# POSTER PRESENTATIONS

# Comparison of Growth of Selected Wood Decay Fungi on Various Agar-Supplemented Media

*Samuel A. Amartey,[1] Miha Humar,[2] and Franc Pohleven[2]*

[1]Division of Biology, Imperial College of Science, Technology and Medicine, South Kensington Campus, Exhibition Road, London, SW7 2AZ, UK; [2]University of Ljubljana, Biotechnical Faculty, Dept. of Wood Science and Technology, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

In order to develop a sustainable waste management system for preservative treated wood waste through bioremediation and biodeterioration (bulk reduction) using wood decay Basidiomycetes, a good solid (agar) medium which supports good growth is required to isolate novel fungi (brown and white rots), screen and evaluate potential strains in terms of growth rate, wood decay ability, tolerance to various preservatives as well as for various laboratory tests using these fungi.

The selection of a suitable media requires a detailed investigation to establish the most suitable medium that meets certain basic requirements. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance. Furthermore, the medium must be cheap, demonstrate a consistent quality, and be readily available.

Wood decay fungi have been found to exhibit different forms on different media. A number of solid agar media have been used by many researchers to grow these fungi. Malt extract agar (MEA) is routinely used as a medium/basal medium for most studies and tests with these fungi. Although it is not the most ideal medium for all fungi species, it is a good general substrate for most organisms and both micro-fungi and Basidiomycetes grow well on it. Other agar-supplemented media such as potato dextrose agar (PDA), YMPG agar (yeast extract, malt extract, bacto-peptone, glucose, asparagines, and thiamine), MEY agar (malt extract, yeast extract), wood extract agar and wood agar, including beech wood agar (BWA), have also been routinely used as media/basal media for various studies and tests involving wood decay fungi.

In this report, the growth rate of a selected brown and white decay fungi on various agar-supplemented media often used to grow these fungi were determined and compared in order to find the best agar medium for growing these fungi.

The media investigated were malt extract agar (MEA), potato dextrose agar (PDA), YMPG agar (yeast extract, malt extract, bacto-peptone, glucose, asparagines, and thiamine), YMPG agar (without amino acids), and beech wood powder agar (BWA).

The wood decay fungi used were *Phanerochaete chrysosporium* BKMF-1767, *Phanerochaete sordida* HHB-8922*, Trametes versicolor* MD-227*, Bjerkandera adusta* FP-135160, *Antrodia vaillantii* FA (F3), and *Leucogyrophana pinastri* HPT-595 (YF). Growth rates were estimated daily by measuring diameters of the colonies of the fungi at right angles along 2 diameters during a period of about 10 days at 22 ± 2°C and 65 ± 5% R.H.

Results obtained show that the growth rates of the white rot decay fungi were higher than the brown rot decay fungi on all the agar media investigated. Among the white rot fungi, *Phanerochaete chrysosporium* consistently showed higher growth rates than the others on all the agar media. YMPG agar was the most preferred medium by all the fungi, and beech wood agar was the least preferred medium. The high growth rates on the YMPG agar could be due to the fact that it is a rich medium containing readily available carbon sources, amino acids, vitamins, and other growth stimulating factors. On the other hand, beech wood agar is a poor medium, since wood is limiting in nitrogen and amino acids, which are required by fungi for the synthesis of protein, enzymes, and the chitin in hyphal cell walls fungi. Growth rates of all the fungi on malt extract agar and potato dextrose agar were comparable.

Finally, the growth rates of both brown rot fungi (*Antrodia vaillantii* and *Leucogyrophana pinastri*) were comparable on all the agar media. It was also interesting to note that both the brown rot fungi tested showed biphasic growth on the beech wood agar. This is a phenomenon known as diauxic growth and could be due to sequential utilization of the carbon and energy sources in the wood agar medium.

1521-9437/07/$35.00

# Biosorption of Heavy Metals in the Ectomycorrhizal Fungus *Tuber borchii*: A Preliminary Molecular Approach

*Antonella Amicucci,[1] Emanuela Polidori,[1] Roberta Saltarelli,[1] Paola Ceccaroli,[1] Alessandra Zambonelli,[2] Emanuela Cerigini,[1] and Vilberto Stocchi[1]*

[1]Instituto di Chimica Biologica "G. Fornaini", University of Urbino, Urbino (PU) 61029, Italy; [2]Dipartimento di Protezione e Valorizzazione Agroalimentare, University of Bologna, Bologna, Italy

The presence of toxic heavy metals in the environment has resulted in the development or acquisition by several bacteria and fungi of genetic systems that counteract their effects. Reactive and potentially toxic cofactors, such as copper ions, are imported into eukaryotic cells and incorporated into target proteins by unknown mechanisms.

Even if Cu is an essential cofactor for mitochondrial, cytosolic, and vesicular oxygen-processing enzymes, nevertheless, it can be toxic even at low concentrations. Cu (I) and Cu (II) ions can bind with high affinity to adventitious sites in partially folded proteins and catalyze auto-oxidation of lipids, proteins, and nucleic acids. To investigate the mechanisms by which cells overcome the dilemma of maintaining Cu availability while controlling deleterious reactivity of the free ions, we have characterized *Tuber borchii* Vittad. *Atx1* cDNA, an intracellular eukaryotic protein implicated in Cu trafficking.

*Atx1* is one of several genes in the Cu dependent, high-affinity iron uptake pathway in yeast. These genes encode Ctr1, a Cu uptake protein in the plasma membrane; Ccc2, an intracellular membrane protein, and; the multicopper oxidase Fet3. Although the role of Fet3 in iron uptake is unclear, Cu loading into this enzyme occurs in a post-Golgi vesicle and is mediated by Ccc2. The *ATX1* gene and its human homolog, *HAH1*, encode cytosolic proteins implicated in Cu trafficking to these Ccc2-containing vesicles. The Ccc2 protein in yeast and its human homologs, the Menkes disease protein and Wilson disease protein are members of the P-type adenosine triphosphatase (ATPase) cation transporter family and are present in the membranes of secretory vesicles. Each transporter contains two or more *Atx1*-like cytoplasmic domains, the functions of which are not known. The conserved MTCXXC sequence (X, any residue), a motif observed in several bacterial Hg (II) transport proteins, is thought to be a Cu binding site, although it does not correspond to known Cu (I) or Cu (II) sites in structurally characterized proteins.

In the present research a molecular approach was aimed at investigating the detoxification of molecular mechanisms in the ectomycorrhizal fungus *T. borchii*, a hypogeous fungus well-known for its edible and highly prized fruit body. Several previous works highlighted that numerous mycorrhizal fungi have a strong ability to absorb different kinds of heavy metals from soil thus permitting the host plant to survive.

In this work different couples of degenerated oligonucleotides deduced from the most conserved *Atx1* amino acid sequences were used in PCR experiments and a fragment of 550 bp highly homologous with *Atx1* gene of yeast was detected. In order to obtain a full-length cDNA encoding a putative Cu transporter from the extra radical mycelium of *T. borchii*, the fragment putatively corresponding to the *T. borchii Atx1* gene was used as a probe to screen a *T. borchii* mycelium cDNA library.

In conclusion, several mycorrhizal fungi protect the host plant from toxic compounds present in the soil and store them in specific subcellular compartments. Additional knowledge of the molecular mechanisms at the bases of the detoxification processes in ectomycorrhizal fungi can be a challenging starting point to develop new strategies and models in mico-remediation projects.

# Antioxidant and Free-Radical Scavenging Activity of Higher Basidiomycetes in Submerged Cultivation

*Mikheil D. Asatiani,[1,5] Lital Sharvit,[2] Vladimir I. Elisashvili,[3] Solomon P. Wasser,[1,4] Abraham Z. Reznick,[5] and Eviatar Nevo[1]*

[1]Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel; [2]Department of Evolutionary and Environmental Biology, Faculty of Science and Science Education, University of Haifa, Mt. Carmel, Haifa 31905, Israel; [3]Durmishidze Institute of Biochemistry and Biotechnology, Academy of Sciences of Georgia, 10 km Agmashenebeli kheivani, Tbilisi 0159, Georgia; [4]N.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev 01001, Ukraine; [5]Faculty of Medicine, Technion, Haifa, Israel

Numerous strains of higher Basidiomycetes, belonging to different ecological groups (saprotrphs, parasites, etc.), were cultivated in submerged conditions in order to produce natural bioactive compounds, including antioxidants. After the fungal biomass production, two solvents with different polarities were used for antioxidant extraction, ethyl alcohol and the culture liquid. The β-carotene bleaching method was used for screening the antioxidant activity of the obtained extracts and measuring the scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals. In this study we investigated the submerged mycelia of 30 Basidiomycetes strains. The antioxidant capability of these extracts significantly depended on mushroom species. A very high AA (more than 85%) was revealed when *Coprinus comatus* 306, *Agaricus nevoi* 408, and *Flammulina velutipes* 104 water (culture liquid) extracts in concentration of 2 mg/ml were used. A slightly lower AA was observed in water (culture liquid) extracts from *Auricularia auricula-judae* 1036, *Cyathus striatus* 978, *Ganoderma lucidum* 545, and *Omphalotus olearius* 1079 mycelial biomasses.

When the ethanol extracts from the same Basidiomycetes strain biomasses were tested for the AA, the highest inhibition values were received with *Agaricus nevoi* 408 ethanol extract followed by *Omphalotus olearius* 1079, and *Auricularia auricula-judae* 1036, 92.1%, 83.4%, and 80.2%, respectively, at an extract concentration of 2 mg/ml. In contrast to these fungi, no antioxidant activity was exhibited for *Coprinus comatus* 906 extract at the same concentration, while *Phellinus robustus* 531 showed only 17.6% inhibition. When the concentration of ethanol extract increased from 2 mg/ml to 4–8 mg/ml, the AA of extracts from *Agrocybe aegerita* 1038 and *Coprinus comatus* 906 increased from 46.6 to 82.7% and from 2.4 to 62.1%, respectively.

The comparison of the antioxidant potential of extracts received from mushroom biomasses with two different solvents show that the water extracts of *Leucoagaricus leucothites* 1075 and *Phellinus robustus* 531 had higher activities than their ethanol extracts. In our experiments the ethanol extracts received from the mycelial biomasses of *Daedalea gibbosa* 514, *Pleurotus citrinopileatus* 435, and *Trametes versicolor* 1013 showed higher antioxidant activities compared with the water extracts.

When the data on free-radical scavenging capacities of the water (culture liquid) extracts measured by DPPH assay were accumulated, again, the highest activity at a sample concentration of 0.5 mg/ml was shown with extracts of *Ganoderma lucidum* 546 (69.0%) and *Daedalea quercina* 943 (51.2%), but the values were much lower than that of the standard (91.5%). It is interesting that the water extract of *Flamullina velutipes* 1046 mycelial biomass exhibited very high scavenging activity at a sample concentration of 3.0 mg/ml, 89.8% of inhibition, which was comparable to that of butylated hydroxyanisole (BHA) standard. The data on water extracts under the same conditions of growth, mycelial biomasses of *Pleurotus citrinopileatus* 435, *Stereum hirsutum* 524, and *Pleurotus nebrodensis* 1019 showed very weak scavenging activity toward DPPH, 8.0, 10.4, and 10.8%, respectively. However, the scavenging activity of *Pleurotus citrinopileatus* 435 and *Stereum hirsutum* 524 water extracts increased with gradual elevation of sample concentrations from 0.5 to 9.0 mg/ml.

When the data on free-radical scavenging capacities of the Et-OH extracts were measured by

DPPH assay, again, the highest activity at a sample concentration of 0.5 mg/ml was shown with extracts of *Ganoderma lucidum* 546 (55.6%) and *Daedalea quercina* 943 (55.2%). However, the inhibition values were much lower than that of the standard. The scavenging activities of these extracts increased by 21 and 11%, respectively, while the sample concentration increased to 1.5 mg/ml. The ethanol extracts of *Stereum hirsutum* 524, *Trametes zonata* 450, and *Pleurotus nebrodensis* 1019 showed a very weak scavenging activity toward DPPH, only 0.6, 1.0, and 3.6%, respectively.

In conclusion, this study shows that the submerged cultivation of Higher Basidiomycetes may be a very promising source for preparing low-cost, effective technology of antioxidant production and for the investigation of extracts that could be suitable as antioxidative agents and bio products.

## Isolation, Characterization, and Cloning of CnSPI, a Serine Protease Inhibitor from *Clitocybe nebularis*

*Petra Avanzo,[1] Sabina Anžlovar,[1] Tatjana Popovič,[1] Janko Kos,[1,2] and Jože Brzin[1]*

[1]Department of Biotechnology, Jožef Stefan Institute, Jamova 39, Ljubljana SI-1000, Slovenia; [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, Lubljana SI-1000, Slovenia

In all groups of organisms the role of protease inhibitors is crucial in the regulation of endogenous proteolytic processes and protection against exogenously introduced proteases of pests and pathogens. Although the number of inhibitory proteins isolated from bacteria, plants, and animals is very large, little is known about their fungal counterparts. Of the few inhibitors observed in mushrooms, only *Lentinus edodes* and *Pleurotus ostreatus* serine protease inhibitors (SPI) have been adequately characterized. In this work, we present the isolation, inhibitory properties, and the full-length sequence of the gene of CnSPI, a serine protein inhibitor from *Clitocybe nebularis* (Batsch: Fr.) Quél.

In order to purify inhibitors, mushroom fruit bodies were extracted in a buffer using a three-step procedure, including affinity chromatography on trypsin- Sepharose. The inhibitory proteins appeared as two closely spaced bands with estimated molecular masses of 15.5 and 16.5 kDa in SDS-PAGE. Similarly, in the isolectric point determination a double band in the acidic range around pH 4 was obtained. The 20 amino acid N-terminal determined by direct protein sequencing revealed weak sequence homology with *Lentinus edodes* inhibitor. In order to test its specificity, various commercially available proteases and partially purified endogenous subtilisin-like protease from *C. nebularis* were compared. Trypsin and chymotrypsin were strongly inhibited, followed by elastase, subtilisin, and kallikrein. The heat and pH stabilities of CnSPI were also tested. It remained relatively stable up to 85°C and survived in a pH range of 3.0 to 11.0.

On the basis of the known N-terminal amino acid sequence of CnSPI and some internal regions within proteins, degenerated primers were constructed. A specific genomic sequence was obtained with two-step PCR amplification using nested primers in the secondary PCR. According to this sequence, specific primers were designed and a gDNA library was constructed using a DNA walking kit. Three different genomic clones were isolated with high identity at the nucleotide level. A cDNA sequence was identified using RT-PCR and complete cDNA sequence was obtained using the rapid amplification of cDNA ends (RACE) method. Comparison of genomic and cDNA sequences revealed that the CnSPI gene is composed of four exons and three introns. Based on CnSPI nucleotide sequence the whole amino acid sequence for the CnSPI protein was deduced. Alignment of deduced amino acid sequence of CnSPI and *Lentinus edodes* proteinase inhibitor shows 36.2% identity.

Besides its regulatory role in intracellular and/or extracellular proteolysis, further experiments are necessary to establish the precise physiological function of CnSPI and its potential in biotechnology and medicine.

# Production and Antitumor Properties of Fruit Body Substances and Submerged Mycelium of *Ganoderma lucidum*

*Anastasia V. Avtonomova,[1] Igor V. Belitskii,[1] Elena B. Isakova,[1] Maria S. Evsenko,[2] Anatolii I. Usov,[2] Vladimir M. Buchman,[1] and Larissa M. Krasnopolskaya[1]*

[1]Gause Institute of New Antibiotics, Russian Academy of Medical Sciences, 11 Bolshaya Pirogovskaya St., Moscow 119021, Russia; [2]Zelinsky Institute of Organic Chemistry, Russian Academy of Medical Sciences, 47 Leninsky Prospect, Moscow 117913, Russia

*Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Lingzhi or Reishi) is one of the most known medicinal species with diverse biological effects on the human organism. As a result of numerous investigations, biologically active substances of *G. lucidum* fruit bodies, basidiospores, and vegetative mycelium were isolated and studied. However, comparison of the literature as well as of our own data is difficult due to strain peculiarities of cultures, differences in the methods of cultivation, and chemical analyses used. The effectiveness of cultivation processes secures high-biological activity of mycelial and fruit bodies substances. At the peak of its physiological activity the mushroom produces a greater quantity of biologically active substances, specifically polysaccharides. Therefore, developing and modernizing known biotechnological methods of *G. lucidum* mycelium and fruit body production became an actual trend in biotechnology.

The present study was designed to develop high-performance methods of previously selected *G. lucidum* strain mycelium and fruit body production and to study their antitumor properties. Such an approach makes it possible to carry out a comparative study of the biological activity of the *G. lucidum* strain on the generative and vegetative stages of ontogenesis.

The developing of submerged cultivation included the study of biomass accumulation including temperature, aeration, medium pH control, and qualitative and quantitative composition of medium. The cultural conditions' effectiveness was judged by air dry biomass accumulation.

The optimal nutrient media was developed on the basis of mathematically designed experiments using full factorial and steepest ascent strategies. As a result, a medium based on 5 nutritional sources was developed, providing the air dry, with less than 6% water content and a biomass yield of 20–21 g/l on the 4th day of cultivation. *G. lucidum* fruit bodies were produced by intensive technology in polypropylene bags with oak sawdust, inoculated with *G. lucidum* submerged culture and grown using previously developed methods.

Antitumor activity of substances obtained from submerged mycelium and fruit bodies was studied *in vivo* on adult male hybrid mice (C57Bl/6 × DBA/2) F1, using transplantable tumor T-cell lymphoma P388. The antitumor activity was evaluated by a tumor inhibition parameter compared to an untreated control group. Freeze-dried submerged culture and total water soluble polysaccharide fractions of mycelium and fruit bodies were used as substances. Freeze-dried submerged culture of the studied *G.lucidum* strain, containing mushroom biomass and cultural liquid solid residue, administered intragastrically, provided 50% significant inhibition of tumor growth. A combined introduction of the studied substance with freeze-dried submerged cultures of mushrooms, e.g., *Hericium erinaceus, Hypsizygus ulmarius,* and *Trametes versicolor* revealed an additive effect expressed in the tumor inhibition growth up to 77%–80%.

The aim of the next stage of the experiment was the isolation and study of the *G. lucidum* submerged mycelium total polysaccharide fraction antitumor activity. To determine the optimal dose of this fraction for intragastric administration the doses of 1, 2, 20, and 200 mg/kg/day were studied. It was shown that all studied doses significantly inhibited tumor growth. The greatest antitumor effect demonstrated a dose of 2 mg/kg/day, comparable with the effect of 200 mg/kg/day dose. However, after the treatment course the antitumor effect of 2 mg/kg/day dose lasted longer than those of 200 mg/kg/day dose. The 20 mg/kg/day dose effect was the least affective.

It should be mentioned that the total polysaccharide fractions of *Armillaria mellea, Hericium erinaceus, Hypsizygus ulmarius,* and *Trametes versicolor* submerged mycelium demonstrated high antitumor activity in the dose of 2 mg/kg/day.

The study of monosaccharide composition of the polysaccharide fraction isolated from fruit bodies and mycelium showed that both fractions contained xylose, mannose, arabinose, galactose, and glucose; the mycelium fraction in contrast to the fruit body fraction also contained rhamnose. The glucose content was higher in the water soluble fraction of the fruit bodies, and the galactose content was higher in the mycelium fraction.

The antitumor activity of fruit bodies and mycelium polysaccharide fractions was studied for the dose of 20 mg/kg/day. The obtained results revealed high and practically equal antitumor effects of both polysaccharide fractions. The fractions inhibitory action lasted after termination in the course of treatment.

Thus the high-performance methods of production of *G. lucidum* biomass, containing biologically active polysaccharides, were developed. *G. lucidum* polysaccharide substances, revealing *per os* antitumor activity were obtained and optimal daily dose of mycelium total polysaccharide fraction was determined. It was proved that water soluble mycelium and fruit body polysaccharides of one strain, despite the determined insignificant monosaccharide composition differences, possessed equal antitumor actions.

## The Influence of Different Factors on the Polysaccharide Accumulation of *Ganoderma lucidum*

*Valentina G. Babitskaya,[1] Nina A. Bisko,[2] Natalia L. Poedinok,[2] and Tatjana A. Puchkova[1]*

[1]Institute of Microbiology, National Academy of Sciences of Byelarus, Minsk, Kuprevicha 2 220141 Byelarus, [2]M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kiev, Ukraine

Different factors that determine the efficiency of polysaccharide biosynthesis by *Ganoderma lucidum* (W.Curt.: Fr.) P. Karst. strains were investigated on glucose-peptone nutrient medium in submerged conditions. It was demonstrated that the best sources of carbon for polysaccharide production from 13 nutrients studied (glucose, arabinose, xylose, fructose, galactose, mannose, lactose, maltose, saccharose, mannit, sorbit, cellulose, and starch) were glucose, lactose, and starch.

The content of endopolysaccharides was 8.5%–9.5% and exopolysaccharides – 4.0%–4.8% on media containing the 13 sources of carbon. The maximum index of biosynthesis efficiency (the ratio of polysaccharide mass to the mass of the carbon source) was observed on the media with 30 g/l of the carbon source. The optimal nitrogen source for polysaccharide accumulation was $(NH)_2SO_4$. The maximum quantity (approximately 18) of endopolysaccharides and exopolysaccharides – approximately 25, was obtained by the correlation between carbon and nitrogen in the nutrient media.

It was determined that temperatures between 25° and 30°C were favorable for polysaccharide biosynthesis. It was demonstrated that a pH of 4.0–6.0 promoted the highest content of exopolysaccharides, and a pH of 5.0–6.5 for endopolysaccharides.

The conditions of aeration of nutrient medium exerted a great influence on the efficiency of polysaccharide biosynthesis. The maximum production of polysaccharides was observed under conditions of aeration 1.0–1.5 l/l/min and mixing 100 rot/min. The radiation of inoculum for the submerged cultivation of *G. lucidum* strains is one of the factors that promote the increase of polysaccharide production.

Radiation of the inoculum by red light increased exopolysaccharides by 47% and endopolysaccharides by 62%. The optimization of cultivation conditions produced the following indexes: the content of exopolysaccharides – 9 g/L, the productivity on exopolysaccharides – 1285 mg/L/day, the content of endopolysaccharides – 1.35 g/L, the productivity on endopolysaccharides – 187.7 mg/L/day.

