. In experiment No. 1, the recalculated PER is better than

Table 3. Diets as percent composition, used in Protein Efficiency Ratio experiments (Table 2).

Ingredients	Experiment No. 1		Experiment No. 2	
	Control	*A.bisporus*	Control	*A.bisporus*
Casein (ANRC)	11.17		11.07	
Dry Mushrooms		37.41		39.68
DL-Methionine		0.57		0.57
N from above	1.60	1.60	1.60	1.60
Corn Oil	8.00	7.33	8.00	6.88
H_2O	4.37	3.94	4.33	2.01
Salts	7.80	3.71	6.49	3.00
Cellulose	3.00	0.61	3.30	0.36
Vitamins	2.00	2.00	2.00	2.00
Corn Starch	20.00	20.00	20.00	20.00
Dextrose	43.66	24.43	44.36	25.50

casein. Digestibility increases when we allow for the 20% of the nitrogen that is apparently in the chitin and nucleic acids, but is never as good as casein. The calculations that I used are questionable, but the whole PER procedure is weak. Table 3 shows the composition of the diets. While the ANRC casein standard is nearly pure protein, and an almost chemically defined diet can be made with it, there are major problems in balancing the experimental diet containing the mushrooms. In their attempt, my colleagues were forced to provide diets of lower energy content with the mushrooms. Of the differences between the diets, the most likely explanation would seem to suggest the rats need some minimum quantity of fat in their diet. The differences in the results, the problems with diet balance and the problems with interpretation of the data should make it clear that there is no absolute answer to nutritional value.

6. CONCLUSIONS

It would be very convenient if all we needed was a Kjeldahl or even an amino acid analysis to determine the nutritional value of mushrooms. Unfortunately, even feeding experiments depend on the degree of destruction of antinutritional factors and on other foods in the diet. We need to pursue all methods that will yield information on the nutritional value of mushroom.

The hemagglutinins and thiaminase may not be the only things found in mushrooms that can reduce their nutritional value and not all species or even varieties may contain them. If all anti-nutritional factors are proteins, then proper cooking may be all that is required to increase nutritional value. Studies need to be run to determine the minimum heating required to destroy the known anti-nutritional factors *in vitro* and the minimum heat necessary to obtain maximum nutritional value from whole mushrooms.

REFERENCES

A.O.A.C. (1990). *Official Methods of the Association of Official Analytical Chemists*. 15th edition. (two volumes) Arlington, VA.

BANO, Z., S. BHAGYA & SRINIVASAN, K. S. (1981). Essential amino acid composition and proximate analysis of the mushroom *Pleurotus eous* and *P. florida*. *Mushroom Newsletter for the Tropics* **1**, 6-10.

BRADFORD, W. CA. (1648). Of Plymouth Plantations: 1620 to 1647. McGraw-Hill, New York.

CHANG, S.T. (1972). *The Chinese Mushroom*. The Chinese University of Hong Kong, Hong Kong.

CONTRACTORS REPORT TO PRODUCE MARKETING ASSOCIATION. (1982). Communicated privately.

CRISAN, E. V. & SANDS, A. (1978). Nutritional Value. p. 137-168. In: *The Biology and Cultivation of Edible Mushrooms*. S.T. Chang and W. A. Hayes, eds. Academic Press, New York.

DOESBURG, J. J. & MEIJER, A. (1965). Analyze van Nederlandse bliconserven. II. Groente en veruchtenproducten. *Voeding* **25**, 258.

ESSELEN, W. B., JR. & FELLERS, C. R. (1946). Mushrooms for food and flavor. *Massachusetts Agricultural Experimental Station Bulletin*, No. 434.

FOOD AND AGRICULTURE ORGANIZATION, U.N. (1970). Amino-acid content of foods and biological data on proteins. F.A.O., Rome.

FOOD AND AGRICULTURE ORGANIZATION, U.N. (1972). Food Composition Table for Use

in East Asia. F.A.O., Rome.
KALBERER, P. & KUNSCH, U. (1974). Amino acid composition of the oyster mushroom (*Pleurotus ostreatus*). *Food Science and Technology* 7, 242.
KOGURE, T. (1975). On the specificity of mushroom *Pleurotus ostreatus* and *Pleurotus spodoleucus* extracts. *Vox Sang* **29**, 221-227.
KURTZMAN, R. H. (1975). Mushrooms as a source of food proteins. p. 305-318. In: *Protein Nutritional Quality of Foods and Feeds, Part 2*. Edited by M. Friedman. New York: Marcel Dekker.
LIENER, I. R. (1975). Effects of anti-nutritional and toxic factors on the quality and utilization of legume proteins. p. 523-550. In *Protein Nutritional Quality of Foods and Feeds, Part 2*. Edited by M. Friedman. New York: Marcel Dekker.
ORTIZ, R., SANCHEZ, R., PÁEZ, A., MONTAÑO L.F. & ZENTENO, E. (1992). Induction of intestinal malabsorption syndrome in rats fed with *Agaricus bisporus* mushroom lectin. *Journal of Agricultural and Food Chemistry* **40**, 1375-1378.
PRESANT, P. & KORNFELD, S. (1972). Characterization of the cell surface receptor for the *Agaricus bisporus* hemagglutinin. *Journal of Biological Chemistry* **247**, 6937-6945.
WAKITA, S. (1976). Thiamine-destruction by mushrooms. *Science Report of Yokohama National University* **2**, 39-70.
WATTS, B. K. & MERRILL, A. L. (1963). Composition of Foods. In: *Agricultural Handbook No. 8*, U. S. Department of Agriculture, Washington.
WITTLIFF, J. L. & AIRTH, R. L. (1970a). Thiaminase I. (Thiamine: base 2-methyl-4-aminopyrimidine-5-methenyl transferase, EC 2.5.1.2). *Methods in Enzymology* **18**, (A) 229-234.
WITTLIFF, J. L. & AIRTH, R. L. (1970b). Thiaminase II. (Thiamine hydrolase, EC 3.5.99.2) *Methods in Enzymology* **18**, (A) 234-238.

CHAPTER 24

NUTRITIONAL IMPORTANCE OF MUSHROOMS

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1. INTRODUCTION

The use of mushrooms as a food item is probably as old as civilization. They were preferred only for their culinary characteristics while the nutritive value of mushrooms was recognized much later (Crisan & Sands, 1978; Chang, 1980; Khanna & Garcha, 1981). In India, information about the food value of mushrooms has been provided by many workers (Samajpati, 1978; Jandaik & Kapoor, 1976; Bano *et al*., 1981; Khanna & Garcha, 1986; Bisaria *et al*., 1987a, b; Hira *et al*., 1990). However, the results reported by most workers are different. These differences have been attributed to variations in the species / strain used, method of composting, composition of compost, age or maturity of fruiting body etc. Moreover, mushrooms have been evaluated for their nutritional status mainly on the basis of their chemical composition. It has now been well established that cultivated mushrooms contain reasonable amounts of proteins, carbohydrates, minerals and vitamins, and nutritionally they rank between low grade vegetables and high grade meats. Although adequate nutrition is furnished by animal products such as fish, meat, eggs or milk, in India these articles are not within the means of the common man. Moreover, animal products are not always acceptable because of many cultural and religious beliefs. Most of the population lives on grains, tubers and small quantities of pulses and vegetables. Vegetables provide hardly any dietary proteins, and proteins from cereals, the staple food, are generally deficient in essential amino acids especially lysine and sulphur-containing amino acids. Pulses, on the other hand, are deficient in other essential amino acids like tryptophan. Mushrooms, which are good protein sources, may be an additional dietary supplement to make up for the protein deficiency of the Indian population in addition to their statiety factor.

Although mushrooms cannot substitute for meat, fish or eggs as far as protein nutrition is concerned, they can serve as a good substitute in preventing protein malnutrition in the Indian dietary pattern. Being rich in minerals, their usefulness becomes still more important.

Mushrooms are rich in proteins and certain essential amino acids (Khanna & Garcha, 1984) but *in vivo* bioavailability of these components is not well established. Although edible mushrooms are apparently innocuous, accumulative side effects caused by constant consumption have not been completely ruled out (Wright, 1978). It often becomes meaningless to compare the results obtained

by different workers with the same mushroom species, since the composition of a given species is affected by its diversity, genetic makeup leading to strain differences, and by environmental factors such as the nature of substrate, method of cultivation, along with relative inaccuracies arising out of the method of analysis. Many workers have reported different mushrooms under a common heading with little or no regard to variety. Being living organisms, they continue to undergo metabolic changes in the post harvest life. This adds to the changes in composition relative to the stage of development, harvest and nature of post harvest storage (Crisan & Sands, 1978).

2. COMPARATIVE NUTRITIONAL ANALYSIS OF *AGARICUS BISPORUS* AND *PLEUROTUS* SPP.

In the northern parts of our country, the most commonly accepted mushroom species are *Agaricus bisporus* and *Pleurotus* (*P.sajor-caju, P.florida* and *P.ostreatus*). So, in our laboratory, work on these mushrooms has been carried out extensively.

In our laboratory, nutritional evaluation of *A.bisporus* and *P.florida* has been done in detail. The results of our studies on *A.bisporus* reveal that its chemical composition is affected by both the stage of maturity and the substrate. The protein content was maximum at the immature primordial stage and slightly decreased on further development of the caps. However, the total lipids and crude fibre contents showed an opposite trend. Mineral content of the sporophores also increased with maturity (Table 1). The results indicate that the protein content continues to be good up to the picking stage.

The composition of *A.bisporus* was also affected when grown on different substrates. Protein content was maximum with compost prepared from wheat straw as the substrate, was lower with compost prepared from a 1:1 mixture of wheat straw and paddy straw, and decreased further if paddy straw alone was used to prepare the compost (Table 2). Mineral content and total carbohydrates were higher with wheat straw as the substrate whereas *A.bisporus* failed to draw nutrients from the compost prepared from paddy straw. Paddy straw did not even yield a reasonable number of fruiting bodies.

Table 3 compares the proximate composition of three species of *Pleurotus* namely, *P.florida, P.sajor-caju* and *P.ostreatus* commonly accepted with the agroclimatic conditions of the state in the months of October to March. The protein contents of *P.florida* and *P.sajor-caju* were comparable

TABLE 1. Percent composition of *A.bisporus* at different stages of growth on wheat straw compost.

Component	Stages		
	Immature (Primordia)	Mature (Unopened)	Fully mature (Opened)
Moisture	89.1	90.2	88.9
Protein (N x 4.38)	27.1	26.7	25.8
Lipids	3.6	3.8	4.1
Crude fibre	6.7	8.0	8.6
Minerals (ash)	9.3	10.5	11.8
Total Carbohydrates	49.2	46.4	45.6
Energy (K cals/100g)	272.2	270.4	260.7

TABLE 2. Percent composition of *A.bisporus* on compost prepared from different substrates.

Component	Stages		
	Wheat Straw	Wheat Straw + Paddy Straw (1:1, v/v)	Paddy Straw
Moisture	90.4	88.1	88.8
Protein (N x 4.38)	26.8	25.3	23.7
Lipids	3.9	4.2	4.0
Crude fibre	8.2	7.3	6.6
Minerals (ash)	10.9	9.8	7.5
Total Carbohydrates	46.8	43.2	41.5
Energy (K cals/100g)	265.6	251.8	240.0

whereas the protein content of *P.ostreatus* was relatively low. *P.florida* had the highest lipid content. On the other hand, the highest crude fibre content was found in *P.ostreatus*.

Carbohydrate content was comparable in all three species. Energy calculations revealed *P.florida* to be the species of choice. Since *P.florida* appeared nutritionally superior, detailed work has therefore been carried out on this species.

3. NUTRITIONAL ANALYSIS OF *PLEUROTUS FLORIDA*

The chemical composition of *P.florida* was affected to a considerable extent with respect to the substrate used (Table 4). Wheat straw not only supported better growth and yields of *P.florida* compared to the other substrates used but also yielded fruit bodies with higher protein content. The protein content of *P.florida* grown on the other substrates was relatively lower. Fibre and mineral contents were affected only marginally. Crude fibre was higher with paddy straw and millet (bajra). Carbohydrates and calorific values were similar for all the growth substrates when calculated according to Crisan & Sands (1978).

The chemical composition of *P. florida* at two different stages of development is shown in Table 5. Moisture content decreased with the maturity of *Pleurotus*. The protein content was also

TABLE 3. Percent composition of *Pleurotus* spp. grown on unfermented wheat straw.

Component	*P.florida*	*P.sajor-caju*	*P.ostreatus*
Moisture	91.5	88.7	89.2
Protein (N x 4.38)	19.1	18.9	15.7
Lipids	5.8	4.8	4.2
Crude fibre	9.5	10.3	12.0
Minerals (ash)	9.2	8.7	7.9
Total Carbohydrates	53.3	52.4	54.4
Energy (K cals/100g)	284.1	272.0	265.5

TABLE 4. Percent composition of *Pleurotus* spp. grown on different substrates.

Component	Substrates			
	Wheat straw	Paddy stalks	Maize stalks	Millet
Moisture	90.8	88.4	91.6	90.4
Protein (N x 4.38)	19.2	16.0	17.0	16.5
Lipids	4.9	4.6	4.3	5.1
Crude fibre	9.8	10.2	9.7	11.1
Minerals (ash)	9.0	10.3	8.2	8.6
Total Carbohydrates	52.6	54.6	54.2	53.4
Energy (K cals/100g)	274.4	270.4	269.2	271.8

lower in the fully mature fruit body compared to the immature stage. The lipid content was higher in immature fruiting bodies compared to mature ones. However, minerals and total carbohydrate levels were higher in mature fruit bodies. Total calories were similar in fruit bodies at both stages of development. Sun drying of this mushroom did not cause any substantial loss of nutrients. Carbohydrates and lipids were marginally lower, whereas minerals and crude fibre were marginally higher, compared to the mature or immature *Pleurotus*. This could be due to the loss of other nutrients.

4. NUTRITIONAL EVALUATION OF *A. BISPORUS* AND *P. FLORIDA*

Since fats and carbohydrates are rarely deficient in the diet, they are not generally considered in the nutritional evaluation of a food class. Protein,of course, is the most critical component contributing to the nutritional value of food. Not only the quantity of protein, but its quality is also important. The quality of a protein is judged by the amino acid composition of the protein. The closer the amino acid composition of the protein is to that of hen's egg protein, the better the quality. Determination of essential amino acid compositions and digestibility of the proteins of mushrooms can give a fair approximation of their protein quality, but the real test would involve actual feeding

TABLE 5. Percent composition of *P.florida* at different stages of maturity.

Component	Stages		
	Immature	Mature (unopened)	Sun dried
Moisture	91.1	87.5	3.2
Protein (N x 4.38)	20.7	17.0	18.4
Lipids	5.2	3.6	3.2
Crude fibre	9.2	10.6	11.5
Minerals (ash)	8.8	9.3	11.2
Total Carbohydrates	52.5	55.6	48.4
Energy (K cals/100g)	280.4	268.2	243.3

TABLE 6. Contents of essential amino acid (g/100g Protein) of *A.bisporus* and *P.florida*.

Amino acid	Hen's egg	*A.bisporus*	*P.florida*
Cysteine	2.4	0.86(0.36)	0.55(0.23)
Methionine	3.1	0.98(0.32)	1.84(0.59)
Lysine	6.4	3.57(0.56)	3.20(0.50)
Tryptophan	1.6	1.87(1.17)	1.08(0.67)

Values in parentheses are amino acid scores.

experiments. Work on the nutritional evaluation of the two mushrooms varieties namely, *A.bisporus* and *P.florida*, was therefore extended.

Table 6 compares the essential amino acids which are normally limiting in the Indian dietary pattern. Most cereals are deficient in sulphur-containing amino acids and lysine whereas pulses which are included in the diet compensate for lysine but do not provide balanced amounts of tryptophan. Therefore, emphasis has been placed on sulphur-containing amino acids, lysine and tryptophan.

The amino acid score and Essential Amino Acid (EAA) index, and Biological Value (BV), were calculated as per Oser (1951, 1959). The BV's or nutritional indices, of these mushrooms are reasonably good. The BV and EAA index of *A.bisporus* was better than that of *P.florida*, However, nutritional indices of the two varieties were comparable (Table 7).

5. COMPARISON OF THE DIGESTIBILITY OF *A. BISPORUS* AND *PLEUROTUS* SPP.

Good quantity and a good EAA index or a calculated BV, are not the true predictors of the

TABLE 7. Essential Amino Acid (EAA) index and Biological Value (BV) of *A.bisporus* and *P.florida*.

	A.bisporus	*P.florida*
EAA	51.10	46.30
BV	45.09	38.77
Nutritional Index	13.69	12.59

$$EAA = \sqrt[4]{\frac{Cys_p \times Lys_p \times Meth_p \times Trp_p}{Cys_s \times Lys_s \times Meth_s \times Trp_s}}$$

$$BV = 1.09\ (EAA\ Index) - 11.7$$

$$Nutritional\ Index = \frac{(EAA\ Index \times \%\ protein)}{100}$$

TABLE 8. Protein digestibility, relative nutritive value and protein efficiency ratio of sporophores of mushrooms.

Species	Digestiblity (%)			
	Pepsin	Pepsin+Trypsin	RNV	PER
P.sajor-caju	73.82 ± 2.03	81.38 ± 3.01	86.90	2.29
P.florida	71.32 ± 1.19	79.07 ± 1.04	97.62	2.43
P.ostreatus	70.09 ± 1.79	77.62 ± 1.84	91.67	2.30
A.bisporus	68.51 ± 2.48	76.43 ± 4.26	ND	ND
Casein	-	-	100.00	2.50

ND - Not determined

$$RNV = \frac{\textit{Tetrahymena} \text{ count with sample protein}}{\textit{Tetrahymena} \text{ count with casein}} \times 100$$

Calculated PER = 0.286 + 0.022 (RNV)

nutritive value of a protein. Digestibility is another limiting factor. A good quality protein based on amino acid composition can only be good if it is easily digestible and its amino acids are available for utilization. Thus, the *in vitro* digestibility of *Agaricus* and *Pleurotus* spp. was also determined.

Table 8 compares the *in vitro* digestibility of sporophores of *Pleurotus* spp. and *A.bisporus*. The data revealed that the digestibility of *Pleurotus* spp. is better than that of *A.bisporus* when pepsin or a pepsin-trypsin combination was used.

Furthermore, when hydrolysates of protein from *Pleurotus* spp. were evaluated using a test

TABLE 9. Fatty acid composition (per cent) of *A.bisporus* and *P.florida*.

Fatty Acid	*A.bisporus*	*P.florida*
Capric acid (10:0)	2.38	-
Lauric acid (12:0)	0.84	Traces
Myristic acid (14:0)	0.25	11.80
Palmitic acid (16:0)	28.12	11.12
Palmitoleic acid (16:1)	4.20	0.36
Stearic acid (18:0)	7.48	-
Oleic acid (18:1)	12.65	13.91
Linoleic acid (18:2)	35.13	72.81
Linolenic acid (18:3)	4.90	-
Arachidonic acid (20:0)	Traces	-
Saturated fatty acids	43.07	12.92
Unsaturated fatty acids	56.83	87.08

involving the growth of the protozoan *Tetrahymena*, their nutritive value appeared to be quite close to that of high quality casein.

6. FATTY ACID COMPOSITION OF *A. BISPORUS* AND *P. FLORIDA*

Fats are not only important as energy yielding substances, but the dietary unsaturated fatty acids add to the hypocholesterolemic/hypolipidemic activity of the food item and, thus, may be of potential biomedical importance. Table 9 compares the fatty acid composition of *A. bisporus* and *P. florida*. *A. bisporus* has most of the common fatty acids with linoleic acid as the main fatty acid followed by palmitic acid and oleic acid. *Agaricus* contained about 57% unsaturated fatty acids. On the other hand, *P. florida* also had linoleic acid as the main fatty acid followed by oleic acid. These represented nearly 87% of the total unsaturated fatty acids. Since unsaturated fatty acids are known to have a hypocholesterolemic effect, a high degree of unsaturation in the lipids of mushrooms, particularly of *P. florida,* is worth mentioning. Although these mushrooms have only small amounts of lipids, they are important as the lipids are rich in unsaturated fatty acids.

7. FEEDING EXPERIMENTS

In order to achieve a real nutritional evaluation of the mushrooms, feeding experiments were also conducted. Both *A. bisporus* and *P. florida* were incorporated in the diet of growing albino rats at 5% or 10% levels. Rats were divided into 5 groups of six animals and were housed individually. Rats of group A were given the basal diet while rats of group B and C were given the basal diet to which 5% or 10% of mushroom on a dry weight basis had been incorporated. Animals of group D and E were given the basal diet containing 5% or 10% *P. florida,* respectively. Inclusion of mushroom increased the food intake substantially. The increase in food intake was more with *Pleurotus* compared to *Agaricus* indicating that these mushrooms add to the palatability of the diet. Therefore, their inclusion in the food formulation may be useful in preventing the protein calorie malnutrition amongst children whose intake is poor because of non-acceptability of the usual diet within the Indian

TABLE 10. Effect of feeding *A. bisporus* or *P. florida* on food intake, gain in body weight, food efficiency and protein efficiency values.

Group	Food intake	Gain in body wt.(g)	FER	PER
A	164.2±4.30	40.66±2.62	0.229±0.025	2.50±0.27
B	183.3±8.51	45.33±3.24	0.236±0.018	2.47±0.34
C	186.2±7.62	43.33±4.55	0.232±0.016	2.31±0.41
D	192.4±8.72	45.24±3.72	0.235±0.026	2.42±0.31
E	212.5±7.36	47.12±4.56	0.221±0.023	2.20±0.25

A : Basal diet
B : Basal diet + 5% *A. bisporus*
C : Basal diet + 10% *A. bisporus*
D : Basal diet + 5% *P. florida*
E : Basal diet + 10% *P. florida*

TABLE 11. Nitrogen excretion of rats fed on diet containing *A.bisporus* or *P.florida*.

Group*	Fecal mass (g/day)	N-Excretion	
		mg/g fecal mass	mg/day
A	1.32±0.089	26.24±0.82	34.52±1.12
B	1.26±0.214	26.16±0.65	32.90±1.94
C	1.28±0.220	29.98±3.61	37.12±2.50
D	1.45±0.270	30.42±2.76	44.11±4.32
E	1.58±0.350	31.68±2.84	50.05±4.21

* As in Table 10

TABLE 12. Effect of feeding *A.bisporus* or *P.florida* on organ weight indices (g/100g body weight).

Group	Liver	Heart	Kidney	Spleen	Lung
A	4.78±0.31	0.300±0.017	0.475±0.810	1.06±0.113	0.908±0.236
B	4.53±0.41	0.297±0.034	0.447±0.045	1.01±0.085	0.807±0.121
C	4.24±0.44	0.281±0.031	0.440±0.070	1.04±0.096	0.870±0.146
D	4.54±0.14	0.278±0.058	0.437±0.048	1.00±0.153	0.876±0.195
E	4.71±0.30	0.320±0.068	0.482±0.051	1.17±0.019	0.890±0.126

Groups as in Table 10.

TABLE 13. Effect of inclusion of *A.bisporus* or *P.florida* on lipid peroxidation (nM MDA/g tissue) of various tissues.

Group	Liver	Heart	Kidney	Spleen	Lung
A	2.64±0.53	2.58±0.06	5.51±0.98	6.96±0.94	2.58±0.46
B	2.90±0.36	2.81±0.65	5.57±0.91	8.51±2.13	6.51±1.26
C	3.22±0.26	3.32±0.34	5.22±0.46	7.22±1.14	5.70±0.83
D	2.85±0.31	3.04±0.44	5.89±0.76	7.34±0.89	4.47±0.43
E	2.78±0.56	2.82±0.46	4.64±0.63	6.72±0.58	3.75±0.43

Groups as in table 10.

dietary pattern. Gain in rat body weight was also higher in mushroom fed groups but was not in concordance with the food intake which resulted in slightly decreased PER and FER (Table 10).

The decrease in PER can be partly attributed to the higher food intake and partly to the decreased digestibility of the dietary proteins by the inclusion of these mushrooms. In fact, this was found to be the case as there was a relatively higher level of fecal N excretion (Table 11).

The rats were sacrificed after 4 weeks of feeding to assess any untoward reaction due to the inclusion of these edible fungi. Organ weight indices (g/100 g body weight) of rats fed on diets

TABLE 14. Effect of inclusion of *A.bisporus* or *P.florida* on the hemoglobin, packed cell volume (PCV), GOT and GPT levels.

Group	Hb	PCV	GOT	GPT
A	13.97±0.42	29.86±1.20	84.6±8.7	32.4±4.1
B	14.03±0.50	31.66±0.42	8.85±7.6	33.6±2.8
C	14.57±1.07	29.33±1.33	96.2±8.6	35.2±3.6
D	15.52±1.17	31.24±1.41	82.4±7.2	31.6±2.9
E	14.20±0.81	28.22±1.18	98.4±8.6	38.2±4.2

Groups as in table 10

containing mushrooms were not affected by either of the mushrooms at either of the levels used in the diet (Table 12).

In order to confirm the innocuous nature of these edible fungi at cellular level, lipid peroxidation in various tissues was also determined (Table 13). The data revealed that these mushrooms did not affect lipid peroxidation in any of the tissues at either of the levels used thereby indicating that they do not affect the antioxygenic potential of various tissues. These observations are also supported by the lack of histopathological lesions in any of the organs.

The innocuous nature of these mushrooms has also been confirmed at the biochemical level. Hemoglobin, packed cell volume (PCV), glutamate-oxoglutarate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) levels were hardly affected by these mushrooms at either of the levels used (Table 14). The innocuous nature of pellets of mushroom mass grown on liquid medium has also been shown by El-Kattan *et al*. (1991a, b).

Thus, in conclusion, these mushrooms are reasonably digestible, providing good amounts of proteins and essential amino acids, unsaturated fatty acids and minerals. They add to the palatability of the diet and do not show any untoward reaction even when consumed regularly in high amounts.

REFERENCES

BANO, Z., BHAGYA, S. & SRINIVASAN, K.S. (1981). Essential amino acid composition and proximate analysis of the mushrooms, *Pleurotus eous* and *P.florida. Mushroom Newsletter for the Tropics* **1**, 6-10.

BISARIA, R., MADAN, N., BISERIA, V.S. & MUKHOPADHYAY, S.N. (1987a). Amino acid composition of mushroom *Pleurotus sajor-caju* cultivated on different agro residues. *Biological Wastes* **20**, 251-259.

BISARIA, R., MADAN, N. & BISERIA, V.S. (1987b). Mineral content of mushroom *Pleurotus sajor-caju* cultivated on different agro residues. *Mushroom Journal for the Tropics* **7**, 53-60.

CHANG, S.T. (1980). Mushrooms as human food. *Bioscience* **30**, 399-401.

CRISAN, E.V. & SANDS, A. (1978). Nutritional value of edible mushrooms. In *The Biology and Cultivation of Edible Mushrooms*, pp 137-168. Edited by Chang, S.T. & Hayes, W.A. New York and London: Academic Press.

EL-KATTAN, M.H., AFFIFY, A.S. & ALY, A.Z.M. (1991a). Evaluation of *Pleurotus sajor-caju*

fungal pellets as food. *Mushroom Journal for the Tropics* **11**, 13-22.
EL-KATTAN, M.H., HELMY, Z.A., EL-LEITHY, M.A.E. & ABDELKANI, K.A. (1991b). Studies on cultivation techniques and chemical composition of oyster mushrooms. *Mushroom Journal for the Tropics* **11**, 59-66.
HIRA, C.K., VADHERA, S. & MANN, A.P.S. (1990). Biochemical changes in sporophores of two mushroom species before and after opening of caps. *Annals of Biology* **6**, 95-99.
JANDAIK, C.L. & KAPOOR, J.N. (1976). Amino acid composition of *Pleurotus sajor-caju* (Fr.) Sing. *Mushroom Journal* **41**, 154-156.
KHANNA, P.K. & GARCHA, H.S. (1981). Nutritive value of mushroom *Pleurotus florida*. *Mushroom Science* **XI**, 561-572.
KHANNA, P.K. & GARCHA, H.S. (1984). *Pleurotus* mushroom - A source of food protein. *Mushroom Newsletter for the Tropics* **4**, 9-14.
KHANNA, P.K. & GARCHA, H.S. (1986). Nucleic acid content and relative nutritive value of sporophore proteins of *Pleurotus* spp. *Mushroom Newsletter for the Tropics* **6**, 17-20.
OSER, B.L. (1951). Method for integrating essential amino acid content in nutritional evaluation of proteins. *Journal of American Dietitians Association* **27**, 396-402.
OSER, B.L. (1959). An integrated essential amino acid index for predicting the biological value of protein. In *Protein and Amino Acid Nutritition*, pp 281-295. Edited by A.A. Albanese. New York: Academic Press.
SAMAJPATI, N. (1978). Nutritive value of some Indian edible mushrooms. *Mushroom Science* **10**, 695-703.
WRIGHT, A.V., NISKANEN, A., PYYSALO, H. & KORPELA, H. (1978). The toxicity of some N-methyl, N-formyl hydrazones from *Gyromietra esculenta* and related compounds in mouse and microbial tests. *Toxicology and Applied Pharmacology* **45**, 429-434.

CHAPTER 25

DIVERSE BIOLOGICAL ACTIVITY OF PSK (KRESTIN), A PROTEIN-BOUND POLYSACCHARIDE FROM *CORIOLUS VERSICOLOR* (FR.) QUEL.

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1. INTRODUCTION

PSK (trade name, Krestin) is a protein-bound polysaccharide, extracted by hot water from the mycelia of *Coriolus versicolor* (Fr.) Quel. (Kawaratake). Kureha Chemical Industry Company Limited paid attention to the effectiveness of oral administration of Polyporaceae (one of the Basidiomycetes) on stomach cancer patients. This company screened over 200 species of the fruit bodies of the Basidiomycetes for their antitumor activity against various tumor cells, including sarcoma-180, and found several promising Polyporaceae strains. Among these strains, *Coriolus versicolor* (Fr.) Quel. was considered to be the most suitable for further fractionation due to its high antitumor activity and stability during serial cultivation. Extracts of cultured mycelia of *Coriolus versicolor* had antitumor activity comparable to that of the fruit body. In 1971, the active principle was precipitated from extracts of cultured hyphae of *Coriolus versicolor* (Fr.) Quel (CM-101 strain) with saturated ammonium sulfate, desalted and named PSK (Hirose, 1988). PSK has been reported to induce host-mediated antitumor activity (Tsukagoshi *et al*., 1984). Clinical application of PSK to cancer patients is only permitted in combination with other chemotherapeutic agents.

PSK has been shown to contain at least four different subfractions which are separable by stepwise filtration through a membrane filter (Tsuchitani *et al*., 1987) or by isoelectric precipitation (Hirose 1988). However, most previous PSK studies have been done using unfractionated samples. This review summarizes data from our groups and others on the biological activity of unfractionated PSK (section 1-3) and fractionated PSK (section 4). Unique biological activities of PSK, in comparison with other glucans, are listed in section 5.

2. CHEMICAL PROPERTIES

PSK is a brownish powder comprised of protein (38%) and polysaccharide. It is highly soluble

in water but only slightly soluble in most organic solvents (methanol, pyridine, chloroform, benzene, hexane). The mean molecular weight of PSK, determined by ultracentrifugation, is 94 kD. The main component of the carbohydrate moiety is glucose, with galactose, mannose, xylose and fucose as minor components. The protein moiety is rich in acidic amino acids (such as aspartic acid, glutamic acid etc.) and neutral amino acids (such as valine and leucine etc.), with basic amino acids (such as lysine, arginine etc.) in lower amounts (Hirose, 1988).

The main component unit of the polysaccharide moiety of PSK is assumed to be a β-glucan, judging from specific rotation and cellulase digestion. Oxidation by periodic acid, Smith degradation and methylation analysis have suggested that the main chain consists of β-(1-4) glucose polymer, branched at positions 3 and 6 of the glucose.

PSK seems to have two forms of binding between the protein and polysaccharide regions: one is O-glycoside bonding between a serine or a threonine residue in the peptide chain and an OH-group in the sugar chain; and the other is N-glucoside bonding between an aspartic acid and an OH-group.

3. INDUCTION OF ANTIMICROBIAL ACTIVITY *IN VIVO*

3.1. Antimicrobial Spectrum

PSK induced potent antimicrobial activity against *Escherichia coli* in ICR mice (Sakagami, *et al.,* 1990d). Significant antimicrobial activity was induced when the mice were pretreated with 40 mg/kg PSK by an intraperitoneal, subcutaneous or intramuscular route, but not by an intravenous route, 48 h before *E. coli* inoculation. Intraperitoneal administration of PSK was most potent. Repeated oral administration of PSK for 2 weeks before *E. coli* inoculation was also effective (Sakagami *et al*., 1990d). It has been reported that orally administered ^{14}C-PSK was absorbed through the digestive tract, and both low and high molecular weight substances appeared in the blood (Fujita, 1988). Undegraded PSK and PSK-derived low molecular weight substances were distributed to various organs (Fujita, 1988).

PSK induced potent antimicrobial activity against other micro-organisms such as *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Klebsiella pneumoniae* (Mayer & Drews, 1980), *Mycobacterium leprae, Listeria monocytogenes, Serratia marcescens, Streptococcus pneumoniae, Bacteroides fragilis, Cryptococcus neoformans* and *Aspergillus fumigatus*.

3.2. Mechanism

Three possible mechanisms of antimicrobial activity induction by PSK will be considered: First, the induction of antimicrobial activity might be mediated via direct bactericidal activity of the induced polymorphonuclear cells (PMN). Administration of PSK rapidly induced the intraperitoneal accumulation of PMN, which generated higher amounts of luminol-dependent chemiluminescence (LDCL)(Sakagami *et al*., 1990d). Although PSK administration stimulated the LDCL generation by peritoneal macrophages, the absolute value of LDCL generated by macrophages was only 5% of that of PMN (Harada *et al*., 1989). Therefore, the major cell population, which is responsible for induction of the antimicrobial activity, seems to be PMN.

Second, the antimicrobial activity of PSK may arise from its ability to induce antimicrobial cytokines, such as tumor necrosis factor (TNF)(Nakane, 1988) and interleukin-1 (IL-1)(Ozaki *et al*., 1987). Intravenous administration of PSK stimulated the endogenous production of TNF and IL-1a

in mouse serum (Sakagami, 1990a; Sakagami & Takeda, 1992c). The amount of TNF induced in the blood was significantly increased when the mice were treated several hours later with bacterial preparations, such as OK-432 (Picibanil)(Sakagami, 1990c) or heat-killed *Lactobacillus casei* (Sakagami, 1992b). The priming activity of PSK was slightly greater than that of other antitumor polysaccharides (Sakagami *et al*., 1990c).

Third, the antimicrobial activity of PSK might come from its direct activation of myeloperoxidase (MPO)-halide antibacterial systems described below.

4. *IN VITRO* EFFECTS

4.1. Stimulation of Monocytes/Macrophages

When mouse macrophages were cultured with 100 ug/ml PSK, they became enlarged and elongated (Kamisato & Nowakowski, 1988). The morphological changes were associated with an increase in NBT-reducing activity and TNF production (Kurakata *et al*., 1991). PSK stimulated the production of IL-1-like activity, assayed with the thymocyte proliferation assay (Kurakata *et al*., 1991). In contrast, natural glucans [native Schizophyllan (MW 6000 kD), lower MW Schizophyllan (MW 450 kD)] and chemically modified glucans [CM-TAK (MW 97 kD), paramylon sulfate (MW 151 kD)] failed to stimulate morphological maturation, regardless of their molecular weights (Kurakata *et al*., 1991).

PSK stimulated the production of IL-1 by human peripheral blood mononuclear cells more efficiently than the production of TNF (Hirose *et al*., 1990; Sakagami *et al*., 1993b) The ability of PSK to stimulate TNF or IL-1 production by mononuclear cells was more potent than that of other natural products such as lignified materials and tannin-related compounds (Utsumi *et al*., 1993; Sakagami *et al*., 1993b). More IL-1α was accumulated in the cells than in the medium fraction after PSK treatment, whereas IL-1β was distributed evenly into both fractions. PSK stimulated the production of adherent mononuclear cells, in which significantly more IL-1α/Il-1β was accumulated per cell than in nonadherent cells. Although IL-1α mRNA synthesis (assessed by a Reverse Transcriptase-Polymerase Chain Reaction) was slightly enhanced, IL-1β mRNA synthesis was not significantly changed by PSK treatment. This suggests that PSK might increase the efficiency of IL-1 mRNA translation or post-translational processing of IL-1 protein (Sakagami *et al*., 1993a). Despite potent cytokine-inducing activity, lipopolysaccharide (LPS) did not significantly stimulate the production of adherent cells. These data suggest that PSK and LPS might stimulate mononuclear cells by different mechanisms.

4.2. Stimulation of PMN and Monocyte Iodination

PSK stimulated the iodination (incorporation of radioactive iodine into acid-insoluble fraction) (Klebanoff & Clark, 1977) of human peripheral blood PMN, monocytes and human promyelocytic leukemic cells of the HL-60 cell line, which had higher amounts of MPO (Ackerman & Douglas, 1986; Bainton, 1981). The PSK stimulation of iodination of both PMN and HL-60 cells was significantly suppressed by the presence of MPO inhibitors (Sakagami *et al*., 1990b). This indicates that the stimulation of iodination by PSK depends on the presence of MPO. The products of MPO reaction (such as hypochlorite), which might be highly antibactericidal, remain to be investigated. In contrast, various glucans such as native (Schizophyllan) and chemically modified glucans

(CM-TAK, paramylon sulfate) did not significantly stimulate the iodination of either PMN or HL-60 cells (Sakagami *et al.*,1990b).

4.3. Stimulation of Cytokine Action

PSK stimulated the action of TNF in various assay systems. TNF has been reported to induce monocytoid differentiation of human myelogenous leukemic cells (Takeda *et al.*, 1986; Weinberg & Larrick, 1987). PSK stimulated the differentiation of human myeloblastic leukemic ML-1 cells induced by macrophage conditioned medium or 10 ng/ml TNF (Sakagami *et al.*, 1989). The extent of differentiation was assayed by NBT-reducing activity and expression of α-napthyl acetate esterase activity. PSK stimulated the cytotoxic activity of PSK against L-929 cells (Sakagami *et al.*, 1991a). It has been reported that TNF is a weak direct stimulant of the PMN respiratory burst and degranulation, as measured by iodination, H_2O_2 and lysozyme release (Klebanoff *et al.*, 1986). PSK further stimulated the iodination, in either an additive or a synergistic manner, whereas human natural interferon-γ (IFN-γ) or recombinant GM-CSF was not stimulatory (Sakagami *et al.*, 1990b).

PSK stimulated the action of IFN-γ. IFN-γ has been reported to induce monocytoid differentiation of the cells of various myelogenous leukemic cell lines, but the differentiation-inducing ability of IFN-γ varies considerably with different target cell lines (Ball *et al.*, 1984; Kim *et al.*, 1990). Upon incubation with IFN-γ, human histiocytic lymphoma U-937 cells produced more differentiated cells, whereas human monoblastic leukemic THP-1 cells produced fewer differentiating cells. PSK further potentiated the differentiation of both of these cells beyond that induced by IFN-γ alone (Kim *et al.*, 1990).

Either increase of cytokine binding to the cellular receptor, or inhibition of receptor down regulation, has been suggested to be a possible mechanism for PSK enhancement of cytokine action (Sakagami *et al.*, 1991a).

4.4. Antiviral Activity

PSK has been shown to inhibit cytopathic effects of the human immunodeficiency virus (HIV) that infects cells of the CD4 positive human T cell line (Tochikura *et al.*, 1987). PSK almost completely blocked the giant formation and HIV-specific antigen expression in both MT-4 cells and MOLT-4 cells at concentrations of 400 and 800 μg/ml, respectively. PSK also non-competitively inhibited reverse transcriptase of avian myeloblastosis virus *in vitro*. One mechanism of the PSK effect is attributed to the inhibition of binding of HIV to the cells (Hirose *et al.*, 1987). However, the anti-HIV activity of PSK (EC_{50}=300 μg/ml) is much lower than that of natural lignin (EC_{50}=6-100 μg/ml) (Sakagami *et al.*, 1992d; Manabe *et al.*, 1992), synthethic lignin (dehydrogenation polymers of phenylpropenoids) (EC_{50}=1.3-13 μg/ml)(Nakashima *et al.*, 1992a), monomeric, dimeric, trimeric, tetrameric hydrolyzable tannins (EC_{50}=2.0-7.0 μg/ml) (Nakashima *et al.*, 1992b) or sulfated polysaccharides (EC_{50}=1.8 μg/ml) (Koizumi *et al.*, 1993). All of these agents significantly inhibited HIV binding to the cells.

However, the antiviral activity of PSK against other viruses is relatively weak. PSK (10 and 100 μg/ml) did not inhibit the plaque formation of the herpes simplex virus (Fukuchi *et al.*, 1989), or the influenza virus (Harada *et al.*, 1991).

4.5. Radical Scavenging Effect

PSK potently inhibited the luciferin-dependent chemiluminescence generated by opsonized

zymosan-stimulated human peripheral blood PMN. The continuous presence of PSK was necessary to express its inhibitory activity. PSK also scavenged the chemiluminescence generated by the xanthine-xanthine oxidase reaction, or by potassium superoxide solution. The inhibitory activity of PSK (EC_{50}=100 μg/ml) was one-order lower than that of tannins and lignins. It is suggested that O_2^- might be scavenged directly by PSK, or by other oxygen radicals produced by activation of the MPO-dependent oxidative process in PMN (Sakagami *et al.*, 1992a).

PSK also scavenged the chemiluminescence generated by the reaction of sodium hypochlorite (a product of MPO reaction) and luminol, although the inhibitory activity of PSK (EC_{50}=26 μg/ml) was much lower that that of tannin-related compounds, or ligninified materials (EC_{50}=0.058-3 μg/ml) (unpublished data).

On the other hand, Schizophyllan and N,N-dimethylaminoethylparamylon did not scavenge O_2^- (Sakagami *et al.*, 1992a) or hypochlorite (unpublished data).

4.6. Inhibition of Poly(ADP-Ribose)Glycohydrolase Activity

Reversible poly-(ADP-ribosyl)ation of chromosomal proteins has been proposed to be important in the regulation of nuclear functions, such as DNA replication, repair and transcription, and thus affect cell growth and differentiation. Poly-(ADP-ribose)glycohydrolase, which splits the glycosidic (1"-2') linkages of poly-(ADP-ribose), is the main enzyme responsible for the degradation of poly-(ADP-ribose). No potent, specific inhibitors of this enzyme have been reported. Recently, it has been reported that dimeric hydrolyzable tannins suppressed this enzyme activity and the glucocorticoid-sensitive mouse mammary tumor virus (MMTV) mRNA in 34I mouse mammary tumor cells (Tsai *et al.*, 1992). Various polyphenols such as tannins (Tanuma *et al.*, 1989a) and lignins (Tanuma *et al.*, 1989b), were found to be potent inhibitors of poly-(ADP-ribose) glycohydrolase. Analysis of the kinetics revealed that inhibition by these polyphenols was competitive with respect to the substrate poly-(ADP-ribose). PSK also inhibited this enzyme activity via the same mechanism, but much less efficiently, whereas glucans were inactive (Tanuma *et al.*, unpublished data).

5. BIOLOGICAL ACTIVITY OF PSK SUBFRACTIONS

PSK was separated into the following four subfractions, of different molecular weights, by successive filtration through membrane filters: F1 (<50 kD), F2 (50-100 kD), F3 (100-200 kD) and F4 (>200 kD), with relative yields of 1 : 1 : 10 : 10 (Tsuchitani *et al.*, 1987). The highest molecular weight fraction (F4), among these fractions showed the greatest activity in the following items: (i) induction of antimicrobial activity in mice by pretreatment to reduce the lethal infectivity of *Escherichia coli* (Harada *et al.*, 1989), (ii) stimulation of the functional maturation (i.e., morphological change, increase of NBT-reducing activity) of mouse peritoneal macrophages (Kurakata *et al.*, 1991), (iii) stimulation of TNF- (IFN-γ)-induced differentiation of the human myelogenous leukemic cell lines, ML-1, HL-60, U-937, THP-1 (Sakagami *et al.*, 1989; Kim *et al.*, 1990), and (iv) iodination of human peripheral blood PMN (Sakagami *et al.*, 1990b).

Material which stimulated PMN iodination was precipitated between pH4.0 and 4.5 (Sakagami *et al.*, 1990b). Further purification and structural analysis of the active subfractions remain to be achieved.

6. CONCLUSIONS

PSK, unlike most of other glucans, displayed various unique biological activity: (i) stimulation of functional maturation of macrophages, (ii) stimulation of PMN iodination, (iii) inhibition of the cytopathic effect of HIV infection, (vi) inhibition of enzyme activity, and (v) ability to scavenge active oxgen (O_2^-, hypochlorite). Lignified materials (Sakagami *et al.*, 1991b), but not most other glucans tested, also displayed these properties. This suggests the presence of some unknown constituent(s), possibly lignin, in the PSK molecule. However, at present, the possibility that some slight difference in structural configuration, including sugar linkage, might confer unique biological activity on PSK cannot be eliminated.

It was suggested that some immunopotentiating activity displayed by PSK might be generated by enhancement of cytokine action, or by stimulation of cytokine production. We demonstrated that PSK, together with bacterial preparations such as OK-432 or *Lactobacillus casei*, stimulated some endogenous cytotoxic factor(s) or TNF. TNF, originally isolated as a macrophage-derived tumoricidal cytokine, has a broad spectrum of biological activity (Old, 1987). However, direct injection of this cytokine into host animals has occasionally induced shock and tissue injury (Tracy *et al.*, 1986). In contrast, administration of various biological BRMs, combined with appropriate eliciting agents of bacterial origin, has been reported to induce endogenous TNF in an amount sufficient to suppress tumor growth, without producing side effects. There is a broad parallel between the capacity of TNF production and the reduction of tumor weight (Haranaka *et al.*, 1988). Therefore, the ability of PSK, in combination with appropriate eliciting agents, to induce endogenous TNF (Sakagami & Takeda, 1992c) suggests the efficacy of PSK for clinical application to cancer patients.

PSK and lignins stimulated the iodination of MPO-positive cells. This suggests that these iodination stimulators might activate the MPO-halogen-H_2O_2 antibacterial system. Thus, it is possible that the antimicrobial activity of these substances might be augmented especially in MPO-positive cells. However, lignins almost equally inhibited acute infection of HIV in both HL-60 cells (which have higher MPO concentrations) and U-937 cells (which have much lower MPO concentrations) (Kunisada *et al.*, 1992). This indicates that the anti-HIV activity of these substances does not depend on the content of MPO in the cells. PSK may interfere with the early stages of HIV infection by modifying the viral receptor (Tochikura *et al.*, 1987), or it may directly inactivate the virus. The possibility that PSK might inhibit reverse transcriptase activity, inhibit viral expression from host DNA, as well as activate host immune functions remains to be investigated. Thus, PSK may be useful in preventing the risk of HIV infection, and in the treatment of AIDS.

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REFERENCES

ACKERMAN, S.K. & DOUGLAS, S.D. (1986). Morphology of monocytes and macrophages. In *Hematology*, pp.837-847, Edited by W.J. William, E. Beutler, A.J. Erslev & M.A. Lichtman (3rd Edition). New York: McGraw-Hill Book Co.

BAINTON, D.F. (1981) Selective abnormalities of azulophil and specific granules of human

neutrophilic leukocytes. *Federation Proceedings* **40,** 1443-1450.
BALL, E.D, GUYRE, P.M., SHEN, L., GLYNN, J.M., MALISZEWSKI, C.R., BAKER, P.E. & FANGER, M.W. (1984). Gamma interferon induces monocytoid differentiation in the HL-60 cell line. *Journal of Clinical Investigation* **73,** 1072-1077.
FUJITA, H. (1988). In *PSK (krestin)*, pp 111-157. Edited by K. Kimura. Tokyo: Excerpta Medica.
FUKUCHI, K., SAKAGAMI, H., IKEDA, M., KAWAZOE, Y., OH-HARA, T., KONNO, K., ICHIKAWA, S., HATA, N., KONDO, H. & NONOYAMA, M. (1989). Inhibition of herpes simplex virus infection by pine cone antitumor substances. *Anticancer Research* **9,** 313-318.
HARADA, H., SAKAGAMI, H., KONNO, K., SATO, T., OHSAWA, N., FUJIMAKI, M. & KOMATSU, N. (1989). Antimicrobial activity induction by PSK subfractions: Dependence on molecular weight. *Infection* **17,** 38-39.
HARADA, H., SAKAGAMI, H., NAGATA, K., OH-HARA, T., KAWAZOE, Y., ISHIHAMA, A., HATA, N., MISAWA, Y., TERADA, H. & KONNO, K. (1991). Possible involvement of lignin structure in anti-influenza virus activity. *Antiviral Research* **15,** 41-50.
HARANAKA, R., HASEGAWA, R., NAKAGAWA, S., SAKURAI, A., SATOMI, N. & HARANAKA, K. (1988). Antitumor activity of combination therapy with traditional Chinese medicine and OK-432 or MMC. *Journal of Biological Response Modifiers* **7,** 77-90.
HIROSE, S. (1988). Structure and property of PSK (in Japanese). In *PSK (krestin)*, pp 3-18. Edited by K. Kimura. Tokyo: Excerpta Medica.
HIROSE, K., HAKOZAKI, M., KAKUCHI, J., MATSUNAGA, K., YOSHIKUMI, C., TAKAHASHI, M., TOCHIKURA, T.S. & YAMAMOTO, N. (1987). A biological response modifier, PSK, inhibits reverse transcriptase *in vitro*. *Biochemical Biophysical Research Communications* **149,** 562-567.
HIROSE, H., ZACHARIAE, C.O., OPPENHEIM, J.J. & MATSUSHIMA, K. (1990). Induction of gene expression and production of immunomodulating cytokines by PSK in human peripheral blood mononuclear cells. *Lymphokine Research* **9,** 475-483.
KAMISATO, J.K. & Nowakowski, M. (1988) Morphological and biochemical alterations of macrophages produced by a glycan, PSK. *Immunopharmacology* **16,** 89-96.
KIM, F., SAKAGAMI, H., TANUMA, S. & KONNO, K. (1990). Stimulation of interferon-γ induced human myelogenous leukemic cell differentiation by high molecular weight PSK subfraction. *Anticancer Research* **10,** 55-58.
KLEBANOFF, S.J. & CLARK R.A. (1977). Iodination by human polymorphonuclear leukocytes: a re-evaluation. *Journal of Laboratory and Clinical Medicine* **89,** 675-686.
KLEBANOFF, S.J., VADAS, M.A., HARLAN, J.M., SPARKS, L.H., GAMBLE, J.R., AGOSTI, J.M. & WALTERDORPH, A.M. (1986). Stimulation of neutrophils by tumor necrosis factor. *Journal of Immunology* **136,** 4220-4225.
KOIZUMI, N., SAKAGAMI, H., UTSUMI, A., FUJINAGA, S., TAKEDA, M., ASANO, K., SUGAWARA, I., ICHIKAWA, S., KONDO, H., MORI, S., MIYATAKE, K., NAKANO, Y., NAKASHIMA, H., MURAKAMI, T. MIYANO, N. & YAMAMOTO, N. (1993). Anti-HIV (human immunodeficiency virus) activity of sulfated paramylon. *Antiviral Research* **21,** 1-14.
KUNISADA, T., SAKAGAMI, H., TAKEDA, M., NAOE, T., KAWAZOE, Y., USHIJIMA, H., MULLER W.E.G. & KITAMURA, T. (1992). Effect of lignins on HIV-induced cytopathogenicity and myeloperoxidase activity in human myelogenous leukemic cell lines. *Anticancer Research* **12,** 2225-2228.
KURAKATA, Y., SAKAGAMI, H., SATO, A., KIKUCHI, K., TAKEDA, M., ASANO, K.& SATO, T. (1991). Functional maturation of monocytes/macrophages induced by PSK subfractions.

Anticancer Research **11,** 1767-1772.

MANABE, H., SAKAGAMI, H., ISHIZONE, H., KUSANO, H., FUJIMAKI, M., WADA, C., KOMATSU, N., NAKASHIMA, H., MURAKAMI, T. & YAMAMOTO, N. (1992). Effects of Catsuaba extracts on microbial and HIV infection. *In Vivo* **6,** 161-166.

MAYER, J. & DREWS, J. (1980). The effect of a protein-bound polysaccharide from coriolus versicolor on immunological parameters and experimental infections in mice. *Infection* **8,** 13-21.

NAKANE, A., MINAGAWA, T. & Kato, K. (1988) Endogenous tumor necrosis factor (catectin) is essential to host resistance against *Listeria monocytogenes* infection. *Infection and Immunity* **56,** 2563-2569.

NAKASHIMA, H., MURAKAMI, T., YAMAMOTO, N., NAOE, T., KAWAZOE, Y., KONNO, K. & SAKAGAMI, H. (1992a). Lignified materials as medicinal resources. V. Anti-HIV (human immunodeficiency virus) activity of some synthetic lignins. *Chemical and Pharmaceutical Bulletin* **40,** 2102-2105.

NAKASHIMA, H., MURAKAMI, T., YAMAMOTO, N., SAKAGAMI, H., TANUMA, S., HATANO, T., YOSHIDA, T. & OKUDA, T. (1992b). Inhibition of human immunodeficiency viral replication by tannins and related compounds. *Antiviral Research* **18,** 91-103.

OLD, L.J. (1987). Tumor necrosis factor. Polypeptide mediator network. *Nature* **326,** 330-331.

OZAKI, Y., OHASHI, T., MINAMI, A. & NAKAMURA, S. (1987). Enhanced resistance of mice to bacterial infection induced by recombinant human interleukin-1a. *Infection and Immunity* **56,** 1436-1440.

SAKAGAMI, H., IKEDA, M. & KONNO, K. (1989). Stimulation of tumor necrosis factor-induced human myelogenous leukemic cell differentiation by high molecular weight PSK subfraction. *Biochemical and Biophysical Research Communications* **162,** 597-603.

SAKAGAMI, H. (1990a). Endogenous production of cytotoxic factor in normal mice by PSK combined with OK-432. *Showa University Journal of Medical Sciences* **2,** 159-164.

SAKAGAMI, H., KIM, F. & KONNO, K. (1990b). Stimulation of human peripheral blood polymorphonuclear cell iodination by PSK subfractions. *Anticancer Research* **10,** 697-702.

SAKAGAMI, H., KOHNO, S., TANUMA, S. & KAWAZOE, Y. (1990c). Induction of cytotoxic factor in mice by lignified materials combined with OK-432 (Picibanil). *In Vivo* **4,** 371-376.

SAKAGAMI, H., KONNO, K., KURAKATA, Y., TAKEDA, M., SATO, T., HARADA, H., OHSAWA, N., FUJIMAKI, M. & KOMATSU, N. (1990d). Effects of pretreatment with PSK, a protein-bound polysaccharide, on *Escherichia coli* infection in mice. *Showa University Journal of Medical Sciences* **2,** 7-10.

SAKAGAMI, H., AOKI, T., SIMPSON, A. & TANUMA, S. (1991a). Induction of immunopotentiation activity by protein-bound polysaccharide, PSK (Review). *Anticancer Research* **11,** 993-1000.

SAKAGAMI, H., KAWAZOE, Y., KOMATSU, N., SIMPSON, A., NONOYAMA, M., KONNO, K., YOSHIDA, T., KUROIWA, Y. & TANUMA, S. (1991b). Antitumor, antiviral and immunopotentiating activities of pine cone extracts: Potential medicinal efficacy of natural and synthetic lignin-related materials (Review). *Anticancer Research* **11,** 881-888.

SAKAGAMI, H., KOHNO, S., TAKEDA, M., NAKAMURA, K., NOMOTO, K., UENO, I., KANEGASAKI, S., NAOE, T. & KAWAZOE, Y. (1992a). O_2^- scavenging activity of lignins, tannins and PSK. *Anticancer Research* **12,** 1995-2000.

SAKAGAMI, H., KUROIWA, Y., TAKEDA, M., OTA, H., KAZAMA, K., NAOE, T., KAWAZOE, Y., ICHIKAWA, S., KONDO, H., YOKOKURA, T. & SHIKITA, M. (1992b). Distribution of TNF endogenously induced by various immunopotentiators and *Lactobacillus casei* in mice. *In Vivo* **6,** 247-254.

SAKAGAMI, H. & TAKEDA, M. (1992c). Induction of endogenous TNF production by biological

response modifiers and bacterial preparations (Review). *International Journal of Oncology* **1,** 283-287.

SAKAGAMI, H., YOSHIHARA, M., FUJIMAKI, M., WADA, C., KOMATSU, N., NAKASHIMA, H., MURAKAMI, T. & YAMAMOTO, N. (1992d). Effect of pine seed shell extract on microbial and viral infection. *In Vivo* **6,** 13-16.

SAKAGAMI, H., SUGAYA, K., UTSUMI, A., FUJINAGA, S., SATO, T. & TAKEDA, M. (1993a) Stimulation of PSK of interleukin-1 production by human peripheral blood mononuclear cells. *Anticancer Research* **13,** in press.

SAKAGAMI, H., UTSUMI, A., FUJINAGA, S., TAKEDA, M., NAOE, T. & KAWAZOE, Y. (1993b). Stimulation of iodination and cytokine production by dehydrogenation polymers of phenylpropenoids. *Anticancer Research* **13,** in press.

TAKEDA, K., IWAMOTO, S., SUGIMOTO, H., TAKUMA, T., KAWATANI, N., NODA, M., MASAKI, A., MORISE, A., ARIMURA, H. & KONNO, K. (1986). Identity of differentiation-inducing factor and tumor necrosis factor. *Nature* **323,** 338-340.

TANUMA, S., SAKAGAMI, H. & ENDO, H. (1989a). Inhibitory effect of tannin on Poly-(ADP-ribose) glycohydrolase from human palcenta. *Biochemistry International* **18,** 701-708.

TANUMA, S., TSAI, Y-J., SAKAGAMI, H., KONNO, K. & ENDO, H. (1989b). Lignin inhibits $(ADP\text{-}ribose)_N$ glycohydrolase activity. *Biochemistry International* **19,** 1395-1402.

TOCHIKURA, T.S., NAKASHIMA, H., HIROSE, K. and YAMAMOTO, N. (1987). A biological response modifier, PSK, inhibits human immunodeficiency virus infection in vitro. *Biochemical and Biophysical Research Communications* **148,** 726-733.

TRACY, K.J., BEUTLER, B., LOWRY, S.F., MERRYWEATHER, J., WOLPS, S., MILSARK, I.W., HAIRIRI, R.J., FAHEY, III T.J., ZENTELLA, A., ALBERT, J.D., SHIRES, G.T. & CERAMI, A. (1986). Shock and tissue injury induced by recombinant human cachectin. *Science* **234,** 470-474, 1986.

TSAI, Y-J., AOKI, T., MARUTA, H., ABE, H., SAKAGAMI, H., HATANO, T., OKUDA, T. & TANUMA, S. (1992). Mouse mammary tumor virus gene expression is suppressed by oligomeric ellagitannins, novel inhibitors of poly(ADP-ribose) glycohydrolase. *Journal of Biological Chemistry* **267,** 14436-14442.

TSUCHITANI, T., NIO, Y., IMAI, S., SHIRAISHI, T., KAN, N., OHGAKI, K. & TOBE, T. (1987). I. Correlation of *in vivo* effects with *in vitro* biological activities. *Journal of Japanese Society for Cancer Therapy* **22,** 1195-1202.

TSUKAGOSHI, S., HASHIMOTO, Y., FUJII, G., KOBAYASHI, H., NOMOTO, K. & ORITA, K. (1984). Krestin (PSK). *Cancer Treatment Review* **11,** 131-155.

UTSUMI, A., FUJINAGA, S., TAKAHASHI, H., SAKAGAMI, H. & TAKEDA, M. (1993). Effect of biological response modifiers on cytokine production in differentiating human myelogenous leukemic cell line (in Japanese). *Show University Journal*, in press.

WEINBERG, J.B. and LARRICK J.K. (1987). Receptor-mediated monocytoid differentiation of human promyelocytic cells by tumor necrosis factor: Synergistic actions with interferon-γ and 1,25-dihydroxyvitamin D_3. *Blood* **70,** 994-1002.

CHAPTER 26

A NEW BIOLOGICAL RESPONSE MODIFIER SUBSTANCE - PSP

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1. INTRODUCTION

Yun Zhi is a kind of Zhi. It is part of Chinese traditional medicine and has the function of promoting one's positive factors, strengthening physique, and increasing energy and spirit (Li, 1991).

One of the main characteristics of Chinese medicines is to promote one's positive factors and to increase or adjust the body's immunological functions to prevent diseases. It is also the direction of research for the preparation and production of new anti-tumor drugs. Recently, the application of Biological Response Modifiers (BRM) in the cure of cancers has become the fourth kind of classical treatment after surgery, chemo- and radio-therapy. Frequently-used BRM substances, such as interferon, interleukin-2,PSK (krestin), lentinan, TNF, etc. are substances for regulating positive factors and removing negative factors (Joji, 1985; Chen, 1989).

From 1983 to 1992, we isolated various strains of *Coriolus versicolor* (L.ex Fr.) Qu'el from many areas in China. Strain Cov-1 was selected for further study and a polysaccharopeptide (PSP) extracted from the mycelia by means of submerged fermentation. This substance has the properties of promoting the positive factors and removing the negative factors of the human body. PSP has been investigated by means of chromatography, mass and nuclear magnetic resonance spectra: it is made up of 90% polysaccharide and 10% peptide and has a molecular weight of about 100kd. The amino acids in the peptide are mostly asparagine and glutamic acid. The main ingredient of polysaccharide is glucose and the main chain is connected by $\alpha 1 \rightarrow 4$ and $\beta 1 \rightarrow 3$ glycosidic linkages. Small amounts of galactose, mannose, xylose, arabinose and rhamnose are also present. The chemical ingredients of PSP are similar to those of Japanese PSK, but the former does not contain fucose and the latter is lacking arabinose and rhamnose (Masayoshi *et al*., 1982; Yang & Jong, 1989).

From research into the pharmacological and clinical functions of PSP, the substrate clearly functions by promoting the positive factors and removing the negative factors of the human body and is a new kind of BRM drug. Its biological regulating functions are reported here.

2. PHARMACOLOGICAL EFFECTS OF PSP

In order to examine the mechanism(s) underlying the promotion of the positive factors and the strengthening of the physique of the organism, we examined several immunological indices in normal mice, tumor-bearing mice and mice undergoing chemotherapy.

2.1. The effects of PSP on the immunological system of normal organisms

2.1.1. The effect of PSP on the phagocytic functions of normal mice. A sample of 100 normal mice (ICR), 1/2 male and 1/2 female, was divided into 10 groups. Half were given PSP by oral administration and half by abdominal injection. There were 3 groups based on high, medium and low PSP dosages. Oral doses (0.5-1.5g/kg) were administered daily for a total of 9 days; intraperitoneal injections (100, 200, 400 mg/kg) were given daily for 4 days. After the last dose, 50% Indian ink was injected into the tail veins of the mice (5ml/kg). After 20 minutes, a 20μl blood sample was taken from behind the eye-socket and added instantly to 2 ml 0.1M Na_2CO_3 solution. The absorbance at 650nm was measured and the carbon clearance rate (K value) calculated. The results showed that the carbon clearance rate of the PSP group was similar to that of the group treated with acanthopanax (Table 1). Thus, whether administered orally or by abdominal injection, PSP clearly increases the carbon clearance rate in mice, suggesting that PSP can appreciably increase the phagocytic function of normal mice (Table 1).

2.1.2. The effects of PSP on the increase of T-lymphocytes. Cell culture solution containing 25, 50, 100, 200, 400 and 800 ug/ml PSP was used to cultivate spleen T-lymphocytes in 5% CO_2 at 37°C. After 66 hr, 0.25 μci of ^{3}H-thymidine (^{3}H-TdR) was added and, after a further 6 hr, the cells were collected and the amount of radioactivity (dpm) determined. Concentrations of PSP in excess of 100ug/ml produced a clear increase in T-lymphocytes, especially in the presence of Con A. The increase in T-lymphocytes was 1.5 to 4.0 times compared to the control group (Table 2).

TABLE 1. The effects of PSP on the carbon clearance rate of normal mice.

Group	Route	Dosage	Number of animals	K value($\overline{X}$±SD)x10^3
Physiological saline	po	0.1ml/10g	10	6.6±2.8
PSP	po	0.5g/kg	10	10.1±3.5*
PSP	po	1.0g/kg	10	11.9±3.5**
PSP	po	2.0g/kg	10	15.0±5.5**
Acanthopanax	po	300mg/kg	10	11.4±3.9**
Physiological saline	ip	0.1ml/10g	10	3.6±2.0
PSP	ip	100mg/kg	10	5.6±2.0
PSP	ip	200mg/kg	10	6.5±2.3#
PSP	ip	400mg/kg	10	6.2±1.3##
Acanthopanax	ip	300mg/kg	10	6.2±2.9#

Compared with the physiological saline group: * P<0.05, ** P<0.01, # P<0.05, ## P<0.01.

TABLE 2. The effects of PSP on the increase of T-lymphocytes.

PSPconcentration μg/ml	^{3}H-Thymidine infiltration capacity(dpm)	
	without Con A	with Con A
0	1174±227	2000±460
25	1079±230	1608±288
50	1521±169	2250±493
100	2191±154**	3435±309*
200	2082±432*	5115±1313*
400	2215±469*	8956±1631**
800	3137±694**	9204±1750**

Compared with the contrast group: *P<0.05; **P<0.01

2.1.3. The effect of PSP on the production of interferon by human blood cells. Human white blood cells ($1x10^7$) were cultivated at 37°C for 24 hr in solutions containing different concentrations of PSP. The cultivation was then continued for one hour after the addition of α-interferon or γ-interferon at low concentrations (200IU/ml). Next, α-interferon and γ-interferon were induced with 64-128 HAV/ml of Newcastle disease virus (NDV-F line) or 100μg/ml PHA, respectively, for 20 or 40 hours at 37°C.

The value of the effect on interferon was determined using the method involving inhibition of pathological changes in the cells, by determining the cells to be human amnionic WISH cells. Vesicular stomatitis virus (VSV) was then used as the challenge virus and the data corrected for values obtained with a standard interferon product and converted into international units. From Table 3, it is clear that PSP induces interferon in human WBC's: γ-interferon levels in the PSP group were twice those of the control group while α-interferon levels were 2-4 times higher.

TABLE 3. The effect of different concentrations of PSP on interferon induction.

Number of cases		Physiological saline contrast group	Effect value of interferon ($1x10^3$ IU/ml) Different concentrations of PSP for the trial group (μg/ml)			
			1	10	100	1000
α-interferon	1	4	4	8	8	16
	2	4	4	8	8	16
	3	4	4	16	16	16
	4	4	4	8	8	16
γ-interferon	1	2	2	2	4	4
	2	2	2	2	4	4
	3	2	2	2	4	4
	4	2	2	2	4	4

2.1.4. The effect of PSP on the secretion of interleukin-2 in mice. $C_{57}BL/6$ mice were administered orally with 1500mg/kg of PSP for 5 days. After dislocating their vertebra cervicalis, the mice were allowed to die and their spleens removed and ground to give a cell suspension. Tris-NH_4Cl (0.17ml) was used to lyse the red blood cells, the spleen cells were washed with RPMI-1640 nutrient solution and the cell concentration adjusted to 1×10^7 cells/ml. Cell survival rate was 90%.

One ml of spleen cells suspended in RPMI nutrient solution containing Con A was added to each well of a 24-well culture plate. The plate was then incubated at 37°C for 24 hr in 5% CO_2. The contents of each well were then centrifuged at 2000 rpm for 20 minutes and the IL-2 content of the supernatant determined as follows:

Nutrient solution with 15% rat factor was used to cultivate IL-2-dependent cells (CTIL-2). The cells were washed twice with RPMI-1640 nutrient solution and then suspended in 10% FCS nutrient solution to a density of 1×10^5 cells/ml. A 100µl aliquot of cell suspension was added to each well of a 96-well culture plate and 1:6, 1:18, 1:54 and 1:162 dilutions of the solutions from the earlier spleen cell cultivations added. The culture plate was incubated for 32 hr at 37°C under 5% CO_2. ^{3}H-Thymidine (0.5 µci) was added to each well and the cultivation continued for a further 6-8 hr after which the cells were collected using a multi-headed cell collector. After drying, the ^{3}H-thymidine infiltration capacity (dpm) was determined using a liquid scintillation counter.

The results showed that the dpm values of all four dilutions of the PSP group were clearly higher than the physiological saline control group (Table 4). Thus, PSP can appreciably increase the secretion of IL-2 in mice.

2.2. The Effect of PSP on the Immunological Function of Mice After Injection of Cyclophosphamide

2.2.1. The effect of PSP on the white blood cells (WBC) of mice injected with cyclophosphamide. 144 Kunming breed mice were divided randomly into normal, negative (physiological saline) and positive (batyl alcohol) control groups, and PSP high, medium and low dosage groups. With the exception of the control group, the mice were given PSP either orally or by intraperitoneal injection once daily for 8 days. On the fifth day, the mice were injected with cyclophosphamide (100mg/kg). On the eighth day, one hour after administration of the drug, blood was taken from the amimals' tail and the number of white blood cells counted under a microscope.

Table 5 shows that the number of WBC's in mice injected with cyclophosphamide was clearly less than the numbers found in the normal group. The number of WBC's ($x10^3/mm^3$) fell from 116

TABLE 4. The effect of PSP on the production of IL-2 by mice.

Degree of dilution	Physiological saline group	IL-2 activity (^{3}H-thymidine activity) dpm($\overline{X}\pm SD$)	
		PSP group	IL-2 group (86µg/ml)
1:6	39155±8655	51639±1090*	138310±16220
1:18	24067±3502	58221±7973*	58343±6433
1:54	12054±679	27741±3322*	16256±9807
1:162	23483±911	7933±248*	1757±270

As compared with physiological saline group, $P<0.01$.

TABLE 5. The effect of PSP on the white blood cells of the mice after injection with cyclophosphamide*.

Route	Group	Dose	Number of animals	Number of WBC ($\overline{X}\pm SD, 10^3/mm^3$)
PO	cyc*+physiological saline	0.1ml/10g	12	41.75±13.88
	cyc+PSP	1.27g/kg	12	66.58±5.48#
	cyc+PSP	2.25g/kg	12	69.58±21.36#
	cyc+PSP	4.00g/kg	12	74.83±19.72#
	cyc+batyl alcohol	200mg/ kg	12	90.50±22.30#
IP	cyc*+physiological saline	0.1ml/10g	12	66.00±12.20
	cyc+PSP	100g/kg	12	73.08±10.98#
	cyc+PSP	200mg/kg	12	80.67±9.21#
	cyc+PSP	400mg/kg	9	86.44±11.33#
	cyc+batyl alcohol	200mg/ kg	12	87.08±17.87#

*: average WBC of this experiment on normal mice without CYC are 116.33±21.01($10^3/mm^3$).
*: cyc=cyclophosphamide; #: compared with cyc+physiological saline; cyc=cyclophosphamide.

to 41 (po) or 66 (ip). However, if the mice were given PSP along with cyclophosphamide, then the drop in WBC count was less. There was a significant difference ($P<0.01$) between the two groups. Thus, with PSP, the mice are able to resist the lowering effect of cyclophosphamide on the WBC count; that is, PSP can partly restore the drop in WBC's caused by cyclophosphamide injection.

2.2.2. The effect of PSP on IL-2 production in mice injected with cyclophosphamide. Using the IL-2 determination method described above, the amounts of IL-2 produced by groups of mice administered physiological saline, cyclophosphamide, or cyclophosphamide with PSP was compared. The results showed that the amounts of IL-2 produced by the group injected intraperitoneally with cyclophosphamide (25mg/kg) were appreciably lower than the control group ($P<0.01$). However, if PSP (25mg/kg) was given simultaneously by intraperitoneal injection, then the amounts of IL-2 produced were significantly higher than those produced by the group injected only with cyclophosphamide ($P<0.01$). This shows that PSP can eliminate the inhibitory effect of cyclophosphamide on IL-2 production (Table 6).

2.2.3. The effect of PSP on the delayed type hypersensitivity (DTH) reaction. 10μl of oil/acetone (1:1) containing 1% dinitrofluorobenzene was applied to the shaved skin of the abdomens ($3x3/cm^2$) of $C_{57}BL$ mice administered either physiological saline (control), cyclophosphamide (25mg/kg), PSP (25mg/kg), or PSP plus cyclophosphamide. Five days later, a second 10μl aliquot was applied on both sides of the right auricle, but not on the left one. Twenty four hours later, pieces of skin (8 mm in diameter) on both ears were punctured with a perforator and the pieces weighed accurately. The weight difference between the two pieces of skin is an indication of the strength of the DTH reaction.

The result shows that the DTH value of the group of mice injected with cyclophosphamide is significantly lower compared with the physiological saline group ($P<0.05$). However, the DTH value of the cyclophosphamide plus PSP group is raised appreciably. Compared with the group injected

TABLE 6. The effect of PSP on the secretion of IL-2 by mice injected with cyclophosphamide.

Group	Dose (mg/kg)	Amount of IL-2 (^{3}H-TdR dpm)
physiological saline	-	2537±66
cyclophosphamide	25x2 days	1557±430*
PSP	25x5 days	3020±150
cyclophosphamide plus PSP		2374±120#

*: Compared with physiological saline group, P<0.01; #: Compared with cyclophosphamide group, P<0.01.

only with cyclophosphamide, the P value is less than 0.05 (Table 7). Thus, it can be seen that PSP restores the DTH reaction inhibited by cyclophosphamide.

2.3. The Effect of PSP on the Immunological System of Tumor Bearing Animals

2.3.1. Effect on immunological organs-thymus. After C_{57}BL mice were inoculated with sarcoma-180, PSP was administered orally for 5 consecutive days. The mice were anatomized on the sixth day and their livers, thymuses and spleens weighed. The result shows that the weights of the thymuses of mice taking PSP were significantly increased ($P<0.05$). Thus, it can be seen that PSP can stop the atrophy of the thymuses of tumor-bearing mice (Table 8).

2.3.2. The effect of PSP on the formation of antibody in sarcoma bearing mice. Several C_{57}BL mice were inoculated with S-180 and then sensitized with sheep red blood cells (SRBC). From the next day, PSP (1g/kg) was administered orally consecutively for 5 days. On the 6th day, blood was removed from the vein cluster behind the eye-sockets, and the amount of anti-SRBC antibody in the serum determined and expressed as half values (HC_{50}).

Using rabbit anti-mouse antiserum, the serum IgG and complement C_3 was determined by the single immunodiffusion method, and the serum IgG and complement C_3 calculated quantitatively from a standard curve. The results show that the HC_{50} values, serum IgG and complement C_3 values are all higher than those of sarcoma bearing mice without PSP. Differences in the serum IgG and

TABLE 7. The effect of PSP on the DTH reaction of mice injected with cyclophosphamide.

Group	Dosage (mg/Kg)	DTH (Strength*)	P value
physiological saline	-	11.0±3.4	
PSP	25	13.3±5.1	
cyclophosphamide	25	7.9±2.3	compared with physiological saline group ($p<0.05$)
PSP + cyclophosphamide	25	13.0±4.1	compared with cyclophosphamide group ($p<0.05$)

*weight of right ear minus that of left ear

TABLE 8. The effect of PSP on the weight of the thymuses of the sarcoma bearing mice.

group	dosage (mg/kg x number ofdays)	route	weight of the thymus (mg/10g,body weight)
control	-	po	18±3
S-180	-	ip	10±4*
S-180+PSP	1000x5	ip+po	14±4#

*: As compared with the normal control group without being inoculated with sarcoma-180 (P<0.01).
#: As compared with the group inoculated with sarcoma-180 (P<0.05).

complement C_3 are especially obvious. Thus, PSP causes a definite improvement in the function of the specific and nonspecific immunity systems of sarcoma-bearing mice.

3. CLINICAL RESEARCH ON PSP

485 tumor patients were given PSP along with chemo- and radiotherapy. Control tests were made on 211 cases. Of the remaining 274 cases, random groups were selected for forward-expecting and double-blind control tests.

Various kinds of cancer were involved: stomach (162 cases), esophagus (172 cases) and primary pathogenic lung cancer (151 cases). Those patients accepted for treatment were all proved positive by esophagoscope, X-ray and cell pathology for esophagus cancer; by operation, pathology and gastroscope or X-ray and by pathology of the inspection of live cells for stomach cancer; and by operation, pathology or bronchoscope or X-ray and CT diagnosis and pathological inspection of live cells for lung cancer. Karnofsky evaluations were all ≥60 points, and both the hemopicture and the liver and kidney functions were all within the normal range.

Chemo- or radiotherapy plans applied: MF chemotherapy plan for stomach cancer (MMC+5-Fu); MAP plan (MMC+ADM+DDP) for lung cancer; and Co^{60}-γray radio-therapy plan (DT65-70, Gy/6-7 months) for esophagus cancer.

PSP dosage: 3 grams daily; batyl alcohol, 450 mg/day was used for the positive control group.

Period of treatment: one month as a period of treatment, altogether 2 periods.

Items and methods of determination: included clinical symptoms, inspection and determination of indices and quality of life. For clinical symptoms we mainly observed indices of weariness and

TABLE 9. The effect of PSP on the weight of the antibody and complement C_3 of sarcoma-bearing mice.

Group	Dosage (mg/kg x number of days)	Route	Half value of hemolysin (HC_{50})	Serum IgG (μg/μi)	Serum complement C_3 (μg/10μl)
control	-	po	565±59	71±2	14±4
S-180	-	ip	316±100	73±8	18±3
S-180+PSP	1000x5	po	422±55	95±12*	21±1*

Compared with the group inoculated with sarcoma-180 (P<0.01)

weakness, poor appetite, nausea and vomiting, dryness of throat and mouth, vexation and insomnia, spontaneous and night sweating, and pain before and after treatment.

The inspection and determination indices are mainly to determine the changes in hemopicture (WBC and platelets) and immunological function (natural killing cells, interleukin-2 and T-lymphocytes) before and after treatment. The quality of life is usually expressed by body weight and Karnofsky evaluation. The method of determination follows that of Li (1991).

The following statistical tests were used: Bonferroi X^2 test (G test) for qualitative materials; Ridit or U test for serial materials, and t test for paired materials. $P<0.05$ is required for marked standard.

Standards of the effect of treatment: the following 3 indices were used for the comprehensive determination of the effect of the treatment:

A. Clinical symptoms:markedly improved (the value of integrated mark lowered $\geq 2/3$)
B. Test indices: hemopictures and immunological indices are stable or with some improvement ($\geq 1/3$)
C. Quality of life:weight or Karnofsky evaluation $\geq$ positive control group

If all the above 3 indices are fulfilled - marked effect; if 2 indices AB or AC are fulfilled - effective; and if all the 3 indices are unfulfilled - not effective.

3.1 The Effect of PSP on Improving Clinical Symptoms of Tumorous Patients

After chemo- or radiotherapy, the tumorous patients usually possess symptoms of tiredness, weakness, poor appetite, dryness of mouth and throat, pain, vexation, insomnia, palpitation, shortness of breath, spontaneous and night sweating, leanness, nausea, vomiting,etc. After taking PSP, all the above symptoms were lessened (Table 10). Appreciable relief was evident especially in the case of poor appetite, weakness, tiredness, dryness of mouth and throat and pain. This shows that PSP is good for invigorating not only one's spirit and energy but also one's spleen and heart.

TABLE 10. Effect on the improvement of symptoms before and after treatment.

Symptoms	Control		Treatment		G value *
	Total number of cases	Effective number	Total number of cases	Effective number	
Poor appetite	106	37	114	87	40.480
Weakness and tiredness	107	42	125	90	25.603
Dryness of mouth and throat	58	15	78	46	16.454
Pain	69	23	67	43	13.555
Palpitation and insomnia	80	20	91	37	5.203
Being short of breath	69	26	75	41	5.000
Spontaneous and night sweat	43	12	56	26	4.042
Leanness	62	9	61	18	3.889
Nausea and vomiting	31	15	44	30	3.368

* According to the 9 tests of Bonferroi X^2: $P=0.05$, $X^2(G)=7.689$.

3.2. The Effect of PSP on the Improvement of the Quality of Life of Tumorous Patients

We investigated the effect of PSP on the quality of life of the patients before and after treatment according to body weight and Karnofsky's appraisal value. The results show that the number of cases exhibiting increased or steady weight, and of raised or steady Karnofsky's marks, were significantly higher than among the control group. ($P<0.01$) (Table 11).

3.3. The Effect of PSP on Improving the Immunological Function of Tumor Patients

The analysis determined the changes in the activities of natural killing cells, the amount of the IL-2 of WBC. and the ratio values of CD_4^+/CD_8^+ of T-lymphocytes before and after treatment. The results show that the above-mentioned indices of the test group were all improved. That is, the number of cases of increased NK cell activity, and of increased IL-2 and CD_4^+/CD_8^+ ratio, were all more than observed in the control group. This shows that PSP has a definite effect on the host's ability to resist immunological inhibition due to chemo- and radiotherapy.

3.4. The Effect of PSP on the Change of Blood Picture of Patients Undergoing Chemo- and Radiotherapy

One of the toxic side-effects of chemo- and radiotherapy is that WBC and platelet counts, and hemoglobin levels, are appreciably lowered. Thus, during the process of chemo -and radiotherapy for the treatment of cancers, it is necessary for the patients to take batyl alcohol in order to prevent worsening of the hemopicture.

We replaced batyl alcohol with PSP, observed the changes in the hemopicture of patients after

TABLE 11. The weights of tumor patients and the Karnofsky evaluation condition after treatment.

Item	Group	Total No.	Raised No. %	Steady No. %	Lowered No. %	U value
body weight	control	135	31 22.9	58 42.9	46 34.1	Test group as compared with control group U=3.185 P=0.0041
	treatment	139	52 37.4	60 43.2	27 19.4	
	PSP control	211	78 37.0	100 47.3	33 15.6	
Karnofsky evaluation	control	135	40 29.6	81 60.0	14 10.4	Test group as compared with control group U=2.957 P=0.0031
	treatment	139	62 44.6	72 51.8	5 3.6	
	PSP control	211	44 20.9	163 77.2	4 1.9	

Body weight: an increase or decrease of 1 kg or more regarded as "raised", an increase or decrease of less than 1 kg as "steady" and a decrease of more than 1 kg as "lowered".
Karnofsky's evaluation: an increase or decrease of 10 or more points regarded as "lowered" or "raised" and the rest regarded as "steady".

TABLE 12. Determination of NK cell activity after treatment with PSP.

Group	No. of cases	No. of raised cases	No. of lowered cases	Rate of increase %
control	135	57	78	42.22
treatment	138	89	49	64.49
PSP control	206	142	42	68.93

Note: comparison between the control and treatment group: X^2=13.6144; P=0.000199.

TABLE 13. Determination of IL-2 levels before and after treatment with PSP.

Group	No. of cases	d±sd	t value	P value
control	81	-0.91±23.56	0.3467	>0.05
treatment	88	7.96±16.85	4.4330	<0.001

Note: apply t test for the paired weighing of the materials (differential statistics) df is n-1.

TABLE 14. Determination of CD_4^+/CD_8^+ ratio values before and after treatment with PSP.

Group	Total No. of cases	No. of raised cases	No. of steady cases	No. of lowered cases
control	133	62	3	68
treatment	133	89	7	37
PSP control	211	133	12	66

Note: comparison between control and treatment group: U=3.158; P=0.006.

treatment, and compared them. The results show that the numbers of WBC and platelets, and hemoglobin levels, all corresponding to those values observed with batyl alcohol (Tables 15,16 and 17). They show that PSP has a definite improving function in preventing the worsening of the hemopicture.

4. DISCUSSION

The pharmacological research described above proves that PSP has the obvious function of fostering the positive forces of one's body. Not only can it strengthen the immunological function of the normal organism but it also adjusts immunological inhibition, caused by tumor-bearing or the use of chemotherapeutic drugs, back to the normal state. The philosophy of Chinese traditional medicine is to foster the positive forces and remove the evil ones in order to effect the cure. "Nei Jin",

TABLE 15. Determination of WBC counts after treatment with PSP.

Group	No. of cases	≥4.0	3.0-3.9	2.0-2.9	1.0-1.9	<1.0
control	135	97	25	12	1	0
treatment	139	105	23	10	1	0
PSP control	211	188	12	11	0	0

Note: the ratio between treatment and control group: U=0.7057; P=0.48.

TABLE 16. Determination of hemoglobin levels after treatment with PSP.

Group	No. of cases	≥11.0	9.5-10.9	8.0-9.4	6.5-7.9	<6.5
control	134	82	36	15	1	0
treatment	139	97	26	15	1	0
PSP control	211	16	34	13	1	0

Note: the ratios between treatment and control group: U=1.3139; P=0.2516.

TABLE 17. Determination of the numbers of platelets after treatment with PSP.

Group	No. of cases	≥10.0	7.5-9.9	5.0-7.4	2.5-4.9	<2.5
control	135	103	22	9	1	0
treatment	139	114	24	1	0	0
PSP control	211	167	37	6	1	0

Note: the ratio between treatment and control group: U= 1.3948; P=0.1633.

the classical Chinese medical compendium states, "with positive forces inside one's body, the evil negative forces can't interfere with them and, with the victory of the positive, the evil ones will disappear". Fostering the positive will surely remove the evil forces in our bodies. Our tumor-inhibiting tests also prove that PSP can inhibit sarcoma-180, Ehrlich ascetic tumor, p388 leukemia, human lung adenocarcinoma, monocytic leukemia, histiocytic lymphoma, stomach, liver, nose and throat cancers (Yang *et al.*, 1992).

In the sarcoma-180 experiments, we devised a proof by contradiction; that was to give anti-lymphocyte serum (ALS) while using PSP. The result showed that if only PSP (50mg/kg ip x 10) is used, the tumor inhibition rate is 44%, whereas if PSP is used together with ALS (0.1 ml x 10), then the tumor inhibition rate falls to 0%. That is to say, the tumor inhibition function of PSP on sarcoma-180 will be cancelled by ALS. This suggests that the removal of the evil forces (inhibition of sarcoma-180) by PSP is related to the fostering of positive forces (activation of the immunological function of the organism) (Zhou *et al.*, 1988).