# Perspectives in the Usage of Bioactive Substances of Medicinal Mushrooms in Pharmaceutical and Cosmetic Industries

*Suzanna M. Badalyan, Narine G. Gharibyan, and Aschen E. Kocharyan*

Laboratory of Fungal Biology and Biotechnology, Faculty of Biology, Yerevan State University, 375025, 1 Aleg Manoogian St., Yerevan, Armenia

Advancements in fungal biotechnology have extended from analysis of nutritional components and benefits to the search for natural bioactive compounds and enzymes, particularly in Basidiomycetes mushrooms, that are used in medicine, the food industry, and cosmetology. Recent scientific publications have documented bioactive compounds of mushroom origin (polysaccharides, glucans, terpenoic, indolic, and phenolic substances, etc.) with antibacterial, antifungal, immune- modulating, antioxidant, hypocholeste-rolemic, hypoglycemic, and other activities. Mushroom-based commercial products and drugs have been successfully used in combination with chemotherapy for the treatment of cancer. Several bioactive compounds isolated from medicinal mushrooms are not considered strictly pharmaceutical products (medicines). They represent a novel class of health-enhancing dietary supplements (DSs) or functional food additives ("nutriceuticals").

Fungal polysaccharides possess immune-stimulating, antioxidant, radioprotective, antibacterial, and antiviral properties. The polysaccharides chitin and chitosan – components of the fungal cell wall have medicinal properties and participate in the regulation of the liver, the gastro-intestinal tract, and kidney functions. Terpenoids comprise the other group of bioactive substances of mushrooms that mainly possess cytotoxic, antibacterial, hypolipidic, antioxidant, antifungal, hypotensive, and hepatoprotective effects. Biologically active proteins - lectins were recently discovered within fungi. As cell receptors, they are able to specifically bind to glucan molecules and form glycoprotein complexes. The fungal pigment - melanin also possesses biological activity. It has antioxidant, immune-modulating, anti-mutagenic, and radioprotective properties.

Nutritive, anti-inflammatory, regenerative, anti-stress, and antioxidant properties of several medicinal and edible mushrooms provide valuable bioactive substances that can be used in food, pharmaceutical industries, and cosmetology. Bioactive compounds and additives of mushroom origin (mycelial biomass obtained by submerges cultivation or 5%–25% aqueous-alcoholic mycelial extract) is used in the formulation of cosmetic compositions. Currently, several mushrooms, particularly *Pleurotus ostreatus, Flammulina velutipes, Lentinus edodes, Ganoderma lucidum,* and *Grifola frondosa* are applicable in the manufacturing of hair and skin care products, nutritive creams, masks, milks, and lotions with anti-inflammatory, protective, and regenerative effects. These products stimulate microcirculation and elasticity of skin capillaries due to carotinoids, vitamins, and phospholipids. Fungal chitosan is widely used in cosmetology as an emulgatory, gel-forming, protective, and anti-bacterial agent. Based on *Lentinus edodes* extract, Ive Roche's "Serum Vegetal de Shiitake" skin care cream is currently available in the world market. The lipid extract of *L. edodes* more actively stimulates the proliferation of epidermal cells and fibroblasts than does the pure ergosterol of synthetic origin.

Recent studies of the *Tremella* mushroom revealed that this species also possesses medicinal properties because of its polysaccharide glucoronoxylomannan, which is a hydrophilic agent largely used in cosmetology. It has anti-inflammatory and anti-allergic effects and could also be applicable for the treatment of neurodermatitis and sclerodermatitis. Glucoronoxylomannan prevents the aging of capillaries and stimulates blood circulation and DNA synthesis of the endothelium of capillaries. Water and ethanol extracts from fruiting bodies of *Tremella* mushrooms have excellent hydrating, film-forming, and moisturizing effects. They also prevent skin from pigmentation.

Further studies of bioactive compounds of medicinal mushrooms are necessary to obtain new biopharmaceuticals, nutriceuticals, and cosmetic commercial products.

## ACKNOWLEDGMENTS

Support by DAAD (#548.104401.174) and NATO (#980764) is gratefully acknowledged.

# Biological Characteristics and Genetic Diversity of Several Basidiomycetes Medicinal Mushrooms

*Suzanna M. Badalyan,[1] Narine G. Gharibyan,[1] Carmen Z. Sakeyan,[1] Hrachuhi K. Avetisyan,[1] Margarita O. Grigoryan,[1] Monica Navarro-González,[2] and Ursula Kües[2]*

[1]Laboratory of Fungal Biology and Biotechnology, Yerevan State University, 1 Aleg Manoogian St., Yerevan 375025, Armenia; [2]Georg-August-University Göttingen, Institute of Forest Botany, Section Molecular Wood Biotechnology, Büsgenweg 2, Göttingen D-37077, Germany

Studying cultural characteristics and optimal growth conditions of mycelia will allow the control of cultivation processes of medicinal mushrooms (MMs) for the purpose of obtaining high-yield biomass and desired health-enhancing mushroom-based biotechproducts.

In our screening, 45 species and 128 strains of higher Basidiomycetes including well known MMs such as *Trametes versicolor, Ganoderma lucidum, Lentinus edodes, Flammulina velutipes, Pleurotus ostreatus,* and *Schizophyllum commune* strains have been involved. The 19 species and 49 strains belong to Coprinoid mushrooms (CMs, species of the genus *Coprinus* s.l.), 10 species and 29 strains to Aphyllophoromycetideae, and 16 species and 50 strains to Agaricomycetideae groups. Among screened collections, 29 species and 95 strains have been genetically identified.

Studies of morphological, ecological, and physiological characteristics as well as definition of growth parameters (growth rate and coefficient) of mycelia were carried out on different nutrient media (MEA, PDA, GPA, etc.), under different pH (3–12), and temperature (5–37°C) conditions. The antifungal (AFA), antibacterial (ABA), antiviral (AVA), immune-modulating (IMA), antioxidant (AOA), antiprotozoal (APA), mitogenic (MGA), and proteolytic (PLA) activities of mycelia and FBs of screened species have also been investigated by appropriate methods. Different tests were applied to detect extracellular phenoloxidase (laccase) activities of mycelia.

Biological characteristics of all screened collections were thoroughly described, their growth parameters were detected. The ranges of growth, temperature, and pH, particularly in CMs and Aphyllophoromycetideae species have been revealed. Based on morphological and ecological studies, as well as *in vitro* fruiting abilities, 2 species-specific morphological types (A, B) and 3 subtypes (A-B, A1, A2) of mycelial colonies within collections of *F. velutipes* were described.

Different levels of AFA of screened mushrooms towards 26 species of filamentous fungi from genera *Acremonium, Alternaria, Aspergillus, Bipolaris, Chrysosporium, Fusarium, Gaeumannomyces, Gliocladium, Microsporum, Penicillium, Rhizoctonia, Trichoderma,* and *Verticillium*) were observed in a dual culture experiment. The activity against *Aspergillus niger, A. versicolor, A. flavus,* and *Penicillium simplicissimum* was particularly detected in *Coprinus cinerea, C. comatus,* and *C. atramentaria. Coprinus disseminatus, C. micaceus,* and *Volvariella bombycina* suppressed the growths of *Aspergillus candidus, A. wentii, F. tricinctum,* and *Ch. keratinophillum* species - potentially pathogenic for humans and animals. Among tested Aphyllophoromycetideae species, the *Daedalea quercina* and *Fomes fomentarius* were more active against filamentous fungi. The highest AFA toward phytopathogens and their antagonists (*G. roseum* and *Trichoderma* spp.) possessed Agaricoid species (*Hypholoma fasciculare, F. velutipes, Lentinus tigrinus, P. ostreatus,* and *Sch. commune).* CMs, particularly *C. disseminatus, C. micaceus, C. strossmayeri,* and *C. domesticus* suppressed the growth of humans, animals, and plant pathogens (except *Fusarium culmorum*); however, *Trichoderma* spp. showed the highest combative activity against these mushrooms.

Among 12 tested species, a relatively higher ABA against *Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhimurium* was revealed in cultural liquid (CL) samples of *Kuehneromyces mutabilis* and *Polyporus varius.*

Strong APA against *Paramecia caudatum* was present in CL of *Lentinus edodes, Coprinus comatus,* and *Flammulina velutipes,* whereas different concentrations of the mycelial extract (ME) showed up to 2.8-fold MGE (Badalyan and Sisakyan, Int J Med Mushr, 2005, 7:382–383). The FB extracts and

isolated polysaccharide protein (PSP) fraction of *F. velutipes* were able to stimulate mitosis in Protozoa by 1.8 and 1.4 times, respectively. The 3- to 4-fold increase of stimulatory effects on wheat and maize seeds' growth of CL samples of *Ganoderma lucidum*, *Daedalea quercina,* and *Piptoporus betulinus* have also been detected.

The FB extract of *F. velutipes* was able to increase the levels of IL-2, IL-12, IFN-α, and IFN-γ cytokines in human peripheral blood mononuclear cells *in vitro*. Inhibition of reproduction of mice's encephalomyocardial and parotid viruses was also observed. The highest AVA showed the PSP fraction obtained from FB extract of *F. velutipes*.

The CL and ME samples of 28 tested species possess certain anti-oxidative potential to inhibit the reactions of free-radical peroxide oxidation of lipids (POL) in rat brain homogenate. The relatively higher indicators of AOA were revealed in CL samples of *Pholiota alnicola, Lepista personata, Trametes versicolor, Volvariella bombycina,* and *Stropharia coronilla*. Significant activity was detected in CL and ME samples of tested CMs (*Coprinus comatus, C. disseminatus, C. domesticus,* and *C. radiate)* and FB and mycelium samples of *F. velutipes*.

A strong PLA was described in CL of *Coprinus xanthothrix*, *C. domesticus, C. disseminatus*, *C. micaceus*, *C. strossmayeri, C. radiata, F. velutipes, L. personata, P. ostreatus,* and *Suillus luteus*. The high PLA showed a water-soluble fraction from FB extract of *F. velutipes*.

Reported medicinal properties of mushrooms have not always confirmed the morphological identification of species, particularly their cultures. In many cases, incorrect species epithets have been used, which makes genetic identification of mycelial cultures necessary. For this purpose, collections of MMs, particularly collections of *F. velutipes* and *P. ostreatus* have been genetically identified. Revealed high genetic variability of Armenian collections of these species within the ribosomal ITS1-5.8S-ITS2 region may be used for improvement of strains by obtaining novel biotech-products. Identification of CMs collections is in progress.

## A Study of the Mitogenic Effect of Cultural Broth of Several Medicinal Mushrooms (Aphyllophoromycetideae)

*Suzanna M. Badalyan and Carmen Z. Sakeyan*

Laboratory of Fungal Biology and Biotechnology, Faculty of Biology, Yerevan State University, 1 Aleg Manoogian St., Yerevan 375025, Armenia

Many Basidiomycetes species belonging to different taxonomical, ecological, and physiological groups, including wood-inhabiting mushrooms (Aphyllophoromycetideae), possess medicinal properties since they represent a natural source of novel bio-pharmaceuticals with immunomodulating, antitumor, antifungal, antimicrobial, hepatoprotective, thrombolytic, hypoglycemic, hypocholesterinemic, and other effects. However, the mitogenic effect of mushrooms has not been sufficiently investigated yet.

The terpenoid, phenolic, and indolic compounds as well as fungal lectins possess significant mitotic activity. However, the mechanism underlying their activity, particularly of lectins, has not been clarified yet.

The aim of our work was to study the mitogenic effect of cultural broth obtained after cultivation of 6 species and 18 strains of medicinal Aphyllophoromycetideae mushrooms, including brown-rot (*Daedalea quercina, Laetiporus sulphureus*, and *Piptoporus betulinus*) and white-rot (*Fomes fomentarius*, *Ganoderma applanatum*, and *G. lucidum*) species. Experiments were carried out on wheat (sort Donskoi) and maize (sort Ani) seeds. Selected seeds were kept in distilled water for one hour, then in cultural broth of tested species for 2 hours. Afterwards, they were placed into Petri dishes (20 seeds per dish) on sterilized sand and watered for 6 days. The experiment was carried out at room temperature (22–24°C) at the day/night regime. The observations were realized daily. The measurements of epigeal and hypogeal parts of tested grown seeds were carried out on the 6th day of growth. The cultural broth samples were obtained after 14 days of mycelial growth in agitated cultural conditions on malt-extract liquid medium.

Two controls—water and malt-extract media—were used in our experiment.

In the experimental plates with wheat seeds up to 27% mitosis-stimulating effect was revealed in *G. lucidum* (Gl/4–14%; Gl/5–27%), *G. applanatum* (Ga/1–11%), and *Daedalea quercina* (Dq/1–22%) strains in comparison with control plates. A growth-inhibitory effect was mainly detected in *Piptoporus betulinus* (Pb/2–87%, Pb/3–64%, Pb/4–74%) and *G. applanatum* (Ga/2-1–8%, Ga-2-2–11%, Ga-2-3– 26%) strains.

A 3- to 4-fold increase of stimulatory effect on the growth of maize seeds was observed in cultural broth of tested mushrooms, particularly of *G. lucidum* (Gl/5), *D. quercina* (Dq/1, Dq/2), and *P. betulinus* (Pb/2, Pb/4) strains in comparison with control plates. Three strains of *G. applanatum* (Ga/1, Ga2/1 and Ga2/3) and one strain of *P. betulinus* (Pb/3) showed up to 54% inhibitory effect on maize seeds growth. No difference between stimulatory and inhibitory effects of epigeal and hypogeal parts of grown seeds was observed.

Within 18 experimental variants, 6/12 ratio of stimulatory and inhibitory effects of tested cultural broth samples on wheat seed's growth were observed, whereas 13/5 ratio was detected in the experiment on maize seeds, respectively.

Thus, the presence of mitosis-stimulating activities within tested mushrooms shows that their further screening is promising for the development of new bio-pharmaceuticals and mushroom-based food supplements, which could be used in the prevention and treatment of wounds, burns, and ulcers as well as other regenerative processes.

## ACKNOWLEDGMENT

Support by DAAD (#548.104401.174) and NATO (#980764) is gratefully acknowledged.

# Medicinal Operculate Discomycetes of Israeli Mycobiota

*Gayane S. Barseghyan,[1] Solomon P. Wasser,[1,2] and Eviatar Nevo[1]*

[1]Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel; [2]N.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev 01001, Ukraine

Only 3.7 percent of Israel's territory is covered by forest, natural or human-made. The Mediterranean climate is predominant, covering about 40% of Israel's territory; up to 70% of human-made forests are located in this region. Thus, the forested areas are proportionately scarce, when compared to other Mediterranean countries such as Spain (13.8%), Portugal (28.7%), Cyprus (16.6%), or Morocco (8.0%). The climate of Israel is optimal for intensive development of general mycobiota. One hundred and twenty-three species of Operculate Discomycetes are known from the forests of Israel. Nine of these species, e.g., *Morchella esculenta* (L.) Pers., *M. conica* Pers., *Helvella crispa* (Scop.) Fr., *H. lacunosa* Afzel.: Fr., *Peziza vesiculosa* Bull.: St. Amants, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Tirmania africana* Chat., *Tuber nitidum* Vittad., and *Terfezia leonis* Tul. contain medicinal substances that play a very important role in pharmacology, as a valuable source for biologically active compounds. Their nutritional value is high, due to different proteins and acids, carbohydrates and dietary fibers, fat, vitamins, minerals, trace elements, etc. In particular, and most importantly for modern medicine, they represent an unique source of polysaccharides with antitumor and immunostimulating properties. Moreover, many *Morchella* species (morels) can be important non-timber forest products because they comprise the most delicious and prized group of edible fungi.

Medicinal properties have been attributed to mushrooms for thousands of years. Mushroom extracts are widely sold as nutritional supplements because of numerous health benefits. Yet, there is no critical review attempting to integrate their nutraceutical potential with basic science. Relatively few studies are available on the biological effects of mushroom consumption, and those have been performed exclusively on murine models. Mushrooms have compounds, in particular (1→3)-β-D-glucans that modulate the immune system and potentially exert tumor-inhibitory effects. Several *Morchella* species have important

antitumor potential. Moreover, the species *Morchella esculenta*, *M. conica*, *M. crassipes* (Vent.) Pers., and *M. deliciosa* Fr. contain brassicasterol, which are widely used in medicine. Some Asian countries have used *Morchella esculenta* as cure and tonic for the intestines and stomach, to reduce phlegm, and to regulate the flow of vital energy. Moreover, this species can be used in tumor inhibition, as Qi regulator, and possesses antioxidant activity.

Also, there are some species from Operculate Discomycetes that produce different hallucinogens, such as gyromitrin and protoplasmic poison monomethyhydrozine (MMH), which are produced by several species in the genus *Gyromitra,* especially by *G. esculenta*.

In recent years, in fermentating and culturing *Sclerotinia sclerotiorum*, Japanese scientists produced *S. sclerotiorum* polysaccharide, a dextrane, which inhibits the growth of Sarcoma 180.

Unfortunately, in Israel only a small amount of data on the species diversity of this class of fungi was studied. There is not enough information on investigations of fungal medicines and food supplements from this group. It is very important to examine the diversity of medical species of the class Operculate Discomycetes.

For this reason, we have conducted our research on cultural characteristics *in vitro* for some species of the genera *Morchella*, *Gyromitra*, *Helvella*, and *Verpa* for further information on the medicinal properties of some species of Operculate Discomycetes. We believe that several species of this class have significant medicinal potential, especially species of genus *Morchella*.

# L-Ergothioneine, a Potent Antioxidant in Cultivated Mushrooms: A Review

*Robert B. Beelman,[1] N. Joy Dubost,[2] and Marvin Hausman[3]*

[1]Department of Food Science, The Pensylvania State University, University Park, PA, USA; [2]PepsiCo Inc., Valhalla, NY, USA; [3]Oxis International Inc., Foster City, CA, USA

L-Ergothioneine (ERGO) is a naturally occurring 2-thio-imidazole amino acid biosynthesized primarily by fungi and few bacteria. ERGO is a potent antioxidant and cellular protector that has been shown to aggressively scavenge singlet oxygen, peroxyl, and hydroxyl radicals. ERGO is concentrated in human tissues that are under the most oxidative stress such as red blood cells, liver, and sperm. ERGO gets into the human food chain mainly through plants that take it up from soil where it is produced by fungi and from meat obtained from animals that absorb it from grazing grasses or plant-based feeds. Recent research demonstrated that ERGO is concentrated in mushrooms in the range of about 0.4–2.0 mg/g. d.w., which makes mushrooms the best source of ERGO in the human diet. Mushrooms provide at least four times more ERGO than chicken livers, previously believed to be the best source of ERGO in the human diet. A preliminary survey indicated that *Agaricus bisporus* strains contained significantly lower levels of ERGO than *Lentinus edodes*, *Pleurotus ostreatus*, *P. eryngii,* and *Grifola frondosa*. Brown strains of *Agaricus bisporus* were found to contain higher levels of ERGO than the more commonly consumed white strains. Hence, studies were conducted to determine the influence of selected cultural practices that affect ERGO content in white button mushroom. Histidine, a precursor of ERGO, added to the substrate significantly increased the amount of ERGO produced in white button mushrooms, especially in the later flushes of the crop cycle. ERGO was also increased when mycelial growth was increased in the substrate. Other studies indicated that ERGO concentration in mushrooms appeared to be increased by several stress factors, such as use of dry substrate, breaking the mycelium in the substrate at casing and harvesting mushrooms from the later flushes of its crop cycle. Recently, research has focused on the nutritional and medical implications of ERGO consumption by animals and humans. A recent study demonstrated that pure ERGO administered to rats protected liver and kidney damage due to lipid peroxidation and also reduced consumption of endogenous glutathione and $\alpha$-tocopherol. The authors suggested that consumption

of mushrooms would be the preferred dietary source of ERGO. Recent research has also demonstrated that ERGO was superior to currently used extenders, to protect sperm cells from oxidative damage that occurs during freezing and thawing. Another study showed that ERGO inhibited TNF-α-mediated HIV-1 LTR activation, thus it could be of benefit in controlling chronic immunodeficiency diseases.

## Aegerolysins: Structure, Function, and Putative Biological Role

*Sabina Berne,[1] Peter Maček,[2] and Kristina Sepčić[2]*

[1]Medical Centre for Molecular Biology, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, Ljubljana 1000, Slovenia; [2]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana 1000, Slovenia

The aim of this contribution is to summarize current knowledge on a novel protein family, called aegerolysins (PF06355 and IPR009413), and to present their structure, function, and possible biological role.

Aegerolysins are highly homologous proteins produced by fungi, bacteria, and plants. Structurally, they are characterized as a single domain, all-β structured proteins, which share several common features: low-isoelectric points, similar molecular weights (15–17 kDa), and stability in a wide range of pH (mainly from 4 to 10). At least for some aegerolysins, interactions with lipids and pore-forming activity have been reported. The toxicity of aegerolysins to different types of cell lines is most probably a consequence of their cytolytic effects. Although the exact function of aegerolysins remains to be explained, their physiological role is manifested in various processes such as modulation of bacterial sporulation, virulence of *Aspergillus fumigatus,* and mushroom fruiting.

Members of the aegerolysin protein family have interesting biological properties that make them applicable from several points of view. Certain aegerolysins, possessing antitumoral, antiproliferative, and antibacterial activities have possible applications in medicine and/or as pharmaceuticals. Furthermore, some of them could be used as drugs in the prevention of atherosclerosis or as vaccines. Besides, aegerolysins may constitute a valuable tool in cell and molecular biology as specific markers for raft-like, cholesterol-rich, membrane domains. Finally, some aegerolysins are specifically expressed during the formation of mushroom fruiting bodies, which makes them interesting from a biotechnological and commercial point of view.

## Ostreolysin Induces Growth Promotion in *Pleurotus ostreatus*

*Sabina Berne,[1] Jure Pohleven,[2,3] Iztok Vidic,[4] Franc Pohleven,[4] Tom Turk,[5] Peter Maček,[5] and Kristina Sepčić[5]*

[1]Medical Centre for Molecular Biology, Faculty of Medicine, University of Ljubljana, Vrazov trg. 2, Ljubljana 1000, Slovenia; [2]Department of Biotechnology, Jožef Stefan Institute, Jamova 39, Ljubljana 1000, Slovenia; [3]Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, Ljubljana 1000, Slovenia; [4]Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Rožna dolina, Cesta VIII/34, Ljubljana 1000, Slovenia, [5]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana 1000, Slovenia

*Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. is an edible ligninolytic basidiomycete with medicinal, biotechnological, and environmental applications. It holds third place in the worldwide commercial mushroom production, after *Agaricus bisporus* and *Lentinus edodes*. Since knowledge on cellular processes leading to the initiation of fruiting body development is lacking in several edible mushrooms, including *P. ostreatus*,

examination of the genes and proteins involved in their fruiting, as well as monitoring of fruiting body formation upon environmental and biochemical treatment of the mycelium, are extremely important from the biotechnological and commercial point of view.

Ostreolysin (Oly) is a 15-kDa acidic protein isolated from the primordia and young fruiting bodies of *P. ostreatus* (Berne et al., Biochem Biophys Acta, 2002, 1570:153–159), and belongs to the aegerolysins – a family of highly homologous proteins found so far in mushrooms, moulds, bacteria, and plants. Its exact biological function has not been resolved yet. The protein is lytic to several types of cell lines, erythrocytes, and artificial lipid vesicles enriched in cholesterol, but it does not lyse vesicles reconstituted from extracted total lipids of *P. ostreatus* (Sepčić et al., Eur J Biochem, 2003, 270:1199–1210), or lipid vesicles enriched with ergosterol (Rebolj et al., Biochem Biophys Acta, 2006, 1758:1662–1670). Its large abundance in the edible oyster mushroom, and rapid heat- and pH-inactivation, suggest that Oly probably does not act as a toxin in its natural environment.

The spatial and temporal expression of Oly in the mushroom reveals its absence at the stage of vegetative mycelium. Its expression is initiated at the stage of rapidly growing primordia and is continued during the maturation of young fruiting bodies. It is preferentially found in the basidia and basidiospores, suggesting its involvement in the initiation of fructification and/or sporulation (Vidic et al., Mycol Res, 2005, 109:377–382).

In this work, Oly was exogenously applied to the solid nutrient medium, inoculated with *P. ostreatus* mycelium, and its effects on growth promotion of the mushroom were studied. Before inoculation with *P. ostreatus* mycelium, Oly (25 μg/cm$^2$, 10 μg/cm$^2$, 1 μg/cm$^2$, and 0.1 μg/cm$^2$) was spread on MEA plates, while the control plates were supplemented with a buffer or with the same concentrations of bovine serum albumin. All the experiments were performed in triplicates. The fructification was induced by lowering the temperature to 8°C at day 18, and monitored for 70 days at 25°C. On the control plates the fructification initiated on the 32nd day after the induction. On the contrary, the formation of primordia and young fruiting bodies on plates treated with higher concentrations of Oly (10 and 25 μg/cm$^2$), initiated 10 days earlier, and resulted also in a much higher production (number) of young mushroom caps.

We conclude that Oly, besides being strongly expressed during the process of fruiting initiation, also promotes earlier production of mushrooms and increases their yield. Therefore, it is indicative that Oly is involved in the stimulation of fruiting body formation of *P. ostreatus*, and possibly of other mushrooms when applied exogenously, which makes this protein and its genetically engineered recombinant forms interesting from biotechnological and applicable points of view.

---

# Solid State Cultivation of *Grifola frondosa* Biomass and Production of Intra- and Extracellular Polysaccharides

*Marin Berovič,[1] Bojana Boh,[2] Anja Menard,[1,2] and Franc Pohleven[3]*

[1]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Askerceva 9, Ljubljana SI-1000, Slovenia; [2]Faculty of Natural Sciences and Engineering, University of Ljubljana, Vegova 4, Ljubljana SI-1000, Slovenia; [3]Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, University of Ljubljana, Ljubljana SI-1000, Slovenia

*Grifola frondosa* (Dicks: Fr.) S.F. Gray, also known as Maitake, is a lignin and cellulose degrading basidiomycete with excellent nutritional and medicinal properties. Its active compounds primarily belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins, and proteins.

As the demands for *G. frondosa* fruit bodies and/or mycelium biomass are constantly increasing, artificial cultivation has become essential. Successful farming of fruit bodies in bags filled with supplemented sawdust has been introduced in several countries, including Japan, China, and Korea.

In our work, *G. frondosa* mycelium biomass with pharmaceutically active polysaccharides was produced by: (1) solid-state cultivation in polyethylene bags, and (2) solid-state cultivation in a horizontal stirred tank

bioreactor. A Slovenian isolate of *G. frondosa* (GF3, Fungal bank of the Biotechnical faculty, Department of Wood Science and Technology, University of Ljubljana, Slovenia) was used in all experiments. Solid substrate mixture consisted of 1000 g of milled whole corn plant (*Zea mays*), 50 mg $(NH_4)_2SO_4$, 200 mg $KH_2PO_4$, 50 mg $CaCl_2 \cdot 2H_2O$, 50 mg $MgSO_4 \cdot 7H_2O$, 150 mg $FeSO_4 \cdot 7H_2O$, 2 g $CaSO_4$, 20 ml olive oil, 0.5 L distilled water, and (optional) 500 g of olive press cake.

In the experiment of cultivating *G. frondosa* mycelium in polyethylene bags, 527.5 g of fresh biomass was produced on 1000.0 g of substrate (yield: 0.53 g of fresh biomass per 1 g of substrate). Dry intracellular polysaccharides (2.86 g) (5.4 mg/g of fresh biomass) and 3.67 g (7.0 mg/g of fresh biomass) of dry extracellular polysaccharides were isolated from the mycelium. Crude polysaccharides were dialyzed and further separated by ion-exchange chromatography on DEAE cellulose, gel chromatography on Sepharose 4B, and affinity chromatography on Concavalin A - Sepharose 4B. Four fractions of pure intracellular β-D-glucans were isolated (total mass: 47.2 mg; 89.6 μg/g of fresh biomass) and four fractions of pure extracellular β-D-glucans were isolated (total mass: 127.2 mg; 241.1 μg/g of fresh biomass).

Three solid-state experiments were carried out in a horizontal stirred tank bioreactor. Optimal cultivation conditions were: temperature: 30°C; air flow: 5 L per minute; periodical mixing after 14 days of cultivation, 1 minute once a week, 80 rpm. The experiments showed the importance of the substrate moisture content. Optimal moisture content for biomass growth and polysaccharide production was 60%–80%, the critical lowest moisture content point was 45%, where the production of biomass and polysaccharides stopped. Fresh biomass (70.0 g) was produced on 1000.0 g of substrate (yield: 0.07 g of fresh biomass per 1 g of substrate). Dry intracellular polysaccharides (38.0 mg/g) and 78.0 mg/g of dry extracellular polysaccharides were isolated from the mycelium.

The above experimental results confirmed that *G. frondosa* biomass can be successfully produced by both cultivation methods: by solid-state cultivation in polyethylene bags and solid-state cultivation in a horizontal stirred tank bioreactor. Both cultivation methods were suitable for the production of fungal polysaccharides, including β-D-glucans, which are the most important active compounds of *G. frondosa*. Compared to submerged cultivation, solid state cultivation seems to have some advantages: higher biomass yield, lesser susceptibility to bacterial infections, and a potential for using agricultural wastes as effective lignocellulosic substrates for the production of *G. frondosa* biomass.

---

# Production of Intra- and Extracellular Polysaccharides of *Grifola frondosa* Biomass by Submerged Cultivation

*Marin Berovič,[1] Bojana Boh,[2] and Jerica Stanisa[1,2]*

[1]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Askerceva 9, Ljubljana 1000, Slovenia; [2]Faculty of Natural Sciences and Engineering, University of Ljubljana, Vegova 4, Ljubljana 1000, Slovenia

*Grifola frondosa* (Dicks: Fr.) S.F. Gray is a white rot basidiomycete belonging to the family *Polyporaceae*. Its main natural habitats include parts of northeastern Japan, northern temperate forests of Asia, Europe, and eastern North America. *G. frondosa* fruit bodies, known as Maitake in Japanese, possess excellent gourmet and nutritional properties. However, *G. frondosa* gained popularity among consumers not only because of its taste and flavor, but also because of its numerous medicinal effects, including anti-cancer activity, enhancement of the immune system, effects on angiogenesis, reduction of benign tumors, such as benign prostatic hyperplasia, antibacterial activity, antiviral effects (including effects against HIV/AIDS), antidiabetic activity, advantageous effects on lipid metabolism and hypertension, antioxidant effects, beneficial cosmetic effects on skin, and enhancement of metabolism and vitality. The most important active compounds of *G. frondosa* are polysaccharides, especially β-D glucans with 1,6-β-D- and 1,3-β-D-chains. Polysaccharides are present in fruiting bodies and in the mycelium (intracellular polysaccharides), as well as in the substrate, to which they are excreted by fungal hyphae (extracellular polysaccharides).

As the demands of pharmaceutical and nutriceutical industries for *G. frondosa* biomass are constantly increasing, submerged cultivation in bioreactors on liquid substrates has been developed recently for small and pilot-plant production of intra- and extracellular polysaccharides.

In our work, *G. frondosa* biomass and its pharmaceutically active polysaccharides were produced by submerged cultivation in a liquid medium in two ways: (1) in 250 mL Erlenmayer flasks, containing 50 ml of substrate, on a shaker, and (2) in a 10-L stirred tank bioreactor. The Slovenian isolate of *G. frondosa* (GF3, Fungal bank of the Biotechnical faculty, Department of Wood Science and Technology, University of Ljubljana, Slovenia) was used in all experiments.

In the production of *G. frondosa* mycelium biomass, Erlenmayer flasks were incubated at 29°C on a shaker at 216 rpm. After 30 days of cultivation in Erlenmayer flasks, 1.8 g of fresh biomass (36 g/L) or 0.9 g of dry biomass (18 g/L) was obtained. The yield of total polysaccharides was 2.9 g/L of the liquid medium.

Optimal growth conditions in a 10-L stirred tank bioreactor were: temperature 28°C, air flow 5 L/min, mixing speed 220 rpm. After 60 days of cultivation in a 10-L bioreactor, the amount of isolated fresh biomass was 307.7 g (30.8 g/L), which corresponded to 167 g of dry biomass (16.7g/L), respectively. Extracellular polysaccharides (3.64 g) were isolated from the mycelium (21.8 mg/g of dry biomass) and 1.29 g of dry intracellular polysaccharides (7.7 mg/g of dry biomass), respectively. Isolated polysaccharides were separated by ion-exchange chromatography on DEAE cellulose, gel chromatography on Sepharose 4B, and affinity chromatography on Concavalin A - Sepharose 4B. Five fractions of extracellular β-D-glucans were obtained, with a total mass of 6.7 mg (40.1 μg/g dry biomass or 4.0 μg/L liquid substrate) and two fractions of intracellular β-D-glucans with a total mass of 4.9 mg (29.3 μg/g dry biomass or 0.5 μg/L liquid substrate).

The above experimental results confirmed that *G. frondosa* biomass can be successfully produced by submerged cultivation on synthetic media. Submerged cultivation was suitable for the production of fungal polysaccharides, including β-D-glucans, which are the most important active compounds of *G. frondosa*.

# The Chemical Composition of the Fruiting Bodies of Selected *Lentinus edodes* Strains

*Nina A. Bisko,[1] Valentina I. Fomina,[2] Nadezda Yu. Mitropolskaya,[1] and Victor T. Bilay[1]*

[1]M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kiev, 2 Tereshchenkivska St., Kiev, GSP-1, 01601, Ukraine; [2]Institute of Forest, National Academy of Sciences of Belarus, Gomel, Belarus

The investigation of fructification peculiarities and productivity of 51 strains of *Lentinus edodes* (Berk.) Singer (Shiitake mushroom) on sterilized mixture which consisted of oak sawdust and wheat bran (4:1) was conducted. Seven strains were selected, which had high yields (49%–54%) and biological effectiveness (120%–125%) on this substrate. The possibility of using oak, ash tree, alder, and birch sawdust as substrate for the spawn production was studied. It was demonstrated that the full consumption of the above mentioned kinds of sawdust as well as wheat grain was observed in 16–20 days. The yield of shiitake using oak–ash tree spawn was 27%–29%, alder spawn – 22%–28%, birch spawn – 19%–24%, and grain spawn – 24%–26% for 60 days. The fruiting bodies of selected strains were analyzed. The considerable strain variability of the crude protein content was demonstrated (see Table 1).

For example, the content of crude protein in the fruiting bodies of strain 368 was higher by 15.7%

**Table 1. Chemical Composition of Fruiting Bodies of *L. edodes* Strains**

| | The content, % a.d.m. | | | |
|---|---|---|---|---|
| **Strain** | **Crude protein** | **Carbohydrates** | **Phosphorus** | **Potassium** |
| 353 | 54.38 | 1.98 | 2.32 | 3.37 |
| 364 | 47.00 | 1.55 | 1.89 | 3.01 |
| 365 | 48.90 | 1.64 | 2.27 | 3.43 |
| 368 | 55.75 | 1.24 | 2.12 | 2.81 |
| 371 | 49.00 | 1.33 | 1.92 | 3.12 |
| 374 | 51.44 | 2.38 | 1.86 | 3.01 |
| 381 | 53.19 | 1.32 | 2.16 | 2.76 |

than in fruiting bodies of strain 364. Fruiting bodies of strains 368, 381, and 353 were characterized as having the highest quantity of crude protein (see table) and surpassed the amounts described in literature. Strain variability on the content of carbohydrates, phosphorus, and potassium in investigated fruiting bodies was significant, too. Also, the content of carbohydrate in the fruiting bodies of strain 374 was higher by 47.9% than in fruiting bodies of strain 368 (see Table 1).

Thus, seven high productive shiitake strains for cultivation on wastes from the forest industry with the addition of wheat bran, which are widespread in the Ukraine and Belarus, were selected. It was demonstrated that oak, ash tree, alder, and birch sawdust may be used as substrates for the cultivation of shiitake spawn.

## Mushrooms Cultivation with the Aim at Developing and Maintaining Psycho-Physical Abilities of Mentally Disabled People

*Irena Borštnik*

CUDV Draga, 1 Draga, 1292 IG, Slovenia

This study is based on the development of oyster mushroom cultivation as part of the Green Program in Center Draga, Slovenia (center for qualifying, work, and care for mentally disabled people), from its beginning, 10 years ago. Many positive effects of the program on our clients were observed such as stimulation and providing conditions for development of oyster cultivation like it is today. The goals of this work were to search for new activities, which would additionally stimulate the potentials of our clients. We used advantages and the knowledge we had at that time—the Center owned a big unexploited place. The methods used made it possible to evaluate the interaction between the working tasks and clients abillities. We developed concepts of activities and technological phases. With a qestionary (individual interviews using photos), we compared their preferences according to other activities in the Green program.

The process of growing oysters appeared to be a good motivational factor, at developing and maintaining the psycho-physical abilities of this population. Personal features and speciality of the people with mental distrubance demand special, adapted manners of gualification and work. The process of oysters growing on wheat straw, satisfied the majority of these special needs. For example, individual phases of cultivation are rather simple, the results of their work are received soon enough, and last but not the least, the production is very tasty.

On the one hand, the quality and quantity of the harvest are important from economic point of view and as motivational factors, but they were not among the goals and objectives of the study. On the other hand, we noticed that this work, affected very well a cartain population of clients. For some of them, that was only a positive experience. Their abillities but not deficiency, accessed expression, which contributed to their positive self-image.

Regarding the positive effects and also the critical avaluation according to the activity, we formed a concept of idividual technological phases and methods of work. The concept was based on the adaptation to the working place, working time, technologies, and methods of work as well as to the available number of professional staff and their working scheduals.

Mushrooms cultivation, as part of the Green Program, is part of a formal special educational program for work and work activities in the Center CUDV Draga, for children, adolescents, and adults with moderate, severe, and profound deficiencies in mental development.

## Insecticidal Proteins from the Mushroom *Clitocybe nebularis*

*Jože Brzin,[1] Lara Kandič,[1,2] Jerica Sabotič,[1] Borut Štrukelj,[1,2] Janko Kos,[1,2] and Spela Schrader[3]*

[1]Department of Biotechnology, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia; [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Lubljana, Slovenia; National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

Edible mushroom *Clitocybe nebularis* (Batsch: Fr.) Quél. found in abundance in Slovenian forests is described as a rich source of three types of proteinous active compounds shown as potential biological insecticides of at least two orders of insect pests, Coleoptera and Diptera.

From its fruiting bodies we isolated two protease inhibitors, clitocypin (*C. nebularis* cysteine protease inhibitor), CnSPI (*C. nebularis* serine protease inhibitor), and several lectins with mono- and disaccharide specificities. Clitocypin is a 16.9-kDa protein, remarkably stable, resisting boiling, pH extremes, and proteolytic degradation. Its inhibition spectrum is broad: several animal cathepsins, plant papain, bromelain, and legumain. Similarly, small 16 kDa protein CnSPI strongly inhibits mammalian trypsin, chymotrypsin, and weakly inhibits elastase. Both inhibitors are members of two novel classes of proteins which so far have been observed only in mushrooms, with yet unknown 3-D structures and mechanisms of action. *C. nebularis* lectins have been obtained using specific affinity chromatographies and are presently only partially characterized. Judging by the determined N-termini, *C. nebularis* lectins cannot be related to any known fungus, mushroom, or other lectins.

To test insecticidal activity of purified inhibitors and lectins two insect feeding assays were designed. Coleopteran Colorado potato beetle (*Leptinotarsa decemlienata*) larvae were reared on fresh potato leaves, coated with tested substances and their number and weight were recorded daily. Both clitocypin and lactosyl lectin showed a deleterious effect on larval development and survival rate in a range of concentrations from 12 μM and 0.12 μM, respectively. In the second toxicological test dipteran fruit fly (*Drosophila melanogaster*) larvae were reared on artificial medium containing different amounts of the tested proteins. CnSPI and all four lectins exhibited high insecticidal action in the $LD_{50}$ range from 10 μg/mL to 200 μg/mL.

The mechanisms of toxicity still have to be elucidated in all cases. Nevertheless, the demonstrated insecticidal effect of both inhibitors and lectins ranks them among the most promising new candidates for efficient and ecological plant protection.

---

## Screening of Medicinal and Culinary Mushrooms in Pure Culture

*Asya S. Buchalo, Natalya L. Poyedinok, Oksana B. Mykchaylova, and Margarita L. Lomberg*

M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev 01601, Ukraine

Special culture collections today are an important prerequisite for carrying out fundamental studies and biotechnological applications of medicinal and gourmet mushrooms. The strategy of the screening program, which was created on the basis of culture collection, depends on their final task: identification of taxonomic position of cultures, selection of producers of mycelial biomass, fruiting bodies, enzymes, polysaccharides, antibiotics, pigments, and other biologically active and pharmacological substances.

As the correct knowledge of the taxonomic position of cultures due to their biotechnological application is a task of paramount importance, the criteria for the identification of mushroom culture were outlined. First, isolates from natural carpophores must be identified as belonging to Basidiomycota or Ascomycota. Clamp connection and type of cell septae is the basic characteristic of mycelial cultures. Identification of isolates at the species level has to be made using the complex of morphological, micromorphological, physiological, and biochemical characteristics. The morphology of mushrooms in pure culture is insufficiently studied, which leads to mistakes in the interpretation of the taxonomic status of cultures and the quality of the final product in the biotechnological process. The teleomorph stage is the most essential criterion for identification of cultures, but every so often mushrooms do not produce fruit bodies in pure culture.

It was shown that for the taxonomic characterization of mushroom cultures, the following criteria should be considered: presence and morphology of the teleomorph stage; morphology and growth rate of mycelial colony on etalon media; type of conidial sporulation; presence, dislocation, and morphology of clamp connections, and other structures of vegetative mycelium; enzymatic reactions of fungal colony; and temperature interval of mycelial growth. A scanning electron microscopic study allowed us to obtain new data on culture morphology in some species of medicinal and culinary mushrooms. Microstructures of more than 150 macromycetes species (Basidiomycota and Ascomycota) were studied using scanning electron microscopy (SEM). New data was obtained on the fine microstructures of medicinal mushroom species.

The radial growth rate on agar media is an important characteristic of selected strains. The data obtained showed sufficient difference, depending on the taxonomic and ecologic position of fungal species. On poor nutritional media (Chapeck, oat meal, compost agar, etc.) mycelial growth mostly is fast but cobweb-like. On reach agar media (MEA, PDA, beer-wort agar, etc.), mycelial colonies were usually dense and high.

The optimal temperature for mycelial growth for most of the tested cultures fell within 26–28ºC. More specific for definite species were the lowest and the highest temperatures of mycelial growth: *Pleurotus djamor*, *P. sajor-caju*, and *Volvariella volvaceae* did not grow under +15ºC. *Schizophyllum commune* grew at 37ºC, while for the most of tested cultures 34ºC was the top temperature of mycelial growth. Representatives of *Morchellaceae* (Ascomycota) demonstrated good, developed mycelial colonies with sclerotia at +4ºC and best radial growth rate at this temperature was slower, about 3.8 mm/day.

Growth characteristics of about 150 medicinal and culinary species belonging to different taxa and trophic groups from the genera *Agrocybe*, *Flammulina*, *Lentinus*, *Lepista*, *Macrolepiota*, *Oudemansiella*, *Panus*, *Pleurotus*, *Marasmius*, *Morchella,* etc.,were studied under submerged cultivation. Most of the tested species and strains with intensive growth in submerged culture were observed among lignotrophes. It was shown that in submerged cultivation, the types of vegetative and asexual reproduction were similar to those on agar media. As a result, producers of mycelial biomass with medicinal properties in submerged culture were selected on different complex media (US Patent Nº 6,372,964 B1. Apr. 16, 2002).

During the screening process it would be beneficial to establish the correlation between definite morphological, physiological, and biochemical characteristics and to determine the desired final properties of the producers. The screening program includes the investigation in culture of enzymes, antibiotics, polysaccharides, and pigment production establishing the best for mycelial growth or metabolite production sources of carbon, nitrogen, minerals, vitamins, pH reactions, etc.

# Identification of a Novel Lectin from the Ascomycetes Fungus *Tuber borchii*

*Emanuela Cerigini, Francesco Palma, Michele Buffalini, Antonella Amicucci, Paola Ceccaroli, Roberta Saltarelli, and Vilberto Stocchi*

Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino "Carlo Bo", Via Saffi 2, Urbino 61029 (PU), Italy

Truffles are hypogeous fungi belonging to the genus *Tuber,* which live in symbiotic associations with the roots of gymnosperms and angiosperms. These ectomycorrhizal Ascomycetes are of considerable interest for their significance in forestry and agronomy. In fact, the plant-fungus interaction provides nutrients for both partners and allows the fungus to accomplish its life cycle. The biological cycle of the fungus culminates in the differentiation of fruiting bodies, some of which are edible and well-known for their organoleptic properties. Truffle ascoma are globular in structure and are made up of several types of tissues. Unfortunately, at present, very little is known about the events responsible for hyphal differentiation in complex structures and in the biochemical and molecular components of these tissues.

In order to obtain information concerning the composition and differentiation of truffle ascoma, an 11.9-kDa fruiting body specific protein, TBF-1, was isolated, purified, and sequenced (Swiss-Prot P80708) from the whitish truffle *Tuber borchii* Vittad.; its related gene, *tbf-1,* (GenBank U83996) was also cloned, but no biological evidence about its role was provided.