According to the above pharmacological experiments and modern tumor immunology research, we consider that the tumor inhibition effects of PSP are brought about mainly through the following

ways (Takemaru, 1991; Takashi, 1992):

PSP→complement C_3→activate macrophages→inhibit cancer cells

PSP→help T-lymphocytes (CD_4^+)→T-killer cells→inhibit cancer cells

PSP→helpT-lymphocytes (CD_4^+)→B-lymphocytes→antibody→inhibit cancer cells

PSP→help T-lymphocytes (CD_4^+→Interleukin-2→LAKcells→inhibit cancer cells

PSP→natural killing cells→inhibit cancer cells

We consider that the above are perhaps the basic mechanisms for PSP to foster the positive forces and remove the negative ones.

The results of the clinical research correspond to the pharmacological experiments. The results of PSP application to 485 cases of stomach, lung and esophagus cancers clearly show that PSP can lower the toxic and side-effects of chemo -and radiotherapy, lessen the seriousness of the symptoms, stabilize the immunological functions and raise the quality of life. The Prince of Wales Hospital in Hongkong applied PSP in cases of breast cancer and discovered that it can appreciably improve the drop in WBC levels due to the use of chemotherapeutic medicines (cyclophosphamide, 4' epidoxorubicin). Chemotherapeutic drugs will decrease the number of WBC's from 6.96 to $5.05 \times 10^3/mm^3$ ($P<0.01$). However, in the group of patients administered PSP, the numbers of WBC's are stabilized at $6.82\text{-}7.02 \times 10^3/mm^3$ ($P>0.05$) (Shiu *et al.*, 1992).

Lessening the seriousness of clinical symptoms, and stabilizing and raising the various pharmacological indices will necessarily raise the effects of treatment and prolong the patients' lives. Clinical tests indicated that, after taking PSP together with chemotherapy, the curative rate in patients with esophagus cancer was raised from 42% in the control group to 72%. The one year survival rate was raised from 40.2% to 51.2% (Liu, 1991; Yao *et al.*, 1992).

5. SUMMARY

The protein bound polysaccharide, PSP, extracted from the mycelia of Yun Zhi Cov-1 is a new BRM substance. Pharmacological and clinical research prove that PSP has the miraculous effect of fostering the positive, and removing the negative, forces of our body. Though the cancer-killing power of BRM drugs is not so powerful as that of chemo- and radiotherapy, they can clearly eliminate the immunosuppression function associated with chemo- and radiotherapy and thereby raise the quality of life. Thus, BRM's have a real value in the comprehensive treatment of tumors. We sincerely hope that BRM substances in Chinese traditional medicine will make greater contributions to the sacred struggle of man's victory over cancer.

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REFERENCES

CHEN, Y.W. (1989). *Oncology of Integrated Western and Chinese Medicine.* Chinese Maps Press.

JOJI, I. (1985). Recent Advances in Chemotherapy Anticancer Section 2, *Proceedings of the 14th*

International Congress of Chemotherapy. University of Tokyo, Japan.
LI, J.T. (1991). *Clinical Pharmacology.* People's Hygiene Press.
LIU, T. (1991). Radiotherapy of carcinoma of the esophagus in China-A Review. *International Journal of Radiation-Oncology-Biology-Physics* **20,** 875-879.
MASAYOSHI, H.,TARO, S., KEIJIRO, T. & MASUMITSU, T. (1982). Drugs in Japan. *Ethical Drugs* 6th Ed. p.230.
SHIU,W.C., LEUNG, T.W.T. & TAO, M. (1992). A clinical study of PSP on peripheral blood counts during chemotherapy. *Physiotherapy Research* **6**, 217-218.
TAKASHI, M. (1992). *Chemistry and Biochemistry of Mushrooms.* Institute of Publishing House,Tokyo.
TAKEMARU, T. (1991). *Basic Sciences and Newest Technology of Mushrooms.* Agricultural Cultural Society,Tokyo.
YAO, W., SHI, X., LIU, T. & HE, S. (1992). The preliminary report of the forward expecting research of the treatment of esophagus cancer by PSP and radiotherapy. *Anthology of the First Session of China's Scientific & Technological Association.*
YANG, Q.Y. & JONG, S.C. (1989). Medicinal mushrooms in China. *Mushroom Science* **12** , 631-643.
YANG, Q.Y., JONG, S.C., LI, X.Y., ZHOU,J.X., CHEN, R.T. & XU, L.Z. (1992). Antitumor and immunomodulating activities of the polysaccharide-peptide(PSP) of *Coriolus versicolor*. *Estratto dalla rivista EOS n.l.* **12**, 29-34.
ZHOU, J., LI, X., SHEN, X., CAO, C., LIANG, H., YANG, Q. & XU, B. (1988). The antitumor and immunomodulating activity of PSP in mice. *Journal of Shanghai Teachers University* **17**, 3.

CHAPTER 27

MEDICAL ASPECTS OF LENTINAN ISOLATED FROM *LENTINUS EDODES* (BERK.) SING.

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1. INTRODUCTION

The most important aim of cancer research is to increase the survival time of cancer patients, keeping their lives comfortable and, if possible, achieve a complete cure of the cancer itself. However, cytocidal anticancer chemotherapeutics generally exhibit severely detrimental side effects, and markedly suppress the host defences against cancer. This hardly meets the purpose of extending the patients survival time except for some specific cancers.

Therefore, new wine is necessary for a new wineskin; i.e. totally new ideas are indispensable for the development of new anticancer drugs. How can these drugs be developed and how can they be used most successfully? This article concerns the evaluation of lentinans as Host Defence Potentiators (HDPs), their mode of action, and their experimental and clinical application to cancer and infectious diseases.

2. LENTINANS AS HOST DEFENCE POTENTIATORS (HDPS)

In studies on the development of anticancer drugs, the concept of killing cancer cells is basically wrong. We should be interested not in such cytocidal chemotherapeutics or immunotherapeutics, but in the augmentation of intrinsic host defence mechanisms against cancer and infectious diseases in the human body, and in the discovery of substances which can enhance this host defence, namely HDPs. This issue concerns the host-tumour relationship in the human body. The most important problem is to understand how the cancer and host compete against each other. When the cancer wins the host dies, and when the host wins the cancer is cured. It is generally accepted that the proliferation and spontaneous regression of tumour cells are balanced at the level of 10^7 cells in immunized mice. If the threshold of the balance is raised up to the level of 10^9, 10^{10} cells or more by augmenting the host defence mechanisms, this will be beneficial for cancer patients. The drugs facilitating this effect should be called HDPs.

The existence of host defence mechanisms against cancer is reflected particularly in the prognosis of patients after surgery. Their existence is also indicated by the spontaneous regression of cancer, or by recurrence even 10-50 years after curative operations on cancer patients. In the latter situation, it is assumed that cancer and host have coexisted for a long time. The most important objective in cancer therapy is, therefore, to markedly augment the host defence activity of patients in every respect. Lentinan is a substances which can improve the physiological constitution of the host against cancer and against various kinds of infections. Thus, it can be said that lentinan can restore or augment the ability of the host to respond to bioactive substances such as lymphokines or cytokines by stimulating maturation, differentiation or proliferation of the cells important for host defence mechanisms.

Agents such as interleukin-2(IL-2) or tumour necrosis factor (TNF) are not categorized as HDPs. Their action is generally localized, not particularly selective, and their half-life in the human body is short. Systemic administration of these agents at large dosage levels is rather against the maintenance of homeostasis of the host and the results are not so different from those following administration of ordinary anticancer chemotherapeutics. Their effectiveness would, therefore, be limited to local administration. These agents appear to have developed from the idea of killing tumour cells and are completely different in nature from lentinan.

3. BIOLOGICAL ACTIVITY OF LENTINAN

Lentinan appears to represent a unique class of HDPs potentiating the physiological constitution of the host against cancer and infectious diseases. This substance was discovered initially in a hot water extract of *Lentinus edodes* (Berk.) Sing., an edible mushroom, and completely inhibited the growth of sarcoma 180 implanted subcutaneously in ICR mice (Chihara *et al.*, 1969). From the extract, we isolated and purified a polysaccharide which showed marked antitumour activity, and named it lentinan.

Lentinan is now proven to exhibit prominent antitumour activity not only in allogeneic, such as sarcoma 180, but also syngeneic and autochthonous hosts without any noticeable side-effects. Lentinan markedly prevents chemical and viral oncogeneses (Suga *et al.*, 1984), and suppresses cancer metastasis and recurrence in animal models (Suga *et al.*, 1989). Therefore, lentinan is not merely a stimulant of homograft rejection but a true antitumour substance.

Furthermore, lentinan was demonstrated to enhance host resistance against infections from various types of bacteria, viruses, fungi and parasites (Chihara, 1990). It suppressed post-chemotherapy relapse in experimental mouse tuberculosis, and proved to be effective against infections from VSV-encephalitis and Abelson virus, *Schistosoma mansoni* and *S. japonicum*. Recently, it has been reported that lentinan and some of its derivatives are effective against HIV-1 or AIDS infections.

4. MECHANISM OF ACTION OF LENTINAN

Lentinan has no direct cytotoxicity to target cells and its action is host-mediated. Why are lentinans referred to as a unique class of HDPs potentiating the physiological constitution of the host against cancer? It is because lentinan is able to augment the host's response through maturation, differentiation and proliferation of lymphoid cells and other physiologically important cells active

in host defence mechanisms.

Lentinan is defined as a T-cell oriented adjuvant, in which macrophages play some part, which displays various kinds of immune reaction in normal and tumour-bearing hosts (Maeda & Chihara, 1971). However, it has not been clear how lentinan affects the host at an early stage, prior to the induction of many immune reactions. We have observed the appearance of various kinds of bioactive serum factors immediately after the administration of lentinan (Maeda *et al.*, 1974). Most of them were induced by macrophages, i.e. acute-phase protein inducing factor (APPIF), vascular dilatation and hemorrhage inducing factor (VDHIF), IL-1 production inducing factor (IL-1PF), interleukin-3 (IL-3) and colony stimulating factor (CSF), which peaked several hours after the administration of lentinan. These bioactive serum factors appear to act on lymphocytes, hepatocytes, vascular endothelial cells or synovial fibrobrasts to cause many host defence reactions associated with inflammation and immunity (Maeda *et al.*, 1984). Moreover, lentinan activates the classical and alternative pathways of the complement system resulting in augmentation of non-specific cytotoxic activity of macrophages. Infiltration of neutrophils into tumour modules should also be noted.

The induction of a remarkable increase in the amount of IL-1, as well as of CSF and IL-3, is also a most important activity of lentinan. This leads to the maturation, differentiation and proliferation of immunologically important cells of the host defence mechanisms such as premature lymphocytes. This, in turn, results in a significant enhancement of the host's capacity to respond to lymphokines and cytokines such as IL-2, and to many other physiologically active substances. Although lentinan does not specifically accelerate the production of IL-2 from helper T-cells, it potentiates the induction of different types of antitumour effector cells, such as killer T-cells, NK-cells and cytotoxic macrophages, as a result of sequential reactions. Under these circumstances, the effector cells may act either selectively or non-selectively on target cells; therefore, it may be wrong to classify lentinan as a non-specific immuno-stimulant. An *in vitro* incubation of thymocytes (immature precursor T-cells) of mice administered a very small amount of lentinan (0.001mg/kg) with IL-2 leads to the production of many allo-killer cells killing EL-4 lymphoma. This result indicates that thymocytes of mice administered lentinan have been enhanced in terms of their responding capacity to IL-2. In other experiments, lentinan did not activate NK cells *in vitro* whereas poly I:C, an interferon inducer, did. However, this experiment showed that, on the basis of enhanced cytotoxicity to YAC tumour cells, NK activity induced by poly I:C *in vitro* was much higher when spleen cells of mice administered lentinan were used instead of normal spleen cells. The same results were obtained using IL-2 instead of poly I:C. Since lentinan did not induce interferon or activate NK cells *in vitro*, these results suggest that, *in vitro*, lentinan markedly enhances the responding capacity of NK cells to IL-2 or NK-activating factors. These are examples showing the potentiation or restoration of the physiological antitumour constitution of the host. In other words, maturation and differentiation of important cells for host defence are essential, which is the most important principle of host defence potentiators (Hamuro & Chihara, 1985).

5. CLINICAL APPLICATIONS OF LENTINAN

Clinical applications of lentinan were examined as a result of the marked antitumour activity, negligible side-effects and the HDP mode of action in experimental studies. Lentinan is worthy of consideration as an effective therapy for cancer patients.

The results of a five year follow-up study on lentinan for advanced and recurrent gastroinstestinal cancer in Phase III have proved to be excellent: a combination therapy of lentinan with tegafur

produced a marked increase in the survival time of patients than tegafur alone. In the case of stomach cancer, the survival rates of patients of one, two and three years after the administration of tegafur alone showed increases of 2.9%, 2.9% and 0% Patients who had been treated with tegafur in combination with lentinan, 1mg twice a week or 2mg once a week administered intravenously, showed survival rate increases of 19.5%, 10.4% and 6.5%, respectively (Taguchi, 1987).

Similar results were obtained for advanced and recurrent colorectal cancer. In these cases, the 50% survival time of patients who had been treated with tegafur alone was 94 days, whereas it increased up to 200 days in the group of patients treated with tegafur plus lentinan. The results were rather disappointing when lentinan was combined with mitomycin C and 5-FU, both of which are more strong anticancer chemotherapeutic agents than tegafur. This finding suggests that lentinan may be more effective when used as a single agent rather than in combination with chemotherapeutic agents. Chemothepeutics may be beneficial when administered once to several times in cases of absolute necessity but not for long term administration. The results for patients with esophageal cancer, squamous cell lung cancer and breast cancer were found to be excellent in combination therapy involving surgery, radiation and lentian therapy without chemotherapy.

6. FUTURE PROSPECTS OF LENTINAN

Suppression of cancer recurrence after surgical resection is another most challenging goal for lentinan therapy. From the above mentioned clinical results and mode of action against tumour cells, lentinan would be expected to bring much better results when used for preventing the recurrence of cancer and the onset of AIDS symptoms in HIV carriers. At present, the five year survival ratio of lung cancer patients after resection is only 51.1% at the National Cancer Centre Hospital, Tokyo, even in cases without any lymphonode metastasis (Watanabe & Suemasu, 1982). Tumour cells are hidden somewhere within the body for the other 48.9% of patients. Even if recurrence is suppressed in 10% or so of these patients, an extremely large number of patients would be affected worldwide. The use of lentinan would be most appropriate and most reasonable for this purpose. In this context, adjuvant chemotherapy using conventional anticancer chemotherapeutics is generally not so effective, and may be harmful by accelerating the formation of metastasis through immunosuppression of the host. This can be realized not by direct attack of cancer cells but by suppression of the proliferation of small numbers of autochthonous cancer cells scattered throughout the body of the host. The best way to attain this may be to potentiate host resistance against cancer so as to suppress proliferation of the cancer cells. There are no magic anti-cancer drugs that are capable of eradicating a large mass of cancer all at once.

There are several reports showing that recurrence after surgery was suppressed by lentinan in animal models. Using MH-134 hepatoma cells, lentinan was shown to completely prevent recurrence of cancer after surgical operation. A similar result was reported using Madison-109 lung carcinoma, DBA/2.MC.CS-1 and DBA/2.MC.CS-T fibrosarcoma. Clinical studies on the prevention of recurrence of many kinds of cancer after surgery are now underway.

The same principle is applicable to AIDS therapy: prevention of clinical manifestation of AIDS symptoms in HIV-1 carriers is most important and this can be realized by HDPs such as lentinan. Recently, our colleagues have reported that lentinan and some of its derivatives are effective against AIDS (Kaneko & Chihara, 1992). Lentinan reduced the toxicity of AZT, and sulphated lentinan inhibited reverse transcriptase activity more strongly than dextran sulphate and proved to be effective against HIV virus. Prevention of the onset of AIDS symptoms through potentiation of host defence

is now being actively investigated experimentally and clinically.

7. CONCLUSION

Any of the physiologically active substances, including lympho-cytokines hormones and neurotransmitters become useful only when the host response to these bioactive substances is fully restored to, or augmented above, the normal state. Lentinan is a substance that potentiates this "physiological constitution of the host". This is particularly important in cancer patients in an immunosupressed state. Agents such as lentinan produce a good response not only to cancer but also to infectious diseases, and might also be associated with protection from aging. The leading principle of the function of lentinan resides in the fact that it can cure patients by restoring their homeostasis, and through enhancement of their intrinsic resistance against such diseases. This implies restoration to a normal from an abnormal state in a relevant area of immunology, endocrinology, neurology or nutrition. Potentiating the physiological constitution in favour of the host defences results in the activation of many kinds of cells that are vitally important for the maintenance of homeostasis. New and widely varying host defence potentiators, having novel characteristics and associated specifically with nervous, endocrine and immune systems, should be further developed. These concepts may also play an important role as a bridge between modern immunology, biology and oriental medicine (Chihara, 1992).

REFERENCES

CHIHARA, G. (1990). Lentinan and its related polysaccharides as host defence potentiator: their application to infectious diseases and cancer. In *Immunotherapeutic Prospects of Infectious Diseases*, pp.9-18. Edited by K. Noel Masihi & W. Lange. Heidelberg, Springer-Verlag.

CHIHARA, G. (1992). Immunopharmacology of lentinan, a polysaccharide isolated from *Lentinus edodes*: Its application as a host defence potentiator. *International Journal of Oriental Medicine* **17**, 55-77.

CHIHARA, G., MAEDA, Y.Y., HAMURO, J., SASAKI, T. & FUKUOKA, F. (1969). Inhibition of mouse sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) Sing. *Nature* **222**, 687-688.

HAMURO, J. & CHIHARA, G. (1985). Lentinan, a T-cell oriented immunopotentiator: Its experimental and clinical applications and possible mechanism of immune modulation. In *Immune Modulation Agents and Their Mechanisms*, pp.409-436. Edited by R.L. Fenichel & M.A. Chirigos. New York, Marcel Dekker.

KANEKO, Y. & CHIHARA, G. (1992). Potentiation of host resistance against microbial infections by lentinan and its related polysaccharides. In *Microbial Infections, Role of Biological Response Modifiers*, pp.201-215. Edited by H. Friedman, T.W. Klein & H. Yamaguchi. New York, Plenum.

MAEDA, Y.Y. & CHIHARA, G. (1971). Lentinan, as a new immuno-accelerator of cell-mediated responese. *Nature* **229**, 634-635.

MAEDA, Y.Y., CHIHARA, G. & ISHIMURA, K. (1974). Unique increase of serum proteins and action of antitumour polysaccharides. *Nature* **252**, 250.

MAEDA, Y.Y., WATANABE, S., CHIHARA, G. & ROKUTANDA, M. (1888). Denaturation and renaturation of a ß-1,6;1,3-glucan, lentinan, associated with expression of T-cell-mediated

responses. *Cancer Research* **48**, 671-675.
SUGA, T., SHIIO, T., MAEDA, Y.Y. & CHIHARA, G. (1984). Antitumor activity of lentinan in murine syngeneic and autochthonous host and its suppressive effect on 3-methyl-choranthrene induced carcinogenesis. *Cancer Research* **44**, 5132-5137.
SUGA, T., YOSHIHAMA, T., TSUCHIYA, Y. SHIIO, T., MAEDA, Y.Y. & CHIHARA, G. (1989). Prevention of tumour emtastasis and recurrence of DBA/2.MC.CS-T fibrosarcoma, MH-134 hepatoma and other murine tumours using lentinan. *International Journal of Immunotherapy* **5**, 187-193.
TAGUCHI, T. (1987). Clinical efficacy of lentinan on patients with stomach cancer: End point results of a four year follow-up survey. *Cancer Detection and Prevention* **1**, 333-349.
WATANABE, H. & SUEMASU, K. (1982). Cancer surgical therapy and lymphonode metastasis. *Oncologia* (Japanese), **1**, 83-89.

CHAPTER 28

PHARMACOLOGY AND CLINICAL USES OF *GANODERMA*

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1. INTRODUCTION

Ganoderma is a medicinal fungus and has been treasured for this value in China for more than two thousand years. This fungus was described as a nontoxic medicine which was beneficial to viscera and could improve intelligence by enhancing memory, hearing, vision and smelling. If taken regularly for long periods, it would also retard aging and prolong life span (Li, 1978). In Chinese folk culture, *Ganoderma* has been regarded as a panacea, curing all kinds of diseases. Regardless of its medicinal value, wild *Ganoderma* were few and very expensive. We have succeeded in the mass cultivation of *Ganoderma* using solid-state-fermentation and submerged fermentation technologies. In practice, only the fruitbodies of *G. lucidum* and *G. japonicum* are used as a medicine, and the spores are discarded. Therefore, we carried out some pharmacological studies on the effects of the spore extracts of *G. lucidum* and mycelial extracts of *G. capense* on various diseases. Some promising results were obtained. This paper mainly reviews the results of our studies on the pharmacology and clinical uses of crude *Ganoderma* preparations.

2. PHARMACOLOGICAL ACTIVITIES OF THE ALCOHOL-WATER SOLUBLE FRACTION

Spores (SGL) and mycelium (MGC) were harvested from submerged cultures of *G. lucidum* and *G. capense*, respectively. Then, alcohol-water soluble and alcohol-ether soluble fractions of SGL and MGC were prepared. When the lipid-soluble fraction was used in aqueous medium, Tween 80 was added as a surfactant. The dosage of the preparations was calculated according to the raw material content.

2.1. Effects on the Central Nervous System in Mice

The following parameters in mice were monitored: spontaneous motor activity, sleeping time induced by barbiturates and salivation induced by pilocarpine and nicotine induced death rate (Liu *et al.*, 1977; 1979a, b; Leng & Liu, 1980a, b). The results are shown in Table 1.

TABLE 1. Effects of the alcohol-water soluble fractions of *Ganoderma lucidum* spores (SAW) and *G. capense* mycelium (MAW) on the central nervous system in mice.

	n	Control	SAW	MAW
Spontaneous motor activity	15	782.0±55	350.0±53*	306.0±62#
Sleeping time (min)				
Pentobarbital	1	51.0±3	69.0±6*	100.0±7#
Barbital	10	65.0±20	149.0±9#	156.0±2#
Nicotine induced death (%)	10	100.0	30.0#	0
Salivation (mg)	10	8.2±0.2	5.4±0.2*	2.6±0.2#

*, $P < 0.05$; #, $P < 0.01$.

For motor activity, adult male mice were injected intraperitoneally with 10gm SAW (from spores of *G. lucidum*) or 5gm MAW (from mycelium of *G. capense*) per kg biomass. Control mice received normal saline instead. The spontaneous motor activity in ten minutes following the injection was recorded by an electron-light apparatus. Both SAW and MAW significantly reduced the spontaneous motor activities in mice.

For sleeping time experiments, a dose of 20-30 gm SAW or MAW per kg biomass was injected subcutaneously to mice 20min before intraperitoneal injection of 50mg pentobarbital or 200mg sodium barbital per kg biomass. The disappearance and recovery of righting reflex was recorded as the sleeping time. SAW and MAW significantly prolonged sleeping time induced by the injection of barbiturates in mice.

For nicotine induced death test, prior to intravenous injection of nicotine (1mg/kg biomass) to the tail vein, 20gm SAW or 10gm MAW per kg biomass was intraperitoneally injected. The injection significantly protected the mice from convulsion and death induced by nicotine.

When SAW or MAW (20gm/kg) was administered subcutaneously 20 min prior to intravenous injection of 2.5mg/kg of pilocarpine to the tail vein, salivation was markedly reduced.

From the above results (Table 1), it shows that SAW and MAW have a sedative effect on the central nervous system in mice. MAW seems to be more potent than SAW.

When the culture broth of *G. capense* was dried and extracted to form the alcohol-water soluble portion (FAW), this portion also affected the central nervous system. FAW, 2.5 - 5gm per kg biomass, when injected intraperitoneally, significantly inhibited the spontaneous motor activities. FAW also potentiated the sedative effects of reserpine and chlorpromazine and antagonized the stimulating effect of amphetamine in mice. This effect was not observed with extract from *G. lucidum* culture broth. FAW, 20gm per kg biomass, when given intraperitoneally, prolonged sleeping time induced by pentobarbital and barbital. Pretreatment using FAW also reduced convulsion and death rate caused by nicotine. However, no such antagonistic effect was observed when convulsion and death were induced by strychnine and ammonium chloride. FAW also significantly inhibited salivation induced by pilocarpine.

In conclusion, the alcohol-water soluble portions of the spores of *G. lucidum*, the mycelium of *G. capense* and the culture broth of *G. capense* have similar sedative effects on the central nervous system in mice.

TABLE 2. Effects of the alcohol-water soluble fractions of *Ganoderma lucidum* spore (SAW) and *G. capense* mycelium (MAW) on cardio-cerebral vascular system on ten mice.

Dosage (gm/kg)	Tolerance to hypoxia (survival min)	Uptake of ^{86}Rb (cpm/g tissue)		Serum total cholesterol (mg/dl)
		Heart	Brain	
Control	47±4.8	3247±179	803±51	165±14
SAW 10	71±8*	3550±370	898±47	237±19
SAW 2	164±26#	2816±286	955±47*	173±10#
MAW	119±11	4271±440*	1223±70#	189±10#
MAW 20	141±19#	5343±649#	1440±87#	180±5#

*, $P < 0.05$; #, $P < 0.01$.

2.2. Effects on Cardio-Cerebral-Vascular System in Mice

The following parameters were examined: tolerance to hypoxia, uptake of ^{86}Rb by heart and brain, experimental hypercholesterolemia in mice and prostaglandin metabolism in ischemic brain of gerbils. Normal and isopropyl adrenaline treated-mice were administered subcutaneously a dose of 10 - 20 gm SAW or MAW 20 min before they were placed in a glass jar with a sealed cover. From Table 2, prior adminstration of MAW significantly prolonged the survival time of normal and isopropyl adrenaline treated-mice under hypoxia, while SAW showed effects only in normal mice but not in isopropyl adrenaline-treated mice under hypoxia (Bao *et al.*, 1988).

The uptake of ^{86}Rb by tissues may be used as an indication of the state of nutritional circulation in the organ. The higher the uptake, the higher the blood flow into the organ. A dose of 20 gm SAW per kg biomass was administered subcutaneously 20 min prior to intravenous injection of 10 uci/kg of ^{86}Rb. The radioactivities in heart and brain tissues were measured 20-30 sec after the injection. Table 2 shows that both SAW and MAW significantly promoted the uptake of ^{86}Rb by the brain, and only MAW increased the uptake by the heart.

Mice received a dose of 400mg triton WR1339 per kg tissue in the tail vein and a dose of 10 - 20 gm SAW or MAW subcutaneously at the same time. Then the mice was fasted for 5 hrs. Table 2 shows that total cholesterol in the serum was higher in the treated groups.

Prostaglandins play some role in the pathogenesis of brain ischemia. Therefore, the effect of *Ganoderma* on prostaglandins PGF1a and TXB_2 in ischemic brain was examined. Both cervical arteria of Mongolian gerbils were ligated 20 min after subcutaneous injection of SAW. 6-Keto-PGF1a and TXB_2 in ischemic cerebral cortex of gerbils were determined 5 min after the ligation. SAW significantly increased the content of 6-keto-PGF1a and decreased TXB_2, thereby increasing the ratio of $PGI2/TXA_2$. This effect of SAW seemed to be more potent than that of nootropil. The results indicate that the two alcohol-water soluble injections of *Ganoderma* have beneficial effects on cardio-cerebral-vascular system and may be useful for the treatment of heart and brain ischemia.

2.3. Effects on Immune Functions in Mice

The immune system is the specific defense mechanism against invaders. Our studies demonstrated that subcutaneous injection of the *Ganoderma* (SAW) once daily for 6 days significantly

TABLE 3. Effects of *Ganoderma* extract (SAW) on acid phosphatase and β-glucuronidase of mouse peritoneal macrophages and ^{3}H-TdR incorporation into spleen DNA in prednisolone treated-mice.

Enzyme	Control	SAW	Prednisolone
Acid phosphatase (u/ 5x10^{6} cells)	1.7±0.35	7.5±0.5#	
Beta-glucuronidase (u/ 5x10^{6} cells)	2.7±0.2	3.8±0.5*	
Spleen DNA (mg/gm tissues)	16.4±0.9	14.5±2.0*	13.4±1.3
^{3}H-TdR (cpm/gm spleen)	2985.0±447	3012.0±525*	2254.0±377

*, P < 0.05; #, P < 0.01.

increased the activity of acid phosphatase and β-glucuronidase of mouse peritoneal macrophages (Table 3) (Liu, 1989). In another test, SAW was given subcutaneously to mice once daily for 7 days, but on day 4 onwards, prednisolone acetate (5mg/kg) was also injected intraperitoneally once daily. Then each mouse received 10μci ^{3}H-TdR/kg tissues one day before the last dose of SAW. Then the spleen DNA content and the incorporation of ^{3}H-TdR into spleen DNA were measured. As shown in Table 3, prednisolone reduced spleen DNA and ^{3}H-TdR was incorporated into spleen DNA. Pretreatment with SAW did not affect prednisolone. It appears that *Ganoderma* extract has some effects on immune function in mice.

2.4. Anti-allergic Myositis in Rats

Serum and muscle creatine phosphokinase (SCPK, MCPK) were determined and served as biochemical parameters for evaluating the efficacy of *Ganoderma* since myopathic patients are usually associated with higher level of SCPK. Male Wistar rats were immunized with leg muscle homogenate of guinea-pig once a week for 4 weeks. One week after the last immunization, rats were killed and SCPK and MCPK were determined. The pathological changes of muscles of hind legs were

TABLE 4. Effects of *Ganoderma* (SAW) on serum and muscle creatine phophokinase (SCPK, MCPK) levels in rats with allergic myositis and mice with myotonia induced by 2,4-dichlorophenoxy acetic acid.

Animal	Group	SCPK	MCPK
Rat	Normal	34.3±3.7	136.0±10
	Immunization	60.6±6.9	91.0±15
	SAW 20gm/kg	43.3±7.9*	121.0±21*
Mice	Normal	25.8±0.4	120.0±12
	Immunization	45.5±10.2	69.6±11.3
	SAW 20gm/kg	30.0±4.8*	99.1±19.3*

*, P < 0.05.

observed by light microscopy. As shown in Table 4, SCPK increased and MCPK decreased significantly after immunization in rats. When the immunized rats were simultaneously injected with 20gm SAW per kg biomass subcutaneously once daily for 30 days, the elevation of SCPK and reduction of MCPK were significantly inhibited. The lesions of leg muscle were also ameliorated.

We have found that subcutaneous injection of 2,4-dichlorophenoxy acetic acid, a herbicide, not only caused myotonia but also induced elevation of SCPK and reduction of MCPK in mice. Therefore, we use this as a model system to evaluate the efficacy of *Ganoderma* extract. SAW was injected subcutaneously to mice after the mice were injected with 200mg herbicide per kg biomass. From Table 4, SAW significantly inhibited the elevation of SCPK and reduction of MCPK induced by the herbicide. However, SAW had no effect on normal mice.

2.5. Anti-oxidant Activity

In order test whether *Ganoderma* exerts its protective effect on experimental myositis through inhibition of lipid peroxidation, homogenates of mouse leg muscles were incubated with various concentrations of SAW for 30 - 60 min at 37°C. Malondialdehyde, a product of lipid peroxidation, initiated by addition of iron and cysteine was assayed. SAW inhibited malondialdehyde formation and the production of superoxide anion in xanthine/xanthine oxidase *in vitro*. These observations provide new evidence for the effects of SAW on several muscle diseases.

3. HEPATOPROTECTIVE ACTION OF THE ALCOHOL-ETHER SOLUBLE FRACTION OF *G. LUCIDUM* SPORES AND *G. CAPENSE* MYCELIUM IN MICE

3.1. Effect on Serum Transaminase

Only the alcohol-ether soluble fraction and not the water soluble fraction from *G. lucidum* spores and *G. capense* mycelium could reduce the elevated serum transaminase level induced by CCl_4 in mice (Liu *et al*., 1979a, b).

3.2. Effect on Toxicity of Indomethacin and Digitoxin

Large doses of indomethacin induces ulceration and perforation of the gastrointestinal tract, and high doses of digitoxin causes cardio-toxicity which usually induces death in mice. When the *Ganoderma* extracts were administered simultaneously, the mortality rate of mice intoxicated with indomethacin and digitoxin decreased. Similar results were obtained with the lipid soluble extracts. In addition, the lipid soluble portion promoted regeneration of the liver in partially hepatectomized mice as revealed by the heavier weight of the liver in the treated group.

4. CLINICAL USAGE

The alcohol-water soluble protions of the *G. lucidum* spores (SAW) and the *G. capense* mycelium (MAW) were tested clinically. Two ml extracts from 400 mg spores/mycelium were administered intramuscularly once daily for a few days. From Table 5, both preparations were shown to have therapeutic effects on dermatosclerosis, dermatomyositis and multiple polymyositis,

TABLE 5. Clinical trials of the alcohol-water soluble preparations of *Ganoderma* on different diseases.

Disease	Preparation	Total cases	Effective rate (%)	Months of Treatment
Dermatosclerosis	MAW	173	79.1	3-6
Dermatomyositis & Multiple Polymyositis	MAW	55	96.4	3-6
Lupus erythromatosis	MAW	84	82.1	3
Alopecia areata	MAW	232	78.9	1-3
Atropic myotonia	MAW, SAW	35	74.3	3-6
Progressive muscular dystrophy	MAW, SAW	121	56.2	3-6

alopecia areata and other diseases.

4.1. Effects on Dermatomyositis and Multiple Myositis

Extracts of *G. capense* mycelium were used. Out of the 55 patients with different disease histories, 36 patients were treated with the *Ganoderma* injection in combination with prednisone. Among them, 53 patients showed improvement to different extents. Eight patients relapsed after the withdrawal of the *Ganoderma* treatment. In the treatment group, 2 patients died but without lung infection and perforation in the gastrointestinal tract. It appears that *Ganoderma* injection not only can arrest the development of the disease but also reduce the mortality rate among the patients.

4.2. Effect on Alopecia

232 patients with different disease histories were treated with *Ganoderma* injection for 3 months. The efficiacy rate was 78.9 - 86%. No side-effects were observed. Both extracts from *G. lucidum* spores and *G. capense* mycelium were effective.

4.3. Effect on Scleroderma

Scleroderma is a collagen disease and its cause is unknown. 173 patients with different disease histories were administered with *Ganoderma* injection or *Ganoderma* tablets for 3 - 6 months. The total rate of effectiveness was about 58-91%. Local injection of the *Ganoderma* extract for localized scleroderma seemed to be more effective than intramuscular injection of the *Ganoderma* for systemic scleroderma. Such local injection promoted blood circulation as revealed by laser instrumentation. Skin biopsy of several patients revealed that the pathological morphology, such as inflammatory infiltration, decreased or disappared. The collagen fibril became aeroses. Therefore, *Ganoderma* injection may have an anti-inflammatory function and loosen the connective tissues.

5. CONCLUSION

Ganoderma has been known as a panacea for thousands of years in China. However, modern

studies on its biology, chemistry, pharmacology and clinical uses has only been carried out within the last two decades in China. From our experience, the different crude preparations of *Ganoderma* extracts have similar pharmacologic activities on animal models and therapeutic effects on patients suffering from different diseases. It is very important to know the action mechanism(s) of the *Ganoderma* extracts and identify the bioactive components. More efforts and co-operation between scientists are needed.

REFERENCES

BAO, T-T. & COLLEAGUES (1988). Pharmacological effects of the spores of *Ganoderma lucidum*. *Pharmacology and Clinics of Chinese Materia Medica* **4**, 16.

CHENG, X-R. (1987). *Journal of Clinical Dermatology* **16**, 122.

LENG, W. & LIU, G-T. (1980a). Some pharmacological effects of fermented fluid of *Ganoderma capense* 1. *Chinese Pharmaceutical Bulletin* **15**, 1.

LENG, W. & LIU, G-T. (1980b). Some pharmacological effects of fermented fluid of *Ganoderma capense* 1. *Chinese Pharmaceutical Bulletin* **15**, 11.

LI, S-Z. (1978). *Ben-Cao Gong Mu*. People's Health Publisher. In Chinese.

LIE, C.R. (1986). *Journal of Clinical Dermatology* **15**, 128.

LIU, G-T. (1989). Pharmacology and clinical uses of *Ganoderma*. *Proceedings of the Second International Symposium on Recent Advances in Natural Products Research*. 12-14th Oct., 1989. Seoul, Korea.

LIU, G-T. & COLLEAGUES (1977). Some pharmacological action of the spores of *Ganoderma lucidum* and the mycelium of *G. capense* produced by submerged fermentation. *Chinese Medicinal Journal* **10**, 607.

LIU, G-T. & COLLEAGUES (1979a). Some pharmacological actions of *Ganoderma lucidum* and *G. japonicum* (Fr.) Lloyd on mouse liver. *Acta Pharmacologia Sincia* **14**, 284.

LIU, G-T. & COLLEAGUES (1979b). A comparison of the protective actions of biphenyl dimethyl-dicarboxylate, *trans*-stilbene, alcoholic extracts of *Fructus schizandrae* and *Ganoderma* against experimental liver injury in mice. *Acta Pharmacologia Sinica* **14**, 598.

CHAPTER 29

HEPATO-PROTECTIVE TRITERPENOIDS FROM *GANODERMA TSUGAE* MURRILL

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1. INTRODUCTION

The traditional Chinese herb, Lin-Chi, played an important role in folk legends as a cure for many unspecified illnesses. However, there was no direct evidence to support this role until, in the past decade, over a hundred novel, highly oxygenated, lanosta-type triterpenoids were isolated from basidiocarps or mycelial mats of *Ganoderma lucidum,* and their chemical structure determined. These compounds were mainly ganoderic acids (Hirotani *et al.*,1985; Kohda *et al.*, 1985), ganodermic acids (Lin & Shiao ,1988; Shiao & Lin, 1989), ganoderenic acids (Nishitoba *et al.*, 1987a; Komoda *et al.*, 1985), lucidenic acids, lucidone (Hirotani *et al.*, 1986), ganoderal (Morigiwa *et al.*, 1986) and ganoderols (Nishitoba *et al.*, 1987a). These findings have led us to consider that the abundant triterpenoids might explain the multiple effects of this fungus. However, the physiological activity of these newly isolated triterpenoids remained unclear and less than 20 of these compounds were reported to be physiologically active (Kohda *et al.*, 1985; Morigiwa *et al.*, 1986).

Therefore, it is becoming important to elucidate the triterpenoid pattern and content of each species in the genus *Ganoderma*. *G. tsugae* was used in the present study because it is one of the most widely cultivated species in Taiwan. Morphologically, it resembles *G. lucidum* (Adaskaveg & Gilbertson , 1986) and crude extracts exhibited pronounced hepato-protective activity in our preliminary screening (Su, 1991).

In the present report, four major compounds were isolated from basidiocarps of *G. tsugae* and they were determined as lucidone A(I), lucidenol(II), ganoderic acid B(III) and ganoderic acid C2(IV). Three of the four isolated compounds were previously reported in *G. lucidum* and compound II was a newly isolated triterpenoid. An *in vivo* bioassay was designed to evaluate the hepato-protective activity of each sample.

2. MATERIALS AND METHODS

2.1. Culture Used and Basidiocarp Production

Ganoderma tsugae Murrill CCRC-37063 was purchased from the Culture Collection and Research Center located in Hsinchu, Taiwan, and a sawdust-polypropylene bag system was employed for fruiting body cultivation in Chiayii county, Taiwan. The basidiocarps were collected during a 6-month period with immediate drying at 45°C after harvest. Totally, a 3000g sample was collected for extraction.

2.2. Extraction and Purification of Triterpenoids

The basidiocarps of *G. tsugae* was sliced, powdered and then immersed in 95% EtOH at room temperature. The ethanolic extract was evaporated to dryness in a rotary evaporator. For the partition of triterpenoids, a modified Kohda (1985) method was followed which is summarized in Fig. 1.

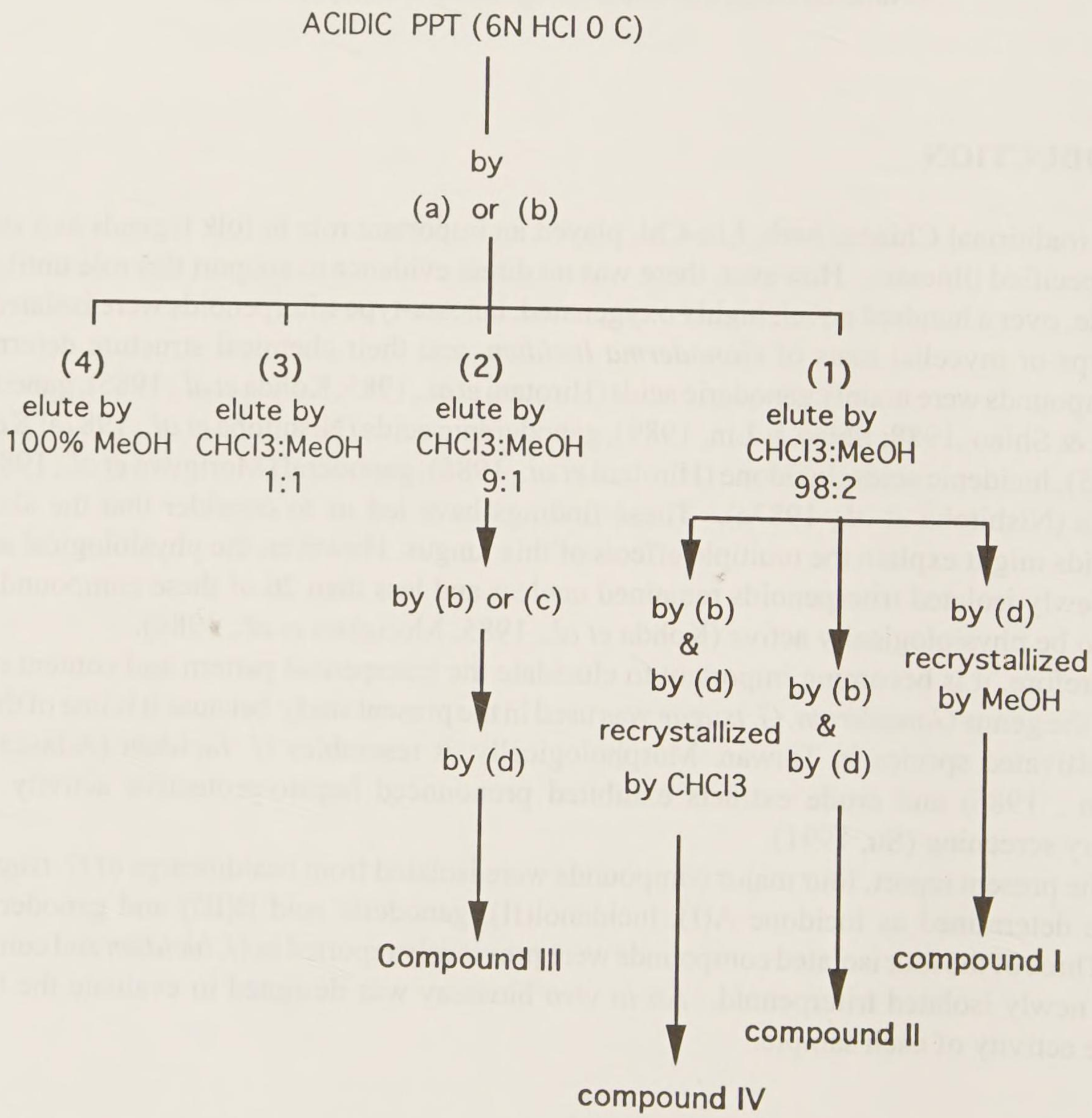


FIGURE 1. Partition of triterpenoids from ethanolic extract of *G. tsugae*. (a) SiO_2 open column chromatography, (b) HPLC, (c) MPLC and (d) TLC.