TBF-1 consists of 108 amino acids nonglycosylated polypeptide chain, including an N-terminal signal peptide of 12 residues. It was localized on the hyphal cell wall but also found as the main soluble protein in fruitbody homogenate.

Recently, TBF-1 was also overexpressed in *E. coli* as fusion protein with GST and purified to homogeneity by glutathione agarose affinity chromatography.

The experiments revealed the identification of the physiological activity performed utilizing both recombinant protein and the protein purified from natural sources allowing us to show TBF-1 as a novel fungal lectin.

The natural and recombinant protein exhibited the same hemagglutination activity toward rabbit red blood cells as well as the same sugar binding specificity. TBF-1 shows specific activity of 178 ± 3 and 175 ± 3 AU/mg for recombinant and natural lectins, respectively.

The *Tuber borchii*'s lectin was inhibited by fetuin, asialofetuin, pectin, and fucoidan but not by any simple sugar. Interestingly, heparin was also able to inhibit the lectin activity; a single case of heparin-binding fungal lectin, from the fruiting body of the Basidiomycetes *Psathyrella velutina* (PVL), was previously described. In fact, this class of lectins is poorly represented in fungi; as heparin is one of the prevalent glycosaminoglycans ($GAG_S$) present in higher animals and produced as capsule components by certain bacteria species. Depending on which literature reports on the requirement of bivalent cations for maintenance of several lectins biological activity, the treatment of TBF-1 with EDTA caused a non-permanent inactivation of protein restorable by $Ca^{2+}$ addition; as expected for a lectin with such a small subunit unlikely to accommodate more than one combining site, TBF-1 was found predominantly in a dimeric form; further studies were additionally performed with the recombinant lectin in assaying pH and temperature stability.

The hemagglutination activity of TBF-1 was unaltered in the range of a pH between 6 and 8, while it lost this activity when the buffer pH was below 4 and above 9.

Lectins are proteins without enzymatic activity that recognize and reversibily bind specific carbohydrate structures; as key components of complex carbohydrate-protein interaction networks, they participate in molecular recognition events of fundamental relevance in a variety of biological processes.

Because of their role in cellular recognition, lectins are also involved in innate defense systems and are endowed with direct antibacterial, antifungal, and antiviral capability. In view of the fact that several heparin binding proteins show antimicrobial activities, TBF-1's heparin-binding ability becomes particularly noticeable.

# Production of Fruit Bodies and Enzymatic Activities of *Agaricus bisporus* on Supplemented Compost

*Oscar Arce Cervantes,[1] Hermilo Leal Lara,[2] Francisco Cruz Sosa,[3] Rebeca Ramírez Carrillo,[2] and Octavio Loera Corral[3]*

[1]Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana, Unidad Xochimilco, Calzada del Hueso 1100, Col. Villa Quietud, Coyoacán 04960, México; [2]Departamento de Alimentos y Biotecnología, Facultad de Química, Edificio E, University of México (UNAM), Ciudad Universitaria Coyoacán 04510, México; [3]Departamento de Biotecnología, Universidad Autónoma Metropolitana, Unidad Iztapalapa, San Rafael Atlixco 186, Col. Vicentina 09340, México

The white bottom mushroom, *Agaricus bisporus* (J.E. Lange) Imbach (Agaricaceae), is a saprophytic fungus commercially cultivated on a substrate prepared with straw and chicken manure as principal ingredients. The main components in the substrate are polysaccharides (i.e., cellulose and hemicelluloses) and the protein-lignin complex. The role of nitrogen as a key nutrient in mushroom compost has been accepted, and nitrogen deficiency in the form of amino acids or amino groupings within the compost has been postulated as a factor that frequently limits yield. Supplementation of substrates has been established as a common practice for commercial cultivation of *A. bisporus*.

In recent years, almost no studies have been published regarding supplements and although it has been generally accepted that supplements should have high protein content, they also usually contain high levels of lipids. Therefore, the beneficial effects of supplementation cannot be only attributable to the addition of protein. Furthermore, the possibility that polysaccharides could be the key limiting nutrient instead of nitrogen has been postulated recently (Dahlberg K.R., Mushr News, 2004, 52(7):6–10). Therefore, the relative importance of the different nutrients in mushroom compost has not been elucidated and the general belief that nitrogen supplementation determines yields is under question. Moreover, if polysaccharides play such a key role in mushroom nutrition, completely new type of supplements could be developed, with additional advantages to conventional supplements, i.e., lower cost, easier to prepare, and the overheating of compost would be less apt to occur.

To elucidate this issue various types of experimental supplements were prepared and added to mushroom compost at spawning (at 3% dry weight basis) and compared to non-supplemented compost (T1). Supplements were elaborated following conventional procedures in regard to antifungal treatment. A control supplement was prepared with cracked soybeans (T2). Experimental supplements were made of corn bran, a material rich in insoluble polysaccharides (cellulose and hemicelluloses). Corn bran was used alone (T3), mixed with corn gluten (T4), with soybean oil (T5), or with corn gluten and soybean oil (T6). Mixtures were elaborated so that its final composition resembled that of cracked soybeans, i.e., 25% protein and 18.05% lipids. Conventional procedures were followed for preparation of substrates, spawning, and cultivation techniques. For each treatment, 18 bags with 3 kg Phase 2 compost were spawned and supplemented with the corresponding material. Bags were randomly allocated in a shelf of a production room in a commercial farm. Production of fruit bodies was recorded for the first 3 flushes.

Table 1 shows that supplementation resulted in higher yields than non-supplemented compost. No significant differences could be established in final yields among the different supplements. However, supplements of cracked soybean and the mixture of Corn bran + Soybean oil produced consistently higher increases in all 3 flushes, mostly on later ones (3rd) in opposition to corn bran alone or in mixtures with either corn gluten or soybean oil, where lower yield increases for 2nd and 3rd flushes were obtained. Samples of substrates (3 bags) were also taken at the time of spawning, primordia formation (3 days after induction), and at the end of the 1st, 2nd, and 3rd flushes. Samples of substrate were analyzed for chemical composition (soluble sugars, cellulose, hemicelluloses, and lignin) and enzymatic activities (celullases, xylanases, laccasses, proteases, and soluble protein). Activity of laccases showed a similar behavior in all treatments; maximal values

were detected at primordia formation, ca. 75 IU compared to 1 IU at spawning and at the end of the 1st flush and 6.3 IU after the 2nd flush. Activity of cellulases showed again a similar behavior in all treatments with maximal values in this case after the 1st flush, ca. 39 IU compared with 1.7 IU and 3.1 IU at spawning and primordia formation, respectively. After the 2nd flush celullase activities lowered slightly to ca. 32.3 IU. Similarly, activities of xylanases showed similar behavior in all treatments with maximal value after the 1st flush, 21 IU compared with 0.19 IU and 1.0 IU at spawning and primordia formation, respectively, and a slightly lower value after the 2nd flush, 15.5 IU. Activity of proteases did not change at different stages of crop, remaining around 4 IU in all treatments. Though no significant differences in yield could be established due to the type of supplementation, a characteristic variation of the activities of substrate degrading enzymes along the crop cycle was observed in this study.

**Table 1. Production of *Agaricus bisporus* Fruit Bodies on Supplemented Compost (g Fresh Mushrooms/100 kg Phase 2 Compost)**

| | | Experimental supplements | | | |
|---|---|---|---|---|---|
| Flush | Nonsupplemented compost | Cracked soybean | Corn bran | Corn bran + Corn gluten | Corn bran + Soybean oil |
| 1 | 13.6 | 16.5 | 16.2 | 14.6 | 15.4 |
| 2 | 6.6 | 9.1 | 6.9 | 9.6 | 8.0 |
| 3 | 1.9 | 5.6 | 2.7 | 2.9 | 3.6 |
| Total | 22.1[a] | 31.2[b] | 25.8[b] | 27.2[b] | 27.1[b] |

[ab] Different letters in the same row indicate significant differences ($P = 0.05$).

# Evidence-Based Potential Benefits of *Ganoderma lucidium*

*Alice W. Chen,[1] John Seleen,[2] and Glen Babcock[3]*

[1]Specialty Mushrooms, Rochester, NY, USA, [2]JHS Natural Products, Eugene, OR, USA; [3]Garden City Fungi, Missoula, MT, USA

Today, a global *Ganoderma* spp. (Ling Zhi or Reishi) market of 2.5 billion in US dollars exists and, indicates the extensive consumption and value of this legendary medicinal mushroom. Searching for evidence- based potential benefits of *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst., we focus on breast cancer from the perspective of patients. We follow a model case study of a stage IIIa breast cancer patient adapting the adjuvant use of *G. lucidum* in conjunction with a standard regimen of breast cancer treatments (pre-surgery ACT chemotherapy, surgery, radiotherapy, and post-surgery chemoprevention). Although some conventional therapies are essential, others produce injury and damaging effects to patients, e.g., toxicity to bone marrow, digestive system, liver, and kidney, and, therefore, we continue to examine the use of *G. lucidum* post-standardized cancer treatment regimen and preventative Herceptin injection. The liver and kidneys are our selected targets.

Concurrent with standard cancer treatments, *G. lucidum* triterpene-enriched polysaccharide extract from the fruiting bodies, containing 12% β-D-glucan and 6% triterpene of a new hybrid, Reishi Gano 161, was beneficial to the patient. Benefits from the medicinal mushroom extract include effective tumor shrinkage, minimal transient side effects from chemotherapy and radiotherapy, and improvement in the quality of life compared to patients without the *G. lucidum* supplement. Supporting evidences are suggested by (1) clinical trials including randomized double-blind, placebo-controlled clinical trials; (2) *in vivo G. lucidum* studies, including rodents bearing malignant human breast-tumors; (3) the use of *G. lucidum* in

conjunction with chemotherapeutic agents; and (4) *in vitro* studies of the effect of *G. lucidum* in breast-cancer cell cultures in the past 5 years.

Of special interest is the finding in our case study of continuous use of *G. lucidum* extract. An alarmingly sizable liver cyst was discovered following extensive chemotherapy in the patient. However, in a later CT scan the cyst could no longer be detected. Recent studies described below support the healing-enhancing property of *G. lucidum* extract to liver and kidney damage.

New extensive studies (including a preliminary report by Chen and Seleen), fresh from the International *Ganoderma* Conference in Taipei, Taiwan (September, 2006), gave strong support to our case study. Sixty breast-cancer patients showed improvement in a study with controls when treatments containing *G. lucidum* were administered. A reduction of white blood cells was found patients as well as a strengthening of immune system, improvement from nausea, vomiting, loss in appetite, and impairment in hearing. In studies on a variety of cancers, the effect of *G. lucidum* was demonstrated in reversing the trend of cancer progression, easing the toxicity of chemotherapy and radiation therapy, such as increasing the tolerance of patients to treatments, reducing the decline in white blood cells, loss of appetite, weight loss, vulnerability to infections, and strengthening patients' immune functions, boosting patients' anti-tumor capability, and improving quality of life.

In conclusion, at the molecular level, the mechanisms of the anti-tumor effect of *G. lucidum* in supressing constitutional activation of the transcription factor, NF-κB is significant. Overexpression of NF-κB activity is characteristic to cancer cells. Translated to visible macro-level for patients, breast-cancer patients not only can potentially benefit from *G. lucidum* extract during the standard cancer therapy regimen, but the extract appears also to accelerate restoration of normal body functions.

## Anticancer Activities of Wild and Cultivated Edible Danish Mushrooms

*Ming Chen,[1] Lin Zhai,[1] Henning Knudsen,[2] Henrik Hoff-Hansen,[3] and Arsalan Kharazmi[1]*

[1]Department of Clinical Microbiology, 7602 University Hospital of Copenhagen (Rigshospitalet), Tagensvej 20, Copenhagen DK-2200 N, Denmark; [2]Botanical Museum, Gothersgade 130, Copenhagen DK-1123 K, Denmark; [3]Rosbaeksvej 4, Copenhagen DK-2100 Ø, Denmark

*Coprinus comatus*, *Meripilus giganteus*, *Flammulina velutipes*, *Grifola frondosa*, and 30 other wild edible mushrooms were collected from Denmark. *Agaricus bisporus, Pleurotus eryngii, P. ostreatus*, and three other cultivated mushrooms were purchased from Danish supermarkets. Fresh mushrooms were dried in an atmosphere of 37°C. After that, water extractions of these mushrooms were made and were sterile filtered through a 0.22-μm pore-size Millipore filter.

The *in vitro* anticancer activity of the water extractions of these mushrooms was tested against four different cancer cell lines, K562, EL-4 (both of them are leukemic cell lines), MCF7 (human breast cancer cell line), and PC3 (human prostate cancer cell line). A standard assay (sulforhodamine B staining method) for anticancer-drug screening recommended by the National Cancer Institute has been employed for the study.

The results show that some of the tested mushrooms exhibit potent inhibitory activity on the *in vitro* growth of the four different cancer cell lines. *Coprinus comatus, Grifola frondosa*, *Meripilus giganteus*, and several others of the tested wild edible Danish mushrooms, show potent *in vitro* anticancer activity against all four cancer cell lines. At the concentration of 0.01g/ml, *G. frondosa* and *F. velutipes* inhibited *in vitro* growth of EL-4 cell line by 92% and 70%, respectively. *Agaricus bisporus, Pleurotus eryngii, P. ostreatus* and other tested cultivated edible Danish mushrooms exhibit a moderate inhibitory effect on *in vitro* growth of the two leukemic cell lines, K562 and EL-4, and a slight effect on PC3, human prostate cancer cell line. However, they have no effect on *in vitro* growth of MCF7, human breast cancer cell line.

The preliminary mechanism of action study indicates that *in vitro* anticancer activity of the tested

edible Danish mushrooms is probably via apoptosis. Identification and purification of active components from the tested mushrooms that show a potent anticancer activity, is currently in progress in our laboratory.

In many parts of the world, especially Europe, wild mushrooms are regularly collected and used directly as a main source of food or added to soups, stews, and teas. Our data indicate that some wild edible Danish mushrooms have potent anticancer activity *in vitro,* and there is a great potential to find new anticancer agents from these mushrooms. Recommendation of these edible mushrooms to cancer patients as functional foods should be discussed.

# Study on the Differentially Expressed Genes in Mice Poisoned by α-Amanitin and Screening the Antergic Materials of Its Toxicity

*Qian Chen,[1] Mei Cao,[1,2] Qun Sun,[1] Jie Zhang,[1] Wen-liang Xiang,[1] Ruo-tong Hou,[1] Zhi-rong Yang,[1] and Jian Zhao[1,2]*

[1]Key Laboratory of Biological Resource and Ecological Environment of the Ministry of Education, College of Life Science, Sichuan University, Chengdu 610064, China; [2]State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China

Some species of the genus *Amanita* are the most lethal species. Their effective toxic agents are amatoxins, phallotoxins, and virotoxins. The major lethal toxins called amatoxins are absorbed in intestines and stomach, and α-amanitin is one of the most toxic amatoxins in wild *Amanita* mushrooms. However, there is no effective therapy for ingesting α-amanitin by mistake at present. The compound α-amanitin chiefly has a direct toxic effect to the liver and kidney of the organism. Besides, it not only noncompetitively affects RNA polymerase, but also changes other important genes.

Studies on those differentially expressed genes will help us to better understand the biological activity and mechanism of action of α-amanitin in organisms and to screen effective materials of resisting α-amanitin toxicity. Because suppression subtractive hybridization (SSH) has high sensitivity, stability and reliability, we select it to study those differentially expressed genes. The α-amanitin was dissolved into normal sodium and injected into BALB/c mice with the $LD_{50}$ dose (0.327 mg/kg) into the caudal vein. The control blood was collected into the test tube with heparin for RNA purification from the left eye of each mouse. The blood of the experimental group was collected from the right eye and divided into two groups. The first part was saved for the assay on biochemical indicators (BUN>15 mmol/L, Crea> 40 μmol/L, ALT> 600 U/L, and AST> 300 U/L), and the second part was collected into the test tube with heparin for the purification of total RNA. The purified RNA was reverse transcripted to cDNA according to the manual of SMART™ PCR cDNA Synthesis Kit (Clontech). The SSH experiment was performed according to the manual of PCR-Select™ cDNA Subtraction Kit (Clontech). The positive cDNA subtractive library was constructed with the tester cDNA synthesized from the poisoned mice, and the corresponding reverse one was constructed with the tester cDNA synthesized from the control mice. The differentially expressed cDNA fragments were cloned, screened, and sequenced, which were checked by dot blot hybridization.

The 41 clones were screened from the positive subtractive library, and 44 clones from the reverse library. The positive genes (NM 013520, AK013055, AK013732, NM 007645, NM 016974, NM 021718, etc.) were only expressed after the effect of α-amanitin. The other genes (NM 013520, NM 008372, D14716, NM 007563, NM 019946, etc.) were only expressed in the control mice. This method suggested that α-amanitin could cause the expression of many relative genes changed. These genes were useful to further understand α-amanitin's biological activity, mechanism of toxic action, improving its clinical diagnosis, and screening antergic materials.

The effects of the antergic materials of α-amanitin toxicity were analyzed among some traditional Chinese medicines (*Silybum marianum* (L.) Gaertn., *Ganoderma sinense*, *G. lucidum*, *Perilla frutescens*

(L.) Britt var. *frutescens, Perilla frutescens* var. *criasta,* and *Glucyrrhiza uralensis* Fisch.), based on the genetic transcription level of the selected differentially expressed genes. Twelve hours after the $LD_{50}$ dose of α-amanitin injection into the caudal vein, the candidate antergic materials of α-amanitin were intragastrically administrated 3 times per day. The change of transcription levels of Catnβ, Flt3L, IL7r, and Rpo2-4 associated with α-amanitin toxicity were analyzed. After intervention by the antergic materials, changes in the clinical symptoms of the mice appeared, especially for the groups of the *S. marianum* and *G. lucidum*. Compared with the poisoned mice, the mice of these two groups were more active.

The results of the semi-quantitative polymerase chain reaction assay indicated that each kind of intervening antergic material had different influences on the mutative genes caused by α-amanitin. *S. marianum*, *G. lucidum, P. frutescens* var. *criasta,* and *G. uralensis* obviously resumed to down regulate Catnβ, and the ratios of the amount of Catnβ transcription to inner standard β-actin were 0.71, 0.80 and 0.85, respectively, which were far below 1.28 of the positive control group. The intervention of *S. marianum* and *G. lucidum* had upregulated the transcripttion of Flt3L gene. After the intervention of these two materials, the ratios of the amount of Flt3L transcription to inner standards β-actin were 0.83 and 0.79, respectively, which were close to 0.88 of the negative control. The transcription of IL7r gene in the intervention group of *S. marianum* reverted to upregulation the most obviously, compared with the group injected by α-amanitin, whose ratio of transcription amount to inner standard increased from 0.36 to 0.78. The ratio was close to 0.83 for the negative control group. In addition, the intervention of *G. lucidum*, *G. sinense,* and *S. marianum* had significant influence on the transcription of Rpo2-4 gene. After the intervention by these traditional Chinese medicines, the ratio of the transcription of Rpo2-4 gene to β-actin increased to 0.58, 0.56, and 0.50 from 0.23 of the group injected with α-amanitin. From these results, *S. marianum* and *G. lucidum* had strong antagonism to α-amanitin toxicity.

# Exploitation and Utilization of Wild Fujian *Ganoderma sinense* (Sect. *Phaeonema*) Resource

*Ti-qiang Chen,[1] Liyi Zhong,[2] Jinzhong Wu,[3] Ye Li,[4] Xingsheng Lin,[1] Peigen Zhu,[1] Jie Xu,[1] and Fuping Qiu[2]*

[1]Fijian Academy of Agricultural Sciences, Fuzhou 350013, China; [2]Fujian Wuping Mushroom Technical Advice Station, Wuping 364300, China; [3]Department of TCM Pharmacology, FCTCM, China; [4]Fujian Xianzhilou Bio-Sci. & Tech. Co., Ltd., China

In recent years, strain "Minzi 96" of *Ganoderma sinense* J.D. Zhao, L.W. Hsu et X.Q. Zhng (with exemplar number HAMS 77207) was successfully domesticated from the wild Zhizi resource (Sect. *Phaeonema,* Subgen. *Ganoderma*) in the Fujian province. Our strain accorded with the statutory origin of Ling Zhi (*Ganoderma*) medicinal material in the Chinese Pharmacopoeia (Ch.Ph). The strain was applied to log-cultivation with laburnum, and on a large scale, in 2004 and 2005. The popularized scale and the cultivated yield were respectively 860 $m^{-3}$ (myceliacolonized wood-logs, $1M^3$ equal to 1100 kg), and the average yield (in primary year) reached 12.70 $kg \cdot m^{-3}$ (dry fruit bodies). The fruit bodies have a moderate size, with 7–9 cm×10–17.3 cm of pileus, while most of the basdiospores are plumpy and intact, with a size of 6.84–7.37 × 10.26–11.05 μm. The fruit bodies were processed into ultra fine powder (through Φ300 R40/3 sieve, GB/T6003.2-1997) by cryogenic vibration mill at a low temperature. The micro-morphology of ultra-fine powder was fiber-form and the context hyphae were disintegrated into fragments, with a length of 10–50 μm, diameter of 1.2 μm–4.5 μm, observed by a scanning electron microscope (SEM). The granularity was tested by Laser grain-size analysis, which specific surface area (BET) was 227.54 $m^2 \cdot kg^{-1}$ and the cumulative distribution data D97 was 63.8 μm (equivalent grain-size). The analytic results showed that the content of moisture, crude protein, fat, crude fiber, total carbohydrates, ash, and polysaccharides in the ultra-fine powder were 4.2

g·100g$^{-1}$, 10.84 g·100g$^{-1}$, 3.71 g·100g$^{-1}$, 36.5 g·100g$^{-1}$, 18.4 g·100g$^{-1}$, 1.7 g·100g$^{-1}$, and 1.3 g·100g$^{-1}$, respectively. The total content of seventeen kinds of amino acids was 9.30 mg·100 mg$^{-1}$ and the ratio of essential amino acids was 65.7%. The fatty acid composition included oleic acid (C18:1, 45.5%), linoleic acid (C18:2, 27.7%), palmitic acid (C16:0, 18.8%), and other, nonsaturated fatty acids that made up the majorities. Moreover, the content of heavy metal elements, such as Pb, As, Cd, and Hg, was less than 0.2 μg·g$^{-1}$, 0.13 μg·g$^{-1}$, 0.072 μg·g$^{-1}$, and 0.24 μg/g, respectively, which coincided with the hygienic standard of *Green Trade Standards of Importing & Exporting Medicinal Plants & Preparations* (WM2-2001).