2.3. Chromatography

For detection of triterpenoids, TLC (Kiesselgel 60 F254, Merck) was used and chromatograms were developed using a $CHCl_3$: MeOH:H_2O (30:4:1) solvent system. The triterpenoids were visualized by UV at 254nm or with 10% H_2SO_4 followed by heating of the TLC plate at 105°C. For a detailed pattern of triterpenoid separation, an HPLC system (LC-4A, Shimadzu) equipped with a UV detector (SPD-6V, Shimadzu) and a column oven set at 45°C was employed. The mobile phase was 40% acetonitrile in 2% methanol with a flow rate of 1 ml/min and a C18 column (30 x 0.5 cm) was used. Similar conditions were used for a medium pressure liquid chromatography system (Bü chi) fitted with a preparative column (60x2.6 cm) packed with C18 gel. However, the flow rate was 5 ml/min. For final purification, a preparative TLC plate (LiChroprep, Merck) was used.

2.4. Structural Analysis

UV (UV-160, Shimadzu), IR (A-100, Jesco), and mass spectrometry (MS, D300, Jeol), and NMR (FT-100, Jeol) were used for spectral analyses.

2.5. Animal Test

Male mice (ICR), 5-6 weeks old, 27± 2 g in weight were subjected to the hepato-protective activity test. CCl_4 (1% in olive oil) was fed to mice at a dose of 2 ml/kg at 0 hr. After 6 hr, the purified triterpenoids were administered at a dose of 0.2 mg/kg (0.1 mg/ml), and a known hepato-protective drug, silymarin, used as a positive control. Blood samples taken from the eye vein was centrifuged for serum separation and then SGOT and SGPT kits (Sigma) were used for analysis at 0, 24 and 48hr, respectively. A dose response test was also carried out in a similar way for compound III with doses of 0.1 mg, 0.4 and 1.0 mg/kg. For each test, 6 mice were used for each group.

3. RESULTS AND DISCUSSION

Around 4.5% (w/w) of crude extract was obtained from the EtOH extraction, but only 27mg, 18mg, 54mg and 28mg of compound I, II, III and IV, respectively, were isolated from each 1000g of *G. tsugae* basidiocarps. The spectra of the four compounds are listed as follows:

Compound I:

UV λ max (nm):	207, 255
IR (cm^{-1}):	2760, 3450-3400, 3960, 1725, 1710, 1660, 1580, 1480, 1360, 1240, 1180, 1090, 1020, 960
Mass spectrum (70ev):	402, 374, 360, 331, 313, 277, 262, 219, 202, 189
^{1}H-NMR (CD30D) (δ) :	0.8342, 0.8794, 1.0223 1.2043, 1.4143, 1.6143, 2.2021, 2.6760 2.7163, 3.1560 3.1963, 4.8317, 4.8746

^{13}C-NMR (CD30D) (δ) : 16.240, 18.800, 25.274, 28.273, 31.327, 36.997, 39.727, 46.196, 54.681, 59.838, 67.794, 78.949, 144.201, 158.338 199.248, 208.374, 216.695

Compound II:

UV λ max (nm): 253

IR (cm^{-1}): 3760, 3500-3300, 1725, 1710 1660, 1580, 1480, 1460, 1440, 1400, 1380, 700

Mass spectrum (70ev): 404, 386, 316, 265, 264, 203, 123, 107, 69, 55

^{1}H-NMR (CD30D) (δ) : 0.7974, 0.8316, 0.9660 1.0136, 1.2457, 1.2982, 1.3018, 1.6084, 2.4805, 2.6934, 3.2915, 3.3085, 3.3122, 3.3391, 3.3427, 4.5153

Compound III:

UV λ max (nm): 206, 253

IR (cm^{-1}): 3760, 1725, 1710, 1690, 1660, 1580, 1480, 1460, 1440, 1420, 1400, 1340,

Mass spectrum (70ev): 516, 501, 500, 482, 402, 389, 374, 359, 344, 279, 262, 207, 189, 167, 149, 139, 121, 105, 95, 83, 69, 57, 55

^{1}H-NMR (CD30D) (δ) : 2.1629, 1.3544, 1.1907, 0.9757, 0.8292

^{13}C-NMR (CD30D) (δ) : 15.972, 17.325, 18.624, 19.794, 24.714, 28.444, 32.888, 35.741, 39.472, 41.615, 46.586, 60.211, 67.709, 78.736, 144, 159.200, 211.218

Compound IV:

UV λ max (nm): 254

IR (cm^{-1}): 3760, 1725, 1710, 1690, 1660, 1580, 1480, 1460, 1440, 1420, 1400

Mass spectrum (70ev): 518, 516, 488, 376, 358, 331, 246, 139, 69, 55

^{1}H-NMR (CD30D) (δ) : 3.6212, 3.3073, 3.2658, 2.6771, 2.3205, 1.7855,

	1.5693, 1.2066, 1.1895,
	1.0698, 0.9647, 0.9269,
	0.8243, 0.8084, 0.7876
^{13}C-NMR (CD30D) (δ) :	16.374, 17.563, 19.995,
	8.700, 33.803, 35.906,
	39.691, 50.445, 50.847,
	53.188, 55.328, 70.159,
	73.213, 78.956,
	143.055, 161.380,
	183.966, 202.400, 212.5

From the spectra obtained, these four compounds were determined as lucidone A (I), lucidenol (II), ganoderic acid B (III) and ganoderic acid C2 (IV). The structures of these four triterpenoids are shown in Fig. 2 and their high performance liquid chromatographs are shown in Fig. 3. Lucidone A (Kohda *et al.*, 1985), ganoderic acid B (Morigiwa *et al.*, 1986) and ganoderic acid C2 (Nishitoba *et al.*, 1987a) are known compounds reported previously in fruitbodies of *Ganoderma lucidum*. However, compound II is a new type of triterpenoid.

The results from animal tests are shown in Figs. 4 to 7. Only compound III showed significant

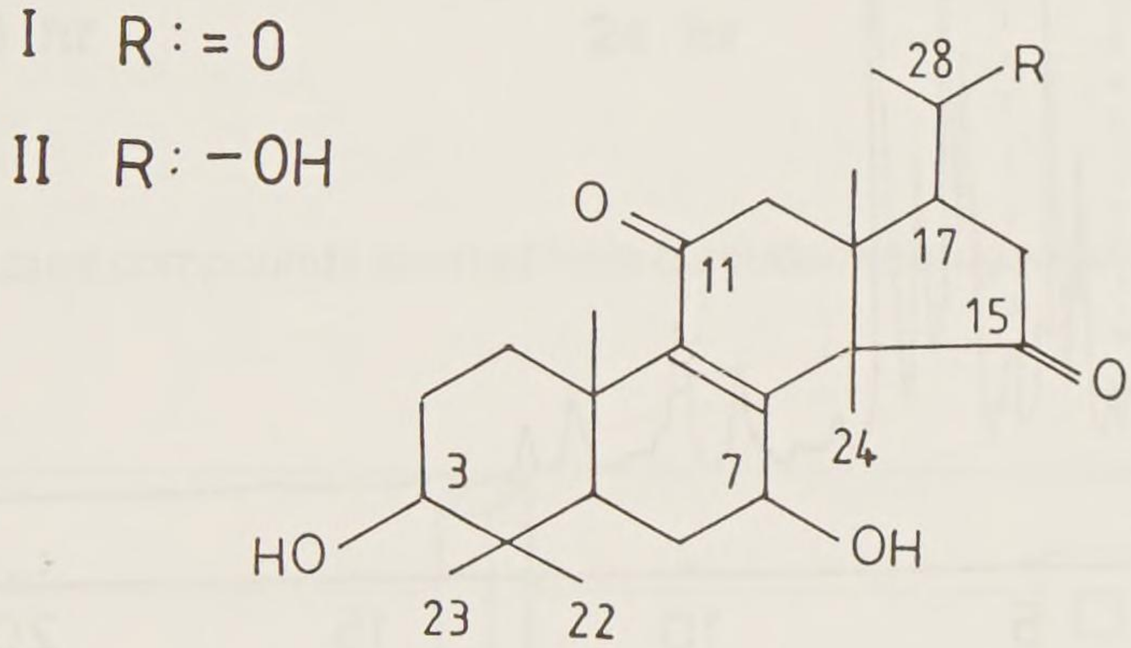


FIGURE 2. Chemical structures of compounds I, II, III and IV isolated from *Ganoderma tsugae*.

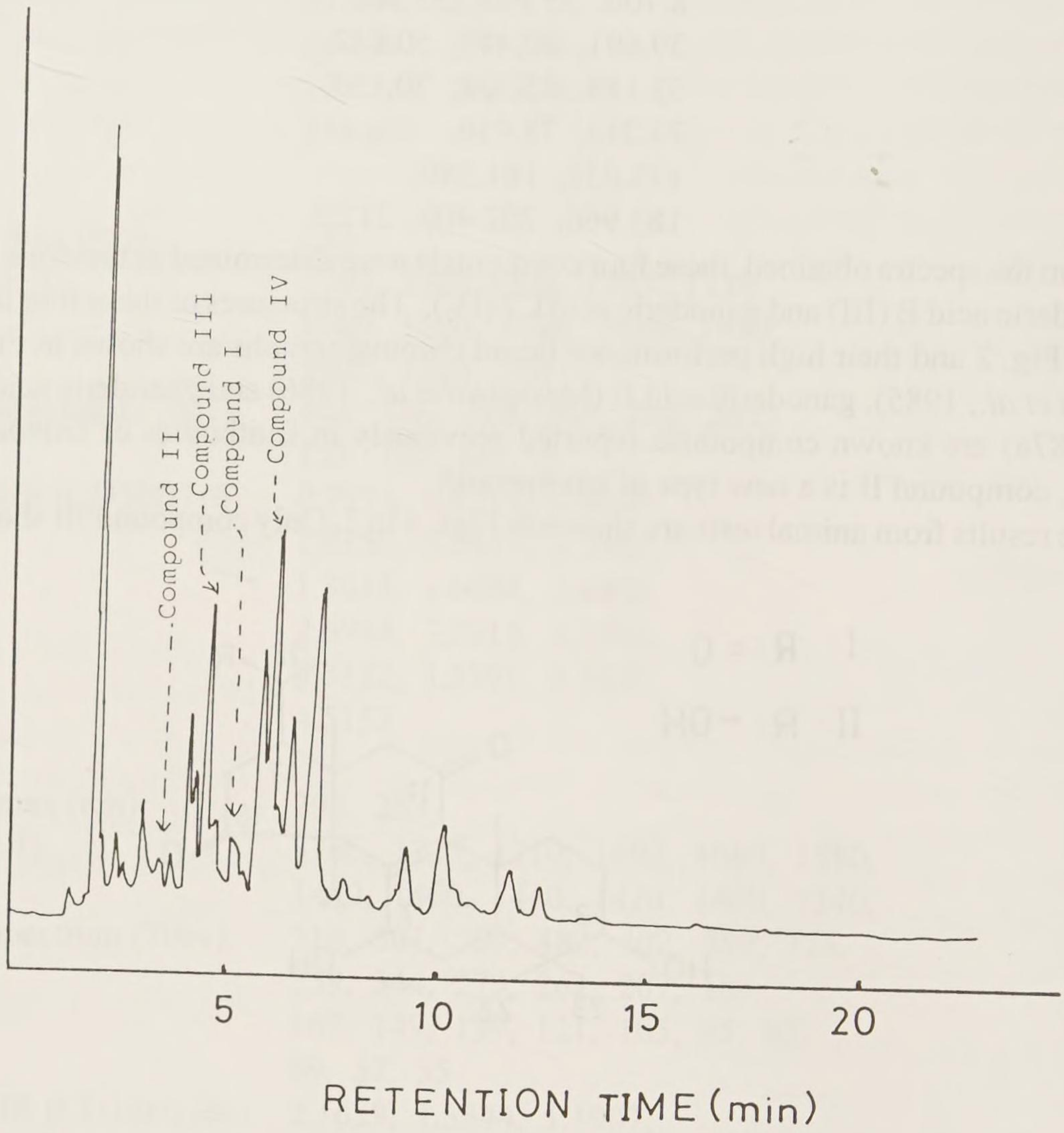


FIGURE 3. Chromatograms of triterpenoids demonstrated by HPLC and the respective retention times of the four isolated compounds.

hepato-protective activity. Only compounds I, II and IV, together with the silymarin group, caused a slight reduction in GPT and GOT levels in serum after 24 hr. The test animals eventually reverted back to the normal condition after 48 hr. However, increased doses of compound III (up to 10 times) did not further reduce GOT/GPT levels in the serum of the mice (Figs. 6 & 7).

Our previous work has demonstrated that a less pure fraction of ganoderic acid B showed a very strong effect in reducing GOT/GPT to almost normal values after 24 hr, but this result was not observed with the pure ganoderic acid B. These controversial results can be explained by the series of triterpenoid analogues functioning as better free radical scavengers than the pure compound in the liver challenged with CCl_4. Further studies on the mechanism of the hepato-protective triterpenoid from *G. tsugae,* and the combined effect of triterpenoid analogues, are now under investigation.

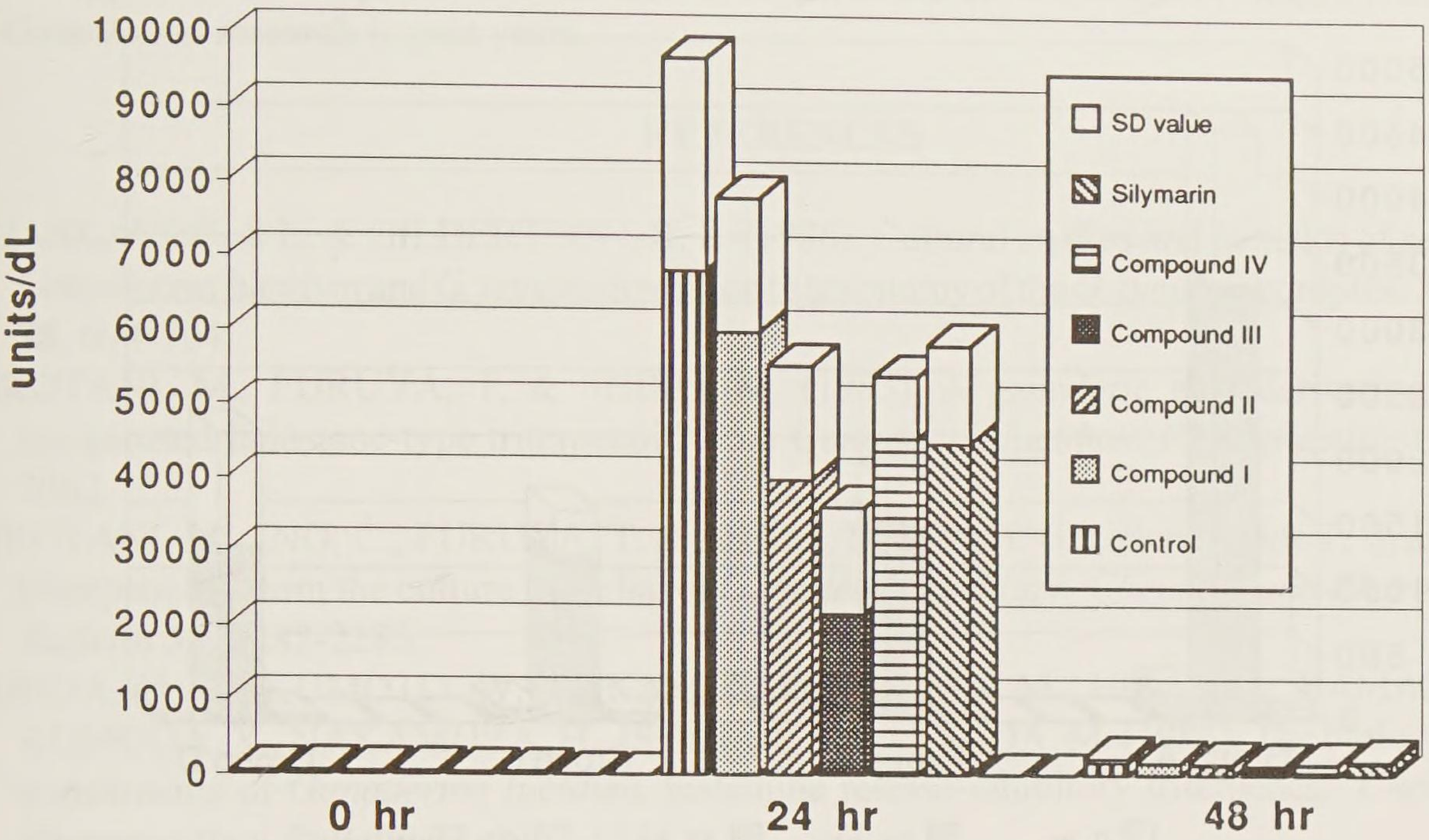


FIGURE 4. The effect of pure compounds isolated from *Ganoderma tsugae* for GPT in mice induced by CCl_4.

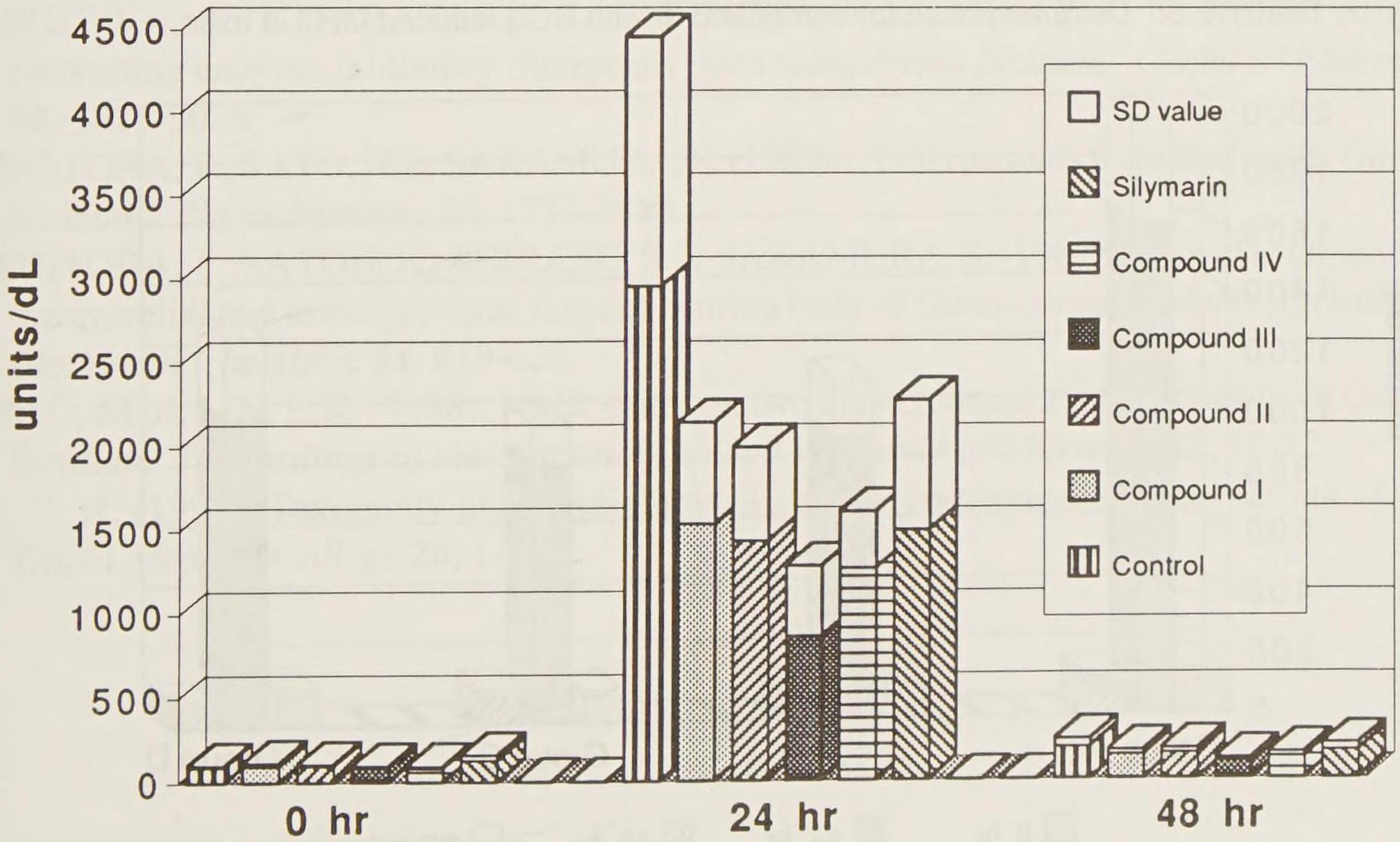
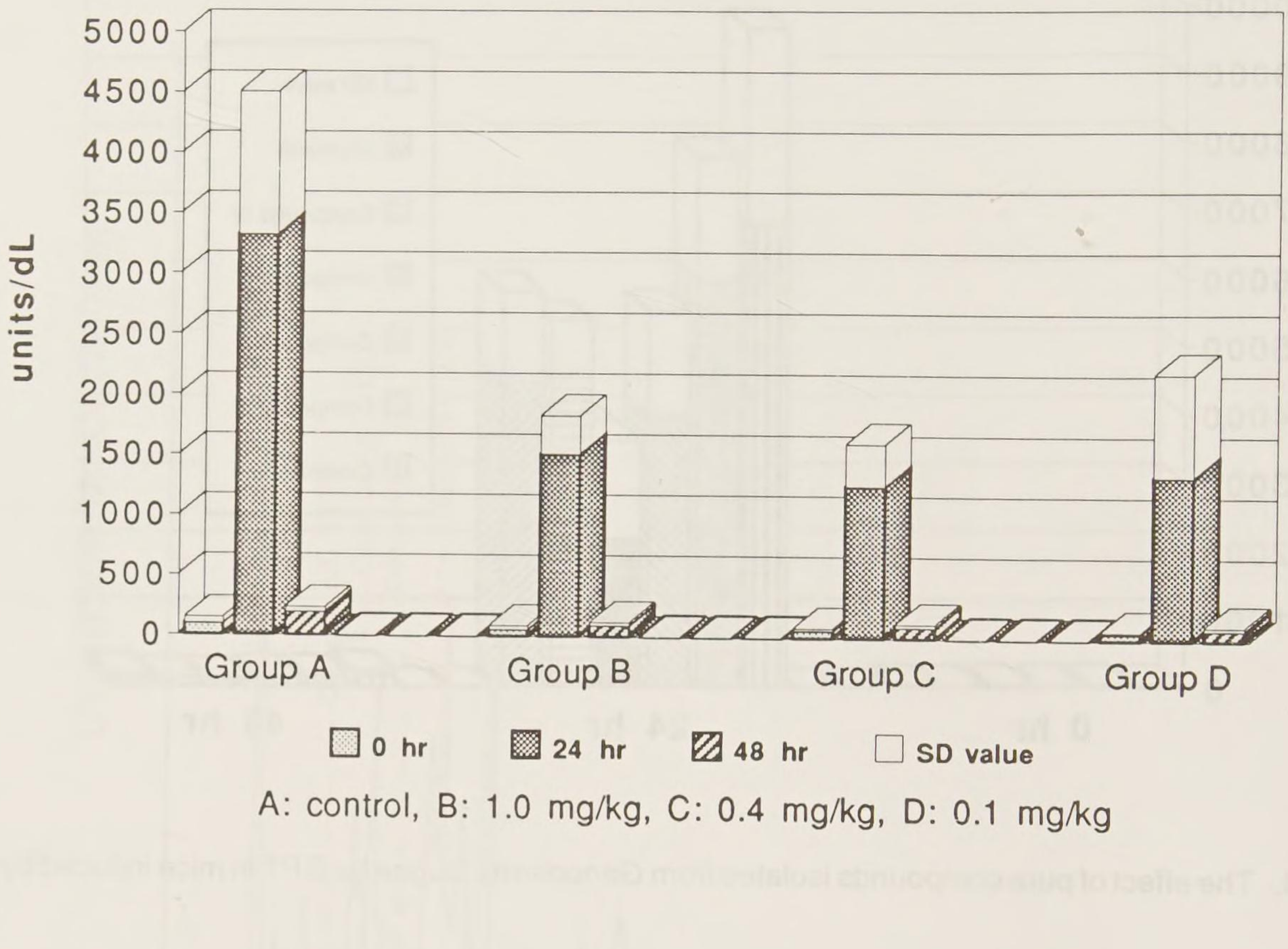
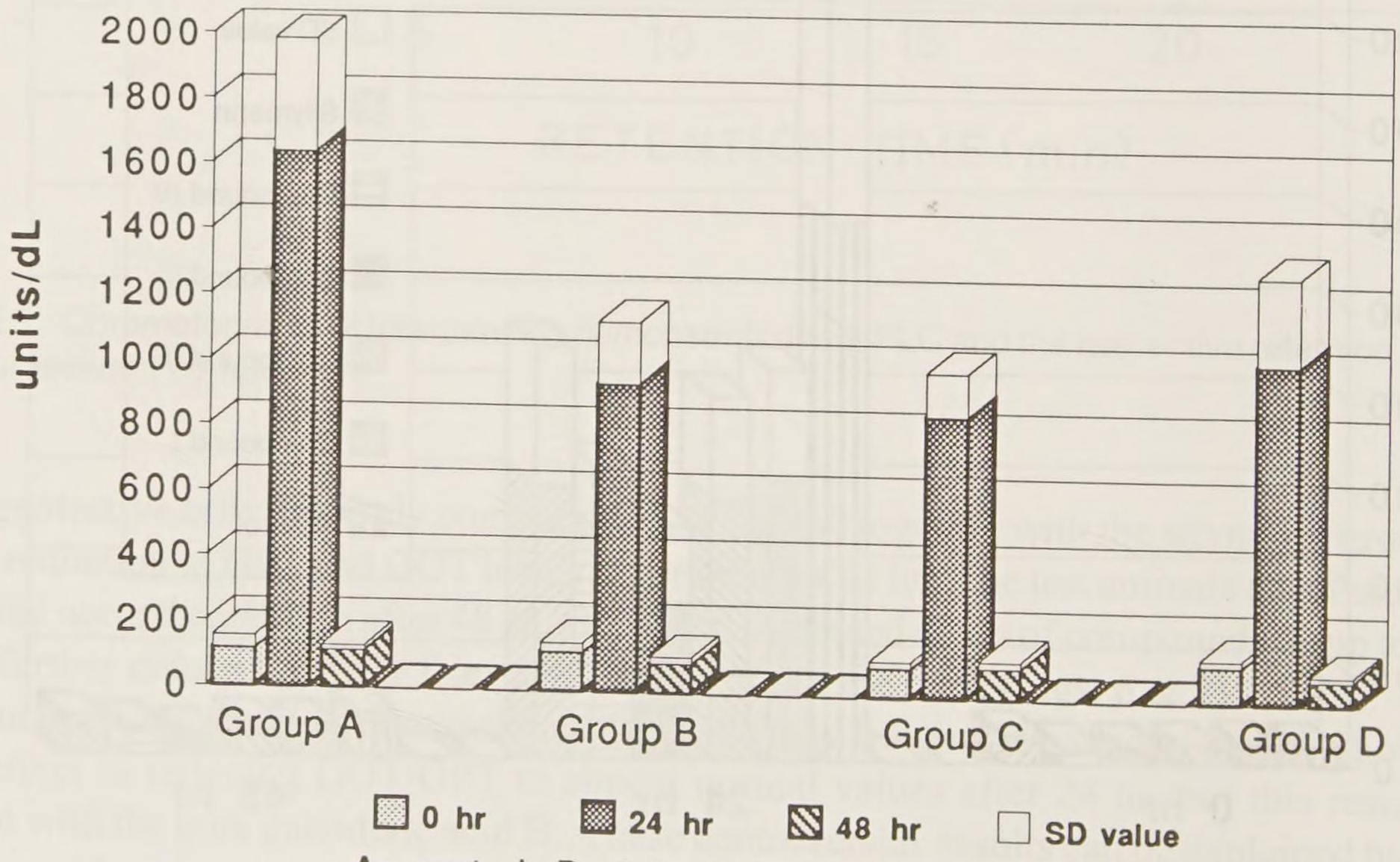


FIGURE 5. The effect of pure compounds isolated from *Ganoderma tsugae* for GOT in mice induced by CCl_4.



A: control, B: 1.0 mg/kg, C: 0.4 mg/kg, D: 0.1 mg/kg

FIGURE 6. Dose response for compound III with CCl_4 induced GPT in mice.



A: control, B: 1.0 mg/kg, C: 0.4 mg/kg, D: 0.1 mg/kg

FIGURE 7. Dose response for compound III with CCl_4 induced GOT in mice.

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REFERENCES

ADASKAVEG, J. E. & GILBERTSON, R. L. (1986). Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to taxonomy of the *G. lucidum* complex. *Mycologia* **78**, 694-704.

HIROTANI, M., FURUYA, T, & SHIRO, M. (1985). A ganoderic acid derivative, a highly oxygenated lanostane-type triterpenoid from *Ganoderma lucidum*. *Phytochemistry* **24**, 2055-2062.

HIROTANI, M., INO, C., FURUYA, T. & SHIRA, M. (1986). Ganoderic acids T, S, and R, new triterpenoids from the culture mycelial of *Ganoderma lucidum*. *Chemical and Pharmaceutical Bulletin* **34**, 2282-2285.

KOHDA, H., TOKUMOTO, W., SAKAMOTO, K., FUGII, M., HIRAI, Y., YAMASAKI, K., KOMODA, Y., NAKAMURA, H., ISHIHAR, S. & UCHIDA, M. (1985). The biological active constituents of *Ganoderma lucidum*, histamine release-inhibitory triterpenes. *Chemical and Pharmceutical Bulletin* **33**, 1367-1374.

KOMODA, Y., NAKAMURA, H., ISHIHARA, S., UCHIDA, M., KOHDA, H.& YAMASAKI, K., (1985). Structures of new triterpenoid constituents of *Ganoderma lucidum*, a polyporeceae, *Chemical andPharmaceutical Bulletin* **33**, 4829-4835.

LIN, J.& SHIAO, M. (1988). Seven new triterpenes from *Ganoderma lucidum*. *Journal of National Products* **51**, 918-924.

MORIGIWA, A., KITABATAKE, K., FUJIMOTA, Y.& IKEKAWA, N. (1986). Angiotensin converting enzyme inhibitory triterpenes from *Ganoderma lucidum*. *Chemical Pharmacology* **34**, 3025-3028.

NISHITOBA, T., SATO, H. & SAKAMURA, S. (1987a). Triterpenoids from the fungus *Ganoderma lucidum*. *Phytochemistry* **26**, 1777-1784.

NISHITOBA, T., SATOH, H., SHIRASU, S. & SAKAMURA, S. (1987b). Novel triterpenoids from the mycelial mat at the previous stage of fruiting body of *Ganoderma lucidum*. *Agricultural and Biological Chemistry* **51**, 619-622.

SHIAO, M,.& LIN, L.K. (1988). NMR study on two diastereometric triterpenes from *Ganoderma lucidum*. *Proceedings of the National Council, Republic of China (A)* **12**, 10-22.

SU, C. H. (1991). Taxonomy and physiologically active compounds of *Ganoderma*. *Journal of Taipei Medical College* **20**, 1-16.

CHAPTER 30

IMMUNOMODULATORY ACTIVITIES OF MUSHROOM MYCELIAL EXTRACTS

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1. INTRODUCTION

Mushrooms are a special group of higher fungi which are considered as a delicious health food in Hong Kong because the nutritive value of high-grade mushrooms almost equals that of milk (Chang & Miles, 1989). They also contains high levels of essential amino acids and are particularly rich in lysine and leucine, unsaturated fatty acids, carbohydrates and many vitamins. Their mineral content is higher than that of meat and fish and almost twice that of vegetables. In addition, mushrooms have long been considered to have medicinal value and are devoid of undesirable effects (Sakagami *et al*., 1991). These special features might be one of the major rationales why the United States National Cancer Institute selects fungi as a source of new drugs. Mushroom extracts have been reported to have hematological, antiviral and antitumor, hypotensive and hepatoprotective effects (Chang & Miles, 1989). In the past two decades, a group of protein-bound polysaccharides have been extracted from an edible mushroom, *Coriolus versicolor*, by scientists in Japan and China, and they are considered by the United States National Cancer Institute to have antitumor activities (Jong & Donovick, 1989). Our previous studies have indicated that the polysaccharopeptide isolated from *Coriolus versicolor* is an immunomodulator which activates the production of antitumor molecules, tumor necrosis factor (TNF), and reactive nitrogen and oxygen intermediates (RNI's, ROI's) by mouse peritoneal macrophages (Liu *et al*., in press). However, most of these protein-bound polysaccharides are extracted from fruitbodies which might be affected by environmental contamination. In order to avoid probable environmental impact on the quality of the mushroom, submerged culture of the mushroom mycelium is recommended to ensure the production of immunopotentiating agents of a consistent quality. We have selected 11 overseas and local mushroom species for submerged mycelial culture, and the preliminary data indicate that the substances released into the culture media possess no cytotoxicity but some of them can activate the defensive cells such as macrophages and lymphocytes.

2. MATERIALS AND METHODS

2.1. Submerged Culture of Mushroom Mycelium

The mycelia were grown in the laboratory in liquid medium at pH 4.5-6 and at 25-30°C for 7 days and their growth rates were monitored. The media were filtered and the filtrates (MCMs) were lyophilised, weighed and reconstituted in mammalian cell culture media RPMI 1640 (GIBCO, USA) culture medium supplemented with 10% fetal bovine serum and antibiotics before use in bioassays.

2.2. Immunological and Antitumor Bioassays

2.2.1. In vitro assays. The filtrate powders were re-dissolved in RPMI 1640, and their cytotoxicity and antitumor actitivity were tested using a wide range of cell lines, including L929 (mouse fibroblast cell line), H3B (mouse hepatoma, ATCC), E367 (mouse neuroblastoma, a gift from Dr. Stark, University of Duesseldorf, Germany) and JAR (human placenta choriocarcinoma, ATCC HTB 144) in a humidified atmosphere of 5% CO_2 for 24 h. The viable cells were stained with crystal violet and their optical density were measured by a microplate ELISA reader (Liu & Ng, 1991). Mouse peritoneal macrophages were collected from male inbred BALB/c mice and incubated with a range of MCMs for 24 h before they were used to measure the production of reactive nitrogen intermediates.

2.2.2. In vivo assays.

i) Macrophages

Inbred male BALB/c mice were provided with three selected MCMs (#4, #19 and #20) in drinking water for 2 weeks and the macrophages were collected for RNI bioassay (Keller et al., 1990, Liu *et al*., 1993).

ii) Lymphocytes

Spleens were removed aseptically from the MCM-treated mice and splenocytes obtained by gently pressing of the spleens through a 100-mesh stainless steel sieve to obtain a single cell suspension. The cells were counted and resuspended in culture medium RPMI for proliferative response to the mitogen, concanavalin A (Liu *et al*., 1989).

3. RESULTS

The mycelia of 11 overseas and local mushroom species have been selected for submerged culture. The growth of the mycelium depends on a wide range of growth conditions, including the type of culture medium, pH, temperature, cultivation period and the shaking speed. The growth rate of these mycelia were different (Fig. 1). An aliquot of each of these submerged mycelial culture media (MCM) has been subjected to the cytotoxicity test and none of them at concentrations below 1 mg/ml possessed *in vitro* cytotoxic effects on normal fibroblast cell line (L929) or tumor cell lines (Figs. 2 & 3). However, at a dose of 10 μg/ml they augmented the production of antitumor molecules, such as reactive nitrogen intermediates, by mouse peritoneal macrophages (Fig. 4). They also stimulated the proliferative response of mouse splenocytes to concanavalin A (Fig 5). It is of particular interest that the MCM with the most potent biological activity (sample #20) is a local *Tricholoma* species which may be unique to Hong Kong. Its immunostimulatory activity is higher than those of the most widely used species, *Coriolus* (sample #19) and *Ganoderma* (sample #4).

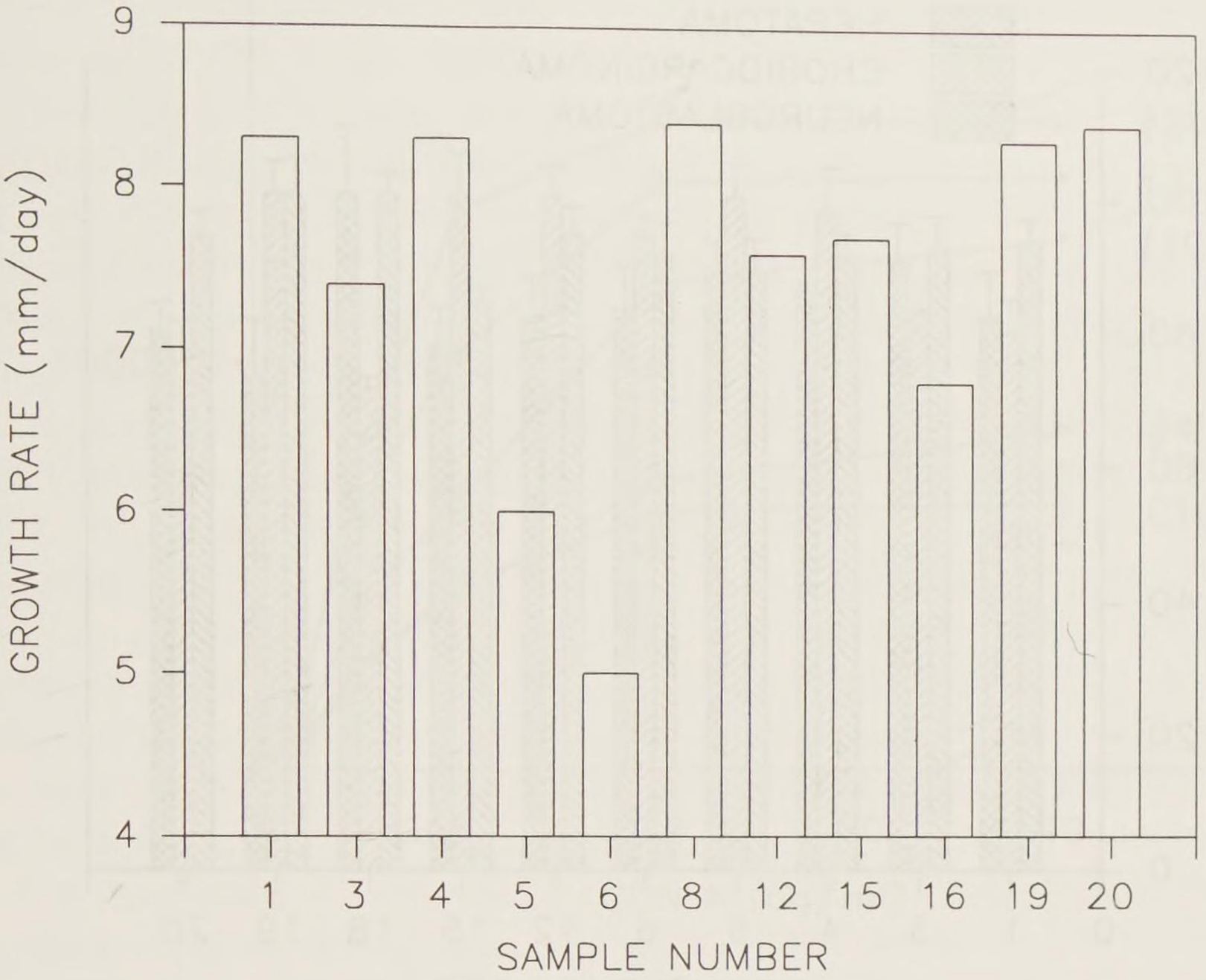

FIGURE 1. Growth rate of selected mushroom mycelia.

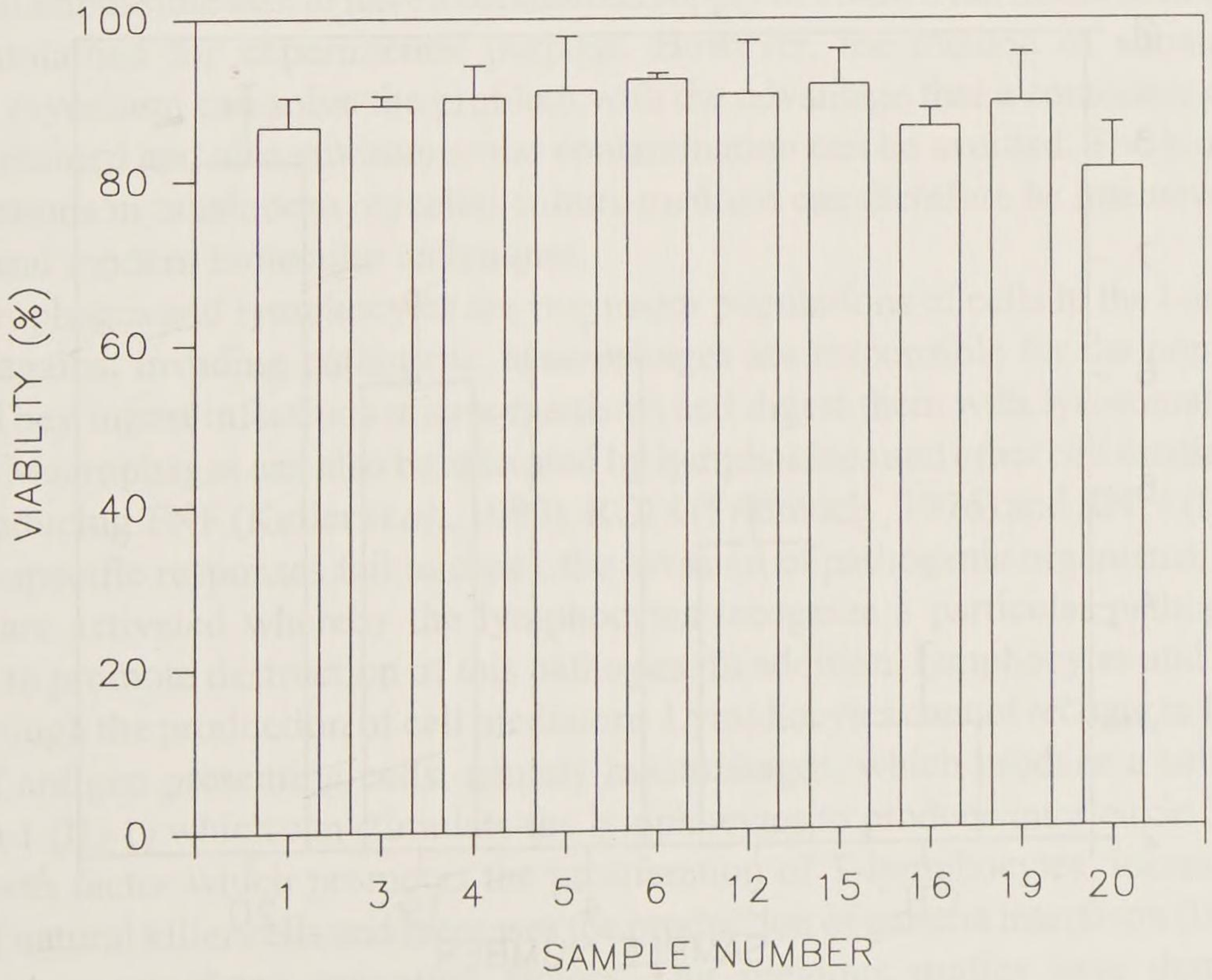

FIGURE 2. Viability of fibroblasts incubated with MCMs (1mg/ml).