# Edible and Medicinal Fungi in Northeast China

*Yu-Cheng Dai[1] and Bau Tolgor[2]*

[1]Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China; [2]Jilin Agricultural University, Changchun 130118, China

Northeastern China is roughly between 39° and 54° N, 118° and 135° E. It includes Heilongjiang, Jilin, Liaoning Provinces, and eastern Inner Mongolia Autonomous Region. Northeast China has the most important forest resources in the country, and therefore, it is an area rich in species of edible and medicinal mushrooms. One hundred and one (101) edible and medicinal fungi are commonly found in the markets, and they are listed below:

*Agaricus campestris* L.: Fr., *Armillaria gallica* Marxm. et Romagn., *A. ostoyae* (Romagn.) Herink, *A. sinapin*a Bérubé et Dessur., *Auricularia auricula* (L. et Hook.) Underw., *A. polytricha* (Mont.) Sacc., *Boletus edulis* Bull.: Fr., *Bulgaria inguinans* (Pers.) Fr., *Calvatia gigantea* (Batsch.) Lloyd, *Cantharellus cibarius* Fr., *Cerrena unicolor* (Bull.: Fr.) Murrill, *Chroogomphus rutilus* (Schaeff.: Fr.) O.K. Miller, *Clitocybe nebularis* (Batsch.: Fr.) P. Kumm., *C. odora* (Bull.) P. Kumm., *Clitopilus prunulus* (Scop.) P. Kumm., *Collybia dryophila* (Bull.) P. Kumm., *Coprinus comatus* (O.F. Müll.) Gray, *Cordyceps militaris* (L.) Link, *Cryptoporus volvatus* (Peck) Shear, *Daedalea dickinsii* Yasuda, *Daedaleopsis tricolor* (Bull.: Mérat) Bondartsev et Singer, *Flammulina velutipes* (W.Curt.: Fr.) Singer, *Fomes fomentarius* (L.: Fr.) Fr., *Fomitiporia hartigii* (Allesch. et Schnabl) Fiasson et Niemelä, *Fomitopsis officinalis* (Vill.: Fr.) Bondartsev et Singer, *F. pinicola* (Sw.: Fr.) P. Karst., *Ganoderma applanatum* (Pers.) Pat., *G. lucidum* (W. Curt.: Fr.) P. Karst., *G. tsugae* Murrill, *Geastrum triplex* Jungh., *Gloeophyllum sepiarium* (Wulfen: Fr.) P. Karst., *Gloeostereum incarnatum* S. Ito et S. Iami, *Grifola frondosa* (Dicks.: Fr.) Gray, *Gymnopilus spectabilis* (Fr.) Singer, *Helvella crispa* (Scop.) Fr., *Hericium coralloides* (Scop.: Fr.) Pers., *H. erinaceus* (Bull.: Fr.) Pers., *Hydnum repandum* L.: Fr., *Hygrophorus chrysodon* (Batsch.) Fr., *H. lucorum* Kalchbr., *H. russula* (Fr.) Kauffman, *Hypsizygus marmoreus* (Peck) H.E. Bigelow, *Inonotus hispidus* (Bull.: Fr.) P. Karst., *I. obliquus* (Pers.: Fr.) Pilat, *Irpex lacteus* (Fr.: Fr.) Fr., *Kuehneromyces mutabilis* (Schaeff.: Fr.) Singer et A.H. Smith, *Lactarius deliciosus* (L.) Gray, *Laetiporus sulphureus* (Bull.: Fr.) Murrill, *Leccinum aurantiacum* (Bull.) Gray, *Lentinus edodes* (Berk.) Singer, *L. lepideus* (Fr.: Fr.) Fr., *Lepista nuda* (Bull.) Cooke, *Lycoperdon perlatum* Pers.: Pers., *L. pyriforme* Schaeff.: Pers., *Lyophyllum decastes* (Fr.) Singer, *L. ulmarius* (Bull.: Fr.) Kühn., *Macrolepiota procera* (Scop.) Singer, *Morchella esculenta* (L.) Pers., *Panellus edulis* Y.C. Dai, G.F. Qin et Niemelä, *Paxillus involutus* (Batsch.: Fr.) Fr., *Perenniporia robiniophila* (Murrill) Ryvarden, *Phaeolus schweinitzii* (Fr.: Fr.) Pat., *Phellinus baumii* Pilat, *Ph. conchatus* (Pers.: Fr.) Quél., *Ph. gilvus* (Schwein.: Fr.) Pat., *Ph. igniarius* (L.: Fr.) Quél., *Ph. laevigatus* (P. Karst.) Bourdot et Galzin, *Ph. laricis* (Jaczewski in Pilát) Pilát, *Ph. lonicericola* Parmasto, *Ph. lundellii* Niemelä, *Ph. pini* (Brot.: Fr.) A. Ames, *Ph. tremulae* (Bondartsev) Bondartsev et Borisov, *Ph. tuberculosus* (Baumg.) Niemelä, *Ph. yamanoi* (Imazeki) Parmasto, *Ph. vaninii* Ljub., *Pholiota adiposa* (Batsch.) P. Kumm, *Ph. alnicola* (Fr.) Singer, *Ph. flammans* (Batsch.) P. Kumm., *Phylloporia ribis* (Schumach.: Fr.) Ryvarden, *Piptoporus betulinus* (Bull.: Fr.) P. Karst., *Pleurotus citrinopileatus* Singer, *Pl. ostreatus* (Jacq.: Fr.) Quél., *Pl. pulmonarius* (Fr.) Quél., *Polyporus umbellatus* (Pers.) Fr., *Pycnoporus cinnabarinus* (Jacq.) Fr., *Rozites caperatus* (Pers.) P.

Karst., *Schizophyllum commune* Fr.: Fr., *Sparassis latifolia* Y.C. Dai et Zheng Wang, *Stereum hirsutum* (Willd.: Fr.) Gray, *Stropharia rugosoannulata* Farl. ex Murrill, *Suillus granulatus* (L.) Roussel, *S. grevillei* (Klotzsch: Fr.) Singer, *S. luteus* (L.: Fr.) Roussel, *Trametes gibbosa* (Pers.: Fr.) Fr., *T. hirsuta* (Wulfen: Fr.) Pilát, *T. versicolor* (L.: Fr.) Pilát, *Tremella fuciformis* Berk., *Trichaptum pargamenum* (Fr.) G. Cunn., *Tricholoma matsutake* (S. Ito et S. Imai) Singer, *T. mongolicum* S. Imai, *T. terreum* (Schaeff.) Quél.

Among these species, 10 mushrooms are widely cultivated in China; 40 species are used as medicines only; 45 species are mainly edible mushrooms, but they have medicinal functions; 16 species are used as food only.

---

# Biomedical Potential of Some Cultures of Basidial Macromycetes

*Nina P. Denisova[1] and Mark M. Shamtsyan[2]*

[1]Laboratory for Biochemistry of Mushrooms, V. L. Komarov Botanical Institute, Russian Academy of Sciences, 2 Prof. Popov St., St. Petersburg 197376, Russia; [2]Department of Technology of Microbiological Synthesis, St. Petersburg State Institute of Technology (Technical University), 26 Moskovsky Pr., St. Petersburg 198013, Russia

We have conducted a long-term research of peptidehydrolases with fibrinolytical action in cultures of Basidiomycetes from the Culture Collections of the V. L. Komarov Botanical Institute of RAS and the Department of Technology of Microbiological Synthesis, St. Petersburg State Institute of Technology (Technical University).

The most active biosynthesis of proteinases with a high level of fibrinolytic activity (**FA**) in Agaricales s.l. is generic for the cultures of species from the genera *Flammulina*, *Lyophyllum*, *Lepista*, *Coprinus*, *Pleurotus,* and several others. Among the Aphillophorales s.l., the marked fibrinolytic activity was observed in the cultures of species from the genera *Trametes*, *Coriolus*, *Cerrena,* and several others.

The cultures of the species *Cerrena unicolor*, *Coprinus lagopides*, and *Flammulina velutipes,* were found to possess high levels of fibrinolytic and thrombolytic activities (**TA**). Polymer substrates, low-molecular specific substrates, and human blood thrombi *in vitro* and *in vivo* were used for evaluation of enzymatic activities. Both direct and activating effects of the fibrino- and thrombolytic activity of the proteinases of these mushrooms were discovered during the research. The activating type of the fibrinolysis was discovered for the first time for basidial mushrooms.

The proteinases of these species possess, along with marked **FA** and **TA**, sensible milk-clotting activity (**MCA**). Such an overlapping substrate specificity of the proteinases was shown also for other species of Basidiomycetes that possess **FA**.

We confirmed the immunoactivating effect of the biomass of *Cerrena unicolor* and *Flammulina velutipes* during *in vitro* experiments. We also confirmed their antitumor effect during *in vivo* experiments (Erlich carcinoma) when the biomass was used as a nutritive supplement *per os*.

Hypolipidemic features of the biomass of cultures of different Basidiomycetes species were examined on experimental animals with a hyperlipidemic diet. For the evaluation of the hypocholesterol effect of the biomass added to the daily ration, we measured the content of total cholesterol, triglycerides (TG), and α-lipoproteids (α-LP) in the blood plasma and also the content of total cholesterol in the liver. The biomass of *Cerrena unicolor, Coprinus lagopides, Flammulina velutipes*, *Pleurotus ostreatus, Trametes hirsuta, Trametes* sp., *Panus conchatus,* and *Bjerkandera adusta* was used as a nutritive supplement for experimental animals. It was manifested that supplementing the animals' diet with biomass of *Pleurotus ostreatus, Trametes hirsuta, Cerrena unicolor, Trametes* sp., and *Panus conchatus* reduced the level of total cholesterol and triglycerides in the blood plasma. The level of α-lipoproteins was also reduced in the group that received mycelium of *Pleurotus ostreatus, Trametes hirsuta,* and *Cerrena unicolor*. It should be noticed that the best results were obtained using *Trametes* sp.

mycelium since it did not only reduce the levels of the total cholesterol and triglycerides, but a high level of α-lipoproteins (the mark of "good" cholesterol) was retained also.

An analysis of the samples from livers of the control (intact and experimental groups of animals) demonstrated that adding mycelia of *Pleurotus ostreatus, Trametes hirsuta, Cerrena unicolor, Trametes* sp., *Panus conchatus,* and *Flammulina velutipes* essentially inhibited the cholesterol synthesis in liver. The level of total cholesterol was lower not only for the control group but for the intact group as well.

Further researches on these cultures of Basidiomycetes will be most fruitful for developing preparations of biomedical interest, such as enzymes with fibrino- and thrombolytic effects, as well as functional nutritive supplements to daily rations for prophylaxis and therapy of hypercholesterolemia and its after-effects.

---

# Cost-Effective Sustainable Cultivation of Some Edible Mushrooms and Assessment of Their Nutritional Values and Immunostimulatory Properties

*Sikha Dutta and Narayan Chandra Chatterjee*

Department of Botany, Mycology and Plant Pathology Laboratory, University of Burdwan, Burdwan 713 104, West Bengal, India

*Pleurotus* species (oyster mushrooms) and *Lentinus edodes* (shiitake mushroom) enjoy a high demand as good sources of non-starch carbohydrates, a higher content of dietary fibers, moderate quantitites of good quality proteins with most of the essential amino acids, minerals and vitamins, and also have pharmaceutical properties like immunostimulation, antimicrobial, antitumor, and anticancer properties.

Composts on which mushrooms colonize depend upon their capacity to degrade complex organic substances into available simple forms for their nutrition, and the yield of mushrooms also varies considerably with the chemical composition contained in the substrates on which they grow. Attempts have been made worldwide for low-cost cultivation of mushrooms on agro-industrial wastes to re-cycle the nutrients and to minimize the environmental pollution. However, a large number of edible and non-edible forms of mushrooms are potentially bioabsorbents of different heavy metals. Though this property of mushrooms is extensively being exploited for bioremediation of heavy metals from natural environments, even then these heavy metals may enter the human food chain on consumption. The present investigation was concentrated towards sustainable production of oyster mushrooms (*Pleurotus sajor-caju, P. ostreatus,* and *P. florida*) and *Lentinus edodes* on agro-industrial wastes (paddy straw, wheat straw, sugarcane bagasse, water hyacinth, saw dust, mustard straw, wastes of rice mill *Azolla*, dry leaves, fibreless jute sticks, etc.) having been confirmed that no or very low amounts of heavy metal accumulation (below detection level) in the fruit bodies raised on them.

Biological efficiency of the fruit bodies of each of the species of mushrooms grown on these agro-industrial wastes was tested in terms of their nutritional values (total carbohydrate, total protein, total nitrogen, soluble nitrogen, protein nitrogen, ascorbic acid, and calorie value) as well as the immunostimulatory properties like the total hemoglobin count, hemolysis, leucocyte count, and the granulocyte-agranulocyte ratio were determined.

The compost beds were prepared using single or combined and cumulative forms of substrates (combined substrates + urea + carbon ammonium nitrate [CAN]). It is evident that all the substrates under trial produced fructifications of all the species tested. Since the spawn run period of *Pleurotus* spp. requires less time than the other, the average yield of *Pleurotus* spp. always remained higher than that of *Lentinus edodes*. Among the *Pleurotus* spp. considered, the yield was highest in *P. sajor-caju* followed by *P. ostreatus* and *P. florida*. The cumulative forms of substrates supplemented with nitrogen-rich organic and inorganic

nutrients offered significant yield of crops with biological efficiency (BE), which remained higher on average than the other forms of compost substrates. Combined substrates responded well next to it.

Studies concerning the bioaccumulation of heavy metals by the fruit bodies grown on these agro-industrial wastes confirmed that fructification when raised on water hyacinth or on dry *Azolla* as compost, the fruit bodies became contaminated with heavy metals. In contrast, when fructifications were grown on a cumulative combined form of substrates, heavy metal contaminations were minimized and the degree of contamination was lower in *Pleurotus* species than that in *Lentinus edodes* and appeared to be the lowest in *Pleurotus sajor-caju*. It is also evident that among the heavy metals detected, levels of $Zn^{2+}$ and $Pb^{2+}$ were significant. $As^{3+}$, $Cd^{2+}$, and $Hg^{2+}$ however, remained below detection levels and as such they were within the permitted limit, as prescribed by the Joint Committee on the Prevention of Food Adulteration of the FAO (1976) and WHO (1972).

It was recorded that the food value of the mushrooms grown on these agro-industrial wastes remained more or less the same and, at the same time, the fruit bodies more or less retained their immunostimulatory properties when compared to the fructifications grown on conventional paddy straw substrate treated as the control. Immunostimulatory properties of *P. sajor-caju* and *L. edodes* appeared to be more than the others. Thus, the present study represents a low-cost sustainable production technology of some selected edible mushrooms having significant medicinal importance.

---

# Quantification of the Bioactive Compound Eritadenine in *Lentinus edodes*

*Josefine Enman, Ulrika Rova, and Kris A. Berglund*

Division of Biochemical and Chemical Process Engineering, Luleå University of Technology, SE-971 87 Luleå, Sweden

Cardiovascular diseases are among the main causes of death in our society, and there is a strong correlation between enhanced blood cholesterol levels and the development of such diseases. The culinary-medicinal mushroom *Lentinus edodes*, is widely cultivated and consumed not only as food but also as a medicine because of its medical properties. Among other things, *L. edodes* have been shown to lower blood cholesterol in both humans and rats. The blood cholesterol lowering compound produced by these fungi is designated eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid. The hypocholeste-rolemic action of this compound has been quite extensively examined in rats, but the exact mechanism by which eritadenine elicits its hypocholesterolemic action is still not fully understood. Eritadenine is suggested to accelerate the removal of blood cholesterol either by stimulating tissue uptake or by inhibiting tissue release; there are no indications of this compound inhibiting the biosynthesis of cholesterol. Furthermore, the hepatic cholesterol levels are not reduced by this substance. In rats, a diet containing 0.005% eritadenine has been shown to lower the serum total cholesterol level significantly.

The amounts of eritadenine in fruit bodies of shiitake mushrooms quantified so far are in the range of 50–70 mg/100 g dry weight in the caps and 30–40 mg/100 g dry weight in the stems, as determined either by column chromatographic fractionation or by GC. No data has been found in the literature pertaining HPLC quantification of eritadenine. In search for a potential hypocholesterolemic product, it is of great importance to determine the content of eritadenine in shiitake mushrooms in order to establish the dose-response effect in humans. Strains producing considerable amounts of eritadenine are favorable, as is a reliable analytical procedure for this substance. This study was conducted in order to quantify the amount of the cholesterol-reducing agent, eritadenine, in the fruit bodies of four different strains of shiitake mushrooms. In order to analyse the target compound, a reliable and reproducible HPLC method for separation, identification, and quantification of eritadenine was developed.

To determine the amounts of eritadenine in fruit bodies of shiitake, methanol extraction was used to recover as much as possible of the hypocholesterolemic

agent from the fungal cells. It was also investigated if the amount of eritadenine released could be increased by pretreating the mushrooms with enzymes involved in the breaking of bonds between the polymers in fungal cell walls. The amounts of eritadenine in the four different fruit bodies of shiitake mushroom were determined, and the strains under investigation exhibited up to 10 times higher levels of eritadenine (317–633 mg/100 g dry weight) than previously reported for other shiitake strains. Furthermore, pretreating the mushrooms with hydrolytic enzymes before methanol extraction resulted in an insignificant increase in the amount of eritadenine released. These results indicate the potential for delivery of therapeutic amounts of eritadenine from ingestion of extracts or dried concentrates of shiitake mushroom strains.

In this study it is clearly shown that HPLC analysis of eritadenine is highly applicable and offers a simple and sensitive method for separation, identification, and quantification of this compound.

---

# *Pleurotus ostreatus* as a Potential Source of Probiotics

*Vladimir Erban,[1] Eliska Komarkova,[1] Oldrich Benada,[2] Andrej Sinitsa,[3] and Jana Čopíková[3]*

[1]Food Research Institute Prague, Radiova 7, CZ 102 31, Prague 10, Czech Republic; [2]Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20, Praha 4-Krč, Czech Republic; [3]Faculty of Food and Biochemical Technology, Institute of Chemical Technology, Prague Technická 5, 166 28 Prague 6, Czech Republic

Mushrooms are widely used in human health care. Mushrooms can be also used as a source of specific polysaccharides – β-glucans. In our study we tested extracts from *Pleurotus ostreatus* for their potential activity to the selected probiotic strains. Extracts were prepared by sequential water and alkaline extraction. Conditions were changed step consecutively. Four following extractions resulted into 14 fractions. Water was used for the first three of them, and the extraction finished with 1 M NaOH. Fractions were observed for β-glucan and protein content. The insoluble fraction still contained a high amount of β-glucan, as described in the respective literature. Mass balance shows that 33% of β-glucans were isolated, 61% stayed in insoluble residue, and only 6% were losses. β-glucans were determined using the Mushroom and Yeast Beta-Glucan kit (MEGAZYME), proteins were determined by applying the Bradford method (Sigma-Aldrich), and the results were verified by FTIR spectroscopy.

The extract with the highest yield contains 40% of β-glucan and was prepared by alkali extraction (4C, 1M NaOH with $NaBH_4$) and precipitated by pH decrease to value 8. This fraction contains a big volume of proteins (10%) also. The proteins are inseparable by normal physicochemical operations. We assume that the isolated compound is a proteoglucan complex. We decided to test it in this form because it can exhibit higher biological activity. A similar situation existed with other extracts; only the amount of components was different. Based on a chemical analysis we selected seven fractions for tests with 15 probiotic strains. Ten probiotics were obtained from a collection (Laktoflora, Czech Republic) and five were isolated and adapted in our laboratory. Maximum growth rate, biomass concentration, and acid production were observed in four independent cultivations. The resulting value was established as the difference between the control cultivation in MRS (oxoid) medium without glucose and the cultivation with extract added to the MRS (oxoid) medium.

Selected water extracts supported the growth of 12 strains out of the 15 tested. The best alkaline extract supported growth of only 8 strains. Others were able to support just 2 or 3 tested strains. If we compare strains, the most universal is the *Bifidobacterium* strain from collection, which utilizes 6 out of 7 extracts. Two *Bifidobacterium* strains and one *Enterococcus* strain showed a good score as well (5 out of 7). *Lactobacillus* strains can utilize just 4 or less extracts. Biomass production is generally lower in medium with extracts, except for one *Bifidobacterium* strain. That strain was isolated as a subpopulation cultivated with an alkaline extract. Only this strain and the original strain from collection

were able to utilize the extract for biomass production higher than the control one. Acid production is a good marker of the extract's metabolic availability. Acid production is also an important characteristic of probiotics. Water extracts support this production for 8–11 strains out of 15. Only two alkaline extracts have a similar characteristic.

Proteo-glucan complex isolated from *Pleurotus ostreatus* can be used for symbiotic construction. Water and alkaline extraction result in a different type of complex because different types of probiotics can utilize them. This exploitation of mushroom extracts continues and extends the use of *P. ostreatus* for human health.

## Extracellular Biosynthesis of Silver Nanoparticles by *Pleurotus* Species

*Aniket Gade, Mahendra K. Rai, Alka Karwa, Prashant Bonde, and Avinash Ingle*
Department of Biotechnology, SGB Amravati University, Amravati 444 602, India

Many scientists across the world have been reporting the ecological, pharmacological, nutritive, and culinary importance of wild as well as cultivated *Pleaurotus* species. *Pleurotus sajor-caju* is an edible species of great biotechnological interest, not only for its ability to grow on numerous agricultural residues to produce mushrooms of high organoleptical quality but also because this fungus produces secondary metabolites with pharmaceutical applications and some proteins for industrial use, amino acids, vitamins, etc.

Mushrooms are unique among microorganisms in that they secrete a variety of extracellular enzymes. These properties significantly increased their commercial value over the last few years. While other lower fungi are subjected to studies of secondary metabolites and nanoparticle synthesis, much less attention in this regard is given to the higher Basidiomycetes. The use of fungi for catalyzing specific reactions leading to inorganic nanoparticles is a new and rational biosynthesis strategy. Recently, mushrooms along with many bacteria and fungi are a potential source of bioremediation of toxic metals through the reduction of metal ions. These dissimilar properties of mushrooms can be exploited for the biosynthesis of inorganic nanoparticles with gold and silver.