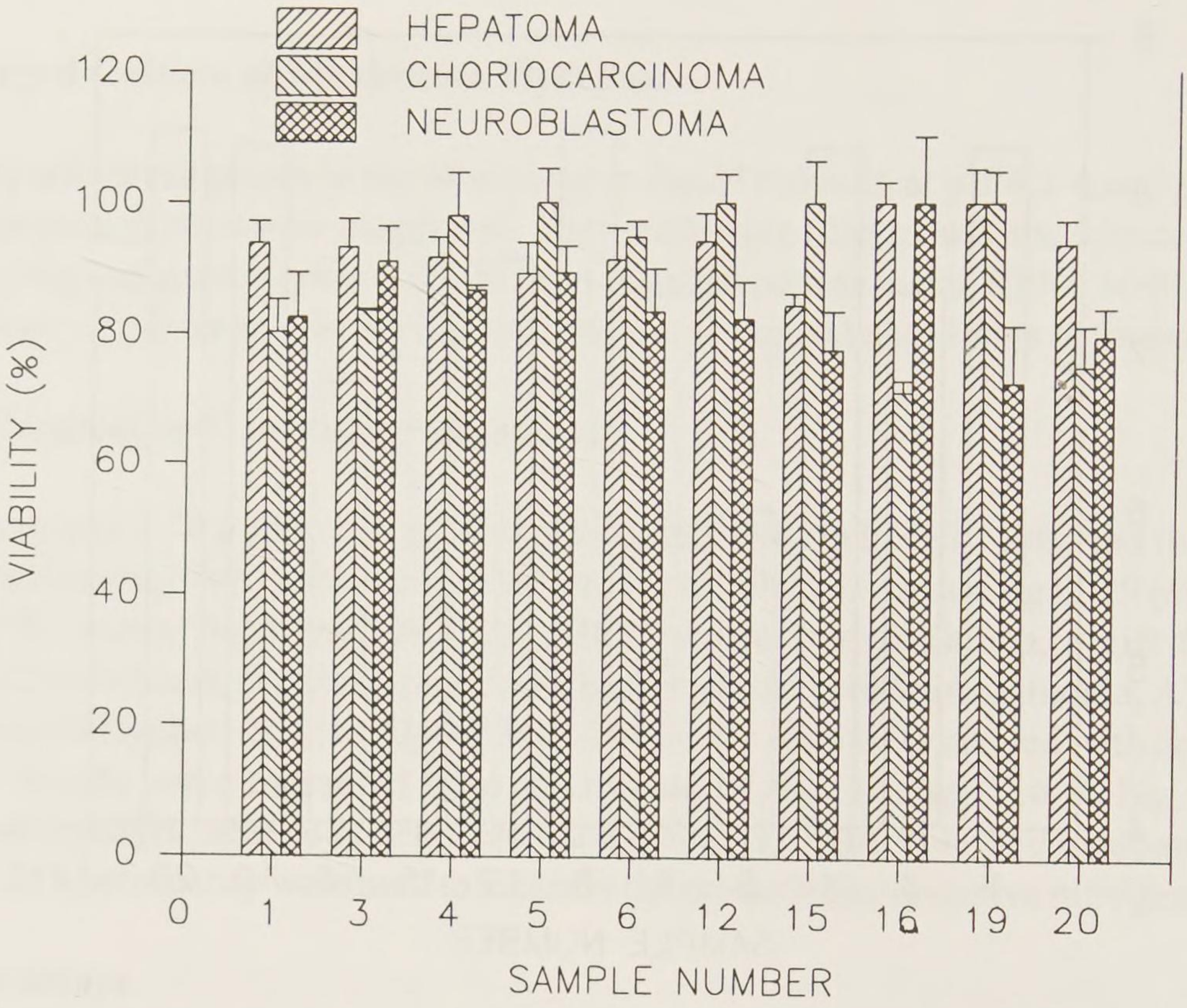

FIGURE 3. Viability of tumor cell lines incubated with MCMs.

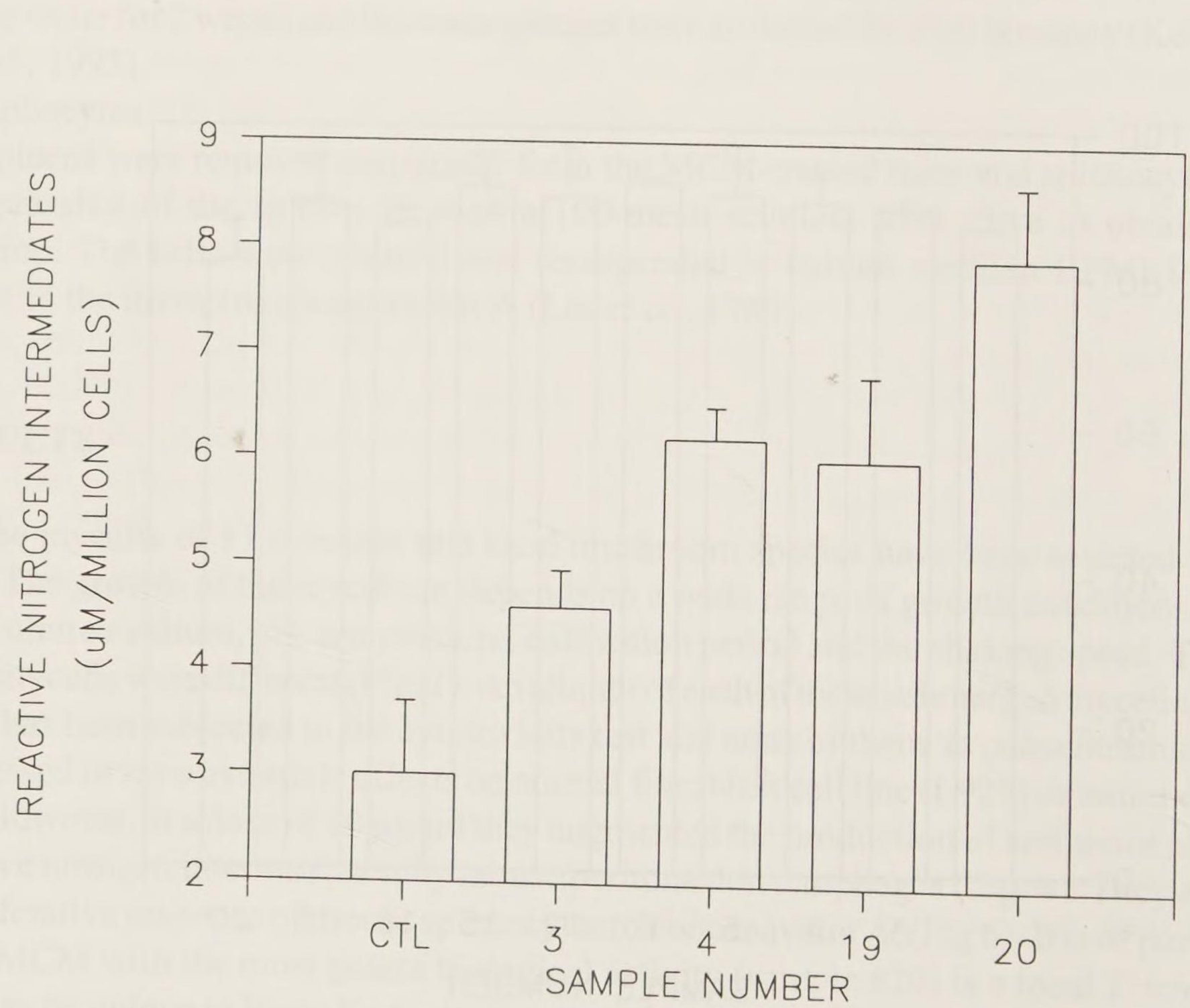

FIGURE 4. Production of reactive nitrogen intermediates by peritoneal macrophages from mice treated with MCMs for 2 weeks.

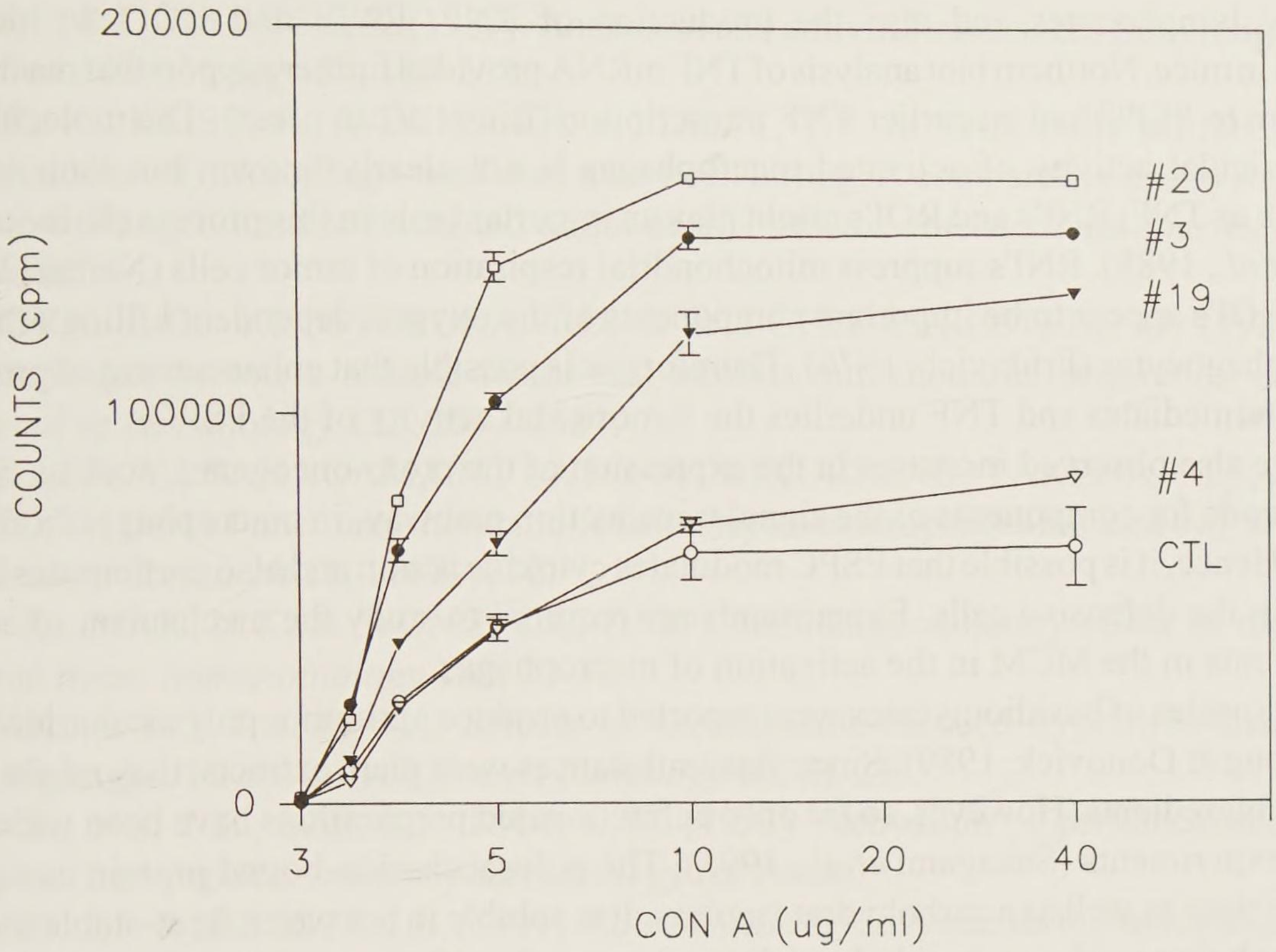

FIGURE 5. Mitogenic responses of splenocytes from mice treated with selected MCMs for 2 weeks.

4. DISCUSSION

It is an impossible task to have a continuous supply of 10-30 fresh mushroom species of various origins maintained for experimental purpose. However, the method of submerged culture of mushroom mycelium can solve the problem with the advantage that a consistent culture condition can be maintained and also environmental contamination can be avoided. The biological activities of the secretions in mushroom mycelial culture medium can therefore be intensively studied using bioassays and modern molecular techniques.

Macrophages and lymphocytes are two major populations of cells in the host defense system which act against invading pathogens. Macrophages are responsible for the non-specific cellular response. They ingest infectious microorganisms and digest them with lysosomal enzymes (Liu & Ng, 1991). Macrophages can also be activated by lymphokines and other cell mediators to kill tumor cells by producing TNF (Keller *et al*., 1990), ROI's (Fridovich , 1976) and RNI's (Hibb *et al*., 1988). When non-specific responses fail to check the invasion of pathogenic organisms, specific immune responses are activated whereby the lymphocytes recognize a particular pathogen and generate antibodies to promote destruction of this pathogen. In addition, lymphocytes and macrophages co-operate through the production of cell mediators. Lymphocytes cannot recognize free antigen in the absence of antigen presenting cells, namely macrophages, which produce a cell mediator called interleukin 1 (IL-1) which can stimulate the lymphocytes to produce interleukin 2 (IL-2). IL-2 is a T cell growth factor which promotes the proliferation of T-lymphocytes, increases the cytotoxic capacity of natural killer cells and increases the production of gamma interferon (IFNγ) which is one of the major macrophage activating factors. Our previous studies have demonstrated that a polysaccharide-protein complex (PSPC) isolated from *Coriolus* augmented the production of IL-2

and IFNγ by lymphocytes and also the production of TNF, RNI's and ROI's by peritoneal macrophages in mice. Northern blot analysis of TNF mRNA provided further support that macrophages upon exposure to PSPC had an earlier TNF transcription (Liu *et al.*, in press). The molecular basis for the tumoricidal activity of activated macrophages is not clearly known, but their secretory products, such as TNF, RNI's and ROI's might play an important role in this process (Kilbourn *et al.*, 1984; Ding *et al.*, 1988). RNI's suppress mitochondrial respiration of tumor cells (Nathan & Hibbs, 1991) while ROI's appear to be important components of the oxygen-dependent killing of ingested microbes by phagocytes (Fridovich, 1976). Therefore, it is possible that enhancement of production of reactive intermediates and TNF underlies the tumoricidal activity of the PSPC.

We have also observed increases in the expression of the proto-oncogenes, such as *c-fos* and *c-myc* which code for components of the signal transduction pathway, in macrophages from PSPC-treated mice. Hence, it is possible that PSPC modulates cytokine action and also participates in signal transduction in the defensive cells. Experiments are required to study the mechanism of action of active ingredients in the MCM in the activation of macrophages.

Several species of basidiomycetes were reported to produce antitumor polysaccharide-protein complexes (Jong & Donovick, 1989). Since these substances were plant extracts, they might contain several active ingredients. However, so far only unfractionated preparations have been widely used in almost all experiments (Sakagami *et al.*, 1991). The polysaccharide-bound protein comprises a polypeptide moiety as well as a carbohydrate moiety. It is soluble in hot water, heat-stable and light-stable. Although these polysaccharide-bound proteins are classified as antitumor chemicals by the United States National Cancer Institute (Jong & Donovick, 1989), the molecular basis of their biological activity is largely unknown. Experiments are in progress to identify the active ingredients and the mechanism of action of these antitumor substances.

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REFERENCES

CHANG, S.T. & MILES, P.G. (1989). *Edible Mushrooms and Their Cultivation*. Florida: CRC Press.

DING, A.H., NATHAN, C.F. & STUEHR, D.J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *Journal of Immunology* **141**, 2407-2412.

FRIDOVICH, I. (1976). In *Free Radicals in Biology, vol 1*, pp. 239-277. Edited by Pryor WA. New York: Academic Press.

HIBBS, J.B. JR., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical and Biophysical Research Communications* **157**, 7-94.

HIRASE, S., NAKAI, S., AKATSU, T., KOBAYASHI, A., OOHARA, M., MATSUNAGA, K., FUJII, M. & OHMURA, Y. (1970). Studies on antitumor activity of polysaccharides. *Proceedings of the 29th Annual Meeting of the Japanese Cancer Association*. p.288.

JONG, S.C. & DONOVICK, R. (1989). Antitumor and antiviral substances from fungi. *Advances in Applied Microbiology* **34**, 183-262.

KELLER, R., KEIST, R., WECHSLER, A., LEIST, T.P. & VAN DER MEIDE, P.H. (1990). Mechanisms of macrophage-mediated tumor cell killing: A comparative analsysis of the roles of reactive nitrogen intermediates and tumor necrosis factor. *International Journal of Cancer* **46**, 682-686.

KILBOURN, R.G., KLOSTERGRAARD, J. & LOPEZ-BERESTEIN, G. (1984). Activated macrophages secrete a soluble factor that inhibits mitochondrial respiration of tumor cells. *Journal of Immunology* **133**, 2577-2581.

KURAKATA, Y., SAKAGAM,I H., SATO, A., KIKUCHI, K., TAKEDA, M., ASANO, K. & SATO, T. (1991). Functional maturation of monocytes/macrophages induced by PSK subfractions. *Anticancer Research* **11**, 1767-1772.

LIU, W.K., MAK, N.K. & WONG, C.C. (1989). Immunomodulatory effect of methimazole on inbred mice. *Immunobiology* **180**, 23-32.

LIU, W.K. & NG T.B. (1991). Effects of methimazole-induced hypothyroidism on alveolar macrophages. *Virchow Arch B. Cell Pathology* **60**, 21-25.

LIU, W.K., NG, T.B., SZE, S.F. TSUI, K.W. (1993). Activation of peritoneal macrophages by polysaccharopeptide. *Immunopharmacology* (in Press).

NATHAN, C.F. & HIBBS JR. J.B. (1991). Role of nitric oxide synthesis in macrophage antimicrobal activity. *Current Opinion in Immunology* **3**, 65-70.

NAKAI, S., AKATSU, T., KOBAYASHI, A., OOHARA, M., MATSUNAGA, K. FUJII, M & OHMURA, Y. (1970). *Proceedings of the 29th Annual Meeting of the Japanese Cancer Association*. p.288.

PICK, E. & MIZEL, D. (1981). Rapid microassays for the measurement of superoxide and peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods* **46**, 211-226.

SAKAGAMI, H., AOKI, T., SIMPSON, A. & TANUMA, S. (1991). Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK (review). *Anticancer Research* **11**, 993-1000.

YANG, Q.Y., YONG, S.C. & YANG, X.T. (1987). In *Report on the polysaccharide-peptide (PSP) of Coriolus versicolor*. pp.1-6, China: Landford.

CHAPTER 31

ADVANCES IN THE PHARMACOLOGY OF *TREMELLA* POLYSACCHARIDES

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1. INTRODUCTION

Tremella, also called Yin er (銀耳) or white jelly fungus, is the dried sporophore of *Tremella fuciformis* Berk. of the Basidiomycetes. It has long been valued in folk medicine as a remedy with nutritive and tonic action in treating debility and exhaustion. It is used in traditional Chinese medicine to nourish yin (陰) essence and moisten the lung, tonify the stomach and promote secretion of saliva. It is now well recognized that *Tremella* polysaccharides (TP) are bioactive components extracted from *Tremella fuciformis* by sequential hot water and ethanol extraction. TP is a protein-binding polysaccharide which consists of mannose, glucuronate, xylose, fructose and glucose with a molecular weight of 3 X 10^5 and is water-soluble (Zhang & Hong, 1984). In order to clarify the therapeutical principle of TP, we have studied the pharmacological effects of TP using modern techniques. The results indicate that TP has various pharmacological effects which are described below.

2. ANTITUMOR EFFECT

Animal experiments have shown that TP exhibited antitumor activities *in vivo*. Zhou *et al* (1987) observed antitumor activity of TP on Ehrlich ascites carcinoma in mice *in vivo* and *in vitro*. It was found that TP (100 mg/kgXd ip for 9 days) significantly decreased the ascites content and inhibited [^{3}H]TdR incorporation into DNA of tumor-bearing mice. On the other hand, TP (100 μg/ml) exerted no distinct effect on [^{3}H]TdR incorporation into DNA of tumor cells *in vitro*. These findings show that TP has antitumor activity *in vivo*, but has not cytotoxic activity *in vitro*. It is believed that the antitumor activity of TP may be related to its immunomodulating effects.

TABLE 1. Effect of TP on phagocytosis of mouse intraperitoneal macrophages.

Group	Dose (mg/kgXd,scXd)	Phagocytic percentage (%)	Phagocytic index
Control	-	30.8±3.5	0.62±0.08
TP	200X4	57.6±3.5***	1.61±0.16***

$\bar{X}$ ±SE, n=12; ***p<0.001 *vs* control.

3. IMMUNOMODULATING EFFECTS

3.1. Effect on Mononucleolar-Macrophage System

Lin and coworkers (1982) studied the influence of TP on the phagocytic activities of intraperitoneal macrophages in mice. The data in Table 1 show that TP (200 mg/kgXd sc for 4 days) markedly increased the phagocytosis of mouse intraperitoneal macrophages on chicken red blood cells (CRBC).

We also demonstrated that TP (100 mg/kgXd sc for 7 days) elevated the clearance rate of intravenous charcoal particles in normal mice and in mice immunosuppressed with cortisone acetate (Lin *et al.*, 1985). The results above suggest that TP can promote the function of the mononucleolar-macrophage system, which may be involved in the antitumor activity of TP.

3.2. Effects on Humoral and Cellular Immunity

Further investigation demonstrated that TP can potentiate humoral and cellular immunity. Lin *et al* (1985) observed the effect of TP on the hemolysin reaction in mice immunized with sheep red blood cells (SRBC). They showed that TP increased the production of hemolysin in SRBC - immunized mice. The same result was seen in mice immunosuppressed with cyclophosphamide(Lin

TABLE 2. Effect of TP on the plaque-forming cell (PFC) response to SRBC in mice aged 3 and 14 months.

Age (month)	Group	Dose (mg/kgXd,ipXd)	PFC/10^6 spleen cells
3	YC	-	1504±420
3	TP	50X5	2671±699**
3	TP	100X5	2734±772**
3	YC	-	1910±473
14	AC	-	995±283**
14	TP	50X5	1464±244++
14	TP	100X5	1148±491

$\bar{X}$±DF, n=6, **p < 0.01 *vs* young control (YC).
++ p < 0.01 *vs* aged control (AC).

TABLE 3. Effect of TP on the plaque forming cell (PFC) response to SRBC in stressed mice.

Group	Dose (mg/kgXd,igXd)	PFC/10^6 spleen cells
Control	-	3068±853
Stress	-	1030±265**
TP+stress	200X8	2894±473++
TP+stress	400X8	2893±538+

$\bar{X}$±SD, n=6,**$p<0.01$ *vs* control
++ $p<0.01$ *v s* stress

et al., 1985). Xia and Lin (1989) studied the effect of TP on the plaque forming cell (PFC) response to SRBC in mice aged 3 and 14 months. TP (50, 100 mg/kgXd ip for 5 days) markedly promoted the PFC response to SRBC in 3 month-old mice. Although the basal PFC response of 14 month-old mice was lower than that of 3 month-old mice, TP (50 mg/kgXd ip for 5 days) clearly enhanced the anti-SRBC PFC response to normal level in 14 month-old mice (Table 2).

Recently, Cui and Lin (1992) observed the effects of TP on immune function in physically-stressed mice. Mice were stressed by swimming in cold water (14±1°C). The maximal decrease of PFC was reached in 9-12 days after stress. The data in Table 3 show that TP (200, 400 mg/kgXd ig for 8 days) can restore the suppressed PFC response in stressed mice.

Xia and Lin (1989) reported the effect of TP on lymphocyte proliferation induced by concanavalin A (Con A) in mice. ConA-induced proliferation was measured by [^{3}H]TdR incorporation into mouse spleen cells from C57BL/6j mice. Table 4 shows that TP (50,100, 150 and 200 μg/ml) significantly increased the ConA-induced lymphocyte proliferation in a concentration-dependent manner. Hydrocortisone can significantly inhibit mouse lymphocyte proliferation induced by ConA. At doses of 25, 50, 100 and 150 μg/ml, TP did not antagonize the suppressive effect of hydrocortisone. It was found that lymphocyte proliferation in 14 month-old mice was lower than that of 3 month-old mice. TP (50 μg/ml) can partly restore the lymphocyte proliferation induced by ConA in 14 month-old mice. TP (100 μg/ml) had no significant effect, but TP (150 and 200 μg/ml) inhibited the lymphocyte proliferation response in 14 month-old mice. This suggests that the optimal dose of TP for lymphocyte proliferation in aged mice was less than young mice.

Cui and Lin (1992) also demonstrated that, at doses of 200 and 400 mg/kgXd ig for 14 days, TP can antagonize the suppressive effect of physical stress on delayed cutaneous hypersensitivity

TABLE 4. Effect of TP on lymphocyte proliferation induced by ConA in mice.

Dose of TP (μg/ml)	[^{3}H]TdR incorporation (cpm)
0	34997±8990
50	58846±8217***
100	64488±11785***
150	72943±10859***
200	81361±11499***

$\bar{X}$±SD, n=12, ***$p<0.001$ *vs* control

TABLE 5. Effect of TP on lymphocyte proliferation stimulated by ConA in physically stressed mice.

Group	Dose (mg/kgXd, igXd)	Uptake of [^{3}H]TdR (dpm)
Control	-	93235±27857
Stress	-	57465±6460*
TP+Stress	200X8	97971±34055+
TP+Stress	400X8	89773±35962

$\bar{X}$±SD, n=6, *p<0.05 *vs* control.
+ p<0.05 *vs* stress control.

(DCH) induced by 2,4-dinitrochlorobenzene (DNCB). As shown in Table 5, lymphocyte proliferation stimulated by ConA was decreased by 38.4% in physically stressed mice. However, TP (200, 400 mg/kgXd, ig for 8 days) can restore the depressed lymphocyte proliferation to normal levels (Cui & Lin, 1992).

3.3. Effect on Interleukin 2 (IL-2) Production and Intracellular Calcium

Ma and Lin (1992) investigated the effect of TP on IL-2 production by mouse splenocytes. TP (1, 5, 10 and 50 μg/ml) significantly increased IL-2 production by mouse splenocytes. In aged mice, the level of IL-2 production was much lower than that in young controls. TP (0.05, 0.5 and 5 μg/ml) restored IL-2 production to the normal levels of young controls (Table 6).

Recently, we examined the effect of TP on intracellular calcium of mouse splenocytes (Cui *et al.*, 1992). The intracellular calcium ($[Ca^{2+}]i$) was measured by the fluorescence probe method with fura-2. Preliminary results revealed that, at concentrations of 8, 24 and 80 μg/ml, TP can increase $[Ca^{2+}]i$ of mouse splenocytes. In the absence of extracellular calcium ($[Ca^{2+}]ex$), TP did not increase $[Ca^{2+}]i$. Therefore, TP may promote transport of $[Ca^{2+}]ex$ into the mouse splenocytes.

4. PROTECTIVE EFFECT ON ARREST OF BONE MARROW

TABLE 6. Effect of TP on IL-2 production by aged mouse splenocytes.

Group	Age (month)	Dose (μg/ml)	[^{3}H]TdR incorporation (cpm)	
YC	3	0	38335±3517	(n=6)
AC	19	0	30572±4394++	(n=6)
AC+TP	19	0.05	39123±1840**	(n=5)
AC+TP	19	0.5	35239±1293*	(n=6)
AC+TP	19	5	35646±2597*	(n=5)
AC+TP	19	50	33682±2137	(n=6)

$\bar{X}$=SD, ++ p<0.01 *vs* young control (YC).,
*p<0.05, **p<0.01 *vs* aged control (AC).

TABLE 7. Effect of TP on arrest of bone marrow caused by ^{60}Co γ–ray irradiation and cyclophosphamide.

Group	Dose (mg/kgXd, scXd)	Myeloid granulocytesX10^{20}	
		^{60}Co γ-ray radiation	Cyclophosphamide
Control	-	10.68±0.48	8.27±0.71
Arrest	-	2.21±0.25**	2.36±0.42***
TP	200X4	6.32±1.37+	4.18±0.62+

$\bar{X}$±SE, n=10, ***p<0.001 *vs* control,
+ p<0.05 *vs* arrest.

Lin, *et al* (1982) have also shown that TP exerted a protective effect on arrest of bone marrow caused by ^{60}Co γ-ray irradiation or by cyclophosphamide. As shown in Table 7, both ^{60}Co γ-ray irradiation and adminstration of cyclophosphamide can decrease myeloid granulocytes. TP (200 mg/kgXd, sc for 3~5 days) can partially improve the arrestment effect on bone marrow induced by ^{60}Co γ-ray irradiation and by cyclophosphamide. These findings indicate that TP may be able to promote hematogenesis in the bone marrow.

5. EFFECT ON THE LIVER

Lin and coworkers (1982) observed the effect of TP on [^{3}H] uridine incorporation into protein, DNA and RNA in mouse liver. This study revealed that TP (200 mg/kgXd, sc for 4 days) increased [^{3}H] leucine incroporation into protein and [^{3}H] uridine incorporation into mouse RNA liver, which suggests that TP can promote the synthesis of protein and RNA in mouse liver cells. However, TP does not exert any influence on DNA synthesis in mouse liver cells (Table 8)

Yue and Cong (1986) reported that TP (200 mg/kgXd, ip or sc for 5 days) increased [^{3}H] leucine incorporation into liver protein and [^{3}H] orotic acid incorporation into RNA in normal and partially hepatectomized mice. But, in both normal and injured liver, TP showed no significant effect on the incorporation of [^{3}H] thymidine into liver DNA. This suggests that promotion of transcription may be the chief mechanism underlying the effect of TP on the synthesis of protein in the liver. However, TP did not alter the glycogen content of liver and did not prevent the decrease in glycogen content induced by CCl_4 or by partial hepatectomy in mice (Yue & Cong, 1986). Cheng *et al* (1984) assessed

TABLE 8. Effect of TP on [^{3}H] leucine, [^{3}H] thymidine and [^{3}H] uridine incorporation into protein, DNA and RNA in mouse liver.

Group	Incorporation of radioactivity (cpm/100 mg liver weight)		
	[^{3}H]Leu	[^{3}H]UR	[^{3}H]TdR
Control	8446±273 (n=10)	4019±241 (n=14)	6087±822 (n=8)
TP	9574±296* (n=10)	5201±458* (n=14)	6236±581 (n=8)

$\bar{X}$±SE, *p<0.05 *vs* control.

the effect of TP on the ultrastructure of liver under the electron microscope using the method of quantitative analysis. The results demonstrated that, after administration of TP (100 mg/kgXd, Sc for 4 days), the rough endoplasmic reticulum (RER) of liver cells was increased from 17% to 26% and liver glycogen increased from 5% to 13%. In contrast, TP decreased the cytoplasmic matrix of the liver cells from 48% to 30%. It is generally considered that the RER can synthesize and secrete protein in liver cells. Therefore, the increase in RER activated by TP may be related to the promoting effect of TP on protein synthesis.

Further evidence revealed that TP (100 mg/kgXd, sc for 7 days) showed no significant effect on the content of cytochrome p-450 in liver homogenates from normal mice but antagonized the decrease in spleen weight and the increase in the content of liver cytochrome p-450 caused by cortisone acetate. At a dose of 200 mg/kgXd, TP remarkably increased spleen weight and reduced the content of cytochrome p-450 in liver homogenates from normal mice. Take together, these findings suggest that the promoting effect of TP on the biosynthesis of nucleic acid and protein is probably an essential mechanism in its liver-protective effect. The effect of TP on the biosynthesis of nucleic acid and protein may also involve the effect of TP on other organs or tissues in the organism (Lin *et al*., 1985).

6. CONCLUSIONS

The studies reviewed in this paper demonstrate the variety of pharmacological effects of TP and provide a theoretical basis for the clinical use of TP. It is believed that TP exerts antitumor effects through its immunomodulating action. TP not only exhibits immunomodulating effects on the humoral and cellular immune functions in normal mice, but also possesses immunorestorative effects on the suppressed immune function in immunosuppressed mice induced by immunosuppressive drugs, antitumor drugs, stress and aging. These results suggest that TP may be used as an immuno-chemotherapeutical or immuno-radiotherapeutical drug to treat cancers, and as an immunorestorative drug to improve immunodeficiency induced by immune deficient disease, including acquired immune deficiency syndrome (AIDS), by stress or by aging. These possibilities need further investigation through clinical trials. Moreover, since nucleic acid and protein play an important role in biological processes, the promoting effect of TP on the biosynthesis of nucleic acid and protein may contribute to the pharmacological mechanism at the cellular and molecular levels.

REFERENCES

CHENG, S., HUANG, J., WANG, Z.M. & LUI, Z. (1984). The effect of Chinese white fungi on ultrastructure of liver cell in mice (quantative analysis by E.M). *Journal of Beijing Medical University* **16**, 208.

CUI, J.Y. & LIN, Z.B. (1992). Effects of *Tremella* polysaccharide on immune function of physically-stressed mice. *Journal of Chinese Pharmaceutical Sciences* **1**, 49.

CUI, J.Y., LIN, Z.B., LUI, Y. & ZHANG, J.T. (1992). Increasing effect of *Tremella* polysaccharide on intracellular calcium of mouse splenocytes. *Information of Chinese Pharmacological Society* **9**, 23.

LIN, Z.B., QIN, Z.L., XIA, H.L., GUAN, H.C. & JIAO, K. (1985). Effects of *Tremella* polysaccharides on immunological function and content of cytochrome p-450 in mouse liver homogenate. *Acta*

Pharmacologica Sinica **6**, 201.

LIN, Z.B., SUN, M.Q., CHAI, B., MA, J.J. & JIAO, K. (1982). Effect of *Tremella* polysaccharides on the activity of macrophages, hemogenic function of bone marrow and synthesis of protein and nucleic acid. *Journal of Traditional Chinese Medicine* **23**, 389.

MA, L. & LIN, Z.B. (1992). Effect of *Tremella* polysaccharide on IL-2 production by mouse splenocytes. *Acta Pharmacologica Sinica* **27**, 1.

XIA, D. & LIN, Z.B. (1989). Effect of *Tremella* polysaccharides on immune function in mice. *Acta Pharmacologica Sinica* **10**, 453.

YUE, W. & CONG, Z. (1986). Effect of *Tremella* polysaccharides on synthesis of protein and on glycogen content in normal and injured livers of mice. *Acta Pharmacologica Sinica*. 7, 364.

ZHANG, Y.J. & HONG, Z. (1984). The study of isolation and properties of *Tremella* polysaccharides. *Journal of Beijing Medical University* **16**, 83.

ZHOU, A.R., WU, Y.K. & HOU, Y.Y. (1987). Studies on antitumor activity of *Tremella* polysaccharides. *Journal of Beijing Medical University* **19**, 150.

CHAPTER 32

ANTITUMOR COMPONENTS OF *COLLYBIA*

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1. INTRODUCTION

Although most of the mushrooms that belong to the genus *Collybia* are edible, they have never been extensively studied for their metabolites. Among the species that are indigenous to Korea, *Collybia confluens* (Fr.) Kummer is fairly common. As part of our research project, we selected to examine the components of this mushroom. When it was examined for pharmacological activity, it showed anititumor activity. In this paper we report the antitumor components and their effects on the immunity of mice.

2. METHODS

2.1. Separation of Antitumor Components

After the mycelia of *Collybia confluens* were grown in a fungal medium of glucose, peptone, yeast extract and seven mineral salts on a rotary shaker, about 3.1 kg (wet weight) of the mycelium were harvested from 50 liters of the culture broth. The high molecular weight components of the mycelia were extracted according to the method of Kim *et al.* (1992). These components of Fraction A were further purified by DEAE cellulose column chromatography and were separated into Fractions B and C. Fraction C was named collyban and was separated by Sephadex G-200 gel filtration into Fractions C-I and C-II.

2.2. Antitumor Test

The tumor cells of S180 were inoculated into the left groin of ICR mice. Those mice in which the solid form of the tumor cell grew were used in the antitumor test. These mice were divided into two groups: control and treated. Two different doses of each fraction were injected daily into the treated group for 10 days, whereas the control group was injected only with saline. After 30 days, the tumors were excised and weighed. The inhibition ratio was calculated by comparing the tumor

weights of the control and treated groups.

After HeLa cells were inoculated into nude mice of BALB/C strain, respective doses of 50 mg and 100 mg/kg/day of collyban were injected into the mice for seven days. The tumor volumes were measured and compared to calculate the inhibition ratio.

2.3. Effects of Antitumor Components on Cancer Cells

To clarify the mechanisms underlying the antitumor activity of the components of the edible mushrooms, their direct cytotoxicity on cancer cells was examined. For this test, S180, P388, L1210 and HeLa cell lines were used (Carmichael *et al.*, 1987).

2.4. Effects of Antitumor Components on Immunity

When macrophages are activated, they release superoxide anion, which can be measured by using colorimetry. The coloration is achieved by glucose, ferricytochrome C and opsonized zymosan. Effects of collyban on macrophage activation were compared according to the method of Richard *et al.* (1975).

Since macrophages also release acid phosphatase when activated, the degree of the activation by collyban was measured according to the method of Suzuki (1989). The effects of collyban on the activity of B lymphocytes were examined by using sheep red blood cells (SRBC) and ICR mice according to the method of Kwag *et al.* (1992). Effects of collyban on delayed-type hypersensitivity (DTH) were evaluated by using SRBC and ICR mice according to the method of Kwag *et al.* (1992). The influence of collyban on natural killer (NK) cells was assessed by the method of Kiessling *et al.* (1976).

3. RESULTS

The tumor inhibition ratios of the five fractions ranged from 46% to 75% against the solid forms of sarcoma 180 in ICR mice at doses of 20 and 59 mg/kg/day when given intraperitoneally. Fraction C especially, which was named Collyban, exhibited a marked life-prolonging effect against ascitic forms of sarcoma 180 at a dose of 50 mg/kg via intraperitoneal administration. To extend the spectrum of the antitumor activities and eliminate the effects of allograft rejection, characterization of the antitumor effects of Collyban was performed in syngeneic host-tumor systems. Collyban did not show antitumor activity against L1210 murine leukemia in CD2F1 mice but prolonged their life span against ascitic forms of MM46 carcinoma in C3H/HE mice. Also, it exhibited antitumor activity against human cervical cancer HeLa cells that were xenografted into nude mice having BALB/c genetic backgrounds following intraperitoneal injection at a dose of 100 mg/kg/day.

When Collyban was examined for immunological activity to elucidate the mechanism of its antitumor activity, it did not show any direct cytotoxicity against S180, P388 and L1210 murine tumor cells except the direct action on HeLa cells. Collyban increased the number of peritoneal exudate cells of mice, especially the mature forms of polymorphonuclear leucocytes. From flow cytometric analysis, Collyban apparently induced the increase of Mac-1 positive murine spleen cells in the early stage of tumor growth. Moreover, at the late stage, it restored the decreased percentages of CD4 and CD8 positive spleen cells in the tumor-bearing mice to normal levels.

Collyban increased the production of acid phosphatase and the superoxide anion released from

activated macrophages in mice. It showed the adjuvant effect by increasing the number of the hemolytic plaque forming cells against sheep red blood cells, and also increased the production of alkaline phosphatase which was an index of the proliferation of B lymphocytes in the murine spleen cells. In cellular immunity, Collyban restored the depressed response of delayed type hypersensitivity in the tumor-bearing mice to the normal level. In nude mice, Collyban potentiated the cytotoxicity of natural killer cells which are one of the important nonspecific effector cells.

In order to characterize the antitumor components, Collyban was examined by chemical analysis. It comprised acidic protein-bound polysaccharides composed of 31% polysaccharide, 27% protein and 3% hexosamine. Collyban was fractionated into two fractions, Fr. C-I (M.W. : 500 KD) and Fr. C-II (M.W. : 30 and 80 KD) by Sephadex G-200 gel filtration chromatography.

Collyban showed a direct effect on HeLa cells, activated various immune effector cells and restored the decreased immune response of the tumor bearing host. Therefore, it was concluded that Collyban acts as an immunomodulator.

REFERENCES

CARMICHAEL, J., DEGRAFF, W.G., GAZDER, A.F., MINNA, J.D. & MITCHELL, J.B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay, assessment of chemosensitivity testing. *Cancer Research* **47**, 936-942.

KIESSLING, R., PERTANY, G & KLEIN, G. (1976). Non-T cell resistance against a mouse moloney lymphoma. *International Journal of Cancer* **17**, 275-284.

KIM, B.K., CHUNG, H.S., CHUNG, K.S. & YANG, M.S. (1980). Studies on antineoplastic components of Korean basidiomycetes. *Korean Journal of Mycology* **8**, 107-113.

KIM, B.K., ROBBERS, J.E., CHUNG, K.S., CHUNG, H.S. & CHOI, H.S. (1982). Antitumor component of *Cryptoporus volvatus*. *Korean Journal of Mycology* **10**, 111-117.

KIM, B.K., KWUN, J.Y., PARK, Y.I., BOK, J.W. & CHOI, E.C. (1992). Antitumor components of the cultured mycelia of *Calvatia craniformis*. *Journal of Korea Cancer Association* **24**, 1-18.

KWAG, S.D., BOK, J.W., HYUN, J.W., CHOI, E.C. & KIM, B.K. (1992). Studies on constituents of higher fungi of Korea (LXXIII). Antitumor components of the cultured mycelia of *Paxillus atromentosus*. *Korean Journal of Mycology* **20**, 240-251.

LEE, C.O., CHOI, E.C. & KIM, B.K. (1987). Immunological studies on the antitumor components of *Lyophyllum decastes*. *Journal of Korea Cancer Association* **19**, 57-62.

RICHARD, B.J., BERNARD, B.K. & HARD, P.M. (1975). The role of superoxide anion generation in phagocytic bacterial activity. *Journal of Clinical Investigation* **55**, 1357-1362.

SUZUKI, I., HARSHIMOTO, K., OIKAWA, S., SATO, K., OSAWA, M. & YADOMAE, T. (1989). Antitumor and immunomodulating activities of a β-glucan obtained from liquid-cultured *Grifola frondosa*. *Chemical and Pharmaceutical Bulletin (Tokyo)* **37**, 410-413.

Part V

FUTURE PROSPECTS FOR RESEARCH AND DEVELOPMENT

Part V

FUTURE PROSPECTS FOR RESEARCH AND DEVELOPMENT

CHAPTER 33

PROMOTION OF MUSHROOM PRODUCTION AND BIOCONVERSION OF WASTES FOR INCOME GENERATION IN RURAL AREAS: CDG-SEAPO'S BIOTECHNOLOGY TRAINING PROJECT

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1. INTRODUCTION

International and/or bilateral development assistance takes many different forms, especially when it comes to involving scientific communities for the purpose of small business promotion in usually underprivileged rural areas of developing countries. Being a very complex issue, every attempt of approaching it will turn out to be quite unique. This paper will try to introduce such a novel approach and the respective experiences made.