We have undertaken this study to determine the extracellular biosynthesis of silver nanoparticles using the commercially cultivated *Pleurotus sajor-caju*. Mycelial cell filtrate of this species showed a change in color from pale yellow to brown upon addition of silver ions. The change in color was due to the excitation of the surface plasmon vibrations. Moreover, it is a clear indication of the reduction of silver ions occurring extracellular through reducing agents released into the aqueous medium by the mushroom. A UV-VIS spectrum of the mycelia filtered aqueous medium showed an absorbance peak at 420 nm indicating the reduction of silver ions. The process is extracellular and fast. In this report we hypothesize that the extracellular nitrate reductase system of the mushroom is responsible for the biosynthesis of silver nanoparticles. This characteristic feature of extracellular activity of *Pleurotus* spp. may open new paradigms in mushroom research and facilitate the biosynthesis of metal nanoparticles in an eco-friendly and novel way. The biosynthesis of silver nanoparticles thus detected can be further characterized by their topology and size using X-ray diffraction and Transmission Electron Microscopic (TEM) studies. Nanotechnology is evolving and expanding in many areas of life sciences, and the achievements are being applied in medicine-like diagnostic purposes and in drug delivery systems. Biologically synthesized silver nanoparticles from mushrooms could have many uses in areas such as aids and cancer, drug and gene delivery, bio-detection of pathogens, probing of DNA structure, tissue engineering, tumor destruction, Magnetic Resonance Imaging (MRI), phagokinetic studies, as non-linear optics, intercalation materials, biolabelling, and as nanobiosensors. Extracellular synthesis of nanoparticles by filamentous fungi like *Pleurotus* spp. offers an advantage of obtaining large quantities in a relatively pure state and at a rate more rapid than that from bacteria or other sources. Furthermore, the extracellular synthesis of nanoparticles would make the process simpler and easier for downstream processing.

In the present study *Pleurotus sajor-caju* exhib-

ited nanoparticle production capacity; however, the technology can be applied on a larger scale once the study of reductase/electron shutter relationships under these conditions is completed. The nitrate reductase is apparently essential for silver ion reduction. Besides the extracellular reductase system, several other metabolites may play the role of electron shuttle in metal reduction. Nanoparticle synthesis in mushrooms provides the potential for significant technological advances in the near future.

## Expression Profile of Extracellular Proteins of *Phanerochaete chrysosporium* RP78 during Biodegradation of Azo Dyes

*Fahimeh Ghasemi and Fatemeh Tabandeh*

National Institute for Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14155-6343, Tehran, Iran

Azo dyes are the largest class of dyes with the greatest variety of colors, which are used extensively in textile industries. The azo dyes have great structural variety, so as a group, they are not uniformly susceptible to microbial attack. The lignin degrading system of the white rot fungus, *Phanerochaete chrysosporium*, is able to degrade a wide range of structurally diverse organic pollutants. Although azo dyes are generally considered to be non-biodegradable under aerobic conditions, the nonspecific nature of the lignin degrading system is reasonably effective in degrading these dyes, especially when it is expressed under nitrogen or carbon limitation.

In this study, the aerobic biodegradation of seven dyes: Reactive Orange 16, Reactive Black 5, Direct Violet 51, Acid Red 88, Acid Red 114, Basic Orange II, and Bismarck Brown R, by the white rot fungus *Ph. chrysosporium,* was investigated. The expression profile of extracellular enzymes during the biodegradation of azo dyes, were determined as well.

All dyes were purchased from Sigma-Aldrich Co. The microorganism *Ph. chrysosporium* strain RP78 was obtained from the Center for Biotechnology, Nagarjuna University, India, and was maintained on potato dextrose agar at 30°C. A nutrient nitrogen-limited medium consisted of 56 mM glucose, 1.2 mM ammonium tartrate, 1.5 mM veratryl alcohol, mineral salts, and thiamin (1 mg/liter) in a 20 mM sodium buffer (pH 4.5). The sterilized medium was inoculated with *Ph. chrysosporium* spores. The medium cultures were incubated at 30°C in 250-ml flasks with two inlets and outlets for oxygen. The cultures were flashed by pure oxygen at the time of inoculation and then every three days. Nitrogen-limited cultures of *Ph. chrysosporium* were allowed to grow for 8 days in 30°C. Then, one of the dyes was added to each culture. All dyes were prepared in water at 0.5 mg/ml and were added to yield a final concentration of 40.483, 25.206, 34.736, 62.4, 30.09, 100.5, and 54.182 μmol for Reactive Orange 16, Reactive Black 5, Direct Violet 51, Acid Red 88, Acid Red 114, Basic Orange II, and Bismarck Brown R. Dye disappearance was detected at or near the wavelength maximum for each dye at specific intervals for 5 days. Lignin peroxidase activity was determined by the method of Tein and Kirk (1983). One unit of enzyme activity oxidized 1 μmol veratryl alcohol in 1 min at room temperature. Equal volumes of extracellular fluid was precipitated by TCA 12% and then subjected to sodium dodecyle sulfate-polyacrylamid gel electrophoresis (SDS-PAGE).

The results showed that Reactive Orange 16, Reactive Black 5, Direct Violet 51, Acid Red 88, and Acid Red 114 were extensively degraded by *Ph. chrysosporium*, evidenced by the decrease in absorbance of the culture medium. Data revealed that Basic Orange II and Bismarck Brown R (basic azo dyes) were not good substrates for the biodegradation system of this fungus. It was observed that more than 90% of decolorization of Acid Red 88, Reactive Orange 16, and Reactive Black 5 occurred within 24 h. Direct violet 51 and Acid Red 114 were degraded after 5 days incubation. Therefore, effectiveness of decolorization depends on structure and complexity of each dye. In a parallel experiment, the cultures were assayed daily for lignin peroxidase activity. Lignin peroxidase activity, measured in terms of veratryl alcohol oxidation, was first observed on day 5. The activity level of lignin peroxidase was increased up to 29 U/ml during the decolorization phase so that

the culture remained ligninolytic up to day 13. The SDS-PAGE gel electrophoresis analysis of the extracellular proteins secreted by *Ph. chrysosporium* in the presence of different dyes showed three major bands around 40 kDa. The expression level of these proteins was also increased through cultivation. There was no significant variation in the protein expression pattern among different biodegradations of azo dyes. In conclusion, *Ph. chrysosporium* RP78 has a lignin degrading system consisting of at least three main enzymes, used efficiently for the biodegradation of reactive and acidic azo dyes.

# Effect of Oregano Essential Oil on Infection of *Agaricus bisporus* by *Mycogone perniciosa in Vitro* and in the Mushroom Growing Unit

*Jasmina Glamočlija,[1] Marina Soković,[1] Jelena Vukojević,[2] Ivanka Milenković,[2] Vele Tešević,[3] and Leo J.L.D. van Griensven[4]*

[1]Institute for Biological Research, Bul. despota Stefana 142, Belgrade, Serbia; [2]Institute of Botany, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia; [3]Faculty of Chemistry, University of Belgrade, Studentski trg 3, Belgrade, Serbia; [4]Plant Research International, Wageningen University and Research, P.O. Box 166700 AA, Wageningen, The Netherlands

*Agaricus bisporus* (J. Lange) Imbach is subject to various diseases and pests. *Mycogone perniciosa,* which causes wet bubble (WBD) disease of champignons, is the most important mycoparasite of this mushroom in Serbia. Although WBD is routinely controlled using different fungicides, *M. perniciosa* remains a constant threat. In addition, the constant use of fungicides causes the development of pathogen resistance. In order to find alternative preventive methods, we tested the antifungal activity of *Origanum vulgare* essential oil against *M. perniciosa,* both by micro-atmospheric method *in vitro* and practically in the mushroom growing unit.

Oregano oil, which contains large amounts of the phenolic components, thymol (15%) and carvacrol (65%), showed very strong antifungal activity *in vitro*. Minimal inhibitory quantity is 0.001 µl/disc and the minimal fungicidal quantity is 0.1 µl/disc. The fungicide prochloraz, which was used as a control in this study, showed a much lower antifungal potential than oregano oil, with MIQ 5.0 µl/disc and MFQ 50.0 µl/disc. It was thought that a vapor treatment would be very effective against *M. perniciosa* allowing the use of only a limited amount of essential oil.

To treat experimentally induced WBD in the growing house we tested the antifungal activity of oregano oil when applied in casing soil in three different ways. In treatment A we added 2.0% oregano oil simultaneously with a *M. perniciosa* spore suspension in casing soil and put it on the compost layer immediately; secondly, 2.0% oil was added to casing soil and 4 h later the spore suspension was applied and put on the compost (B); finally we added different concentrations (0.1%, 1.0%, 2.0%, and 5%) oil and 4 h later the spore suspension was applied to the casing soil and incubated at room temperature for 12 h, after which the casing mixtures were put on compost (C). We used prochloraz as a no-disease control and had two extra controls, i.e., one with spores of *M. perniciosa* and another without spores.

The best results were achieved in experiment A with 2% of oregano oil and simultaneous application of the spore suspension where oregano oil completely inhibited the growth of *M. perniciosa*. The treatment of casing soil with oregano oil in the growing house (B) showed only slight inhibition of the pathogen. The handling of casing soil, in this case, might be the reason for evaporation of oil and subsequent loss of activity. In experiment C no inhibition of the pathogen was observed, except with 5% oil where the symptoms were observed later and were less severe than in the control. Commercial fungicides usually show carcinogenic, teratogenic, and residual effects. Risk of pathogen resistance is highly present. As essential oils are largely nontoxic and easily biodegradable, we advise to disinfect commercial casing soil with 2% oregano oil before applying the casing to the compost.

# Membrane Activity of Ostreolysin, a Cytolytic Protein from the *Pleurotus ostreatus*, Is Inhibited by Lysophospholipids

*Dejan Gmajner,[1] Katja Rebolj,[2] Peter Maček,[2] and Kristina Sepčić[2]*

[1]Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia; [2]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

Ostreolysin is a 15 kDa cytolytic protein found in large amounts in young fruiting bodies of the edible oyster mushroom, *Pleurotus ostreatus*. It belongs to the aegerolysin protein family of closely similar proteins that can be found also in bacteria and plants. Many of these proteins exert cytolytic and hemolytic effects that are the consequence of pore formation and can be responsible for their toxicity. In lipid membranes, ostreolysin specifically recognizes cholesterol rich liquid-ordered ($l_o$) domains, which are the basis for the formation of cellular lipid rafts. Rafts are enriched in cholesterol, sphingomyelin, and specific proteins and are involved in several important biological functions such as exocytosis and endocytosis, signal transduction, pathogen entry, and attachment of various ligands. They exist as nanoscale clusters of various sizes and are believed to be transient, dynamic, and unstable membrane entities with a lifetime of less than 0.1 ms. Lysophospholipids are single-chain, water-soluble surfactants that were found to enter the $l_o$ phase in artificial vesicles and cell rafts. They are present in biological membranes in low concentrations, where they are synthesized *de novo* or are generated by enzymatic cleavage of glycerophospholipids and sphingomyelin. Their role is to regulate and maintain the organismal homeostasis. Many lysophospholipids affect fundamental cellular functions like proliferation, differentiation, survival, migration, adhesion, invasion, and morphogenesis. Their signaling activity is mediated by the activation of transmembrane G-protein coupled receptors. Impairment of their signaling functions is associated with several metabolic and physiological disorders. The novel mode of activity of lysophospholipids *via* changing properties of lipid rafts has emerged recently, opening new questions about the interaction of different proteins with raft domains. Consequently, the aim of our study was to determine the mode of modulation of ostreolysin membrane activity by lysophospholipids in large unilamellar vesicles (LUVs).

Binding of ostreolysin to LUVs composed of lipids, resembling those of lipid rafts and $l_o$ domains, was studied with surface plasmon resonance (SPR) technique on the Biacore X refractometer, using a L1 sensor chip. Addition of lysophospholipids, especially lysophosphatidylinositol (LPI) and lysophosphatidylcholine (LPC), drastically inhibited the binding of ostreolysin. The experiments revealed kinetics of lysophospholipid association with, and dissociation from vesicles composed of cholesterol and sphingomyelin (1:1, mol/mol). The dissociation of lysophos-pholipids was found to be rather slow, which allowed ostreolysin to be applied on the sensor chip at various times during the dissociation process. The binding signal of ostreolysin was weak during the initial period of dissociation of lysophospholipids, and increased on further dissociation from the vesicles. We further studied the pore formation of ostreolysin by the release of fluorescence dye calcein from LUVs. Addition of LPC in micromolar concentrations strongly inhibited the capacity of ostreolysin to permeabilize lipid vesicles composed of cholesterol and sphingomyelin. The increased concentration of LPC in the lipid vesicles decreased the ostreolysin activity even further in a dose-dependent relationship.

In conclusion, our results show that incorporation of lysophospholipids into the lipid bilayers of large and giant unilamellar vesicles markedly reduces the binding and pore forming activity of ostreolysin. It suggests that this inhibition is a consequence of changed properties of $l_o$ domains and the structural changes of cholesterol-rich raft domains induced by lysophospholipids.

# Two Control Strategies of Oxalate Concentration in White-Rot Fungi: *Bjerkandera fumosa* and *Abortiporus biennis*

*Marcin Grąz*

Department of Biochemistry, Maria Curie-Sklodowska University, Sklodowska Square 3, 20-031 Lublin, Poland

White-rot fungi are considered as the most efficient lignin decomposers in nature. They secrete an array of oxidases and peroxidases involved in lignocellulose complex degradation. The three main enzymes are laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). This is well established that enzymes cannot initiate lignolysis due to their excessive size in comparison to wood pores. The low-molecular-weight compounds are responsible for initiation of lignocellulotic complex degradation. Among different low-molecular-weight compounds involved in the initiation of ligninolytic processes, organic acids are important factors. It has been reported that oxalic acid is a predominant organic acid in wood-rotting fungi cultures. In the wood degradation system oxalate can play a role as a proton and electron source, a strong metal chelator, a factor which stabilizes osmotic potential, and in the pH of fungal growth environment. Oxalic acid can also facilitate the catalytic cycle of MnP by chelating $Mn^{3+}$ ions. Due to its numerous roles, the concentration of oxalic acid must be controlled by the fungus. In the regulation process of oxalate concentration two enzymes can be involved - oxalate decarboxylase (OXD) or oxalate oxidase (OXO). The OXD decomposes oxalate to formate and carbon dioxide, the OXO to carbon dioxide and hydrogen peroxide. Formate as well as hydrogen peroxide can play important roles in fungal metabolism. Both enzymes belong to the same protein family and probably originated from gene duplication or deletion. Most typical for fungal is the oxalate degradation by decarboxylation, but, recently, there are findings of oxalate oxidase existence in fungi, which generally was considered plant enzyme taking part in plant fungal resistance.

In our study we have investigated the secretion of organic acids in two white-rot fungi, *Bjerkandera fumosa* and *Abortiporus biennis,* and the enzymatic control of oxalate concentration, which was the main organic acid detected. It was ascertained that *Bjerkandera fumosa* degrades oxalate *via* the decarboxylation process, but oxalate metabolism in *Abortiporus biennis* is *via* the oxidation process. The novel role for oxalic acid as a factor providing initial concentration of $H_2O_2$ by enzymatic degradation *via* oxalate oxidase in *A. biennis* cultures is proposed. Relevance of oxalic acid secretion with iron ions chelation ability in both fungi was studied. Correlation between MnP, Lac activity, $H_2O_2$ concentration and secretion of oxalic acid and its enzymatic degradation in *A. biennis* and *Bjerkandera fumosa* liquid cultures were investigated.

---

# Potential Use of Agricultural By-Products for Medicinal Mushroom Cultivation

*Andrej Gregori,[1] Bojan Pahor,[2] Roman Glaser,[2] and Franc Pohleven[3]*

[1]SRC Bistra Ptuj, Slovenski trg 6, 2250 Ptuj, Slovenia; [2]Perutnina Ptuj d.d., Potrčeva cesta 10, 2250 Ptuj, Slovenia; [3]Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Rožna dolina, Cesta VIII/34, Ljubljana 1000, Slovenia

The agricultural by-products used in this study have low or no economical value and olive oil press cakes (OOPC) also presented an ecological problem. Pumpkin (POPC), sesame (SEOPC), olive (OOPC), sunflower (SUOPC), rape (ROPC), and soy (SOOPC) oil press cakes are generally used as animal feed. Due to

high concentration of saturated fatty acids and particles of broken and undeveloped seeds, leftover hemp seeds (LHS) are not appropriate for oil production but are used mostly for animal feed.

The aim of this study was to determine suitability of the abovementioned by-products for cultivation of five culinary-medicinal mushroom species (*Grifola frondosa, Pleurotus ostreatus, Lentinus edodes, Flammulina velutipes,* and *Ganoderma lucidum*).

Substrates consisted of 49% air-dry agricultural by-product, 49% air-dry beech sawdust, and 2% $CaCO_3$. Corn plant was used alone (98%) with 2% of $CaCO_3$. Water was added to all the mixtures to achieve 60% water content. Mixtures were filled into racing tubes (25 mm in diameter, 175 mm in length) with uniform pressure of 400 g, covered with polypropylene caps on both sides and sterilized for three hours at 121ºC.

One mycelium-covered PDA disc with a 9 mm diameter was used for inoculation of each racing tube and incubated at 20ºC in the dark. Growth of mycelium was measured after 14, 20, 22, 25, 27, 30, and 35 days on the fastest and slowest growing point. The average distance of mycelium growth rate was calculated. Mycelium density was determined visually and estimated regarding the growing criterion, ranging from 1-the weakest to 10-the strongest growing. All the experiments were conducted at the mycological laboratory of the Institute for Natural Science, Ljubljana, Slovenia.

The highest *G. lucidum* mycelium overgrowth was measured on substrates with LHS, OOPC, and WGCP. The intermediate growth rate occurred on SUOPC substrates. Compared to other tested substrates, mycelium growth rate was the lowest on SOOPC and ROPC, and completely repressed on POPC- and SEOPC-containing substrates. The strongest mycelium growth was detected on LHS and WGCP.

The colonization rate of *P. ostreatus* mycelium was the highest on substrates containing LHS, WGCP, and SEOPC; intermediate growth rates occurred on OOPC, ROPC, and SUOPC, and; lowest on SOOPC. Growth was completely repressed on POPC-containing substrates. The strongest mycelium growth appeared on LHS and WGCP; the weakest on OOPC-containing substrates.

*Flammulina velutipes* mycelium growth rate was the greatest on substrates with LHS and SUOPC; intermediate growth rates were observed when substrates contained SEOPC, WGCP, ROPC, and OOPC. The lowest growth rates appeared on substrates containing SOOPC. *F. velutipes* mycelium does not grow on substrates containing POPC. The strongest mycelium growth was observed on LHS and WGCP, while the weakest was on OOPC.

*Lentinus edodes* mycelium grew on substrate with LHS and OOPC only, while on substrates containing WGCP, SOOPC, SEOPC, ROPC, SUOPC, and POPC no growth appeared. The greatest growth rate was evaluated on OOPC. Mycelium density on LHS was stronger in comparison with OOPC.

The greatest growth rate of *Grifola frondosa* mycelium was measured on OOPC and grew also on substrate with LHS. No *G. frondosa* mycelium growth appeared on substrates containing SEOPC, SUOPC, SOPC, ROPC, WGCP, and POPC. Mycelium growth was stronger on substrates containing LHS compared with OOPC-containing substrates.

According to these results, substrates containing LHS were probably the most suitable agricultural by-product for potential mushroom cultivation out of all tested materials. Mycelium growth rate and density of all tested species are faster on these media in comparison with other tested by-products. In comparison with LHS-containing substrates, mycelium growth and density on WGCP is also efficient.

Mycelia of *L. edodes* and *G. frondosa* grew only on LHS and OOPC. Growth on OOPC substrate was weak and almost invisible, however faster than on LHS-containing media. Although mycelium growth was completely suppressed by POPC in high proportions, these substrates were not suitable for potential mushroom cultivation.

## ACKNOWLEDGMENT

This research work was financed by Slovenian Research Agency (applicative research project No. L2-7598), Perutnina Ptuj d.d., and Municipality of Ptuj.

# Spread of the *Fomitopsis officinalis* Inoculated in Stems of Living Larch in Slovenia

*Andrej Gregori,[1] Barbara Piškur,[2] Marija Gregori,[1] and Dušan Jurc[2]*

[1]Institute for Natural Sciences, Podkoren 72, SI-4280 Kranjska Gora, Slovenia; [2]Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia

The fungus *Agaricum,* or *Agariokon,* was used by ancient Greeks and Romans for tuberculosis treatments. Throughout history, known also as "quinone conk", "ghost bread", "tree biscuit", and white agaric, this fungus is the larch polypore *Fomitopsis officinalis* (Vill.) Bondartsev et Singer (=*Laricifomes officinalis* (Vill.) Kotl. et Pouzar) noted for its medicinal properties. *Fomitopsis officinalis* is usually associated with *Larix* trees, but it is reported to occur also on different coniferous species. The fungus infects the trees through broken branches and causes intensive brown rot in wood. Growth of *F. officinalis* is slow and the fruiting bodies vary in shape. They can live up to 70 years and weigh up to 10 kg. *F. officinalis* is characterized by diminishing distribution from south to north and has a tendency to mainly inhabit mountainous regions. In Slovenia, the total of five locations of *F. officinalis* are known. *F. officinalis* is a very rare species and has already been suggested to enter it on the list of protected species. It has been placed on a list of 33 endangered fungi in Europe, which was proposed by the European Council for Conservation of Fungi.

The purpose of this study was to determine the success of larch tree inoculation with *F. officinalis,* in order to use it for spreading this fungus in nature, thus promoting and securing its survival in Slovenia.

Two healthy larches, with diameters at breast height of 89 cm and 58 cm, were inoculated with larch wood sticks overgrown with *F. officinalis* mycelium at different tree heights. Mycelium culture was obtained from the Institute for higher fungi, Vinje pri Ljubljani, Slovenia (ZIM culture collection No. 1024). The culture was isolated from a conk of *F. officinalis* collected at Podolševa, Slovenia (Longitude: 14° 14' 28'', Latitude: 46° 26' 12'') in the year 2000 and has been kept under oil on potato dextrose agar. One larch tree was inoculated in Ljubljana and one in Gozd Martuljek. Selected sites are distinct in climate conditions: 1) mean annual air temperature (Ljubljana: 10.2°C; Gozd Martuljek: 6.1°C); 2) mean annual precipitation (Ljubljana: 1377 mm; Gozd Martuljek: 1604 mm) and; 3) max/min air temperature in the year 2006: (Ljubljana: 35.9/15.7°C; Gozd Martuljek: 32.6/20.4°C).

In April 2006 and December 2006, 3 years after the inoculation, wood core samples were extracted with an increment borer from the standing trees, at different distances from the primary inoculation points. The wood core samples were sectioned at a distance of 3 cm, followed by placing obtained wood particles on the malt extract agar (MEA) plates and incubated at 24°C. The wood material was treated with sterile equipment, and sterile techniques were used throughout the isolation procedure. After mycelium had outgrown from the incubated wood samples, the fungus was determined by its microscopic characteristics. Spore and mycelium characteristics of the original *F. officinalis* strain were compared with isolates obtained from our inoculated trees. The inoculation was considered successful, if the above-mentioned parameters were comparable.

Maximum length of tree trunks overgrown with *F. officinalis* mycelium was 9 cm below, 10 cm above the inoculation point (Ljubljana, April), 20 cm left, 10 cm below, 10 cm above the inoculation point (Ljubljana, December), and 10 cm above the inoculation point in December at Gozd Martuljek as was showed with laboratory tests.

Inoculation of larch trees with *F. officinalis* was successful using the abovementioned method, even on trees in rural and urban areas, where this species was not found. After 3 years of larch tree inoculation, re-isolation of *F. officinalis* mycelium was successful from 83.3% wood core samples. This percentage declined to 8.3% re-isolation success, when isolations following the same procedure were done in the winter time. We can only speculate that this decline occurs due to the influence of weather conditions to mycelium activity inside the tree trunk. Slow rates of mycelium progress in living trees were

also revealed in the experiment in Slovenia – 6 cm per year, perpendicular to the trunk axis and around 3 cm per year in the direction parallel to the tree axis. The technique of artificial inoculation could be used for preventing imminent extinction of *F. officinalis* in Slovenia as was also indicated with obtained results in our experiment.


## ACKNOWLEDGMENTS

We thank Prof. Franc Pohleven for helping us to organize and plan our experiment; Andrej Piltaver for providing us with valuable *F. officinalis* cultures; Aleš Jezeršek for donating one of the experimental trees; and Vesna Rajh for laboratory technical support.


# Enzyme Activities and Growth Rate of *Pleurotus ostreatus* Mycelium on Substrates Composed of Wheat Bran, Wasted Brewery Grains, and Beech Sawdust


*Andrej Gregori,[1] Mirjan Švagelj,[2] Bojan Pahor,[3] Marin Berovič,[2] and Franc Pohleven[4]*

[1]SRC Bistra Ptuj, Slovenski trg 6, 2250 Ptuj, Slovenia; [2]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, 1000 Ljubljana, Slovenia; [3]Perutnina Ptuj d.d., Potrčeva cesta 10, 2250 Ptuj, Slovenia; [4]Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Rožna dolina, Cesta VIII/34, 1000 Ljubljana, Slovenia


One of the main by-products of brewery industry is wasted brewery grains (WBG), which also present a large economical burden to this industry. Big efforts are made to use wasted grains as potentially valuable secondary raw material, including usage as components of substrates for mushroom cultivation. The presented study was focused on enzyme activities and growth rates of *Pleurotus ostreatus* (oyster mushroom) mycelia on substrates containing SBG: to determine the best substrate for mycelium overgrowth.

Substrates were prepared with different proportions of wasted brewery grains - WBG (0 and 10%), wheat bran - WB (10, 20, and 30%), beech sawdust, and 2% $CaCO_3$. The moisture content was adjusted to 65%. Substrates were transferred into racing tubes (25 mm in diameter, 175 mm in length) with uniform filling of 40 g per racing tube. Racing tubes were steam sterilized at 121°C for three hours and inoculated at one side with a 9 mm disk of actively growing *P. ostreatus* mycelia on potato dextrose agar and sealed with cotton stoppers. The tubes were incubated in a dark controlled environment at 24°C. After 33 days of growth, the extracellular enzymes were extracted from 5 g of overgrown substrate with 10 mL of extraction buffer (0.1M sodium phosphate (pH 6.5) with 5% Tween-80) and screened for total peroxidase (TP), manganese-independent peroxidase (MiP), lignin peroxidase (LiP), and laccase (Lac) activity. Lac activities were determined, as described in Podgornik et al. (Enzyme Microb Technol, 2001, 29(2–3):166–172). TP, MiP, and LiP activities were determined, as described in Podgornik et al. (Lett Appl Microbiol, 2001, 32(6): 407–411). Growth and enzyme activity experiments were performed at least in triplicates.

Mycelium growth rate was about 10% slower in the substrate with 30% wheat bran with the addition of wasted beer grains compared to the substrate that had no additional SBG (135 mm/3 days for 30% of WB in the substrate compared to 122 mm/33 days in the substrate with 30% WB and 10% SBG). The enzyme activities were rising as the level of supplementation with WB and/or SBG raised. The highest enzyme activities were found in the substrate with 30% WB and 10% SBG. Lignin peroxidase activity was not detected in any of the prepared substrates.

Wheat bran enhances the growth rate of *P. ostreatus* mycelium and correlates well with the induction of peroxidase type enzymes. The addition of SBG (10%) stimulates the growth rate only in the substrates with 10% and 20% WB and is higher than in the substrates with no addition of SBG. The comparison of the substrates with 30% WB and 10% SBG with the substrates with only 30% WB showed an inversion in the growth rate. The unexpected decrease in the growth rate could be attributed by nutrient overload and should be investigated in more detail in the future.

*Pleurotus ostreatus* displays a markedly higher lignin peroxidase-type activity compared with laccase-type enzyme activity in all of the substrates. There is, however, only a small difference in enzyme activity (TP, Lac, and MiP) if we compare the substrate with 20% WB with no addition of SBG to the substrate with 30% WB and no added SBG. This implies that WB and SBG are interchangeable in the respect to enzyme production.

Our method for the determination of LP activity is based on the detection of veratryl aldehyde using the oxidation of veratryl alcohol by the enzyme veratryl alcohol oxidase. The fact that we did not detect any LP activity is in agreement with Vyas and Molitoris (Appl Environment Microbiol, 1995, 61:3919–3927), even though lignin peroxidases in *P. ostreatus* were discovered and characterized.

## ACKNOWLEDGMENTS

This research work was financed by Slovenian Research Agency (applicative research project No. L2-7598), Perutnina Ptuj d.d., and Municipality of Ptuj.

# Effect of *Hericium erinaceus* Extracts on Physiological Functions, Growth, and Development of Nerve and Glial Cells, and Myelination of Nerve Fibers

*Andrii P. Gryganski,[2] Mykhaylo G. Moldavan,[1] Olena V. Kolotushkina,[1] Burkhard Kirchhoff,[3] Galina G. Skibo,[1] and Paola Pedarzani[4]*

[1]A.A. Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, 4 Bogomoletz St., Kiev 01024, Ukraine; [2]National Agrarian University, 13 Gerojiv Oborony St., Bldg. 4, Kiev 03041, Ukraine; [3]Company Weser-Champignon, Neue Heerstr. 35, Hessisch-Oldendorf 31480, Germany; [4]University College London, Gower St., London WC1E 6BT, UK

The neurotropic and trophic effects of extract obtained from the edible and medicinal mushroom *Hericium erinaceus* (Bull.: Fr.) Pers. (Yamabushitake) on nerve cells were studied.

Spike reactions of hippocampal neurons during application of *H. erinaceus* fruiting bodies extract were studied on rat brain slices *in vitro,* using whole-cell patch clamp recording. Spike activity was inhibited in a concentration-dependent reversible manner in 34% up to 90% of studied neurons by extracts obtained with ethanol, ether, or broth. The extract suppressed the excitation of neurons caused by L-glutamic acid application. The assumption is made that the *H. erinaceus* extract contains substances that may activate receptors, causing an inhibition of spike activity. The inhibitory effect of extract was not induced by GABA and serotonin receptor activation or activation of M- and N-cholinoreceptors. Inhibition of spike activity was caused by hyperpolarization of the neuronal membrane during extract application. The hyperpolarization was accompanied by an increase of apamin-sensitive Ca-activated $K^+$ current ($I_{AHP}$) and apamin-insensitive, slow Ca-activated $K^+$ current ($sI_{AHP}$), but was not caused by an increase of inward rectifier $K^+$ current (I(ir)) or by changes of hyperpolarization-activated cationic currents I(h). The effect of the extract was observed in the presence of tetrodotoxin that has proved its effect on the postsynaptic cell membrane. Extract application did not suppress biochemical processes in cell respiratory circuits. The extract did not affect regenerating abilities of neurons and glial cells of the cerebellum and hippocampus.

It was demonstrated that the *H. erinaceus* extract concentrate exerted neurotropic action and improved the myelination process in the mature myelinating fibers, did not affect the nerve cells growth *in vitro,* and did not evoke toxic effect or nerve cell damage.

# Natural Inhibitors of Asparagine Proteases from Mushrooms as Bioactive Metabolites of Potential Medicinal Value

*Krzysztof Grzywnowicz, Krzysztof Sobczyk, Katarzyna Socala, and Magdalena Jaszek*
University of Maria Curie-Sklodowska, Department of Biochemistry, Lublin 20-031, Polska

During the last several years peptidases have been firmly established not only as digestive enzymes, but also as main regulatory components in a number of cellular, tissue, and physiological processes. Activity of proteolytic enzymes is regulated by limited proteolysis of inactive precursors – zymogens, by specific, sometimes multistage, degradation of mature enzymes, pH of the surroundings, and by protease inhibitors. The most important factors influencing the proteolytic enzymes themselves and pathways of proteolysis are natural protease inhibitors which form complexes with target proteases to inactivate and/or to regulate their activity. They are important factors in controlling proteolysis, as indicated by their abundance in many cells and tissues as well as by the variety of their molecular forms characterized from all groups of organisms, especially from animals and plants. These proteins function as regulators of endogenous, and possibly exogenous also, proteolytic enzymes as well as defense molecules against pathogens and pests. They have been extensively investigated from the viewpoint of physiological functions, tools for analyzing protease enzymology, models for protein-protein and protease-protein interactions, and on medical applications, also. They can be grouped as asparagine, glutamine, cysteine, serine, threonine, and metalloprotease inhibitors. There is growing interest in new inhibitors of proteases, not only synthetic but also naturally occurring (mushrooms' ones among them), due to their role in various human diseases including cancer (chemopreventive), neurodegenerative diseases, hemorrhagic disorders and thrombophilia (anti- and pro-coagulant), or infections (antibacterial, antiviral, or antiparasitic).

Searching for new bioactive metabolites of higher Basidiomycetes we isolated and recently characterized some low- and high-molecular, proteinaceous, natural protease inhibitors of asparagine proteases. While the number of these protease inhibitors isolated from bacteria, plant and animals is large, little is known about their fungal equivalents. The detailed study on asparagine protease inhibitors from fungi is still limited, according to our knowledge, only to several proteins. Asparagine proteinase inhibitors are produced by yeast and a wood degrading basidiomycete, *Ganoderma lucidum.* We decided to investigate mushrooms from various life strategy groups. Asparagine protease inhibitors were preliminarily isolated from fruit bodies, coremia and mycelia of edible, cultivatable and wild mushrooms, and non-edible but potentially medicinal mushrooms, also cultivatable and wild ones. Isolation of inhibitors was achieved by typical ion exchange chromatography and size exclusion chromatography. Preliminary characterization of their inhibitory activity (against appropriate enzymes), pH optimum of action and temperature optimum of action, and molecular mass were classically analyzed. It appeared that inhibitors of asparagine proteases were typical for both mycelium and fruit bodies, but they were a little bit different.

Exact physiological function of the described proteins in mushrooms itself is yet unknown. But these inhibitors of asparagine proteases can be used as potential pharmacological substances, considered for treatment of Alzheimer's disease (inhibitors of β-secretase - BACE1), for treatment of pepsin-like digestive enzymes (enzymes of fungal origin are used now), and as regulators of activity of asparagine-type cathepsins. Further investigations on the most promising mushrooms and inhibitors from this work are in progress.

# Contribution of Dectin-1 on Immunomodulating Activity of Soluble Beta-Glucan SCG from *Sparassis crispa* in Mice

*Toshie Harada,[1] Hiromi Kawaminami,[1] Noriko N. Miura,[1] Yoshiyuki Adachi,[1] Mitsuhiro Nakajima,[2] Toshiro Yadomae,[1] and Naohito Ohno[1]*

[1]Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [2]Minahealth Co., Saitama, Japan

SCG is a major 6-branched 1,3-beta-D-glucan in *Sparassis crispa* Wulf:Fr. SCG shows antitumor activity and also enhances the hematopoietic response in cyclophosphamide (CY)-treated mice. Splenocytes from naive DBA/1 and DBA/2 mice are potently induced by SCG to produce IFN-gamma, TNF-alpha, IL-12p70, and GM-CSF, which plays a key biological role among these cytokines. Cell-cell contact involving ICAM-1 and LFA-1 was an essential step for the induction of GM-CSF and IFN-gamma by SCG but not for the induction of TNF-alpha or IL-12p70 by SCG. SCG directly induced adherent splenocytes to produce TNF-alpha and IL-12p70. GM-CSF was required for the induction of TNF-alpha by SCG, and in turn, TNF-alpha enhanced the release of GM-CSF and thereby augmented the induction of IL-12p70 and IFN-gamma by SCG. Neutralization of IL-12 significantly inhibited the induction of IFN-gamma by SCG. We concluded that induction of GM-CSF production by SCG was mediated through ICAM-1 and LFA-1 interaction; GM-CSF subsequently contributed to further cytokine induction by SCG, and reciprocal actions of the cytokines were essential for enhancement of the overall response to SCG in DBA/2 mice. In dectin-1 KO mice, almost all of the cytokine productivity and induction of co-stimulatory molecules were disappeared, strongly indicated contribution of such molecule on major SCG mediated signaling.

In CY-treated mice, the levels of IFN-gamma, TNF-alpha, GM-CSF, IL-6, and IL-12p70 were significantly increased by SCG. GM-CSF production in splenocytes from CY-treated mice was higher than that in normal mice, regardless of SCG stimulation. Neutralizing GM-CSF significantly inhibited the induction of IFN-gamma, TNF-alpha, and IL-12p70 by SCG. The level of cytokine induction by SCG was regulated by the amount of endogenous GM-CSF produced in response to CY treatment in a dose-dependent manner. The expression of beta-glucan receptors, such as CR3 and dectin-1, was up-regulated by CY treatment. Blocking dectin-1 significantly inhibited the induction of TNF-α and IL-12p70 production by SCG. All together, these results suggest that the key factors in the cytokine induction in CY-treated mice were the enhanced levels of both endogenous GM-CSF production and beta-glucan receptor expression.

# Contribution to Understanding the Biodegradation Mechanism of Wood Sterilized by Gamma Radiation

*Marin Hasan,[1] Radovan Despot,[1] Miha Humar,[2] Franc Pohleven,[2] and Gregor Rep[2]*

[1]University of Zagreb, Faculty of Forestry, Svetosimunska 25, P.O. Box 422, HR-10002 Zagreb, Croatia; [2]University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Jamnikarjeva 101, SI–1000 Ljubljana, Slovenia

An easy, fast, and effective sterilization method – gamma radiation – changes molecular structure not only in living cells of pests, but also in wooden cell walls. Radiation-induced depolymerization causes significant changes in wood properties crucial for laboratory testing of natural wood durability and wood-preservative effectiveness. Natural durability of wood against rot fungi under lab conditions depend on different sterilization methods was researched and the data presented. In the research, white rot fungus *Trametes versicolor* (L.: Fr.) Pilat and brown rot fungus *Gloeophyllum trabeum* Pers.: Fr., were tested on the Scots pine. Cobalt $^{60}$Co was

used as a gamma radiation source at the dosage of 30 and 150 kGy. Control non-irradiated specimens were sterilized using an autoclave.

Statistically significant differences in mass loss between gamma irradiated and autoclaved specimens were established only after 4 and 8 weeks of exposure to *G. trabeum*. Irradiated specimens had greater mass loss. During further exposure, the differences decreased and became insignificant. On the other hand, during the first 12 weeks of exposure to *Trametes versicolor*, no significant differences in mass losses between irradiated and autoclaved specimens were determined. After 16 weeks of exposure with 30 kGy, irradiated specimens lost significantly more mass, while the specimens irradiated with 150 kGy lost significantly less mass than the control ones.

---

# A PCR-Based Test for the Rapid Detection of *Trichoderma pleurotophilum* and *T. fulvidum*, the Causative Agents of the Newly Emerged Worldwide Green Mold Disease of *Pleurotus ostreatus*

*Lóránt Hatvani,[1] Sándor Kocsubé,[1] László Manczinger,[1] Irina S. Druzhinina,[2] Christian P. Kubicek,[2] Csaba Vágvölgyi,[1] and László Kredics[1]*

[1]Department of Microbiology, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary; [2]Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166.5, A-1060 Vienna, Austria

*Pleurotus ostreatus* is the third, most important commercially grown, edible mushroom worldwide. Since the early 1980s severe green mold infections affecting *Agaricus bisporus* have been reported in Europe and North America. The causative agents were described as *Trichoderma aggressivum* f. sp. *europaeum* and f. sp. *aggressivum*, respectively. In recent years, case reports have come up about serious green mold epidemics causing significant losses in the cultivation of *P. ostreatus* in South Korea, Italy, Hungary, and Romania. The organism responsible for the disease has proven to be different from *T. aggressivum* and was identified by *TrichO*KEY as the phylogenetic, but formally yet undescribed species, *Trichoderma* sp. DAOM 175924.

Following the detection of the oyster mushroom green mould disease, we isolated several *Trichoderma* strains from *Pleurotus* substrate samples derived from Hungary and Romania (Transylvania). Internal transcribed spacer (ITS) 1 and 2 sequences of the isolates proved to be highly similar to or identical with those for *Trichoderma* pathogens of *P. ostreatus* in South Korea, indicating that the green mold disease of *P. ostreatus* in Hungary and Romania is due to the same *Trichoderma* species found in South Korea. The group of isolates co-specific to *T.* sp. DAOM 175924 can be divided into two alleles of ITS2 sequences based on a single A/C transversion. Interestingly, all the representatives of this species that were isolated from the winter wheat rhizosphere of South Hungarian agricultural fields contain "A" at this position, while the isolates deriving from Hungarian and Transylvanian *Pleurotus* substrate samples belong, almost exclusively, to the other, "C" type. The subsequent sequence analysis of translation elongation factor 1-alpha (*tef1*) and endochitinase *chi18-5* genes showed that these two types of isolates belong to two clearly diverged phylogenetic species. They also differ from each other based on morphological and physiological features and therefore have recently been described as the new species *T. fulvidum* and *T. pleurotophilum*.

Since the green mold disease of *P. ostreatus* is spreading fast worldwide, there is an emerging need of a rapid method for detection of pathogens in order to be able to control the disease properly. Therefore, the aim of this work was to develop a PCR-based technique for the rapid detection of *T. pleurotophilum* and *T. fulvidum*.

PCR primers were designed based on the 4th big intron, the 5th exon, and the 5th small intron of

*tef1*, which are specific for both *Pleurotus* pathogenic *Trichoderma* species as well as only for *T. pleurotophilum*. Besides *T. pleurotophilum* and *T. fulvidum,* we have tested the primers in a multiple PCR with the DNA samples of several *Trichoderma* species (*T. aggressivum* f. sp. *europaeum, T. harzianum, T. atroviride, T. longibrachiatum,* and *T. ghanense*). The results show that *T. pleurotophilum* and *T. fulvidum* can be distinguished unequivocally from each other as well as from other fungal species by the application of our three-primer set.

Based on our results, the two recently emerged *Pleurotus* pathogenic *Trichoderma* species can be detected rapidly without the need of ITS sequence analysis. This finding may help to recognize and control green mold disease of *P. ostreatus* in its early phase.

## ACKNOWLEDGMENTS

This work was supported by the Hungarian Government with the Research Grant NKFP OM-00083/2004, by the Austrian Science Foundation grants FWF P-16601 and P-17895-B06, as well as by NKTH (National Office for Research and Technology, Hungary) and ÖAD (Österreichischer Austauschdienst, Austria) under the bilateral project TéT A02/2006 - WTZ HU 03/2007. László Kredics is the grantee of the János Bolyai Research Scholarship (Hungarian Academy of Sciences).

# Mushroom-Derived Supplements for the Control of HIV and Type 2 Diabetes

*John C. Holliday*

Aloha Medicinals Inc. P.O. Box 686 Haiku, Hawaii 96784, USA

Aloha Medicinals has developed dietary supplements for humans and pets, based upon traditional medical knowledge. The present work focuses on the potential use of *Cordyceps* derived supplements in the control of HIV and type-2 diabetes. Also, an economic aspect of *Cordyceps* is given as an example for developing nations.

**Mushroom-Derived Supplements for the Control of HIV.** As a global epidemic, the world has never before known anything as devastating as HIV and AIDS. It is estimated that there are currently more than 40 million people infected with HIV in sub-Saharan Africa. The current anti-retroviral (ARV) treatment of choice used in developed nations do not work very well in the rural African setting, due to the lack of an infrastructure of specialty trained staff and facilities to handle the severe side effects associated with standard ARV therapy. This has prompted science to look into other ways of treating these and related viral infections. In a comprehensive search of the world's medicinal practices, it was noted that effective antiviral therapy has long been practiced in Chinese and Tibetan medicines. These treatments revolve around the use of *Cordyceps sinensis* and other medicinal mushrooms. Armed with this knowledge, we looked into the mechanisms of anti-viral action involved, and found that *C. sinensis* contains naturally occurring ARV compounds of the altered nucleoside class. These naturally occurring compounds have an identical mechanism as some of the most well known of the synthetic ARV drugs. They work by a process known as reverse transcriptase inhibition, but with virtually none of the toxicity associated with pharmaceutical ARV drugs. Compared to the synthetic drugs, this is indeed good news, since the limiting factor in the application of ARV drugs in Africa is their extreme toxicity. One commercial product developed from this research gained approval for use in South Africa in early 2006, where it is showing very good results at a cost and safety level heretofore unknown. This represents an entirely new generation of anti-HIV treatment potential. We look at those mechanisms of action and the potential for domestic production of these natural, non-toxic ARV drugs in African nations and elsewhere.

**Mushroom-Derived Supplements for the Control of Type 2 Diabetes.** Diabetes is a worldwide problem growing by epidemic proportions. In 1999, when 135 million people had this disease, it was estimated that the number of people affected would double by 2025. However, science now recognizes

that the rate of increase for diabetes is accelerating, rather than remaining steady, and the growth of new cases is exponential. Compounding this problem is a newly recognized disease called Syndrome-X. This is a cluster of diabetes-like symptoms that is effecting more than just the genetically predisposed. It affects the majority of obese people living sedentary lifestyles. In America the current statistics are more than 25 million people (1 in 12) have diabetes. This is a disease that frequently causes extreme suffering, disability, and death. It is responsible for the majority of cases of blindness, end stage kidney disease, and lower limb amputations, and it increases the risk of stroke, high-blood pressure, high-blood cholesterol levels, and heart disease. Aloha Medicinals has developed a dietary supplement based upon traditional medical knowledge, which combines several medicinal mushrooms, along with an extract of the herb *Salacia oblonga,* the B vitamin biotin, and the mineral Chromium for use in this condition. Taken as part of a daily regimen, this is showing effectiveness in controlling high-blood glucose in greater than 80% of type 2 diabetics. We look at the various mechanisms of action involved in the new therapeutic approach.