1.1. Background

Having been convinced in early 1982 of the prospects of biotechnology as "the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services" (Bull *et al.*, 1982), I - as a layman in this field - had the feeling that this emerging "complex web of enabling technologies" (Antebi & Fishlock, 1986) could evolve into a fascinating development potential for developing countries, especially the ones rich in renewable natural resources in the tropics. By "utilizing living organisms or their biological functions" (Tomita, 1990) for generating higher-value added products and services, biotechnology is not only comprising millenia-old simple fermentation technologies for food production, e.g. bread, wine, beer, cheese, soy sauce, etc. (Holt, 1990) but also a set of powerful new high technologies with potential industrial applications. It is even perceived as "soft technology" of multidisciplinary nature that 1) saves non renewable resources, 2) causes "lowest possible negative impact on the environment" and 3) would even ensure, euphorically stated, "harmony between human welfare and the economy" (Tomita, 1990).

When I initiated, in September 1985, CDG-SEAPO's involvement in biotechnology promotion

for small enterprise development in rural regions of developing countries in Southeast Asia, this step was taken on the rationale of eventually contributing to some form of eco-development in the long term through the promotion and development of Small and Medium Industries (SMI) in socio-economically poorer rural regions of the developing world (Setty, 1991a). By focussing this overall eco-goal direction of SEAPO's SMI promotion efforts, among others, on biotechnology applications, essentially five major development aspects were to be combined, such as:

(a) SMI support and development (APO, 1992b);
(b) for rural/regional development (Setty, 1991b; APO, 1992a);
(c) through utilization and improvement of simple and cheap traditional biotechnologies (Chaiwongkeit, 1988, Espiritu, 1992);
(d) and their upgrading through transfer and infusion of modern bio-science and technology knowledge (Suwana-Adth, 1990);
(e) to be complemented by practical small entrepreneurship development schemes (Setty, 1991a).

At that time, this specific direction of SEAPO's entering the biotechnology field was based on my assumption that it should be practical, realistic, relevant and timely to the particular development needs, prospects and constraints of the developing world. However, knowing very little about biotechnology itself, that it should work and become a meaningful engagement was then just a hypothesis. Even though some disconcerting signals were received during the following years, I remained convinced being on the right track with the idea. For instance, I encountered some discouraging feedback from a microbiologist friend in Germany (about being unrealistic because of trends there to swallow up all small dairies due to their non-competitiveness against the larger and more modern ones, etc), the quite strong reservation of most of the Thai biotechnologists at an information and planning meeting in Bangkok in September 1986 who emphasized their prime interest in high technology trends (CDG-SEAPO, 1987), the Asian Productivity Organization's unexpected reluctance to take up my initiative of early 1986 in Kuala Lumpur for working together in this newly emerging technology field even though many delegates of the APO member countries then supported the idea (APO, 1986), and the German government's indecisiveness in approving the initiative which lasted about two years.

Nevertheless, the overall very encouraging feedback stimulated during the 1988 Symposium on this topic in Bangkok provided us with the green light for going ahead in the anticipated direction. Already existing working contacts between parts of the science and technology community and small or medium sized producers of soy sauce, edible mushrooms and bio-organic fertilizers were utilized for launching in 1990 the still ongoing "Training and Technology Transfer Project on Application of Biotechnology for Small Industries Development, 1990-1994" (CDG-SEAPO, 1990) in Thailand and the Philippines.

1.2. Rationale of Field Selection

The process of selecting the targeted fields of small biotechnology industries was influenced by existing working contacts of SEAPO with R & D centers and, interrelated with these, the prime interest of the researchers and their respective working experiences with small industries. As a result, even small-scale soy sauce production in Thailand was taken up as one of the fields despite its relative insignificance in economic, social and environmental terms. Nevertheless, the promotion of small -scale mushroom production was on top of the agenda for the following reasons:

(a) Edible mushroom production constitutes a formidable food industry sector with worldwide

production in 1986 estimated at over 2.2 million tons (Chang, 1990);

(b) Mushroom consumption rates in more affluent industrialized countries are rapidly increasing (Zadrazil & Grabbe, 1983);

(c) In tropical developing countries, there exists considerable potential for economic expansion especially in mushroom cultivation (Bergmann *et al*, 1981; Li, 1991); due to

(d) Abundance of easily available agricultural and agro-industrial waste materials since 75% of plant production output remains unutilized biomass most of which can be (re-)used as substrates for mushroom production (Zadrazil & Puniya, 1992);

(e) Capacity of mushrooms to supply a cheap source of protein from organic wastes to alleviate still existent malnutrition in poorer rural regions (Chang, 1990);

(f) High unemployment rates in the rural regions with associated social problems of poverty, poor health, etc. (Ghose, 1992);

(g) Relatively high probability of improving and disseminating small-scale mushroom production in rural areas through injection of compatible advances in biotechnology as well as good housekeeping practices for desired changes in products, processes, substrates as raw (waste) materials and selective spawn production to achieve higher productivity and product quality (Thaithatgoon, 1991); and

(h) Urgent needs for better utilization of natural resources, including agricultural residues which consists mainly of lignocellulosic materials, for environmental protection purposes (Sasson, 1988).

What is exciting about small-scale mushroom production is its high environmental and socio-economic value. It is possible to combine traditional composting technology with modern biotechnology on a small-scale, involving larger numbers of people who generate higher income from putting value to erstwhile organic waste materials. In addition, the spent mushroom substrates provide a good fertilizer, thereby closing the ecological cycle. Edible mushroom cultivation is even considered as "one of the most economically viable bioconversion processes of lignocellulosic residues known for centuries" (Borromeo *et al.*, 1992).

In line with SEAPO's general eco-development orientation, the extension from mushroom cultivation to bio-organic fertilizer production as the major activity areas of the project was entirely logical since both fields have much in common. While edible mushroom growing itself is an economical bioconversion process, usually of otherwise unused organic waste materials, bio-organic fertilizer production also employs fungal species, albeit of different types, e.g. the cellulolytic fungi *Trichoderma* spp., for the enhanced composting of organic residues (Lynch & Wood, 1984). In both cases, abundant organic waste matter is converted to higher-value products: nutritional food (mushroom) and recycled soil-nutrients (bio-fertilizer) resulting in increased food production with lower negative impacts on the natural environment. Both production processes serve the environment in multiple ways.

2. PROJECT FRAMEWORK

To provide an overview of the project set-up, the following deliberations touch upon the salient points.

2.1. Goals and Objectives

The overall goals which the project sets out to achieve are an increased competitiveness and

viability of small biotechnology industries (SBI) on the one hand, and, to make it work, successful commercialization of technology generated by science and technology (S&T) institutions on the other. This is to be accomplished through improving the linkage between the research community and small biotechnology industries and enhancing the R&D capability of S&T institutions. Both should be geared to higher productivity of respective SBI through improved process standardization and scaling-up, better products and service quality through more efficient quality control and packaging, and diversification into new value-added goods and services. To complement the more technical efforts above, improved small business management, product marketing and distribution as well as access to venture capital financing, are additionally intended effects. In order to achieve all of this, the project purpose is twofold: a) improving the production and management capabilities of small and medium mushroom, bio-organic fertilizer and soy sauce producers in Thailand and the Philippines; and, as a prerequisite, b) an improved R&D as well as technology transfer competence of concerned S&T institutions to better utilize their potential contribution to small industries development in rural regions. These two mutually related project objectives constitute the following aspects:

- suitable biotechnology transfer programs for the targeted small industries through technical training and consultancy, together with entrepreneurship development training and support programs;
- manpower development programs for target S&T institutions through advanced training and applied research and consultancy;
- North-South and South-South dialogue, exchange of experiences and cooperation among S&T institutions;
- effective information packaging and dissemination of suitable biotechnology (improved traditional and injected advanced technology) for small industries promotion;
- institutional arrangements to facilitate implementation and management of the project including mechanism(s) for sustainability of project results.

Altogether four distinctive project outputs, derived from the objectives mentioned above, are to be achieved:

(1) The establishment and functioning of four Quality Control and Training Centers (QCTCs) the concept of which is central to the whole project as they have to serve as technology transfer focal points for respective industries and provide the link between their host S&T institutions and the industry as well as other government agencies. Furthermore, they act as project management centers of their respective components, i.e. mushroom, bio-fertilizer or soy sauce;
(2) Staff training and development programs for S&T personnel;
(3) Training and extension programs for SBI personnel;
(4) Information dissemination programs.

2.2. Target Groups

At least two different groups of people to be targeted by the project can be distinguished: 1) the ultimate target group of owner/managers of SBIs as well as their technical staff, if the latter one can be separated at all from the former one in small enterprises, and 2) the intermediate target group of involved S&T personnel, above all at the respective QCTCs as the focal points of the project.

However, a closer look at the project reveals that the intermediate group is even larger than originally anticipated and planned as a number of key staff of various NGOs and cooperatives became

interested in the project and linked up with it, to further disseminate and facilitate needed technology transfer among their members and clients at village level. This has happened in the Philippines especially since late 1991, not only because of strong-felt needs and the interest generated by the project but also because of numerous initiatives taken by the project managers there and the SEAPO project coordinator. Furthermore, to increase and strengthen the outreach capacity of the project, active partnership and cooperation has been sought and achieved with a number of academic as well as promotional institutions in the Philippines. Among these should be mentioned the following:

- Mushroom Training Laboratory of Central Luzon State University (CLSU)
- Extension Office of the Camarines Sur Stage College of Agriculture (CSSAC)
- Department of Science and Technology (DOST), offices in Regions 3, 5, 7 & 11 (i.e. in different parts of the country)
- Technology and Livelihood Resource Center (TLRC)

Additionally, even larger agro-industries, e.g. piggeries, approached the project for help regarding their huge waste loads, i.e. primarily as a matter of economical and environmentally safe waste management and only secondarily as another business venture, but with the side-effect of making bio-organic fertilizer cheaper and, thus, more attractive to small farmers.

Even the ultimate target group consists of more people than just the SBI owners and technical staff. To this group also belong the potential small entrepreneurs in the mushroom or bio-organic fertilizer industries, rural cooperatives, as well as the indirect beneficiaries of employed workforce, whether family members or outsiders.

Thus, both the intermediate and ultimate target groups each represent several layers of actively involved participants besides the even larger groups of more passively benefitting people. If the momentum already gained, especially in the Philippines, can be maintained, strengthened and consolidated, then the positive socio-economic and even environmental impacts must, after all, become noticeable.

2.3. Partner Institutions

The core partner institutions are where the respective QCTCs are located, i.e.

- the National Institutes of Biotechnology and Applied Microbiology, University of the Philippines Los Banos (UPLB-BIOTECH) for both the mushroom and bio-fertilizer components in the Philippines;
- the Center for Biotechnology, Mahidol University (MU-CB) in Thailand for the soy sauce component in Thailand only;
- the Soil Microbiology Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives for the mushroom-cum-fertilizer components of Thailand.

As the mid-evaluation report suggested (Suwana-Adth & Illy, 1993), the former might eventually be shifted to the Arunjik Mushroom Center outside Bangkok.

Besides these project focal points, several other working partnerships with respective extension services of other universities, regional government offices, cooperatives, NGOs and companies have been developed and utilized. This applies, above all, to the project operation and networking in the Philippines.

Last, but not least, the project avails itself of advanced staff training opportunities through the ongoing working relationship with the German Agency for Biotechnology Research (GBF) in Braunschweig which accepts about 2 core staff to its annual Industrial Biotechnology Training Program for the purpose of staff development as well as the tackling of directly relevant applied

research problems.

2.4. Organizational Structure

As already mentioned, the four QCTCs under the leadership of their project managers perform the main role in project implementation and operation. Further essential coordination and steering efforts are provided by SEAPO: its Project Coordinator, the Management Assistant - Administration (above all, for all financial management matters) and the Head.

The two Project Directors, one for each target country, have so far performed more in the role of academic representatives and facilitators of project implementation. Eventual adjustments might be considered in due course.

The National Advisory Boards are important policy support committees comprised of leading representatives of target industries, different government agencies, involved universities and the key project staff who meet once a year in Thailand and the Philippines respectively.

2.5. Project Resources

Being in several respects a pilot project, project funding is fully provided by the German government's Federal Ministry for Economic Cooperation and Development (BMZ) through CDG-SEAPO. Total budget for the period of four years is roughly 2.8 million DM. Although almost all the money, except the portion for evaluation missions, flows through SEAPO, the office itself spends only about 7% of total budget while almost all the rest is handled by the QCTCs which are accountable to SEAPO for their expenditure.

2.6. Methodology

Whereas the overall mission is to generate and strengthen the commercialization of biotechnology for small enterprise development in rural regions of developing countries, such technology transfer is to be effected primarily through suitable technical training, follow-up and consultancy coupled with needed applied research focussing on encountered problems in the SBI sectors. To be more effective, complementary small entrepreneurship development training and support services are also provided.

2.7. SEAPO's Role and Approach

Although I once initiated and secured the specific focus of SEAPO's involvement in the biotechnology field, SEAPO never intended playing more than a supporting role. While SEAPO is concentrating more on the strategic project management, the QCTCs are relatively free to execute their operational project management tasks as they are much closer to the real needs in the countryside. Thus, SEAPO's project input is more of an initiating, guiding and facilitating nature while the QCTCs handle basically indigenous development processes in participating developing countries. SEAPO exerts more a kind of remote control only to keep the whole undertaking going as originally anticipated and mutually agreed upon. By also trying to encourage cross-fertilization, not only between the different project components but also between this biotechnology project and others initiated and managed by SEAPO, synergistic effects could be generated. This happened in many ways, the more notable one of which is the utilization of SEAPO's long experiences in small

entrepreneurship development for respective Project Casework action training on small business management of SBIs, etc. (Tharun, 1989).

Flexible project management is a definite need to better cope realistically with necessary changes not only because of encountered problems but also to fully utilize emerging opportunities. Therefore, the original project plan is primarily used as an overall guideline open to adjustments, not more. This enabled SEAPO to incorporate almost instantly the bio-organic fertilizer component with very encouraging results so far.

Also believing in the full utilization of locally, nationally or regionally available expertise, SEAPO relies primarily on the resources existing in the region. Working partnerships should not only be strengthened but expanded further through additional networking efforts for better technology dissemination results. In line with this is SEAPO's attempt to encourage and intensify South-South dialogue, mutual learning and exchange of experiences and cooperation. As an integral part of this cooperative scheme, study tours of entrepreneurs to the other partner country are arranged and partially supported. For instance, Philippine mushroom producers went recently to Thailand to observe the mushroom industry there and exchange related opinions and expertise with their Thai counterparts.

At the QCTCs, data bases are updated and exchanged. The same applies to the involvement of respective Asian experts.

3. PROSPECTS AND CONSTRAINTS

According to available records and our own observations, quite a number of good results have already been achieved. Tables 1-6 provide a glimpse of the activities carried out by the QCTCs since early 1991 with the exception of QCTC mushroom and bio-organic fertilizer in Thailand which started operation according to plan only in the spring of 1992.

3.1. Reaching and Serving the Ultimate Target Groups

Judging from the results of the surveys carried out by the QCTCs and the Project Coordinator on SBI's needs, the responses received and the number of training activities accomplished up until April 1993, and the respective number of participants trained (see Table 1 for more details), one can assume that the project, in general, is reaching and serving its ultimate target groups. This is further supported by Table 2 giving an account of PR activities and publications. More evidence is provided in Table 5, on follow-up and feedback, and in Table 6 on enterprise support programs.

3.2. Mobilizing and Enabling S&T Personnel for Technology Transfer

The commercialization of biotechnology for the benefit of rural SBI requires not only technology transfer from the usually urban S&T community to the small and medium industries, but also from a more advanced urban to a more traditional rural setting. Assuming that the numerous studies started under the applied research program (see Table 4) are really focussing on the particular needs of cooperating SBIs, then one can also conclude that the achieved R&D results are beneficial to the SBI.

Through intensive networking efforts (see Table 3), the cooperation and partnership of other academic, government and non-governmental organizations, cooperatives, as well as industrial

TABLE 1. QCTC's Training Activities, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
Technical:				
Total no. of training materials:	9	8	2	30
• adjusted	1	-	-	8
• developed	8	8	2	22
Total no. of courses	31			
planned	24		3	8
held	31	32	2	7
Total no. of participants:				
• seminars	209	(1) 60	(2) 525	(1) 135
• training courses (TRC)	335	(32) 301	(2) 67	(7) 122
• individual or small group coaching	5	32	2	13
Grand total number of courses held	31	33	4	8
No. of participants	549	361	594	270
Entrepreneurial:				
No. of training packages developed:				
planned	3	3	3	2
accomplished	2	2	-	-
No. of TOT courses planned	-	1	-	1
held	-	1	-	-
Total no. of participants planned	-	20	30	20
already achieved	-	18	-	-
No. of entrepreneurs' TRC planned	2	2	-	1
held	1	1	-	-
Total no. of participants planned	60	60	-	25
already achieved	36	27	-	-

Legend: BOF = Biofertilizer M = Mushroom
SS = Soy Sauce
PH = Philippines Th = Thailand

associations, is sought and forged for sharing resources for the benefit of all involved. South-South, as well as North-South, dialogue and exchanges, including staff development, are further enabling measures for effective technology transfer and dissemination.

3.3. Securing Sustainability

Considering the fact that some SBI associations were founded already, there is some hope that

TABLE 2. QCTC's PR works and publications, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
Promotional and training materials:				
• newsletters				
No. of issues	9*	9*	3	9
No. of copies distributed	2,250*	2,250*	1,600	1,620
• posters	3	4	-	7
• videos	-	1	-	1
• • held	-	-	-	-
• manuals	2	1		2
• • used in training	2	-	2	-
• Proceedings	1	-	-	-
no. of issue printed/	1	-	-	-
no. of copies distributed/sold	400	-	-	-
Exhibitions: (booth set-up)				
o within own programs	2	3	2	3
o as part of other activities	2	3	-	10
Radio and Broadcast:				
o no. of times QCTC's activities broadcasted	4	2	2	5
Print media:				
o no. of articles prepared/	10	6	1	15
published	6	6	1	15

* As both Philippine project components share the newsletter, the number is the same.

they might carry forward the project's mission. However, it remains to be seen whether these associations will be able and willing to finance QCTC activities later on. While their financial support would be crucial for continuing the work done so far, it would also avoid, or at least minimize, the straying from practicality by the R&D community. Most likely, a tripartite partnership between government, industry and academia as the support base for QCTCs would be one of the best conditions for sustaining their work (Lu, 1989). However, besides paying lip service, nothing has yet come forward from the government side. When I asked deputy ministers of science and technology some time ago what their departments would contribute financially if convinced that the project and its results are really beneficial to their country's socio-economic and environmental development, their replies were evasive. Nevertheless, as most of the work has been carried out by indigenous staff attached to indigenous R&D centers, the only problem would be the funding source after completion of the project. Somehow they might be able to solve this problem.

TABLE 3. QCTC's Networking efforts, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
No. of contacts initiated	70	2	7	74
received	11	123	3	-
monthly average	2.9	7	0.8	2.6
Total	81	125	10	74
No. of meetings with cooperating partners	70	162	-	10
No. of cooperating partners	20			
- agencies				
• national	3	3	3	-
• regional	2	4	4	-
• local	4	3	-	2
- academic institutions	6	2	-	3
- cooperatives	2	5	-	-
- NGOs	2	4	-	-
- companies	1	6	4	74
No. of on-going cooperative arrangements	13	17	1	2
No. of additional partnerships planned, if any	5	-	1	-

3.4. Potential Model Role and Transferability

When UNIDO HQ in Vienna approached SEAPO in summer last year, it was mentioned that they considered the project as a model case for regionalization of their biotechnology program. The aspect of successful commercialization of biotechnology R&D results seemed especially to impress the UN organization (UNIDO, 1992). As the project was designed as a de-centralized cooperative

TABLE 4. Applied research programs, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
No. of studies / started	13	7	7	19
completed	6	3	4	11
Ave. man-months / study	3	-	18	6
Ave. US$ / study	150	-	-	-
Total man-months	18	-	72	66
No. of AR results transferred to industries	2	-	1	11
effective so far	2	-	1	-
No. of field trials started /	1	8	4	-
completed	-	6	2	5

TABLE 5. QCTC's follow-up and feedback, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
Number of inquiries				
received /	841	123	44	440
served	800	112	44	440
Total	841	123	44	440
monthly average	30	7	3.4	15.7
No. of visits to SBIs	43	144	2	71
No. of visitors from:				
- industries	10	51	1	42
- others	280	72	-	86
No. of producers' association established	4	-	-	1
strengthened	-	12	-	-
- national	1	-	1	-
- local / regional	4	-	-	-

TABLE 6. QCTC'sEnterprise Support Program, January 1991 - April 1993.

Services	Center			
	M-Ph	BOF-Ph	M/BOF-Th	SS-Th
Number of enterprises assisted	36			
Total:				
medium /	32	2	1	3
small	0	14	19	70
	4			
No. of newly established business (as result of project)				
Total:	3			
- micro (cottage)	-	1	-	
- small	3	3	1	3
- medium	-	2	-	
- cooperatives	1	5	-	
- part of cooperative	-		1	
- new product line of other business (e.g. piggery)	-	1	-	1 (spore inocula prod'n)
No. of loan guarantees facilitated	-	1	-	
No. of regional demonstration centres planned	4	-	4	
established	1	-	2	

scheme, there exists enough scope for other interested developing countries to link up, provided they bring along the necessary resources.

The overall framework and direction of the project might be easily transferable to other countries as long as they already possess similarly developed S&T institutions to carry out the necessary technology transfer work. Otherwise, quite major adjustments will be required. Concerning the fields targeted, the promotion of mushroom as well as biofertilizer production might be feasible for many countries although detailed conditions and requirements will differ and will have to be taken into account. Lessons learned from the existing pilot project might be very helpful in any such endeavor.

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REFERENCES

ANTEBI, E. & FISHLOCK, D. (1986). *Biotechnology Strategies for Life*. MIT. Cambridge, Massachusetts.

APO (1986). *Proceedings of APO 26th Workshop Meeting of Heads of NPOs in Kuala Lumpur*, 27-39 January 1986.

APO (1989). *Industry-Academic-Government Collaboration for Technology Development*. APO: Tokyo.

APO (1990). *Biotechnology in Asia. Development Strategies, Applications and Potentials*. APO: Tokyo.

APO (1992a). *Rural Poverty Alleviation Programmes in Asia*. APO: Tokyo.

APO (1992b). *Policies and Programmes for Rural Employment Generation in Asia*. APO: Tokyo.

BERGMANN, C. *et al* (1981). Nutzungsmoeglichkeiten moderner Erkenntnisse der Biotechnologie fuer Entwicklungslaender. Forschungsberichte des Bundesministeriums fuer wirtschaftliche Zusammenarbeit, Bd. 18, Muenchen, Koeln, London.

BOI (1992). *The Status of Biotechnology Research and Industry in Thailand*. Office of the Prime Minister, Bangkok, February.

BORROMEO, E.S., DZOGBEFIA, V. & ESPIRITU, B.M. (1992). *Mushroom Group Project Report. International Training Program*. GBF, Braunschweig.

BULL, A.T., HOLT, G. & LILLY, M.D. (1982). *Biotechnology-International Trends and Perspectives*. OECD, Paris.

CDG-SEAPO (1987). *Proceedings of CDG-MU-NCGEB-DIP Information and Planning Meeting on Application of Biotechnology for Small Industries Development in Developing Countries*. Bangkok, 25 September 1986.

CDG-SEAPO (1990). *Project Proposal on Training and Technology Transfer Project on Application of Biotechnology for Small Industries Development*, 1990-1994, Bangkok, March.

CHAIWONGKEIT, D. (1988). *Development of Mushroom Production Industries in Thailand.* Bangkok.

CHANG, S.T. (1990). In *Biotechnology for Mushroom Cultivation in Developing Countries*, pp. 92-97. Edited by Flegel *et al.*

ESPIRITU, B.M. (1992). Bio-organic Fertilizers (BOF): Benefits of Microbial Inoculation in Organic Fertilizers and the Steps to Mass Production. *Paper presented at the National Conference on Conversion of Agricultural and Agro-Industrial Wastes into Fertilizers: Concrete Experiences and Potentials*, 27-28 June 1992. Manila.

FLEGEL, T.W., THARUN, G. & YUTHAVONG, Y. (ed.) (1990). Biotechnology for Small Industries in Developing Countries. *Proceedings of the International Symposium on Application of Biotechnology for Small Industries Development in Developing Countries.* Bangkok, 21-24 September 1988.

GHOSE, A.K. (1992). In *Rural Employment in Asia,* pp. 15-21. Edited by APO.

HOLT, G. (1990). In *Prospects of Biotechnology*, pp. 5-12. Edited by Flegel *et al.*

LI, S. (1991). Marketing Schemes for Mushroom Growers. In *Proceedings of a Mushroom Technology Update Seminar,* pp. 31-33.

LU, W.M. (1989). Industry-University-Government Linkage in R&D Development. In *The ROC Experience*, pp. 23-47. Edited by APO.

LYNCH, J.M., & WOOD, D.A. (1984). Controlled Microbial Degradation of Lignocellulose: The Basis for Existing and Novel Approaches to Composting. In *Composting of Agricultural and Other Wastes*. Edited by J.K.R. Gasser. London.

SASSON, A. (1988). *Biotechnologies and Development*. UNESCO, Paris.

SETTY, E.D. (1991a). *Rural Industrialization, Small-Scale and Cottage Industries in Asia.* UNCRD Working Paper No. 91-2. Nagoya.

SETTY, E.D. (1991b). *Rural Development: Problems and Prospects*. New Delhi.

SUWANA-ADTH, M. & ILLY, H.F. (1993). *A Mid-Term Evaluation of the Training and Technology Transfer Project on Application of Biotechnology for Small Industries Development*. UNIDO/ ABI, Vienna/Freiburg.

THAITHATGOON, S. (1991). Hygiene Practice and Experiences at the Arunyik Mushroom Center. In *Proceedings of a Mushroom Technology Update Seminar*, pp. 17-26. Nakorn Pathom, Thailand.

THARUN, G. (1989). Suitable Action Training for Small Entrepreneurship Development. The Project Casework Approach, *Paper presented at Workshop on "Project Casework Training Approach"*, Bangkok, 17-28 April.

TOMITA, F. (1990). In *New Trends in Biotechnology and Future Potential*, pp. 371-379. Edited by APO.

UNIDO (1992). *The Establishment of the South East Asia Regional Training Center for Commercialization Opportunities in Biotechnology*. Vienna.

ZADRAZIL, F. & GRABBE, K. (1983). Edible Mushrooms. In *Biotechnology,* pp. 145-187. Edited by H.-J. Rehm & G. Reed. Weinheim.

ZADRAZIL, F. & PUNIYA, A.M. (1992). Advances in Mushroom Production Technology and Waste Management. *Paper presented at National Seminar on "Mushroom Industry in Thailand: Production and Waste Utilization"*. Bangkok, 7 August.

CHAPTER 34

PROSPECTS AND PROBLEMS IN COMMERCIALIZATION OF SMALL-SCALE MUSHROOM PRODUCTION IN SOUTH AND SOUTHEAST ASIA

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1. INTRODUCTION

Mushroom production can be a lucrative cottage industry for low-income rural households in developing countries (Lelley, 1988). The activity is labor-intensive, and can provide full- or part-time employment. A small mushroom production business can be established with low capital investment and with minimal requirements for space and equipment. As with any business, availability of inputs (for mushrooms, agricultural wastes/by-products such as straw and manure) and access to markets are essential. In addition, training and a source of spawn are necessary.

The mushroom market has grown rapidly in recent years. In the United States, fresh mushroom production more than quadrupled in 15 years, from 1975-90; total annual production was 743 million pounds (337,727 Mt) in 1991-92; 68% was for the fresh market, and 32% was processed (USDA, 1992). Imports of canned mushrooms was 53,338 Mt in 1989, accounting for about 40% of all processed mushrooms sold. Although button mushrooms (*Agaricus bisporus*) accounted for 99% of U.S. commercial production, specialty mushrooms (shiitake, oyster and others) accounted for about 5% of dollar sales (McClure, 1990). The average price received by *Agaricus* growers was 87.7 cents per pound in 1991-92, whereas the average price for the specialty mushrooms was US$3.87.

In spite of its growth, the U.S. *Agaricus* mushroom industry is highly competitive, and works on slim and declining margins of profit. In the 1980s, two-thirds of the mushroom growers in Pennsylvania, which accounts for 48% of total U.S. production, went out of business. The labor-

1 Appropriate Technology International (ATI) is a not-for-profit development assistance organization, headquartered in Washington, D.C., with projects in Asia, Africa, Latin America, and the Caribbean. ATI works with, and on behalf of, small farmers and entrepreneurs in developing countries to boost productivity and income, foster new enterprises, and generate economic growth. ATI's portfolio of projects in Asia includes the application of biotechnologies, such as biofertilizers, biopesticides, plant tissue culture, and mushroom production, for small farmer benefit.

intensive nature of the business has resulted in the increasing use of low-paid Mexican workers; the poor working conditions have been the cause of recent labor strikes which threaten to drive up production costs, and owners are increasingly worried about foreign competition (Hinds, 1993).

Mushroom production represents an important opportunity for developing countries, particularly in Asia. In 1990, world production of edible mushrooms was estimated at 3.8 million tons, with *Agaricus* spp. accounting for 38% (Chang & Miles, 1991). Between 1986 and 1989/90, the worldwide annual increase in all mushroom production exceeded 24%. The most spectacular increases in production have occurred for the specialty mushrooms. For example, the oyster mushroom (*Pleurotus* spp.), which is more readily grown in tropical climates, had an increase in production of 438% over the three year period. China had a 289% increase in overall mushroom production from 1980 to 1986; export earnings for 1986 were $160 million (Chang & Miles, 1989). Tourism can be a major factor in stimulating the development of local markets, such as in Nepal.

Innovations in cultivation and post-harvest processing also make possible new opportunities. For example, growers can use low value agricultural residues instead of hardwood logs for cultivating shiitake (Worrall & Yang, 1992), and fastidious species usually collected from the wild, such as morels or truffles, can now be cultivated, or semi-cultivated in association with trees. New process technologies such as vacuum-drying can be used to prepare a high quality export product.

Non-governmental organizations (NGOs) and development assistance can facilitate the introduction of technologies for mushroom production.

2. THE ROLE OF DEVELOPMENT ASSISTANCE ORGANIZATIONS

In addition to fixed and working capital requirements, a mushroom production facility, and indeed any business, will require other kinds of support, principally in the areas of:

1) Reliable source of inputs/raw materials (for mushroom production, spawn and agricultural wastes);
2) Technical assistance and training;
3) Business skills, including entrepreneurship, administration, and marketing capability;
4) Access to and analysis of markets.

Indeed, it is likely that the most serious obstacles to business success will arise from these areas, and it is particularly here that assistance may be of the most benefit. These areas should ideally be analyzed and presented in a business plan prior to investment, since they contribute so much to the success or failure of a venture. However, access to the information necessary for adequate analysis is often not readily available.

In the case of very small (single owner/operator) facilities, which might be desirable for mushroom production in a developing country, the producers can meet some of the requirements for success through informal cooperative efforts or by formal cooperatives, which combine their skills, and by linkages to public institutions such as universities and/or agricultural institutes for technical assistance and spawn.

Both information and low-cost capital are in very limited supply in the rural areas of most developing countries. In summary, a development assistance organization can play a catalytic role in small business development in a variety of ways:

- Providing expertise to help develop a business plan;
- Improving access to investment capital, either through grants, loans, or venture capital;

- Fostering linkages to technical expertise in universities and research institutions, both in-country and internationally;
- Supporting technical training programs;
- Stimulating and improving business skills and entrepreneurship through several avenues, such as training, guidance, monitoring and evaluation;
- Assisting with market analysis.

3. MUSHROOM PROJECT ACTIVITIES IN THE PHILIPPINES

In 1984, ATI developed an experimental program with the Ayala Foundation, Inc. (AFI, and formerly, the Filipinas Foundation, Inc.). Equity investment financing for seed capital, and technical assistance and training costs, were provided for the establishment of five small-scale commercial enterprises. Two of enterprises established under the program were for mushroom production. The purposes of the program were to test a rural small-scale industries strategy aimed at creating jobs and increasing incomes for poor people, by (1) helping set up new manufacturing enterprises that employed innovative appropriate technologies and could eventually be replicated, and (2) developing a new financing mode using a venture capital mechanism. This program and an early case study on the Antipolo project (discussed below) have been discussed by Cuano (1990).

The equity partner, either a local entrepreneur or a community group, was required to invest a minimum of 30% of the required capital, either in cash or in kind; AFI's equity investment share could be up to 70%. A business plan was developed for each venture, including market assessments. Enterprises selected for investment were expected to have a positive cash flow within one year of start-up, and an average internal rate of return of at least 15%. The venture capital company under the auspices of AFI would exit from a project after 5 years, recouping its investment and share of the returns for re-investment in other enterprises.

The two mushroom projects were developed at different times near the cities of Antipolo and Cebu. Both were to involve a medium-scale mushroom enterprise that produced spawn, and small-scale contract outgrowers recruited from local low-income families, who would be provided with training and hardware, and given a guaranteed price for mushrooms produced. The outgrowers would be given an option to buy shares in the company. This linkage was seen as the key to the sustainability and socio-economic benefits of the enterprise after completion of the projects.

3.1. Antipolo

The first mushroom business established was Valley High Products, Inc. (VHPI), located near Antipolo, Rizal, about 30 to 40 km from Metro Manila. The site was selected because it was close to market outlets (Manila, which represented 80% of the total Philippine market), and because the average temperature (25°C) and humidity conditions (70-90%) were judged to be conducive to growing the selected mushroom varieties. Also, the joint venture partner was a local landowner/ entrepreneur, whose contribution to the equity consisted of land, a growing house and some equipment, and labor (as Manager of VHPI). Total venture capital needed was estimated to be a maximum of US$60,300, recoverable in 3.1 years. VHPI began operations in January of 1986.

The business intended to produce *Auricularia* spp. spawn and mushrooms (dried), and then expand to fresh abalone and oyster mushrooms, and possibly button and shiitake mushrooms. The substrate would be composed principally of composted sawdust, rice bran, sugar, and lime. The

technology had been pilot-tested for two years by the AFI's Science and Technology Research Center, and used successfully by other mushroom growers. Actual biological efficiency or yield for the proposed varieties ranged from 40-75% in pilot test studies. For cost-benefit estimates of profitability, 40% was used (estimates for a worst-case biological efficiency of 25% indicated that income would be reduced by 53% and return on equity would decrease from 21.8% to 13.1%).

The market share aim of the enterprise was 14.7% of the total market for these mushrooms; most of the dried *Auricularia* currently available in the market was imported from China and Hong Kong. Market analysis estimated the demand for *Auricularia*, on a fresh weight basis, at 30,200 kg/month (over 80% by restaurants), and for abalone, at 15,500 kg/month. Market growth was estimated at 10-20%/yr. Oyster mushrooms were not yet commercially available; however, limited test marketing was done, which found high consumer acceptance.

The start-up goal was to produce 20,000 spawn bags and 6,000 kg of mushrooms per month (50% *Auricularia*) by the end of the first year, through in-house production (16 workers plus management staff) and outgrower contracts (20). The plan called for in-house production of 50% and 25% in years 1 and 2, respectively; in year 3, 100% of production would be by the 20 contract growers.

After the first quarter of operation, only oyster mushrooms were being produced. However, financial break-even was achieved after 8 months, and the project financial statements showed a loss of only US$142 for the first year of operation. At the end of the first year, monthly production was approximately 9,000 spawn bags and 4,000 kg of oyster mushrooms. The mushrooms were packaged for the market in styrofoam with a clear plastic cover. Since the average biological efficiency achieved was only about 20%, technical corrections were introduced.

In the fifth and sixth quarters, technical problems, attributed to the use of sawdust that had been chemically treated, caused financial losses. After 18 months of operation, no contract growers had as yet been recruited, but negotiations were ongoing. By the end of the second year, however, production levels varied monthly but were still about the same as at the end of the first year. There were now 4 growers who purchased spawn bags.

The project began to incur losses in the third year, as competitors entered the market. Profitability was lower because of a 20% reduction in the market price of fresh oyster mushrooms (although market volume demand appeared to increase). Staff turnover and a prolonged illness of the Manager created problems. In addition, a typhoon destroyed two of the nine growing houses in the second half of 1988. In March 1990, after just over 4 years of operation, a decision was made by AFI to close the venture for the following reasons:

1) The biological efficiency did not improve. It was concluded that the altitude was too high and the climate was not conducive to oyster mushroom production;
2) The enterprise continued to be a financial drain. A total of US$42,100 had by now been invested;
3) The social objectives were not being achieved. This was partly due to the resistance of the venture capital partner to having contract growers, who were perceived as a threat to profitability.

3.2. Cebu

The second mushroom business, Cebu Farmers Agri-Venture Corporation (CFAVC), started commercial operations in April 1991. It is located about 12 km from Cebu City, and employs 13 people. CFAVC produces *Volvariella* spawn and mushrooms, which are suitable to the local climate. The straw mushroom substrate is simple, consisting of outdoor raised beds of dried banana leaves and dirt, designed to encourage more easier involvement of contract growers.

The equity partner (30%) is a local NGO which shares the same social objectives as ATI and AFI, and can monitor day-to-day operations. The initial capital stock was set at US$50,000 (in cash and kind). In the business plan, the target was to recruit and train 200 contract growers. In-house growing was intended to serve as a model production area, and contribute about 10% to total production. In addition, the plan called for the outgrowers to hold 80% of the equity in five years time.

A market study in 1987 indicated good potential. There was strong demand locally for straw mushrooms, projected to be 3000 kg per month, while supply was only about 15 kg/day. Technical problems with spawn production by an existing grower appeared to be a significant obstacle to increased availability. The business plan indicated that at the then current price of P80/kg, the venture would become viable by the second year at a production level of 1300 kg/month, assuming a median biological efficiency of 15% (10-20% range).

Production performance in 1991 was unimpressive. A typhoon that struck the Philippines had greatly reduced the available supply of banana leaves for the beds for a period of about nine months. Preventing contamination of spawn by bacteria and other fungi was also a problem. Also, the project manager failed to use the substrate recipe from AFI, and instead used one recommended by the local Department of Agriculture office, which was less productive. In late 1991, the project manager was replaced, and new spawn introduced. In-house production for early 1992 (Feb.-Apr.) increased to an average of about 235 kg/month, with an acceptable biological efficiency of 10-20% (given the simple, outdoor beds). However, drought and heat in late April 1992 contributed to a subsequent production failure in May.

By May 1992, only 10 outgrowers had been contracted, and they were producing about 28 kg/ month. Mushrooms are a non-traditional crop, and local farmers were reluctant to grow them because of unfamiliarity with the product and the technology. Some initial failures in production increased this reluctance. Consequently, additional land was leased for production by CFAVC to sustain the project.

Increased monitoring and a technician were provided in mid-1992 by AFI to further rehabilitate the project technically and socially. A mid-1992 evaluation pointed out particular problems to be addressed: strain selection and maintenance, improving spawn production technology, disease management, improving yield, increasing the number of contract growers, expansion (labor and equipment), and improved packaging, marketing and delivery. The evaluation also recommended that, if expansion occurred, indoor cultivation be implemented (as suggested by the original plan) and equipment improved; otherwise, for a low-cost venture, production should be restricted to 1500 kg/ month and 10-20 outgrowers at a single site, and that more sites in different areas be developed. As of June 1992, approximately $30,000 of the initial capital allocation had been spent. Changes were made to introduce corrective measures, and the project is ongoing as of June 1993; a new evaluation is presently underway.

3.3. Lessons Learned

The business plans and marketing assessments which preceded the projects were important both in the selection of projects and as guidelines to measure success. It is also clear that the Cebu project is attempting to remedy some of the problems uncovered by the Antipolo project experience. Nonetheless, project problems in both cases were related to both quantifiable and non-quantifiable factors, to which more attention should be paid. Perhaps most importantly, the complexity of the projects was underestimated, since so many new and interrelated variables were introduced: in

technology, financing mechanisms, management structure, and social impact.

The mushroom technologies involving spawn production, while having been proven under controlled laboratory conditions and in a very few small-scale commercial activities, are by no means simple. They require a significant amount of technical expertise, e.g., training in sterile technique, and familiarity with mushroom growing conditions, such as substrate mixes, climate, and disease control. These problems plagued both of the projects. At Antipolo, there was no practical evidence that the choice of location would provide good growing conditions. Assumptions regarding production schedules were overly optimistic. It became clear that technical issues must not be underestimated in moving from demonstration and pilot projects to commercialization, and that linkage to a non-profit institution involved with mushroom research is important for troubleshooting technical problems.

The financing mechanism, venture capital, is experimental in the Philippines, and especially so at a small-scale level. Because there has been so little experience with it, a trial-and-error process is expected. For example, choices that prove unsatisfactory can easily be made in terms of project partners. In these particular cases, choosing a project partner with entrepreneurial/management skills, with a social conscience, and at the same time willing to take personal financial risk is a difficult task. Responsibilities, risks and benefits were also poorly defined and/or were difficult to communicate. A simpler supportive financing mechanism, such as low-interest loans, may be more effective, will be easier to understand, and may enlist more committment to the enterprise.

Management and monitoring problems affected both projects, and probably caused the demise of the Antipolo project. Technical problems were not addressed soon enough to be effective. Marketing skills were insufficient. The equity partners were somewhat removed from direct responsibility, benefit and hands-on daily management, and therefore did not have the motivation of a truly profit-motivated entrepreneur. Monitoring by AFI, particularly at Antipolo, was inadequate, and insufficient attention was given to acquainting all parties involved with the complexities of the project.

Finally, the projects were not purely business ventures, but had the principal objective of providing socio-economic benefits for poor people, primarily small farmers with little education. However, new, non-traditional technologies, requiring unfamiliar skills, were introduced to the intended beneficiaries. In similar case histories in development, technology dissemination generally proceeds at a slow pace, and rarely at the rate that the project designers intend. The Cebu project, while more successful than Antipolo, found it necessary to significantly scale back this objective. Community organizing skills were inadequate for the task at hand. Commercialization of a complex technology may be best achieved on a purely for-profit basis, which may better serve socio-economic objectives than a commercial venture with mixed objectives.

It is evident that more attention must be given to these complex factors in future project design, particularly when one of the goals of a program is to achieve replicability in a project.

4. ASSESSMENTS IN SOUTH ASIA

ATI conducted preliminary studies in 1992 to assess the potential for small-scale mushroom production in Nepal, Bangladesh, and India. The studies reported on the market overview, producers and exporters, types and value of mushrooms, processing requirements, and potential for small farm cultivation. Individuals from mushroom laboratories, Departments of Agriculture, growers and processors, and small farmers were interviewed, and numerous site visits were made. The study led

to suggestions for possible project designs. A brief summary is presented here.

The mushroom market study was conducted in order to assist the newly formed Asia Network for Small-Scale Agricultural Biotechnologies (ANSAB), headquartered in Kathmandu, in identifying projects for technology dissemination and commercialization. Experts from nine countries had begun to assess this and other biotechnologies for potential commercialization for the benefit of small farmers during the inaugural ANSAB workshop held in the spring of 1992 (Ferchak & Ribeiro, 1992).

4.1. Nepal

In Nepal, there are numerous microclimates that are quite suited to mushroom cultivation. In the last few years, several hundred small farmers in the Kathmandu Valley (where 90% of production is presently located) produce button mushrooms in small quantities during the cool season and oyster mushrooms during the warm season for the local fresh market, primarily the international hotels. Production of these varieties in Nepal was estimated at 80-100 Mt in 1991. Imports are estimated at 250 Mt, principally canned button mushrooms from China, but also some dried mushrooms from India.

Mushroom activity first began in Nepal at the Entomology Division of the National Agricultural Research Council in 1975. Mushroom spawn is now produced at the Central Plant Pathology Division, which makes it available at subsidized prices to small farmers (Dongol, 1991). However, facilities are inadequate and the spawn is of variable quality. About 8,000 bottles of spawn are sold (70% *Agaricus*); however, the potential demand is estimated to be 50,000 bottles. Distribution of spawn outside the valley remains a difficulty. Yields are also low due to the lack of sterile substrate and poor technique used by the farmers. Paddy straw is in high demand for uses such as thatching, and therefore inferior wheat straw is used for substrate. There are no mushroom processing facilities. Marketing is not organized, and packaging is neglected, which reduces the farmers' market availability, penetration and selling price. No promotion of the product takes place. At least one private commercial venture, which produced its own spawn, is known to have failed; however, new ventures are under consideration.

The climate of the Kathmandu valley is too cold for straw mushrooms. Shiitake has the disadvantage of requiring wood or sawdust, which is in short supply. The cultivation of *Pholiota* is at a research stage. High value mushrooms, particularly morels, are picked in the wild by rural peoples for sale.

The favorable climate in Nepal, coupled with low labor cost and substrate availability, are suitable to expanded mushroom cultivation and export. Small-scale farmers can be assisted in forming cooperatives to develop a quality product in higher quantities, through improved methods of cultivation. A commercial spawn production center is necessary, linked to a strengthened government research institution. A processing facility can be encouraged to develop the mushroom export market.

4.2. Bangladesh

In Bangladesh, initial work began at the Mushroom Culture Centre, Savar, in the mid-1970s to develop and supply spawn. However, commercial cultivation of mushrooms did not begin until 1987. The Mushroom Centre has developed cultivation methods for straw, oyster, and Jew's Ear (*Auricularia)* mushrooms, and has begun to produce spawn for small-scale growers.

Mushrooms are now principally imported, and are primarily consumed at the international

hotels. However, local people have started to produce and use mushrooms in their diet. This start has prepared the way for expanding mushroom growing activities in other regions and potentially for export. A recent economic study on straw mushroom cultivation in Saturia, Manikganj, found that all the mushroom growers were women, who began cultivation as a supplementary work activity for added income (Raquib, 1991).

At present, production of mushrooms and availability of spawn is highly localized. Availability of spawn should be expanded to reach a wider range of growers. Growers need training and assistance in production techniques, and in developing cooperatives and markets.

4.3. India

The mushroom industry in India has grown slowly since its beginning in the 1960s; total production was estimated at 6,000 tons for 1991. Reasons for this slow growth were such factors as traditional fears of mushrooms because of poisonous species, and avoidance by Brahmin classes; lack of government support for extension; lack of quality spawn and substrate; and lack of capital, expertise, and access to foreign markets for entrepreneurs. However, a new National Centre for Mushroom Research and Training was establishing in 1986, and a Mushroom Society of India was formed in 1991. These new developments will encourage mushroom cultivation in India.

5. SUMMARY

Commercialization of new developments in mushroom production and processing requires links between agricultural research and the farmer. Production of high-quality mushrooms needs the support of a well maintained, publicly financed laboratory for the collection, preservation, maintenance, and breeding of varieties, for research into low-cost production techniques, and for support of agricultural extension work. One approach to cultivation is by small farmers in association with a spawn production center, cooperatively owned or in a university laboratory; this system is being applied in Nepal and India. Production is labor-intensive and land conserving, utilizes agricultural wastes, and is suitable for small-scale enterprise. A farmers' cooperative can be linked to international markets.

Some obstacles to increased commercialization of mushrooms by small-scale farmers include lack of access to quality spawn, poor information distribution and lack of training programs, and low productivity due to unsterilized growth substrates. Local markets are frequently limited due to high prices and consumer unfamiliarity or prejudice. Often, small-scale farmers are in the situation of having to sell at low prices to a single canner/wholesaler. Other difficulties include lack of support from government for research, need for national and regional laboratories, shortage of technical expertise, and a lack of regional conferences and cooperative activities (Chang & Miles, 1991).

REFERENCES

CHANG, S.T. & MILES, P.G. (1989). *Edible Mushrooms and Their Cultivation*. Boca Raton: CRC Press.

CHANG, S.T. & MILES, P.G. (1991). Recent trends in world production of cultivated edible mushrooms. *The Mushroom Journal* **503**, 15-18.

CUANO, M.G. (1990). Ayala Foundation Incorporated Venture Capital Program. In *Seed Enterprise Equity Development (SEED) Fund Program*. Proc. Workshop 12-16 June 1989, Manila. Washington, D.C.: AT International.

DONGOL, R.H. (1991). Prospect of mushroom cultivation in Nepal. National Agricultural Research Centre, Khumanlar, Lalitpur, Nepal.

FERCHAK, J. & RIBEIRO, S., eds. (1992). *Lab to Land: Biotechnology for Sustainable Agriculture in Asia.* Washington, D.C.: AT International.

HINDS, M. deC. (1993). A Strike Agitates Mushroom Country. *New York Times* **142** (49,340), 26 (May 23).

LELLEY, J. (1988). Growing Edible Mushrooms - Still a Generally Neglected Opportunity. *Gate* **4**, 30-34.

MCCLURE, B.H. (1990). Mushrooms Grow into Mainstream Profitability. *Supermarket Business* **45**, 48-49.

RAQUIB, A. (1991). *Technologies relevant to women: mushroom production*. Mushroom Culture Centre, Savar, Dhak, Bangladesh.

USDA (1992). Mushrooms. Report released by the *National Agricultural Statistics Service*, August 19, 1992. Washington, D.C.: United States Department of Agriculture.

WORRALL, J.J. & YANG, C.S. (1992). Shiitake and Oyster Mushroom Production on Apple Pomace and Sawdust. *Horticulture Science* **27**, 1131-1133.

CHAPTER 35

MUSHROOM CULTIVATION: ENVIRONMENTAL PROBLEM TO ENVIRONMENTAL BENEFIT

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1. INTRODUCTION

The mushroom industry has for a long time been considered problematic from an environmental standpoint. The use of organic manures as substrates, the operation of open composting yards, and the uncontrolled disposal of spent compost create an environmental nuisance, including air pollution (bad odours), water pollution, flies and other harmful insects, and even possible vectoring of diseases. In recent years, worldwide mushroom production has increased dramatically (Chang, 1991). A considerable proportion of this production takes place in urban areas with increasingly dense populations. At the same time, environmental awareness has grown, with the result that the enforcement of pollution control laws has become more and more effective.

This situation forces the mushroom industry to develop technologies that ensure production with the least possible harmful effects on the environment. This process will continue in the future, with the techniques developed for "clean" mushroom production providing applications in other fields of waste recycling. There is, in addition, a potential for the development of environment-related technologies that exploit certain special biological features of mushrooms for pollution control.

The present review deals with a number of aspects of interaction between mushroom production and environmental issues, on the basis of previous experience, together with extrapolations for the future.

2. RECYCLING ORGANIC WASTES VIA MUSHROOM PRODUCTION

Most of the substrates used for mushroom production are organic wastes or by-products, involving agricultural, municipal or industrial wastes. The exploitation of such wastes by the mushroom industry could be environmentally beneficial, provided that a number of basic aspects are taken into account, so that proper environmental concepts underpin each case. The following are the major aspects to be considered:

2.1. Characterization and Qualification

There is a need to define standard chemical, physical and biological parameters for the characterization and qualification of wastes intended to serve as substrates for mushrooms. Such parameters must be based on the nutritional requirements of each specific variety of mushroom. Chemical and physical parameters have been defined by Levanon *et al.* (1984; 1988), for example, in the search for alternative recycled materials for use as casing soils for *Agaricus bisporus*. The solid fraction of the digested slurry resulting from the thermophilic, methanogenic fermentation of cattle manure and slaughter-house wastes were found to be comparable to peat-moss, the material most commonly used as casing soil (Marchaim *et al.*, 1991). Chemical parameters were also defined to select substrates for the production of composts for *Agaricus bisporus*. With the decreasing availability of horse manure, the traditional substrate, chicken manure and cereal straws were found to provide useful alternatives (Levanon *et al.*, 1983).

The examination of chemical parameters was found to be necessary to develop alternative substrates for wood-degrading mushrooms: cotton straw was found to be suitable for the production of both *Pleurotus* spp. and *Lentinus edodes* (Levanon *et al.*, 1989; 1993a). This suitability is due to the chemical composition of the straw, and especially that of the lignocellulose fraction (Silanikove & Levanon, 1986; 1987), that meet the nutritional needs of these mushrooms.

It should be emphasized that the establishment of such parameters is essential, for each variety and each location, in order to select the optimal substrate for production. Proper use of these parameters is beneficial both to the industry and to the environment.

2.2. Collection and Storage

The methods of collection and storage of wastes is an important component in the recycling concept. While manures are usually produced steadily throughout the year, and supply is therefore less problematic, plant residues, especially straws, are mostly produced during short seasons, giving rise to the need for special collection and storage systems. Clearly, the most viable solution for harvesting and transporting straws is the use of the same farm machinery as is in actual use on local farms. Thus, cotton straw was harvested and transported by the machinery in local use for forage crops used for animal feed (Levanon *et al.*, 1988). Storage of plant material can be problematic and expensive. Cereal straws should be stored under cover, to avoid contamination resulting from excess humidity, especially rain. This is not the case with cotton straw because it contains 50-60% humidity at harvest time. Drying the straw in the field is impossible because of the rain that can be expected during the fall. The practical solution that was developed involves the preservation of cotton straw under anaerobic conditions (Silanikove & Levanon, 1986; 1987). Scaling up the process was performed by storing the cotton straw in farm facilities originally built for silage (Silanikove *et al.*, 1988). Such silage was successfully used as a substrate for *Pleurotus* spp. and *Lentinus edodes* (Levanon *et al.*, 1989; 1993a).

2.3. Selection of Mushroom Strains

One of the most important conditions for successful mushroom production is to find maximum compatibility between the fungal strain and its substrate. In their search for the strains of *Lentinus edodes* most compatible with cultivation on sawdust, Royse and May (1987) suggested the identification of genetic differences between different fungal strains by isoenzyme electrophoresis.

This step was used in the selection process to reduce the number of strains that would later be tested for their biological efficiency (i.e. yield/dry weight of substrate). Schmidt and Dittberner (1989) studied variations through mycelial growth rates and the capacity to degrade wood. These two methods were combined in a two-step method for selection by Levanon *et al.* (1993b), when searching for the strain of *Lentinus edodes* best suited to cultivation on a mixture of cotton and wheat straw: esterase isoenzyme assay was used to study genetic variations, after which the mycelial growth rate was used to determine strain/substrate compatibility among the strains short- listed by the first step.

2.4. Handling and Processing

Composting facilities are known to cause an environmental nuisance, by emissions to the air from volatile compounds and through leachates that run out, which are not only unpleasant in themselves, but can contaminate surface or even ground water.

In a study by Camp *et al.* (1991), a number of volatile compounds were identified in air samples taken above compost windrows, including several that contain sulfur and are the source of bad odours. The concentration of volatile sulfur compounds in the evolving air during windrow composting ranged from 1-35 mol/m^3. It was found that indoor composting in tunnels reduced odour emission by 90%. Considerable amounts of ammonia are also released during open composting, as a result of ammonification. This was the only odourous gas identified in significant quantities during indoor experiments in tunnels (Gulliver *et al.*, 1991). Ammonia emission can be minimized, however, by the manipulation of working conditions.

Other developments in the indoor composting process arise from a drastic reduction of the period (Kurtzmann, 1991), and the provision of special inoculation to enhance the process (Nair & Price, 1991; Houdeau *et al.*, 1991). In the near future, most of the industry will be forced to go over to indoor composting by increasing concern for clean air. Consequently, more efforts will be made to develop methods of indoor composting that are less expensive and more productive. The processing of lignocellulosic wastes for the production of wood-degrading mushrooms usually does not include composting. As a result, the substrate is more susceptible to contamination by pests, and especially by competing fungi. Hence, the heat treatment that such substrate undergoes becomes more crucial. The cultivation of *L. edodes* on combinations of sawdust and agricultural wastes (the "synthetic log" method) is limited to small units (1-2 kg), due to the need to sterilize the substrate. This method is excessively labour-intensive. A more efficient method was recently reported (Levanon *et al.*, 1993a) by which bulk pasteurization of a cotton and wheat straw mixture replaced sterilization, allowing treatment of 20 kg units of substrate for the cultivation of *L. edodes*.

2.5. Recycling Spent Substrate

Public awareness and the pressure of environmental laws is forcing the mushroom industry (like others) to take responsibility for the disposal or recycling of its own spent raw materials. An outlet for used substrate is becoming an essential component for every mushroom production plant. A number of methods for recycling have already been developed, and new ideas are being studied constantly. Spent compost that has been used for *A. bisporus* can be used as a soil conditioner and as an organic manure for agriculture or horticulture. Spent compost was found to be useful for land reclamation, and as an organic manure for the cultivation of corn, without any adverse effect on runoff or subsurface water quality (Wuest & Fahy, 1992). It is also being tested as a substitute for peat as

a growth medium (Chong *et al*., 1991; Maher, 1991). Spent mushroom compost can also be recycled within the industry itself, as a casing soil, usually mixed with other organic materials, mostly peat.

The used substrate of wood-degrading mushrooms can be considered as a feed for ruminants. The digestibility of lignocellulosic waste by ruminants is inversely proportional to its lignin content. Many white rot fungi consume components of the plant material (e.g. cellulose) while breaking down the lignin. Since the loss of organic matter is higher during the short stage of fructification than during the longer spawn run phase, the potential feed value of the used substrate decreases after harvesting the mushrooms. According to Rajarathnam *et al*. (1992), the ability of *Pleurotus* spp. to utilize a wide range of substrates and grow in a wide range of temperatures gives these species an advantage over others for this purpose.

Kerem *et al*. (1992) studied the degradation of lignocellulose in cotton straw by *Pleurotus ostreatus* and *Phanerochaete chrysosporium*. They found that, while *P. ostreatus* showed selectivity for lignin degradation, *P. chrysosporium* showed more rapid degradation of the total organic matter unselectively. The use of cotton straw as a cattle feed, after its use to produce *Pleurotus* spp., was reported by Silanikove *et al*. (1988).

3. UNCONVENTIONAL USES OF MUSHROOM TECHNOLOGIES AND PRODUCTS

The mushroom industry continually faces the challenge of producing fresh, clean food products from "dirty" organic wastes. The ability to meet this challenge arises first and foremost from the catabolic and anabolic systems of the organism itself. The technology that is evolving for mushroom production creates the basis for the optimal exploitation of these features in the production system.

There are a number of tasks in which the organism and its production technology can be exploited to reduce or solve ecological problems.

3.1. Composting as an Aid to Waste Management Authorities

A solid waste management facility for recycling and composting consists of three major elements (unit processes). The first completes the job of separation, started in the household, to yield fractions for recycling, landfilling and composting. The second element size-reduces and homogenizes the fraction to be composted. The third element is the composting system itself, in which the waste is transformed through biological action to a lesser weight and volume of stabilized, sanitized process residue that is storable, transportable and usable as a compost.

A number of methods for composting the organic ingredients of the solid waste are available to waste management authorities. In a comparative study, Feinstein (personal communication) found that the composting systems offered by the mushroom industry are superior to other methods that are available. The advantages of the mushroom composting tunnel lie in the following categories: general concept, materials handling, biological process control, exhaust air scrubbing, monitoring progress, and system track record. He concluded that:

"certain `tunnel` systems offered in the mushroom growing industry, which relies on composting, are technologically mature, conform to settled scientific understanding of composting, and are well-proven in the routine processing of a variety of demanding agricultural residues. This situation reflects the highly competitive nature of the mushroom business, which encourages excellence in composting system design. For waste management purposes, such systems are distinctly superior to those originating in

the waste field."

The growing demand for recycling and composting of solid wastes will therefore create more opportunities for technologies developed by the mushroom industry.

3.2. Bio-Accumulation of Heavy Metals

Metals are among the most commonly used raw materials in an industrial society. Aqueous effluents emerging from the mining and metal refining and other industries contain dissolved heavy metals, which are toxic to living organisms, and pose a biological threat to man as they can cause serious hazards to public health. One of the most important features which distinguishes metals from other toxic pollutants is their non-biodegradability. To avoid environmental hazards it is necessary to remove the metal contaminants from the effluents at source. Conventional techniques for metal removal, prior to discharge, include chemical precipitation, electrolysis, ion exchange, solvent extraction and evaporative extraction, but these techniques are likely to become increasingly expensive and inefficient when the anticipated stricter statutory limits are imposed.

Using microorganisms as biosorbents for metals offers a potential alternative to existing methods for detoxification and for recovery of toxic or valuable metals from industrial discharge water (Sahoo *et al.*, 1992).

A variety of mechanisms exists for the removal of heavy metal from solutions by biomaterials, ranging from purely physico-chemical interactions, such as adsorption to cell walls, to mechanisms dependent on metabolism, such as transport, internal compartmentation and extracellular precipitation by excreted metabolites (Fourest & Roux, 1992).

The use of living organisms or dead biomass are both possibilities. Numerous publications have appeared, describing studies of different organisms for this purpose: bacteria (Sahoo et al., 1992; Remacle, 1992); filamentous fungi (Fourest & Roux, 1992); the water fern, *Azolla* (Tel-Or & Sela, 1992); yeasts (Yanni, 1992); marine algae (Volesky, 1992).

The bioaccumulation of heavy metals is a known phenomenon in basidiomycetes, which has been studied principally because of its harmful effect on mushroom quality. However, the rates and concentrations of metals accumulated by different species can be predicted only to a limited extent from the taxonomic status of the species or the properties of the substrate (Rajarathnam *et al.*, 1992). More research is needed to explore what advantage can be gained from this phenomenon and to develop the technique to implement such a process.

3.3. Detoxification of Organic Chemicals

Toxic organic chemicals of agricultural, industrial and municipal origin are an environmental hazard all over the world. The possible use of microorganisms to detoxify xenobiotic chemicals is confined mainly to bacterial species. However, in a study of the biodegradation of pesticide residues in the soil by fungi and bacteria, a specific role for both groups was demonstrated (Levanon, 1993).

In recent years, the ability of white-rot fungi to biodegrade toxic chemicals has also been studied. These studies were based on the hypothesis that ligninolytic systems of fungi are capable of degrading a broad spectrum of xenobiotic chemicals whose structure resembles that of lignin (Boominathan & Reddy, 1991). Numerous reports have been published indicating that white-rot fungi are capable of degrading highly toxic pesticides and xenobiotic chemicals. The fungus most widely studied for this purpose has been *P. chrysosporium* (Aust, 1990). The lignin-degrading system of this fungus include two families of H_2O_2-requiring extracellular hemeperoxidases,

designated lignin peroxidase (LIP) and manganese-dependent peroxidase (MNP), together with H_2O_2-producing enzymes (Boominathan & Reddy, 1991). In a recent study on the regulation of these enzymes, overproduction of LIP was demonstrated by Dosoretz *et al.* (1993) under non-limiting nutrient conditions.

The ligninolytic enzyme system of *Pleurotus* spp. differs from that of *P. chrysosporium*, in that no evidence has been found of the presence of LIP and MNP in the former (Sannia *et al.*, 1991; Kerem *et al.*, 1992; Masaphy & Levanon, 1992). A veratryl alcohol oxidase was found in cultures of *P. ostreatus* (Sannia *et al.*, 1991) and there is also evidence of a probable role for polyphenol oxidase (PPO) and, especially, laccase in the ligninolytic system of this fungus (Kerem *et al.*, 1992; Masaphy & Levanon, 1992). The ligno-cellulolytic activities of this fungus were induced by the presence of lignocellulose in the growth medium (Masaphy & Levanon, 1992).

The ligninolytic system of *Coriolus* spp. involves both lignin- degrading oxidase and peroxidase activities. *C. versicolor* secretes LIP, MNP and at least three major laccase-type phenol oxidases. Several studies have appeared, reporting the use of this fungus for the efficient degradation of bleach-plant effluents from the paper industry (Archibald *et al.*, 1990).

The variations in extracellular ligninolytic enzymatic systems among species, and the possibility of regulating their activities, create opportunities for the development of techniques to utilize these features for the biodegradation of recalcitrant environmental pollutants.

4. CONCLUSIONS

a. The mushroom industry, like other industries, must develop its overall environmental concepts. This is essential in an industry that is largely located in populated areas, and must comply with ever stricter environmental laws and regulations.
b. This environmental thinking must be based on "zero leakage of pollutants", at all stages of production and substrate recycling. The elimination or recycling of all by-products and used materials must be an integral part of planning for production.
c. The evolution of environmental concepts for each production plant must be "tailored" to local conditions (environment, species of mushroom, substrate, etc.) but based on a number of basic aspects that have to be taken into consideration under all conditions.
d. When the industry is built on the foundation of the above criteria, it will be environmentally beneficial, because its utilization of problematic organic wastes will neutralize them and thereby contribute to a cleaner environment.
e. Since the mushroom industry has to face and overcome the challenge of recycling wastes with minimal pollution, the answers that it develops will be superior in efficiency and cost-effectiveness to other techniques, and can be used in waste management facilities.
f. Mushrooms will be found useful in the fast-growing environmental cleaning and protection industry, due to their particular biological features and the techniques developed for their production. These uses will include the biosorption of heavy metals and the biodegradation of toxic organic chemicals. It would not be too visionary to suggest that research will discover further uses in this field. I would like to throw out the challenge of degrading plastic wastes, the purification of contaminated water and reclaiming polluted soils as possible targets for such research.

REFERENCES

ARCHIBALD, F., PAICE, M.G. & JURACEK, L. (1990). Decolorization of kraft bleachery effluent chromophores by *Coriolus (Trametes) versicolor*. *Enzyme and Microbial Technology* **12**, 846-853.

AUST, S.D. (1990). Degradation of environmental pollutants by *Phanerochaete chrysosporium*. *Microbial Ecology* **20**, 197-209.

BOOMINATHAN, K. & REDDY, C.A. (1991). Fungal degradation of lignin: biotechnological applications. In *Handbook of Applied Mycology, Vol. IV: Biotechnology*, pp. 763-822. Edited by D.K. Arora, K.G. Mukerji & R.P. Elander. New York: Marcel Dekker, Inc.

CAMP, H.J.M. OP DEN, DERIKX, P.J.L., DRIFT, C. VAN DER, VOGELS, G.D. & GRIENSVEN, L.J.L.D. VAN. (1991). Odorus sulfur compounds emitted during conventional outdoor- and during indoor-composting. *Mushroom Science* **13**, 147-154.

CHANG, S.T. (1991). Mushroom Biology and Mushroom Production. Ad-Hoc Research Group on Mushrooms. FAO-CNR, Rome, Italy.

CHONG, C., RINKER, D.L. & CLINE, R.A. (1991). A comparison of five spent mushroom composts for container culture of ornamental shrubs. *Mushroom Science* **13**, 637-644.

DOSORETZ, C., ROTHSCHILD, N. & HADAR, Y. (1993). Overproduction of lignin peroxidase of *Phanerochaete chrysosporium* (BKM-F) under non-limiting nutrient conditions. (in press)

FEINSTEIN, M.S. (1990). Comparative evaluation of compost systems offered in the waste and mushroom industry. Personal communication, Rutgers University, New Jersey, U.S.A.

FOUREST, E. & ROUX, J.C. (1992). Heavy metal biosorption by fungal mycelial by-products: mechanisms and influence of pH. *Applied Microbiology and Biotechnology* **37**, 399-403.

GULLIVER, A., MILLER, F.C., HARPER, E. & MACAULEY B.J. (1991). Environmentally controlled composting on a commercial scale in Australia. *Mushroom Science* **13**, 155-164.

HOUDEAU, G., OLIVIER, J.M. & CHABBERT, B. (1991). Improvement of indoor short composting. *Mushroom Science* **13**, 215-220.

KEREM, Z., FRIESEM, D. & HADAR, Y. (1992). Lignocellulose degradation during solid state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. Applied Environmental Microbiology **58**, 1121-1127.

KURTZMAN, R.H.JR. (1991). One-day Phase I & II: Mushrooms from less. *Mushroom Science* **13**, 165-168.

LEVANON, D. (1993). The role of fungi and bacteria in the mineralization of the pesticides atrazine, alachlor, malathion and carbofuran in the soil. *Soil Biology and Biochemistry*. (in press).

LEVANON, D., DOSORETZ, C. & MOTRO, B. (1983). Chemical and biological qualification of synthetic composts for mushrooms (*Agaricus bisporus*). *Mushroom News* **31**, 16-19.

LEVANON, D., DOSORETZ, C., MOTRO, B. & COHEN, I. (1984). Recycling agricultural wastes for mushroom casing. *Mushroom Journal* **133**, 13-17.

LEVANON, D., DANAI, O. & MASAPHY, S. (1988). Chemical and physical parameters in recycling organic wastes for mushroom production. *Biological Wastes* **26**, 341-348.

LEVANON, D., DANAI, O. & SILANIKOVE, N. (1989). Cotton straw silage as substrate for *Pleurotus* sp. cultivation. *Mushroom Science* **12**, 81-90.

LEVANON, D., ROTHSCHILD, N., DANAI, O. & MASAPHY, S. (1993a). Bulk treatment of substrate for cultivation of shiitake mushrooms (*Lentinus edodes*) on straw. *Bioresource Technology*. (in press).

LEVANON, D., ROTHSCHILD, N., DANAI, O. & MASAPHY, S. (1993b). Strain selection and

improved cultivation method for shiitake mushrooms (*Lentinus edodes*) on straw. *Bioresource Technology*. (in press).

MAHER, M.J. (1991). Spent mushroom compost (SMC) as a nutrient source in peat based potting substrate. *Mushroom Science* **13**, 645-652.

MARCHAIM, U., LEVANON, D., DANAI, O., MASAPHY, S., CHEN Y., INBAR, Y & KLINGER, I. (1991). A suggested solution for slaughterhouse waste: uses of residue materials after anaerobic digestion. *Bioresource Technology* **37**, 127-134.

MASAPHY, S. & LEVANON, D. (1992). The effect of lignocellulose on lignocellulolyic activity of *Pleurotus pulmonarius* in submerged culture. *Applied Microbiology and Biotechnology* **36**, 828-832.

NAIR, N.G. & PRICE, G. (1991). A composting process to minimise odour pollution. *Mushroom Science* **13**, 205-206.

RAJARATHNAM, S., SHASHIREKHA, M.N. & BANO, Z. (1992). Biopotentialities of the basidiomacromycetes. *Advances in Applied Microbiology* **37**, 234-361.

REMACLE, J. (1992). Heavy metal trapping by gram negative and gram positive bacteria. In *Harnessing biotechnology for the 21st Century*, pp. 458-462. American Chemical Society, Washington, DC, U.S.A.

ROYSE, D.J. & MAY, B. (1987). Identification of genotypes by multilocus enzyme electrophoresis: Catalog of lines. *Biochemical Genetics* **25**, 716-719.

SAHOO, D.K., KAR, R.N. & DAS, R.P. (1992). Bioaccumulation of heavy metal ions by *Bacillus virulans*. *Bioresource Technology* **41**, 177-179.

SANNIA, G, LIMONGI, P., COCCA, E., BUONOCORE, F., NITTI, G. & GIARDINA, P. (1991). Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Biochimica Biophysica Acta* **1073**, 114-119.

SCHMIDT, O. & DITTERBERNER, D. (1989). Biological and enzymatic variation in strains of the cultivated mushroom Shiitake, *Lentinus edodes*. *Agnew. Botanik*. 63, 55-65.

SILANIKOVE, N. & LEVANON, D. (1986). Cotton straw: composition, variability and effect of anaerobic preservation. *Biomass* **9**, 101-112.

SILANIKOVE, N. & LEVANON, D. (1987). Interrelationships between acidic and alkali treatments of cotton and wheat straw and fiber chemical properties. *Journal of Science of Food and Agriculture* **38**, 117-124.

SILANIKOVE, N., DANAI, O. & LEVANON, D. (1988). Composted cotton straw silage as a substrate for *Pleurotus* sp. cultivation. *Biological Wastes* **25**, 219-226.

TEL-OR, E. & SELA, M. (1992). Bioremdiation of heavy metals and radioactive wastes. *3rd US-EPA-Israel Workshop on Bioremediation*. Ministry of Environment, Jerusalem, Israel.

VOLESKY, B. (1992). Removal of heavy metals by biosorption. In *Harnessing biotechnology for the 21st Century*, pp. 462-466. American Chemical Society, Washington, DC, U.S.A.

WUEST, P.J. & FAHY, H.K. (1992). Spent mushroom compost: its origin, components and impact on water quality. *Mushroom News* **40**, 27-33.

YANNI, S. (1992). Removal and reclamation of toxic heavy metals from industrial effluents by preparation made from spent yeast. *3rd US-EPA-Israel Workshop on Bioremediation*. Ministry of Environment, Jerusalem, Israel.

CHAPTER 36

EXTRACELLULAR ENZYMES AS TARGETS FOR STRAIN IMPROVEMENT IN *AGARICUS BISPORUS*

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1. INTRODUCTION

The cultivation of *Agaricus bisporus* represents the largest proportion of mushroom production in the world. In common with the production of other edible fungi this output is dependent on bioconversion of low cost waste lignocellulosic residues into a useful human foodstuff (Wood, 1989).

Agaricus bisporus production is carried out in a two-stage process. The first is the controlled composting of cereal straw with suitable nitrogen sources such as manures or inorganic nitrogen fertilisers. The second stage is the **partial** utilisation of the compost by the mycelium of the fungus. This is followed by various cultural manipulations to promote production of fruit bodies (Wood & Smith, 1987).

Extensive nutritional and enzymological studies have revealed which nutrient fractions of the compost are utilised by the fungus and the likely extracellular enzymes used (Wood *et al*., 1990; Wood *et al*., 1991). The fungus has been shown to be able to degrade both plant and microbial biopolymers such as lignin, cellulose, hemicelluloses, protein and microbial cell walls (Durrant *et al*., 1991; Manning & Wood, 1983; Fermor & Wood, 1981). The secreted enzymes produced by *Agaricus* include both hydrolases and oxidases. These include laccases, cellulases, hemicellulases, proteases and other enzymes.

When cropping is terminated the quantity of substrate finally utilised by the fungus is about 25-30% of the starting dry matter present after the preparation of compost. This is partitioned into mycelial biomass, respiratory products such as CO_2 and fruit body biomass (Wood, 1979; Wood & Smith, 1987). There is thus a considerable quantity of non-degraded organic material still available from the compost after fruit body production has finished.

Scope exists, therefore, for approaches aimed at increasing the efficiency of compost utilisation. One route would be to increase the level of extracellular enzyme secretion into the compost and then to determine if this change could effect an increase in mycelial biomass and greater fruit body yield.

As part of a programme aimed at this approach to strain manipulation, two extracellular enzymes of *Agaricus bisporus*, laccase and cellulase, have been studied in considerable detail. Both

of their activities are strongly regulated in the substrate during fruit body development (Wood & Goodenough, 1977). Their regulatory control indicates that these enzymes are associated with both the nutritional and developmental physiology of the fungus. Studies have been carried out on the production, enzymic properties, physiological role, protein structure, antigenicity and molecular genetics of both enzyme activities (Wood & Thurston, 1991; Raguz *et al.,* 1992; Perry *et al.,* 1993a & b). The aim of this work is to provide suitable enzyme targets in the life cycle that could be manipulated by genetic engineering methods, particularly by the use of DNA transformation techniques. Transformed strains would then be analysed for any useful effect of increased enzyme level on mycelial biomass and fruit body yield, and regulation of the "flushing" response. Additionally, the analysis of the molecular genetics of these enzymes could provide useful DNA sequences to assist the development of a workable DNA transformation system for the fungus.

2. LACCASE

2.1. Regulation, Structure and Biosynthesis

Laccase activity is strongly regulated during fruit body production on compost. Its activity increases during colonisation and then declines rapidly when fruit bodies develop (Wood & Goodenough, 1977). The use of axenic cultures and strains of *Agaricus* genetically blocked in fruiting shows that regulation of this decline in activity occurs at the stage of fruit body **enlargement**, not initiation. A useful by-product of this work was the observation that laccase activity can be used (in cultures before fruiting occurs) to estimate the yield of mycelial biomass (Wood, 1979). Analysis of the loss of activity showed this was due primarily to inactivation followed by enzyme protein loss.

The molecular properties of laccase protein from both liquid and compost cultures have been extensively analysed (Wood, 1980a, b; Wood *et al.*, 1991; Perry *et al.*, 1993a). The enzyme is abundant, forming over 2% of cell protein and its various molecular forms have been purified from malt extract medium and from compost cultures at maximal activity (high activity, HA form) and low activity (low activity, LA form).

The purified liquid culture form is a laccase of 100 kDa molecular weight, with substrate specificities, kinetic properties and inhibitor profile similar to other fungal laccases but with lower copper content and is yellow. The HA laccase from compost has considerable enzyme and structural similarities to the liquid culture form of the enzyme, but has more copper and consequently is blue. The LA form differs considerably, is nearly copper deficient and has a very different amino acid profile.

The HA and LA forms show considerable differences on SDS-PAGE analysis. Various antibodies have been prepared to native enzyme forms, denatured forms, and to an N-terminal 15 amino acid sequence of the major polypeptide (Perry *et al.,* 1993a). The enzyme forms present in liquid and compost cultures have been analysed by Western blotting techniques. The different band patterns revealed are probably due to the production of partially cleaved enzyme molecules associated with differing extents of glycosylation.

Studies on biosynthesis in liquid culture show that a single major excreted protein product of 68 kDa is made. The higher molecular weight of purified enzyme may be due to dimerisation. Poly(A) containing mRNA from these cultures translates an immunoprecipitable product of 57 kDa, consistent with a carbohydrate (glycosylation) level of 15% for the final form of the exported enzyme.

Biosynthesis of laccase protein in compost cultures shows that maximal biosynthesis occurs at the veil break stage when enzyme activity is rapidly **declining**.

The physiological role of laccase is unknown. Its abundance indicates a key role. It may be associated with the ability to release recalcitrant or bound nutrients linked to phenolic polymers, and thus contributes to the 'selectivity' of mushroom compost as a growth medium for the fungus (Wood & Smith, 1987).

2.2. Molecular Genetics

Two distinct laccase genes were identified when an expression library was screened with anti-laccase antibody (Perry *et al.*, 1993b). Identification of certain of these clones as laccase genes was confirmed by demonstration of two characteristic copper binding motifs in the deduced amino acid sequence and by alignment of deduced sequence with amino acid sequence of a cyanogen bromide fragment of pure laccase protein.

One cDNA clone was used to identify further cDNA clones and a genomic clone. The coding sequences showed two related but non-identical genes were present. The two genes, lcc1 and lcc2 are non-allelic as they did not segregate in Southern blot analysis of DNA from the uninucleate spores of a 4 spored basidium. Both laccase genes encode a sequence expressed as a 2.3 kb mRNA specifying a 520 residue polypeptide including a 19 amino acid residue signal peptide not present in the mature protein. The laccase genes mapped to the 2 smallest *Agaricus* chromosomes separable by electrophoretic karyotyping (Lodder *et al.*, 1993).

3. CELLULASE

3.1. Regulation, Structure and Biosynthesis

Regulation of endocellulase activity is associated also with fruit body development (Wood & Goodenough, 1977). Endocellulase activity increases and decreases in parallel with harvested fruit body biomass in each 'flushing' cycle (Claydon *et al.*, 1988). By use of different harvesting techniques such as continuous picking, or allowing large fruit bodies to reach senescence on the production beds it was shown that fruit body biomass positively regulates the quantity of substrate located extracellular endocellulase activity (Claydon *et al.*, 1988). It is also known that various **intracellular** fruit body carbon metabolising enzymes undergo cyclic variation in activity associated with flushing (Hammond, 1985; Wells *et al.*, 1987). This regulatory pattern has allowed the formulation of a simple physiological model to account for the observed enzyme activity changes (Wood *et al.*, 1988). It is assumed that the physiological role of endocellulase is to act on cellulose in the compost. This produces soluble carbohydrates which can be assimilated by the mycelium. When fruit bodies subsequently form on the mycelium the levels of intracellular mycelial carbon compounds are depleted due to translocation of these to the developing fruits. This depletion activates a putative signalling system which eventually results in more cellulase being produced to replenish carbohydrate levels in the mycelium. When fruit bodies are harvested there is no requirement for increased cellulase level and enzyme activity is down regulated.

Endocellulase activity has been purified from bulk liquid cultures grown on cellulose as sole carbon source (Manning & Wood, 1983). Purification was achieved by successive ultrafiltration, ion exchange and preparative gel electrophoresis steps.

Unpurified and purified liquid culture enzyme activity and compost extracts show five activity bands of increasing mobility when stained by zymogram techniques on cellulose with Congo Red counterstaining. These purified bands have been used to prepare an anticellulase antibody. Each

isoform of native endocellulase resolves into further multiple bands using isoelectric focusing, and each isoform has different structural properties. Analysis by SDS-PAGE gels shows that each band differs quantitatively in polypeptide composition but that they all share certain common bands. The isoforms 1, 2 contain predominantly polypeptides of 56 and 40 kDa, isoforms 4 and 5 of 22 kDa. Amino acid sequence analysis shows that the 56 and 40 kDa bands share a common N-terminal sequence of up to 15 amino acids.

Biosynthesis of endocellulase protein in compost cultures parallels expression of enzyme activity, with maximum biosynthesis occurring at the senescent stage of maximal fruit body biomass, and synthesis declines rapidly on harvesting of fruit bodies. Successive flushes are associated with new rounds of enzyme biosynthesis.

3.2. Molecular genetics

The anticellulase antibody has been used to isolate cellulose induced genes. RNA was prepared from cellulose grown mycelium. *In vitro* translation was then used to identify three main polypeptides of 38, 58 and 60 kDa. No such products were found from mycelium grown on fructose. The same antibody was used to screen an expression library, and two related cDNA clones were isolated. One clone selected for an mRNA that translated a 38 kDa product. A gene, cel 1, was sequenced from a genomic fragment hybridising to this cDNA clone. Sequence analysis shows this gene encodes for a cellulase, as shown by identification of a cellulose binding domain, and a separate proline-threonine-serine rich region known as the 'hinge' region by homology with other glycanases. The remaining core region is not homologous to any other -glycanase so far sequenced. A further gene cel 3, has been identified, and is being sequenced.

4. FUTURE WORK

This is aimed at studying gene expression during growth on liquid or compost cultures to determine if regulation of laccase or cellulase activity is associated with transcriptional regulation. Sufficient genomic DNA sequence has been obtained to allow identification of regulatory regions upstream to the coding region for both sets of enzyme protein genes. This information should aid development of a workable efficient DNA transformation system by allowing homologous genes and their associated upstream regulatory regions to be placed in suitable transforming vector DNA sequences. Once this tool is in place it may be possible to use further genetic manipulation technology specifically to amplify or delete the production of laccase and cellulase to examine the possibility of producing novel mushroom strains with improved agronomic qualities.

REFERENCES

CLAYDON, N., ALLAN, M. & WOOD, D.A. (1988). Fruit body biomass regulated production of extracellular endocellulase during periodic fruiting by *Agaricus bisporus*. *Transactions of the British Mycological Society* **90**, 85-90.

DURRANT, A.J., WOOD, D.A. & CAIN, R.B. (1991). Lignocellulose degradation by *Agaricus bisporus* during solid substrate fermentation. *Journal of General Microbiology* **137**, 751-755.

FERMOR, T.R. & WOOD, D.A. (1981). Degradation of bacteria by *Agaricus bisporus* and other fungi. *Journal of General Microbiology* **126**, 377-387.

HAMMOND, J.B.W. (1985). The Biochemistry of *Agaricus* fructification. In: *Developmental Biology of Higher Fungi*, pp. 389-401. Edited by D.A. Moore, L.A. Casselton, D.A. Wood & J.C. Frankland. Cambridge: Cambridge University Press.

LODDER, S., WOOD, D.A., & GULL, K. (1993). An electrophoretic karyotype of the cultivated mushroom *Agaricus bisporus*. *Current Genetics* (in press).

MANNING, K.M. & WOOD, D.A. (1983). Production and regulation of extracellular endocellulase by *Agaricus bisporus*. *Journal of General Microbiology* **129**, 1839-1847.

PERRY, C.R., MATCHAM, S.E., WOOD, D.A. & THURSTON, C.F. (1993(a)). The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. *Journal of General Microbiology* **139**, 171-178.

PERRY, C.R., SMITH, M., BRITNELL, C.H., WOOD, D.A. & THURSTON, C.F. (1993(b)). Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. *Journal of General Microbiology* **139** (in press).

RAGUZ, S., YAGUE, E., WOOD, D.A. & THURSTON, C.F. (1992). Isolation and characterization of a cellulose-growth-specific gene from *Agaricus bisporus*. *Gene*. **119**, 183-190.

WELLS, T.K., HAMMOND, J.B.W. & DICKERSON, P.G. (1987). Variation in activities of glycogen phosphorylase and trehalase during periodic fruiting of the edible mushroom *Agaricus bisporus* (Lange). *New Phytologist* **105**, 273-280.

WOOD, D.A. & GOODENOUGH, P. (1977). Fruiting of *Agaricus bisporus*. Changes in extracellular enzyme activities during growth and fruiting. *Archives of Microbiology* **114**, 161-165.

WOOD, D.A. (1979). A method for estimating biomass of *Agaricus bisporus* in a solid substrate, composted wheat straw. *Biotechnology Letters* **1**, 255-260.

WOOD, D.A. (1980(a)). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology* **117**, 327-338.

WOOD, D.A. (1980(b)). Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. *Journal of General Microbiology* **117**, 339-345.

WOOD, D.A. & SMITH, J.F. (1987). The Cultivation of Mushrooms. In *Essays in Agricultural and Food Microbiology,* pp. 309-343. Edited by J.R. Norris & G.L. Pettipher. Chichester: Wiley.

WOOD, D.A., CLAYDON, N., DUDLEY, K., STEPHENS, S. & ALLAN, M. (1988). Cellulase production in the life cycle of the cultivated mushroom *Agaricus bisporus*. In *Biochemistry and Genetics of Cellulose Degradation*, pp. 53-70. Edited by J.P. Aubert, P. Beguin & J. Millet. London: Academic Press.

WOOD, D.A. (1989). Mushroom biotechnology. *International Industrial Biotechnology* **9**, 5-9.

WOOD, D.A., PERRY, C., THURSTON, C.F., MATCHAM, S.E., DUDLEY, K., CLAYDON, N. & ALLAN, M. (1990). Molecular analysis of lignocellulolytic enzymes of the edible mushroom *Agaricus bisporus*. In *Biotechnology in Pulp and Paper Manufacture,* pp. 659-666. Edited by T. Kent Kirk & H-M. Chang. Boston: Butterworth.

WOOD, D.A. CLAYDON, N., BURTON, K.S., MATCHAM, S.E., ALLAN, M., PERRY, C., THURSTON, C.F., RAGUZ, S. & YAGUE, E. (1991). Molecular Analysis of Enzymes of *Agaricus bisporus*. In *Science and Cultivation of Edible Fungi,* pp. 43-50. Edited by M.J. Maher. Rotterdam: Balkema.

WOOD, D.A. & THURSTON, C.F. (1991). Progress in the molecular analysis of *Agaricus* enzymes. In *Genetics and Breeding of Agaricus,* pp. 81-86. Edited by L.J.L.D. van Griensven. Wageningen: Pudoc.

CHAPTER 37

MUSHROOMS AS A SOURCE OF NATURAL FLAVOR AND AROMA COMPOUNDS

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1. INTRODUCTION

Flavors, aromas, and fragrances are extremely important in the food, cosmetic, and pharmaceutical industries. A flavor is a volatile compound in a foodstuff, whose aromatic properties are detected by the nose. It may be inherently present or produced during processing by chemical reactions or microbial action. Some flavors such as peach, anise-licorice, and almond are due to a single impact flavor compound, 4-decalactone, anisaldehyde, and benzaldehyde, respectively. Others, like mushroom flavor or fruity flavor, are due to a mixture. Flavor compounds are usually present in very low concentrations. Flavor assessment is somewhat subjective; organoleptic evaluation is routinely performed along with chemical analysis. The term fragrance is usually reserved for volatile compounds with applications in the perfume and cosmetic industries.

2. SOURCES OF NATURAL FLAVOR COMPOUNDS

In the past, the only natural sources of flavors and essential oils were derived from plants. While production of flavor compounds from the distillation of plant material does have the advantage of using renewable resources, the products themselves may vary in quality and consistency. Active components often occur in minor quantities or in bound form or only in exotic plants, making isolation difficult and the products expensive. These limitations, combined with the fact that plant growth is subject to seasonal, climatic, and geographical variations and diseases, have resulted in an almost total shift to chemical synthesis for production of flavor and fragrance compounds. Nonetheless, chemical synthesis of compounds also has drawbacks, including the formation of undesirable racemic mixtures (isomers) and the growing aversion of consumers to chemical additives in food, cosmetics, and other household products (16, 30).

While fungi have long been utilized in the food industry, their potential for the industrial production of natural flavor compounds has been overlooked. Many fungi are capable of *de novo* synthesis of flavoring compounds when grown on standard culture media. In fact, sensory properties,

such as sweet, fruity, musty, earthy, and antiseptic, have long been used as taxonomic markers for classification (3). With advances in analytical techniques, it has become evident that the chemical structures of many volatile fungal metabolites are similar to those of well-known fragrance chemicals.

Odor-producing fungi occur in each of the classes (with the possible exception of the Myxomycetes), but the largest number is found within the Basidiomycetes, particularly the fruiting stages commonly referred to as mushrooms. This is not surprising, since Basidiomycetes have enzyme systems capable of catabolizing aromatic substrates. The Ascomycetes and Fungi Imperfecti also contain a large number of aroma-producing organisms. The Phycomycetes have not received much attention.

When a characteristic odor is absent from a mushroom, it may be because the specimen is immature, over-mature and/or incomplete. Many mushroom species, whose natural fruit bodies have no detectable odor, will produce a distinctive scent from the mycelium on agar (3) or in liquid culture (10). Edible and wood-decaying mushrooms and their respective aromatic notes are listed in Table 1.

TABLE 1. Mushrooms and their aromas.

Mushromms	Aromas	References
Agaricus bisporus	strong mushroom	20, 21, 25, 26
Antrodia malicola	faintly fragrant	3, 18, 30
Antrodia xantha	limonene, lemon	3, 18, 30
Armillaria lutea	honey, caramel, whey, meal, fatty, mushroom, blue cheese	10
Armillaria mellea	anise, licorice, fruity grapefruit,	10
Armillaria ostoyae	fruity, quince, fresh, leek, citrus, floral burnt, mushroom	10
Auriporia aureas	weet	15
Boletus edulis	dried mushroom	26
Cantharellus cibarius	mushroom	26
Ceriporiopsis subvermispora	berry, fruity, pineapple, carrot, cheese, burnt caramel, fresh, apple, medicinal, floral, citrus, strawberry	10
Chondrostereum purpureum	moderately fragrant	3, 18,
*Coprinus micaceus*strong	mushroom	3, 18, 30
Cystostereum murrayi	vanilla	3, 18, 30
Daedalea quercina	apples	3, 18, 30
Datronia scutellata	hydrocyanic acid, cherry laure	l3, 18, 30
Dichomitus squalens	almond, bitter almond, fruity	10

TABLE 1 continued.

Mushromms	Aromas	References
Dichostereum effuscata	vegetable soup, boiled carrot, floral	10
Fibroporia vaillantii	faint mushroom	3, 18, 30
Fomes fomentarius	oily, tallow	3, 18
Fomes fraxineus	faint sardine	3, 18, 30
Fomitopsis officinalis	faintly fragrant	3, 18, 30
Fomitopsis pinicola	fish, tallow	3, 18, 30
Ganoderma applanatum	faint tallow	3, 18
Ganoderma oregonense	faintly fragrant	3, 18, 30
Gloeophyllum odoratum	pleasant, fruity, honey, rose, anise, sweet	3, 7, 13, 15, 18, 19, 30
Gloeophyllum sepiarium	slightly spicy	3, 18, 30
Gloeophyllum striatum	iodine, bitter	3, 18
Gloeophyllum trabeum	iodine, bitter, balsamic terpenic, lemon, fruity, resin, wax polish, apple, floral, rose, citrus, mango, peach	10, 18
Grifola frondosa	hydrocyanic acid, cherry laurel nut oil then blue cheese, herbaceous, whey, musty, wild hyacinth, cereals, hyacinth, yeast, cereals then citrus	3, 10, 18, 30
Gyromitra esculenta	mushroom	26
Hapalopilus croceus	narcissus	3, 18, 30
Hapalopilus rutilans	slightly fragrant	3, 18, 30
Haploporus cytisinus	faint sardine	3, 18, 30
Hebeloma sacchariolens	sweet, fragrant, aromatic, fruity orange blossoms	34
Heterobasidion annosum	mushroom, fruity, floral, pungent, peppery, hay, celery	10
Hirschioporus pergamenus	leek, boiled carrot, orange, honey, fruity, sweet, burnt	10
Inocybe corydalina	fruity, jasmine, rose	15, 19, 30, 31
Inocybe pyriodora	fruity, jasmine, rose	15, 31
Inocybe odorata	fruity, jasmine, rose	15, 31
Ischnoderma resinosum	benzaldehyde, anisaldehyde	3, 18, 30
Kuehneromyces mutabilis	earthy then fragrant	3, 18

TABLE 1 continued.

Mushromms	Aromas	References
Lactarius rufus	mushroom	26
Lactarius torminosus	mushroom	26
Lactarius trivialis	mushroom	26
Lentinellus cochleatus	anisaldehyde	3, 15, 18, 30
Lentinula edodes	mushroom, floral, almond, fruity, apple	10
Lentinus lepideus	anisaldehyde, fruity, aromatic, cedar-wood	3, 18, 30
Lenzites betulina	fish, tallow, bitter cocoa, coffee grounds, fruity, bitter almond, camphorated, minty, walnut	3, 10, 18, 30
Lenzites flaccida	tallow	3, 18
Lenzites tricolor	slightly pepper	3, 18, 30
*Lepista irina*iris	oil, orange blossom	2
Lyophyllum ulmarium	slightly fragrant	3, 18, 30
Marasmium alliaceus	garlic	3, 18, 30
Merulius confluens	moderately fragrant	3, 18, 30
Merulius himantioides	iodine, bitter	3, 18
Merulius rufus	faint mushroom	18, 30
Merulius tremellosus	burnt toffee	3, 18, 30
Mycoacia uda	almond, floral, sweet, fruity, faint grassy, hyacinth, gardenia, alcoholic	14, 18, 19, 30
Nigroporus durus	alcoholic, sweet, fruity, fruity-flowery, estery, fragrant, coconut, peach, quince	3, 30
Ossicaulis lignatilis	new meal	3, 18, 30
Paxillus atrotomentosus	unbleached calico	3, 18, 30
Paxillus panuoides	unbleached calico, slightly fragrant	3, 18, 30
Perenniporia subacida	grapefruit, resin, fruity, floral, bitter almond, fermented, soup, cereal, sour, burnt, almond, orange, fruity apple	10
Phaeolus schweinitzii	pleasant, anise	3, 18, 30
Phellinus contiguus	pineapple, menthol, floral, fruity, citrus	10
Phellinus igniarius	faint, pleasant, fruity, floral, sweet fruity, wintergreen, rose	8, 18, 19
Phellinus laevigatus	faint, pleasant, fruity, wintergreen, rose	8, 18, 19

TABLE 1 continued.

Mushromms	Aromas	References
Phellinus pini	fruity, cereals, bitter cocoa, vegetable soup	10
Phellinus robustus	fruity, painting, floral, earthy	10
Phellinus tremulae	faint, pleasant, fruity, floral, sweet, wintergreen, rose	8, 18, 19
Phlebia radiata	butanol, harsh, herbaceous, orange, waxy, sweet, mirabelle, floral, cheese, pungent, fruity, citrus, cauliflower, mushroom, fatty, pineapple, roasted, faded rose, dandelion, burnt wood, musty, peach, creamy, fatty, lavender, almond, heavy, warm, roasted meal	12
Pholiota adiposa	earthy	3, 18, 30
Pholiota lucifera	earthy	3, 18, 30
Pholiota spectabilis	sickly sweet	3, 18
Pholiota squarrosa	strong, earthy	3, 18, 30
Piptoporus betulinus	apples, cereals, pineapple, fruity, coffee, pungent	3, 10, 18, 30
Pleurotus euosmus	fragrant, sweet, floral	3, 18, 30
Pleurotus ostreatus	slightly fragrant, mushroom, fruity, bitter cocoa, anise, almond, yeast	3, 10, 18, 30
Pleurotus sapidus	fragrant	3, 18, 30
Polyporus adustus	slightly fragrant (terpenoid)	3, 18, 30
Polyporus badius	slightly fragrant, resinoid1	8, 30
Polyporus obducens	hydrocyanic acid, cherry laure	l3, 18, 30
Polyporus obtusus	moderately fragrant, jasmine	3, 18, 30
Serpula lacrymans	very slight fungus odor	3, 18
Stereum gausapatum	faintly fragrant	3, 18
Stereum hirsutum	faintly fragrant	3, 18, 30
Stereum ostrea	faintly sweet	3, 18
Stereum rugosum	fruity, banana	3, 18, 30
Stereum sanguinolentum	fragrant	3, 18, 30
Trametes rubescens	faint tallow	3, 18
Trametes suaveolens	strong, anise, almond	3, 18, 19, 30

TABLE 1 continued.

Mushromms	Aromas	References
Trametes versicolor	fish, tallow	3, 18, 30
Tricholoma matsutake	mushroom	19
Tyromyces sambuceus	peach, passion fruit	15
Xylobolus frustulatus	fruity, rotting apples, spicy	3, 18, 30
Xylobolus illudens	sweet, banana	3, 18, 30

3. TYPES OF AROMATIC COMPOUNDS

Aromatic compounds are usually classified into two groups: pleasant and unpleasant. Pleasant aromatic compounds include balsamic, almond-, or anise-like odors, and floral or fruity odors suggesting pineapple, honey, lemon, or orange; unpleasant include sulfurous, rubbery, oily, alliaceous, ammoniacal, and putrid (11).

The most important components responsible for the characteristic odors or flavors of essential oils are terpenes. They are hydrocarbons built from a basic isoprene unit with structures that may be open chain, closed chain, cyclic, saturated, or unsaturated. Odor descriptions associated with monoterpenes include rose-like (geraniol), sweet (linalool), and sweet, rose-like (nerol). Sesquiterpenoids, which are generally oxygenated, possess intensely balsamic, citrus or floral odors.

Esters are important aroma compounds of fruits, in which they are present in fairly low concentrations. Fruity flavors are attributed to ester formation caused by the reaction of organic acids with ethanol. Produced as the first synthetic flavors a century ago, esters can also be synthesized by fungi.

Lactones, widely used by the flavoring industry, are characterized a pleasant odor and flavor. They can be formed naturally in foods through a variety of chemical reactions or through biological processes and are described as fruity, coconut-like, buttery, sweet, or nut-like. Although chemical synthesis remains the most practical method of production at this time, optically active lactones in a relatively pure form can be naturally synthesized in fewer steps by fungi.

Pyrazines are the typical aroma components of heated foodstuffs, to which they give a characteristic roasted or nutty flavor. Normally, these heterocyclic, nitrogen-containing compounds are formed through nonenzymatic browning, whereby a carbonyl group and an amine group condense when heated above 100°C. Pyrazines have been isolated from vegetables, and associated sensory properties include bell pepper, peas, potatoes, vegetable, nutty, popcorn, pineapple, grassy, pungent, and earthy.

The same volatile metabolites that contribute to the flavor in some foods, such as edible mushrooms, can also be responsible for off-flavors in others, such as bread, cereals, and dairy products (17). Oxidation of terpenes also causes the formation of off-flavors. Table 2 lists aromatic compounds detected in mushrooms and their characteristic odors.

TABLE 2. Aromatic compounds produced by mushrooms and their characteristic odors.

Compounds	Odors	References
Alcohols		
methanol	alcoholic	18
ethanol	alcoholic	18
propanol	alcoholic	18
1-butanol	butanol	10, 12
1-pentanol	rubbery, harsh, alcoholic	10, 12, 18
1-hexanol	sweet, slightly fruity	4, 9, 10, 12
1-heptanol	herbaceous	10, 12
1-octanol	fruity-flowery, sweet soap, orange, waxy, sweet	4, 9, 10, 12, 27
1-nonanol	sweet, floral, soap	27
1-decanol	sweet, mirabelle	12
1-dodecanol	floral	12
isopropanol	alcoholic	18
isobutanol	alcoholic	18
isopentanol	alcoholic	18
2-methyl-1-propanol	alcoholic, butanol, harsh	4, 9, 10, 12
2-methyl-1-butanol	pungent	10, 12
3-methyl-1-butanol	alcoholic, cheese, pungent	4, 9, 10, 12
2,2-dimethyl-1-hexanol	herbaceous, pungent	10
3-octanol	fruity, cod liver oil, citrus, weakly nutty, fungal	4, 9, 12, 19, 27
4-nonanol	heated plastic	10
3-methyl thiopropanol	sulfuraceous, pungent	10, 12
1-octen-3-ol	mushroom, butter, resinous	9, 10, 12, 19, 20, 21, 26, 27, 28
2-octen-1-ol	fruity	12
trans-2-octen 1-ol	medicinal, oily	27
cis-2-octen-1-ol	oily, musty	19
5-octen-3-ol	floral	12
7-octen-4-ol	cauliflower	10, 12

TABLE 2 continued.

Compounds	Odors	References
2,2-dimethyl-1-hexanol	herbaceous, pungent	10
3-octanol	fruity, cod liver oil, citrus, weakly nutty, fungal	4, 9, 12, 19, 27
4-nonanol	heated plastic	10
3-methyl thiopropanol	sulfuraceous, pungent	10, 12
1-octen-3-ol	mushroom, butter, resinous	9, 10, 12, 19, 20, 21, 26, 27, 28
2-octen-1-ol	fruity	12
trans-2-octen 1-ol	medicinal, oily	27
cis-2-octen-1-ol	oily, musty	19
5-octen-3-ol	floral	12
7-octen-4-ol	cauliflower	10, 12
2-decen-1-ol	floral	12
2,3-butanediol	fatty	10, 12
1,2-heptanediol	herbaceous, bay	10
1,9-nonanediol	pineapple, citrus	12
4,5-dimethyl-1,3-benzenediol	warm, floral	10
phenyl methanol	roasted then harsh	10, 12
3-hydroxyphenyl methanol	peach, fruity	10
3,4-dimethoxyphenyl methanol	milky, vanilla, pungent	10
2-phenyl ethanol	faded rose	8, 10, 12, 18, 19
3-ethoxy-1-propanol	fruity	12
2-butoxy ethanol	roasted	12
2-phenoxy ethanol	dandelion, burnt wood	12
4-hydroxyphenyl ethanol	fruity	10
2-butoxyethoxy 2-ethanol	musty	12
3-phenyl propanol	floral	10
3,4-dimethyoxyphenyl propanol	herbaceous	10
benzyl alcohol	faint, pleasant fruity	8, 18, 19
p-methylbenzyl alcohol	hyacinth, gardenia	18, 19

TABLE 2 continued.

Compounds	Odors	References
p-α-dimethylbenzyl alcohol	grassy	14
p-α-dimethylphenyl alcohol	faint grassy	18
α,4-methylcyclohex-3-ene ethyl alcohol	light, sweet fruity	14, 18, 19
cis-cinnamic alcohol	warm, balsamic, sweet	8, 10
Phenols		
5-methoxy-2,3-dimethylphenol	herbaceous	10
Ketones		
3-hydroxy 2-butanone	Creamy, fatty	10, 12
2-heptanone	fruity, chemical	10
1-octen-3-one	boiled mushrooms, metallic, fungal, wild mushroom	9, 26, 27
2-octanone	floral, musty	10
3-octanone	fruity, sweet, musty, floral, lavender, sweet ester	4, 9, 10, 12, 19, 27
2-nonanone	blue cheese	10
3-ethyl-4-methyl-3-penten-2-one	potato	10
acetophenone	meal	10
4-methyl acetophenone	warm, floral	10
1-phenyl-1,2-ethanedione	camphorated	10
1-phenyl-1,2-propanedione	cake	10
4-hydroxy-3-methyl 2-phenyl-2-cyclopenten-1-one	chlorophyll, chewing gum	10
p-methylacetophenone	sweet, floral, fruity	14, 18, 19
3,6-dihydro-3-methyl acetophenone	sweet, fruity	14, 18, 19
3-methyl-8-hydroxy 3,4-dihydro (1H) 2-benzopyran-1-one	rubbery, balsamic, floral	10

TABLE 2 continued.

Compounds	Odors	References
Esters		
ethyl acetate	solvent, glue	10, 12
1-octen-3-yl acetate	mushroom, soapy water	27
ethyl heptanoate	floral, fruity wine	10
ethyl octanoate	estery	4, 9, 10
ethyl decanoate	estery	4, 9
ethylhydroxy butanoate	pineapple	10
linalyl acetate	floral, fruity	10
methyl benzoate	floral, heavy, pungent, fruity	8, 10, 12, 18, 19
ethyl benzoate	warm, citrus, floral, fruity	8, 10, 12, 18, 19
methylphenyl acetate	fruity, honey	10, 13, 18, 19
1,3-propanediol diacetate	roasted meal	12
1-octen-3-yl propionate	sweet, fruity, herbaceous, medicinal, mushroom	27
2-phenylethyl acetate	rose	10
methyl-4-methoxybenzoate	floral, hyacinth	10
methyl-*p*-methoxyphenyl acetate	anise	13, 18, 19
methyl salicylate	wintergreen	8, 18, 19
methyl anisate	anise	18, 19
methyl cinnamate	strawberry, fruity, jasmine	10, 30
ethyl cinnamate	fruity	10
Lactones		
γ-butyrolacetone	faint odor	10
γ-hexalactone	floral, herbaceous	10
4-hexanolide	fragrant	4, 9
4-heptanolide	fragrant	4, 9
4-octanolide	coconut	4, 9
4-nonanolide	fruity	9
4-decanolide	peach	4, 9, 12

TABLE 2 continued.

Compounds	Odors	References
2-hepten-4-olide	fragrant	9
2-octen-4-olide	fruity, coconut	4, 9
5-octen-4-olide	coconut	4, 9
6-octen-4-olide	coconut	4, 9
2-nonen-4-olide	fruity	4, 9
5-methyl (2H) 3-furanone	rubber, harsh	10
4,6-dimethyl (2H) 2-pyranone	fruity	10
4-methyl-5-hydroxy hexanoic acid lactone	grassy	10
Aldehydes		
hexanal	freshly cut grass	10
heptanal	oily, fatty	10
nonanal	fatty, floral	10
4-nonenal	watermelon	10
2,4-decadienal	fatty	10, 12
trans-2-octenal	sweet to phenolic	27
benzaldehyde	almond, sweet, phenolic	10, 12, 18, 27
2-aminobenzaldehyde	pungent, bad perfume, strong, sweet fruity, orange blossom	12, 34
p-tolualdehyde	almond	14, 18, 19
anisaldehyde	anise	10, 18, 19
phenyl acetaldehyde	oily, green, floral	10
3,4-dimethoxybenzaldehyde	milky, chocolate	10
Pyrazines	dried mushroom	32
Terpenes		
citronellol	rose	13, 18, 19
nerol	sweet, floral, slightly bitter, rose	13, 16, 18, 19
geraniol	rose, sweet	13, 16
linalool	light, sweet, fresh, citrus, floral	10, 16, 18, 19

TABLE 2 continued.

Compounds	Odors	References
γ-nerolidol	woody, floral warm	10
α-bisabolol	floral	10, 12
lepistirone	iris oil, orange blossom	2
Hydrocarbons		
o/m-diacetyl benzene	rubber	10
α-guaiene	balsamic	10
α-gurjunene	fatty	10
α-muurolene	fruity	10
limonene	citrus	10
β-bisabolene	orange, fruity	10
γ-β-farnesene	warm	10
Miscellaneous		
trans-furan linalool oxide	burnt	10
cis-pyran linalool oxide	herbaceous	10
trans-pyran linalool oxide	herbaceous	10
4-methyl-5-thiazole ethanol	animal, sweet	10
Compound A	balsamic, camphor	10

4. MUSHROOMS KNOWN TO PRODUCE AROMATIC COMPOUNDS

Many mushroom species possess an odor that can be described as "mushroom-like," and this special aroma is an essential part of their appeal. Mushroom-like flavor is due to the presence of aliphatic eight-carbon compounds, although other compounds contribute to the flavor. The mycelium of edible mushrooms cultivated by submerged fermentation can be used for the production of mushroom aroma (29), and this process has been patented (28). Other mushroom species have additional distinct aromas that uniquely flavor foodstuffs (19). Specific mushrooms and the aromatic compounds that they have been shown to produce are found in Table 3.

About 50 volatile compounds have been identified in each of seven edible fresh mushrooms: *Cantharellus cibarius, Gyromitra esculenta, Boletus edulis, Lactarius trivialis, Lactarius torminosus, Lactarius rufus,* and *Agaricus bisporus* (26). The C_8 alcohols and aliphatic methyl ketones (C_5 to C_{11}) together with benzyl alcohol, benzaldehyde, hexanol, and 3-methyl butanol comprise around 90% of the total volatile compounds extracted. General mushroom-like aroma is due to 1-octen-3-ol, formed by the enzymatic breakdown of linoleic acid, while 1-octen-3-one seems to be

TABLE 3. Mushrooms and their aromatic compounds

Mushrooms	Compounds	References
Agaricus bisporus	1-octen-3-ol 1-octen-3-one *trans*-2-octenal *trans*-2-octen-1-ol benzaldehyde benzyl alcohol phenylacetaldehyde octanol 3-methylbutanal 3-octanone 3-octanol 1-octen-3-yl acetate 1-octen-3-yl propionate nonanol furfural	9, 15, 19, 21, 25, 26, 27
Armillaria lutea	3,4 dimethoxyphenyl methanol 3,4 dimethoxy benzaldehyde methyl ketones	10
Armillaria mellea	3,4 dimethoxyphenyl methanol 3,4 dimethoxy benzaldehyde	10
Armillaria ostoyae	1-octanol	10
Auriporia aurea	2-octen-4-olide	15
Boletus edulis	1-octen-3-ol 1-octen-3-one *trans*-2-octen-1-ol *trans*-2-octenal 3-octanol 3-octanone octanol 1-octen-3-yl acetate 1-octen-3-yl propionate nonanol pyrazines 2-formylpyrroles lactones	9, 15, 26, 27, 33
Cantharellus cibarius	1-octen-3-ol 1-octene-3-one *trans*-2-octenal *trans*-2-octen-1-ol 3-octanol	26, 27

TABLE 3 continued

Mushrooms	Compounds	References
	3-octanone octanol 1-octen-3-yl acetate 1-octen-3-yl propionate nonanol	
Ceriporiopsis subvermispora	methyl benzoate methylphenyl acetate linalool linalool oxide phenyl acetaldehyde methyl cinnamate 4-methyl-5-thiazole ethanol ethyl cinnamate *cis*-cinnamic alcohol	10
Clitocybe illudens	sesquiterpenes: δ-cadinol [(+)-torreyol] protoilludane skeleton sesquiterpenes: illudalic acid, illudinine	15, 22, 23
Dichomitus squalens	benzaldehyde 3,4 dimethoxyphenyl methanol 3,4 dimethoxy benzaldehyde	10
Gloeophyllum odoratum	methylphenyl acetate geraniol methyl-*p*-methoxyphenyl acetate nerol citronellol linalool drimenol 1-octen-1-ol 1-octen-3-ol anisaldehyde d-limonene drimenol methyl-3-hydroxy-3,7-dimethyl-6-octenoate	13, 15, 18, 30
Gloeophyllum trabeum	methyl cinnamate sesquiterpenoids compound A	10
Grifola frondosus	benzothiazole 3-methyl-8-hydroxy-3,4-dihydro (1H) benzopyran-1-one	10

TABLE 3 continued

Mushrooms	Compounds	References
Gyromitra esculenta	1-octene-3-one *trans*-2-octenal *trans*-2-octen-1-ol 3-octanol 3-octanone octanol 1-octen-3-yl acetate nonanol	26, 27
Hebeloma sacchariolens	2-aminobenzaldehyde	34
Heterobasidion annosum	4-hydroxy-3-methyl 2-phenyl 2-cyclopenten-1-one	10
Hirschioporus pergamenus	1-octanol 3,4 dimethoxyphenyl methanol 3,4 dimethoxy benzaldehyde methyl cinnamate	10
Inocybe corydalina	methyl cinnamate	15, 31
Inocybe pyriodora	methyl cinnamate	15, 31
Inocybe odorata	methyl cinnamate	15, 31
Lactarius rufus	1-octen-3-ol *trans*-2-octen-1-ol 1-octene-3-one *trans*-2-octenal 3-octanol 3-octanone octanol nonanol	26, 27
Lactarius torminosus	1-octen-3-ol *trans*-2-octen-1-ol 1-octene-3-one *trans*-2-octenal 3-octanol 3-octanone octanol	26, 27
Lactarius trivialis	1-octen-3-ol *trans*-2-octen-1-ol 1-octene 3-one *trans*-2-octenal 3-octanol 3-octanone	26, 27

TABLE 3 continued

Mushrooms	Compounds	References
	octanol 1-octen-3-yl acetate	
Lactarius uvidus	drimenol (a bicyclofarnesol)	15
Lentinellus cochleatus	*trans*-nerolidol fokienol 6-formyl-2,2-dimethylchromene	15
Lentinula edodes	lenthionine 4-methyl acetophenone 3-methyl-8-hydroxy-3,4 dihydro (1H) 2-benzopyran-1-one	5, 10
Lentinus lepideus	methyl cinnamate sesquiterpenes: cadinols muurolols cubenols farnesol drimenol terrestrol linalool geraniol	1, 15, 18, 32
Lenzites betulina	linlool linlool oxides linlool acetate 3-hydroxy-2-butanone 2,3 butanone 2,3-butanediol 3-octanone methyl benzoate 2,4-decadienal	10
Lepista irina	bisabolane derivative: lepistrone	2, 15
Mycoacia uda	*p*-tolualdehyde *p*-methylacetophenone α-4-methylcyclohex-3-ene-ethyl alcohol *p*-tolyl-1-ethanol *p*-tolylaldehyde *p*-methylbenzyl alcohol *p*-α-dimethylbenzyl alcohol iso-butanol 3,6-dihydro-3-methylacetophenone methanol	6, 7, 14, 15, 18, 30

TABLE 3 continued

Mushrooms	Compounds	References
	ethanol iso-pentanol pentanol isopropanol propanol	
Perenniporia subacida	benzaldehyde 1-octanol methyl cinnamate Compound A	10
Phellinus igniarius	benzyl alcohol ethyl benzoate linalool methyl benzoate methyl salicylate phenylethyl alcohol phenol	8, 10, 15, 18
Phellinus laevigatus	benzyl alcohol methyl benzoate methyl salicylate phenylethyl alcohol	8, 15, 18
Phellinus pini	phenyl methanol 3-phenyl propanol	10
Phellinus robustus	1-octanol	10
Phellinus tremulae	benzyl alcohol ethyl benzoate linalool methyl benzoate methyl salicylate phenylethyl alcohol phenol	8, 10, 15, 18
Phlebia radiata	aromatic primary alcohols α-bisabolol1	12, 15
Pleurotus euosmus	linalool coumarine cis- and trans-linalooloxides	10, 15, 18
*Pleurotus ostreatus*se	squiterpenoids	10
Polyporus betulinus	1-octanol	10
Polyporus durus	2-methyl-1-propanol	4, 5

TABLE 3 continued

Mushrooms	Compounds	References
	3-methyl-butanol 1-hexanol 3-octanol 1-octanol 3-octanone ethyl octanoate ethyl decanoate 4-hexanolide 4-heptanolide 2-hepten-4-olide 4-octanolide 2-octen-4-olide 5-octen-4-olide 6-octen-4-olide 4-nonanolide 2-nonen-4-olide 4-decanolide 4-butanolide 4-pentanolide 2-hexen-4-olide 5-hexen-4-olide 2,5-en-6-octan-4-olide 2-decen-4-olide	
Stereum subpileatum	cinnamic acid derivatives	10
Trametes suaveolens	anisaldehyde benzaldehyde methyl anisate δ-cadinene	13, 18
Tricholoma matsutake	1-octen-3-ol	22
Tyromeces sambuceus	4-decalactone (4-decanolide)	15

important in producing the more specific aroma of cooked mushrooms. The major compounds found in uncooked *Agaricus bisporus* are 3-octone, 3-octanol, 1-octen-3-ol, benzaldehyde, octanol, and 2-octen-1-ol. After cooking, 1-octene-3-one is found in addition to the others (25). The flavor of the shiitake mushroom *Lentinula edodes* is due to a cyclic sulphur compound lenthionine, which is produced during drying and processing (5). An analysis of the dried mushroom *Boletus edulis* showed the presence of approximately 70 constituents, including nine pyrazines and seven 2-formylpyrroles (33).

Gloeophyllum odoratum (=*Trametes odorata*) produces sweet or rose-like citronellol, nerol,

and geraniol, methylphenylacetate with a honey-like odor, and methyl-*p*-methoxyphenylacetate with an anise-like odor. *Trametes suavolens* produces methylanisate, anisaldehyde, and benzaldehyde (13). In the culture broth of several *Phellinus* strains, methylbenzoate was discovered as being the main compound along with methyl salicylate, benzyl alcohol and linalool (8). *Mycoacia uda* synthesizes a pleasant fruity aroma. Thirty-one compounds have been detected in surface cultures of this strain with *p*-tolualdehyde, *p*-methylacetophenone, *p*-α-dimethylbenzyl alcohol, and *p*-methylbenzyl alcohol being the main volatiles (14).

Fruit bodies of *Lentinus lepidus*, which produce a characteristic anise-like odor, contain cinnamic acid derivatives, sesquiterpene hydrocarbons, and alcohols (1, 32). The source of the sweet odor of *Hebeloma sacchariolens* is 2-aminobenzaldehyde (34). The fruity, jasmine aroma of *Inocybe corydalina* and *Inocybe pyriodora* is due to methyl cinnamate (31). The lactones of *Nigroporus durus* (=*Polyporus durus*) have a pineapple or coconut aroma (4).

5. CULTURE CONDITIONS FOR PRODUCTION OF AROMATIC COMPOUNDS

Production of volatiles and the resulting flavors by mushrooms depends on the composition of the growth medium, growth conditions, genetic variation of the strains, and the subjectivity of sensory perception. Modifications of culture conditions, especially the choice of nitrogen and carbon sources appear to influence, at least quantitatively, the composition of the fungal odorous profile. Gallois and coworkers (10) investigated the volatile metabolites produced by 29 ligninolytic strains, representing 22 species, on six different liquid media, two natural and four synthetic, with and without agitation. They identified 113 compounds in 25 strains. Detectable odorous notes generally appeared after seven to ten days and lasted for two to ten days. Aromas were generally more intense in stationary culture. The mycelium morphology in agitated media seems to affect odorous metabolite production; strains that form numerous small pellets of regular size were odorless. One that formed long-hair fur balls produced a strong odor. The production of pigment is sometimes associated with aroma production. At different stages of growth, the same mushroom can produce different odors (3).

Odorous profiles of mushrooms are generally determined through the use of gas chromatography coupled with infrared, mass spectroscopy or nuclear magnetic resonance analysis. Results obtained by different laboratories using the same species often differ in both composition of the profile and the concentration of the volatile compounds. More than 150 compounds have been detected, one-third of which possessed an aromatic skeleton. The most numerous are alcohols, aldehydes, and ketones (10, 19).

6. DISCUSSION

A wide variety of compounds with unique and potent flavor notes have been isolated as naturally occurring metabolites of mushrooms. The aroma profiles for individual strains vary according to the medium used, growth conditions, genetic variation, and sensory evaluation of the investigators. With the help of analytical techniques an increasing number are still being identified and characterized. Further research in this area should be most rewarding.

Although mushrooms produce desirable aromatic compounds, they have not yet become a commercial source of flavor materials. One of the limiting factors is the time required for growth and the low yield of flavor material. Due to the flavor potency of certain flavor compounds, low yields

can be tolerated. Other problems are technical. The volatility and low solubility of aromatic compounds in water makes their recovery difficult. However, aromatic metabolites, which are produced only to a small extent on the natural substrate, can be enormously enriched with the selection of suitable strains and appropriate growth conditions. Purification of flavor compounds from mushrooms that produce complex volatile mixtures can be difficult, but a systematic screening for mushrooms in culture that produce a few primary volatiles could help to solve that problem.

In the search for naturally-occurring flavor substances, strains for screening are available from culture collections. The American Type Culture Collection (ATCC), for example, maintains more than 6,000 authenticated strains of edible and wood-decaying mushrooms from all over the world for use by research and bioindustry.

In addition to opening up new possibilities for the production of aromatic materials, mushrooms can also provide a system for studying the biosynthetic pathways involved in flavor formation. In the future, biotechnologists will be able to choose from a large variety of biocatalysts for the total biosynthesis of aromatic compounds from simple precursors. Further development of these processes, which could translate into billions of dollars worth of flavoring materials for the food and cosmetic industries, depends on a thorough knowledge of mushroom potentialities.

REFERENCES

1. ABRAHAM, W.-R., HANSSEN, H.-P. & MÖHRINGER, C. (1988). Novel sesquiterpene ethers from liquid cultures of the wood-rotting fungus *Lentinus lepidus*. *Zeitschrift für Naturforschung* **43c**, 24-28.
2. ABRAHAM, W.-F., HANSSEN, H.-P. & URBASCH, I. (1991). Lepistirones, major volatile metabolites from liquid cultures of *Lepista irina* (Basidiomycotina). *Zeitschrift für Naturforschung* **46c**, 169-171.
3. BADCOCK, E.C. (1939). Preliminary account of the odour of wood-destroying fungi in culture. *Transactions of the British Mycological Society* **23**, 188-245.
4. BERGER, R.G., NEUHÄUSER, K. & DRAWERT, F. (1986). Biosynthesis of flavorcompounds by microorganisms. 6. Odorous constituents of *Polyporus durus* (Basidiomycetes). *Zeitschrift für Naturforschung* **41c**, 963-970.
5. CHEN, C.-C., LIU, S.-E., WU, C.-M. & HO, C.-T. (1986). Enzymatic formation of volatile compounds in shiitake mushroom (*Lentinus edodes*). In: *Biogeneration of Aromas,* ACS Symposium Series 317. Edited by T.H. Parliament & R. Croteau. pp. 176-183. Washington, DC, American Chemical Society.
6. COLLINS, R.P. (1976). Terpenes and odoriferous materials from microorganisms. *Lloydia* **39**, 20-24.
7. COLLINS, R.P. (1979). The production of volatile compounds by filamentous fungi. In: *Developments in Industrial Microbiology vol. 20*. Edited by the Society of Industrial Microbiology. pp. 239-245. Arlington, VA, SIM.
8. COLLINS, R.P., & HALIM, A.F. (1972). An analysis of the odorous constituents produced by various species of *Phellinus*. *Canadian Journal of Microbiology* **18**, 65-66.
9. CRONIN, D.A., & WARD, M.K. (1971). The characterization of some mushroom volatiles. *Journal of Science of Food and Agriculture* **22**, 477-479.
10. GALLOIS, A., GROSS, B., LANGLOIS, D., SPINNLER, H.-E. & BRUNERIE, P. (1990). Influence of culture conditions on production of flavor compounds by 29 ligninolytic

Basidiomycetes. *Mycological Res*earch **94**, 494-504.
11. GILBERT, E.J. (1934). Odor properties of fungi fruiting bodies. In: *Methode de Mycologie Descriptive*, Paris.
12. GROSS, B., GALLOIS, A., SPINNLER, H.-E. & LANGLOIS, D. (1989). Volatile compounds produced by the ligninolytic fungus *Phlebia radiata* Fr. (Basidiomycetes) and influence of the strain specificity on the odorous profile. *Journal of Biotechnology* **10**, 303-308.
13. HALIM, A.F. & COLLINS, R.P. (1971). Analysis of the odorous constituents of *Trametes odorata*. *Lloydia* **34**, 451-452.
14. HALIM, A.F. & COLLINS, R.P. (1975). Characterization of the major aroma constituents of the fungus *Mycoacia uda*. *Lloydia* **38**, 87-91.
15. JANSSENS, L., DEPOOTER, H.L., SCHAMP, N.M. & VANDAMME, E.J. (1992). Production of flavours by microorganisms. *Process Biochemistry* **27**, 195-215.
16. KEMPLER, G.M. (1983). Production of flavor compounds by microorganisms. *Advances in Applied Microbiology* **29**, 29-51.
17. KINDERLERER, J.L. (1989). Volatile metabolites of filamentous fungi and their role in food flavor. *Journal of Applied Bacteriology Symposium Supplement.* 133S-144S.
18. MAGA, J.A. (1976). The potential of certain fungi as sources for natural flavor compounds. *Chemical Senses and Flavor* **2**, 255-262.
19 MAGA, J.A. (1981). Mushroom flavor. *Journal of Agricultural and Food Chemistry* **29**, 1-4.
20. MAU, J.-L., BEELMAN, R.B., ZIEGLER, G.R., & ROYSE, D.J. (1991). Effect of nutrient supplementation on flavor, quality, and shelf life of the cultivated mushroom, *Agaricus bisporus*. *Mycologia* **83**,142-149.
21. MAU, J.-L., BEELMAN, R. B. & ZIEGLER, G.R. (1992). 1-Octen-3-ol in the cultivated mushroom, *Agaricus bisporus*. *Journal of Food Science* **57**, 704-706.
22. NAIR, M.S.R. & ANCHEL, M. (1973). Metabolic products of *Clitocybe illudens*. X. (+)-Torreyol. *Lloydia* **36**, 106.
23. NAIR, M.S.R., TAKESHITA, H., MCMORRIS, T.C., ANCHEL, M. (1969). Metabolites of *Clitocybe illudens*. IV. Illudalic acid, a sesquiterpenoid, and illudinine, a sesquiterpenoid alkaloid. *Journal of Organic Chemistry* **34**, 240-243.
24. NEIDLEMAN, S.L. (1989). The microbial production of biochemicals. In: *A Revolution in Biotechnology,* pp. 56-70. Edited by Jean L. Marx. Cambridge: Cambridge University Press.
25. PICARDI, S.M., & ISSENBERG, P. (1973). Investigation of some volatile constituents of mushrooms (*Agaricus bisporus*): Changes which occur during heating. *Journal of Agricultural and Food Chemistry* **21**, 959-962.
26. PYYSALO, H. (1976). Identification of volatile compounds in seven edible fresh mushrooms. *Acta Chemica Scandinavica B* **30**, 235-244.
27. PYYSALO, H. & SUIHKO, M. (1976). Odour characterization and threshold values of some volatile compounds in fresh mushrooms. *Lebens.-Wiss. u. -Technol* **9**, 371-373.
28. SCHINDLER, F. (1989). Method of producing a mushroom aroma in mushroom cellmasses. US Patent 4,810,504.
29. SCHINDLER, F. & SEIPENBUSCH, R. (1990). Fungal flavour by fermentation. *Food Biotechnology* **4**, 77-85.
30. SCHINDLER, J., & SCHMID, R.D. (1982). Fragrance or aroma chemicals - microbial synthesis and enzymatic transformation - a review. *Process Biochemistry* **17**, 2-8.
31. SCHMITT, J.A. (1978). Über den Duftstoff zweier Rißpilzarten (Agaricales, Basidiomycetes).

Zeitschrift für Naturforschung **33c**, 817-819.

32. SPRECHER, E. & HANSSEN, H.-P. (1982). Influence of strain specificity and culture conditions on terpene production by fungi. *Planta Medica* **44**, 41-43.
33. THOMAS, A.F. (1973). An analysis of the flavor of the dried mushroom, *Boletus edulis*. *Journal of Agricultural and Food Chemistry* **21**, 955-958.
34. WOOD, W.F., BROWNSON, M. & SMUDDE, R.A. (1992). 2-Aminobenzaldehyde: The source of the "sweet odor" of *Hebeloma sacchariolens*. *Mycologia* **84**, 935-936.

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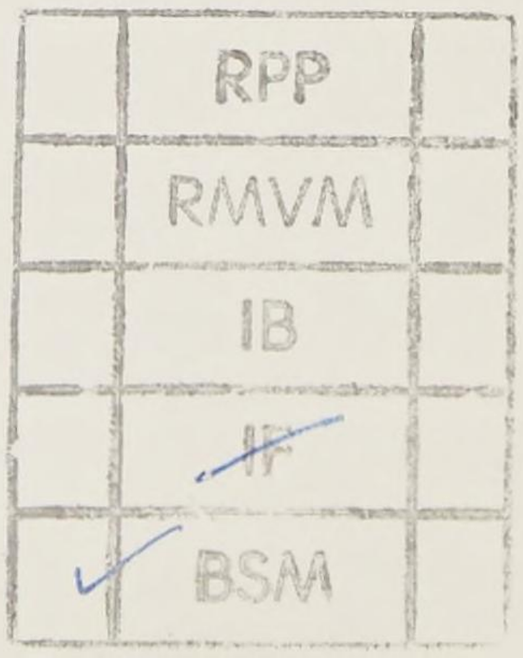
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The use of mushrooms and mushroom cultivation have a long history in human development. During the past decade, mushroom production and research into various aspects of mushroom biology have increased dramatically. This has been due largely to a growing awareness that mushrooms, apart from their delicacy, have a high nutritional value, can serve as a cheap source of protein, and possess important tonic and medicinal properties. In recognition of the expanding scientific and economic importance of mushrooms, the First International Conference on Mushroom Biology and Mushroom Products was held in Hong Kong from 23–26 August 1993. This volume represents an anthology of contributions from the Keynote, Plenary and Round-Table Speakers participating in the Conference.

A wealth of new information encompassing many facets of mushroom biology and mushroom products is to be found in this book. Recent experimental advances have been integrated with exisiting knowledge by leading investigators in such areas as:

(1) Fundamental Aspects of Mushroom Biology,
(2) Mushroom Cultivation and Bioconversion Technology,
(3) Post-harvest Processing and Quality Control,
(4) Nutritional and Medicinal Attributes of Mushrooms,
(5) Future Prospects for Research and Development.

The papers provide an up-to-date account of current research trends and advances in mushroom biology and mushroom products, and highlight some of the problem areas which remain to be resolved.

Mushroom Biology and Mushroom Products will be of interest to mushroom research scientists and growers, as well as to teachers and students of mushroom biology and biotechnology.

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