**Economic Aspect of Wild *Cordyceps* Collection in Tibet.** An Example for Developing Nations. In China, one of the most common fungi used in medicine is known as *Cordyceps sinensis*. This important herbal medicine has been well known and used by traditional healers for more than 1,500 years. A fascinating creature, this mushroom-like fungus inhabits the larvae of the Himalayan ghost moths of the genus *Thitarodes* (formerly *Hepialus*). *Cordyceps sinensis* is found only in the high-altitude grasslands of the Qinghai-Tibetan plateau, where this fungus has become a major component of the economy of the region. Collection and trade of this "Caterpillar Fungus" has become one of the most important sources of income for most farmers in the pastoral Tibetan communities. Known as "Yartsa Gunbu" to the Tibetans, literally translated as summer grass winter worm, the trade in this important medicinal typically accounts for at least 50% of the annual income of this region. Most of the *Cordyceps* harvested in Tibet goes to China, Hong Kong, and the United States, where demand is increasing exponentially year by year, and where prices have increased by over 800% in the last 5 years. The collection and distribution of this mushroom form a classic example of the potential for wild mushroom collection and marketing by developing nations. Looking closely at this industry could yield an important model to follow for other nations, and with other species, in the development of sustainable forest income.

---

# PCR-RFLP Database: Its Application in Ectomycorrhizal Identification and Assessment of Fungal Molecular Diversity

*Melita Hrenko, Barbara Štupar, Tine Grebenc, and Hojka Kraigher*

Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia

The traditional method for identification of fungi in ectomycorrhiza involves analysis of macro- and microscopic characteristics of the type of ectomycorrhiza. Incorrect identification of ectomycorrhiza can be avoided through the use of molecular markers. PCR-RFLP (Polymerase Chain Reaction in combination with Restriction Fragment Length Polymorphism) approach proved to be a successful method for the identification of ectomycorrhizae, allowing fast and large-scale identification projects possible. Fungi in ectomycorrhiza can be determined by comparing the digestion patterns of the ectomycorrhizae with the PCR-RFLP pattern of previously identified sporocarps. Frequently, rDNA ITS regions are used due to their tandem repeats and easy amplification. The use of specific primers enables us to selectively amplify a particular group of fungi: Ascomycetes (A) and Basidiomycetes (B) A+B, or Basidiomycetes (B) only. Here we present both, the Ascomycetes and Basidiomycetes (A+B, and B only) PCR-RFLP database built at the Slovenian Forestry Institute in the last decade and its application in the identification of ectomycorrhiza from various symbiotic tree partners and research plots, including an example of intraspecific variation of the analyzed rDNA region.

Types of ectomycorrhizae were separated based on morphological and anatomical characteristics and were briefly described and identified following the published procedure and identification keys. Reference sporocarps of mycorrhizal species were collected and identified, including sporocarps from excursions, exhibitions, and target site mappings.

Total DNA was extracted from a single fresh and vital ectomycorrhizal root tip or part of the sporocarp (hymenium) homogenized with a micropestle in 2% CTAB (Cetyl Trimethyl Ammonium Bromide) buffer. DNA was amplified with 1 µl of template DNA in a total volume of 40 µl. PCR was performed with an annealing temperature of 53°C using direct primer ITS1F and reverse primers ITS4 or ITS4B, to amplify both ITS regions including the 5.8S of the ribosomal RNA gene cluster and small flanking parts of the SSU and LSU genes of Ascomycotina (ITSF/ITS4) or Basidiomycotina (ITS1F/ITS4B). PCR products for RFLP analysis were cleaved in a single enzyme digest, with *Hinf I*, *Mbo I* and *Taq I* endonuclease following instructions of the manufacturer. Gels and fragment size data were read from Polaroid® or Gel-Doc (BioRad) digital photos, processed using Adobe Photoshop® software, and analyzed in the Taxotron® software system, especially designed for RFLP data processing.

Two separate databases were established using two sets of primers, a database for Basidiomycetes, and Ascomycetes + Basidiomycetes. Currently, there is, 1,046 records in the Basidiomycetes database; 547 derive from the reference sporocarp RFLP patterns. The success of the Basidiomycetes ectomycorrrhizal identification was about 50%. The A+B database comprises 256 records, mainly from ectomycorrhiza. The success of identification with this database is lower, about 25%. Both databases were successfully used for identification of ectomycorrhizae on spruce, beech from a provenance test trial, mixed beech-silver fir forest from different management regimes, and from an ozone fumigation experiment. The approach was successfully used for confirmation of the identification or for identification of as yet undescribed types of ectomycorrhiza. The remaining unidentified ectomycorrhiza belonged mainly to corticiaceous fungi and some under-sampled groups of Ascomycetes (e.g., Pezizales).

The method is fast and reliable although more than just one sample of reference of sporocarps should be included into the database to test for possible intraspecific variation, such as was observed in some *Russula* spp. and *Hydnum rufescens*. Both databases are useful in the identification of ectomycorrhiza, unknown fungi from ectomycorrhiza, or from any other sources, especially when compared to appropriate reference material; this can all be done at a relatively low price and on a large scale.

## ACKNOWLEDGMENTS

The study was co-financed by 5th FP EU project NatMan (QLRT1-CT99-1349, CASIROZ (EVK2-2002-00165, Ecosystem Vulnerability), and the Ministry of Higher Education, Science and Technology (MEST) through the Young Researchers Scheme (for TG), the Research Programme Forest Biology, Ecology and Technology (P4-0107), and several research projects co-financed by MEST and the Ministry for Agriculture, Forestry, and Food.

# Reactivity of Anti-Beta-Glucan Antibody in Sera to Edible Mushrooms

*Ken-Ichi Ishibashi,[1] Mayu Morita,[1] Masuro Motoi,[2] Noriko N. Miura,[1] Yoshiyuki Adachi,[1] and Naohito Ohno[1]*

[1]Tokyo University of Pharmacy and Life Science, Tokyo, Japan; [2]Toei Pharmaceutical Co., Tokyo, Japan

The systems recognizing immunomodulating beta-glucan have been examined extensively. Some of the cell surface molecules such as Dectin-1, complement receptor 3, and lactosylceramide were cited as candidates for beta-glucan receptors. Specific antibodies are key molecules in the acquired immunity and have been shown to promote phagocytosis, enhancing the presentation of antigens and co-stimulatory molecules

and modifying the production of cytokines, and so on. However, there are few reports on the specific antibody to beta-glucan. Recently, anti-beta-glucan (BG) antibody was detected in sera from human volunteers. Also, it was suggested that anti-BG antibody formed an antigen-antibody complex and participated in the immunopharmacological activity of beta-glucan. An anti-BG antibody could play a role in the recognition of beta-glucan, and induce biological activity in collaboration with other molecules, such as the beta-glucan receptor, and complement human health. In the present study, we have examined the acquired immune response to the fungal cell wall beta-1,3-/1,6-glucan (BG) by measuring specific antibodies in sera.

Anti-BG antibodies in sera were measured by ELISA-plate coated with *Candida* solubilized BG (CSBG). A class of antibodies was detected by anti-human IgG+M+A, IgG, IgM, and IgA. Sera from 77 human volunteers were tested for anti-BG antibody. Polyclonal human immune globulin preparation was also tested. All of the sera and the globulin contained anti-BG antibody. Titer of antibody significantly varied (highest: 14,000 unit; lowest: 240 unit). To test the specificity of anti-BG antibody, the sera of healthy volunteers were tested with hot water extract of the naturally outdoor-cultivated *Agaricus brasiliensis* (KA21) fruit bodies, in which AgHWE was mainly composed of beta-1,6-glucan. The sera were also tested with ASBG, mainly composed of β-1,3-glucan from *Aspergillus niger*. On the other hand, it showed only a weak response to GRN, a 6-branched β-1,3-glucan prepared by fermenting mycelium of *Grifola frondosa*. The highest reactivity was shown to AgHWE in IgG and ASBG in IgM. Of interest, a part of the volunteers showed high titer of anti-BG IgM or IgA isotypes.

Considering the data shown in the present study, the host established acquired immune response to fungi and kept it for a lifetime. Various fungi are present in the environment and some colonized in mucosa. Continued stimulation by such fungi would induce specific immunity to fungi.

---

# Preliminary Studies on Fruit Body and Lignocellulolytic Enzyme Production by *Pleurotus ostreatus* on Solid Waste from Anaerobic Digestion of Poultry Litter

*Omoanghe S. Isikhuemhen,[1] Nona A. Mikiashvili,[1,2] and Jonathon M. Chatfield[3]*

[1]Mushroom Biology & Fungal Biotechnology Laboratory, North Carolina A&T State University, Greensboro, NC 27411, USA; [2]Faculty of Exact and Natural Science, Iv. Javakhishvili Tbilisi State University, 1 Chavchavadze Avenue, Tbilisi 0128, Republic of Georgia; [3]Gus R. Douglass Land Grant Institute, Agricultural and Environmental Research Station, West Virginia State University, 129 Hamblin Hall, Institute, WV 25112-1000, USA

Broiler production waste comprised of wood chips and chicken droppings poses a major disposal problem for the poultry industry. This waste became the subject of research on how to utilize and recycle it; leading to its use as a feed stock in anaerobic digestion to produce biogas and fertilizer.

White-rot fungi belonging to the Basidiomycetes are the most efficient and extensive lignin degraders. White rot fungi have evolved to effectively use lignocellulose that is both relatively poor in nitrogen and other macro-elements content as substrate. In the case of solid waste (SW), we have lignocellulose material that has abnormally high levels of calcium, nitrogen, phosphorus, and potassium. We tested the ability of an edible, white rot mushroom (*Pleurotus ostreatus* 400) to grow and degrade SW as a sole substrate and in other combinations with wheat straw and millet (Table 1).

Results showed that substrate combinations consisting of up to 20% SW had higher fruit body yield than the wheat straw (80%) and millet (20%) control. Substrate combination consisting of 75% solid waste did not support the mushroom yield.

Carboxyl methyl cellulase (CM-cellulase) was the most active in the substrate combination A, which was 100% wheat straw (42 U/l) followed by combination I (25 U/l), which happened to give the highest fruit body yield. Substrate combination F (100% SW) had the least CM-cellulase activity (10 U/l). CM-cellulase

**Table 1. Substrate Combinations with Solid Waste Material**

| Substrates | Combinations |
|---|---|
| **A** | Wheat straw 100 % |
| **B** | Wheat straw 90% and SW 10% |
| **C** | Wheat straw 75% and SW 25% |
| **D** | Wheat straw 50% and SW 50% |
| **E** | Wheat straw 25% and SW 75% |
| **F** | SW 100% |
| **G** | Wheat straw 80% and millet 20% |
| **H** | Wheat straw 70%, SW 10% and millet 20% |
| **I** | Wheat straw 70%, SW 20% and millet 10% |
| **J** | Wheat straw 80%, SW 10% and millet 10% |

activity was not detected after fruiting. 1,4-β-glucosidase activities, increased significantly during fruiting, and in the case of substrate combination E (83 U/l) reached a 3.3-fold increased value.

Xylanase activity was the highest after fruiting, except for substrate combination A. Production of xylanase in substrate combination A showed maximum activity (73 U/l), which decreased to 35 U/l after fruiting. In contrast, in all other substrate combinations, the xylanase activities were significantly increased. For example, in substrate combinations B, C, and D enzyme activities increased for 4-fold, 16-fold, and 7-fold, respectively. Another hemicellulose enzyme detected was xylosidase, which showed higher enzyme activities before fruiting, except in substrate combinations B and C.

Laccase, manganese peroxidase (MnP), and manganese independed peroxidase (MnIP) were the lignin degrading enzymes detected. Generally, laccase activities in all substrates decreased significantly after fruit bodies were formed and harvested. The highest performing substrate combination (E) had 165 and 24 U/l laccase activities before and after fruiting, respectively. The substrate combination H showed the least laccase activities among all substrate combinations tested.

MnP and MnIP activities peaked substrate combination F, 29 U/l and 41 U/l, respectively before fruiting. This was followed by the substrate combination E, 22 U/l and 35 U/l, respectively. The control, substrate combination G showed the least MnP and MnIP activities. It is interesting that the substrate combination, which have no solid waste as component showed relatively low MnP and MnIP activities.

The data obtained in this work showed that fungal substrates with up to 20% SW supported higher fruit body yield than the control. Enzyme activities were affected differently by the amount of SW in the substrate. In the case of lignin degrading enzymes, the higher the SW content was, the higher enzyme activity was exerted. However, this trend does not correlate with fruit body production.

The dynamics of production of several cellulose and hemicellulose degrading enzymes was also detected. The pattern in which these enzymes appear during the process of cultivation is a reflection of the effect of SW component compared to the control. However, fruit body yield did not always correlate with ligninolytic enzyme activities. Experiments including post-cultivation, substrate analysis to determine the relationship between enzyme production, substrate degradation and utilization as well as fruit body production are ongoing.

## ACKNOWLEDGMENT

This work was supported by a USDA CSREES grant entitled "Agricultural Waste Treatment Utilizing Thermophilic Anaerobic Digestion (Bioplex Phase 6)" CSREES Award # 2005-38850-02292.

# Pelletized Plant Waste—A Perspective Source of Substrate for Growing Wood-Inhabiting Cultivated and Medicinal Mushrooms

*Ivan Jablonský,[1] Zdeněk Blažek,[1] and Václav Šašek[2]*

[1]VALMYC, Ltd., Dolecká 160, CZ-55101 Jaroměř, Czech Republic; [2]Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic, Vídenska 1083, CZ-14220 Prague 4, Czech Republic

A great number of various agricultural and forestry waste products/secondary raw materials can be used for cultivation of wood-inhabiting mushrooms. The type of the respective base material depends on the situation in individual countries. In the Czech Republic, with the aim of using renewable resources

for energy production, different agriculture and forestry waste materials are modified to improve their properties for usage in incineration houses. The basis of this treatment is to finely crush the plant waste products and then to strongly press them into pellets. This method was used to determine, if such modified plant residues could serve as input raw material for mushroom substrate preparation.

Modified waste of different plants (wheat, rape, soya-bean, corn, sweet sorghum, and sorrel "Uteusa" – *Rumex* sp. hybrid) in the form of small (0.6–0.8 cm in diameter), dry pellets was used to prepare substrates for mushroom growing. The oyster mushroom *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm., king oyster mushroom *Pleurotus eryngii* (DC.: Fr.) Quél., shiitake *Lentinus edodes* (Berk.) Singer, and viscid mushroom *Pholiota nameko* (T. Ito) S. Ito and S. Imai were cultivated in plastic bags, where the pellets, originally prepared for combustion purposes, were placed deep into hot water (60–90°C); the optimum ratio was 1 part pellets in 2 parts water (w/w). The material in the closed bags was jumbled and the next day the cooled substrate was inoculated *in situ* through the sheet of the plastic bag by mycelium grown on wooden plugs. Different sizes of bags, water temperature, and humidity of the substrate was tested. Mycelium growth rate and yield of mushroom fruit bodies on different substrates were compared. The first mushroom fruit bodies appeared 5–6 weeks after inoculation depending on the mushroom species and blend of the substrate mixture.

Our preliminary results indicate that pre-treated wheat straw, rape straw, and sorrel waste serve as good raw materials for the most growth in the fungal species tested. On the contrary, substrates based on pretreated soya-bean, corn, or sweet sorghum waste were often spoiled by molds. In the case of wheat straw, the quality of harvested straw after storage played an influential role; the pellets made of unclean straw gave unstable results. Cost-wise, even though the pre-treatment of raw materials increases the substrate price somewhat (the final cost of 1 ton is around EUR 100), the pre-treated substrate is still one third cheaper than the cost of substrate made by conventional procedures.

## ACKNOWLEDGMENT

The work was supported by Grant Agency of the Czech Republic grant no 525/05/0273 and grant no 203/06/0528.

# Treating Human Cancers with Medicinal Mushroom Preparations (Croatian Experience)

*Ivan Jakopovich*

Dr Myko San – Health from Mushrooms, Ltd., Zagreb, Croatia

Despite all advances in modern medical science and practice, the crucial problem of malignant tumors in humans is still far from being definitively solved. Although medicinal mushrooms – whose powerful antitumor effects are known and used in traditional (predominantly Far Eastern) medicine(s) from ancient times, have been scientifically verified in research during the last half a century as well – are certainly not "magical bullets"; mycotherapy, with scientifically-based extracts and compounds from some medicinal mushrooms, could be a very significant additional weapon in fighting human cancers.

An important part of the quest for the proper mycotherapy of human cancers could be the experience from practice of DR MYKO SAN – HEALTH FROM MUSHROOMS company (Zagreb, Croatia). During the 15 years of operation, the company developed 5 antitumor mushroom preparations, all of them proprietory blends of various mushroom extracts. These preparations are sold as "over-the-counter" dietary supplements for additional treatment (and sometimes as the only treatment) of (most often) advanced, recurrent, and/or metastatic cancers. Another inovation in DR MYKO SAN's approach to mycotherapy of human cancers is the application of massive doses of medicinal mushroom extracts in almost every case, except the early stage of cancers. Until now, these antitumor mushroom preparations have been used by several thousand people, mainly in Croatia, but also in other countries of the former Yugoslavia, other

European countries (Germany, Switzerland, Italy), as well as the USA and Canada.

Due to predominantly immunostimulating and immunomodulating action of antitumor mushroom compounds, these preparations are applicable for virtually all types of cancers. In our practice they have been used in fighting a wide range of cancers, particularly colorectal, breast, lung, ovary, hepatic, pancreatic, prostate, testicular, bone and soft tissues, melanomas, malignant brain tumors, lymphomas, leukemias, etc., mainly in adults, but in children, too.

The proposed presentation, based on analysis of the official medical records, provides the exact data/conclusions that certain medicinal mushroom extracts

- inhibit the tumor growth and can cause the tumor regression in human malignancies;
- improve the effectivness of standard oncological therapies (surgery, chemotherapy, radiotherapy);
- facilitate the bearing of aggressive standard cancer therapies and alleviate their side-effects;
- boost the immunological and performance status of cancer patients, improve as well as prolong their quality of life.

## Major Diseases and Pests on Mushroom Beds in Korea

*Chang Sung Jhune,[1] Won Sik Kong,[1] Young Bok Yoo,[1] Jong Cheon Jung,[1] and Se Chul Chun[2]*

[1]Applied Microbiology Division, Agricultural Sciences and Technology Institute, R.D.A. Suweon 441-707, Korea; [2]KonKuk Univ. Life Environment Science, Crop Science, Seoul 143-701, South Korea

The major diseases of oyster mushrooms in Korea are green mold, hypocrea disease, bacterial brown blotch, viruses, and malformed mushrooms caused by abiotic factors. The incidence and severity of diseases varies from time to time. In Korea, *Trichoderma* disease and pink mold were most commonly found from 1970 to 1980, but from the late 1980s to 2000 bacterial brown blotch predominated over the oyster mushrooms. At present, bacterial brown blotch, malformed fruiting bodies and viruses have become more common and caused severe damages. Morphological characteristics of malformed fruiting bodies are sclerodermoid masses, very thick stipes, long and small caps, lighter or dark cap color, etc. The major causes of abnormality in fruiting bodies are stress of carbon dioxide, low/high temperature, low/high humidity, low/high air velocity, and low water content of substrates. The major pests of oyster mushrooms reported in Korea were five kinds of mushroom flies, two types of mites, and nematodes.

The major diseases of the common mushroom in Korea are wet bubble, dry bubble *Trichoderma* disease, false truffle, mummy disease, bacterial brown blotch, viruses, and malformed mushrooms caused by abiotic factors. Abiotic diseases are sectoring, crack pileus, mass pinning and swollen stems, etc.

The major diseases of the *Agaricus brasiliensis,* which needs high temperature for cultivation, are false truffle and *Trichoderma* disease. At present, new, unknown diseases predominated over the mushroom farms since 2002. Symptoms of new, unknown diseases were crowded mycelial growth, lysis or necrosis of pileus surface, color change of inner tissue of fruiting bodies, and depression of one division of pileus. The damage by new diseases has become very severe affecting yield and quality of this mushroom.

# *In Vitro* Study of Neuroprotective Activity of Extracts Isolated from *Piptoporus betulinus*

*Józef Kaczor,[1] Agata Mitura,[1] Wojciech Brzana,[2] Małgorzata Juszczak,[2] Martyna Kandefer-Szerszeń,[1] and Wojciech Rzeski[1,2]*

[1]Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland; [2]Department of Toxicology, Institute of Agricultural Medicine, Lublin, Poland

*Piptoporus betulinus* is one of the commonest polyporous bracket fungi growing on the wood of dead birches. It has a long tradition in folk medicine for the treatment of cancer and stomach diseases as well as an anti-inflammatory agent. It was found experimentally that extracts isolated from fruiting bodies of *P. betulinus* exert miscellaneous effects including antibacterial, antiviral, antiparasite, antiproliferative, and immunomodulatory activity.

The aim of the study was *in vitro* characterization of extracts from *P. betulinus* as neuroprotective agents.

Extracts were prepared from fruiting bodies in a Soxhlet apparatus after ether and ethanol extraction. After evaporation of diluents, the dry residual was dissolved in DMSO (stack solution 100 mg/ml). Neuronal cell cultures were prepared from retinoic acid-induced neural differentiation of P19 mouse embryonal carcinoma cell line. Neuronal differentiation was induced in α-MEM culture medium supplemented with 5% FBS and 0.5 μM of retinoic acid. Neurons were plated on poly-l-lysine coated 96 multiwell plates in culture medium consisting of neurobasal medium and 2% B-27 supplement. Neuronal cultures were incubated at 37°C in a humidified 95% air and 5% $CO_2$ atmosphere. Cell viability was determined by means of MTT and LDH assay.

In order to characterize the neuroprotective effects, neuronal cultures were exposed to neurodegenerative agents like serum deprivation, glutamate, and cisplatin alone and combined with tested extracts (5–100 μg/ml). Applied agents induced a prominent neurotoxicity, which was further significantly ameliorated by co-exposure with extracts from *P. betulinus*. It should be stressed that at the same concentrations these extracts produced strong antiproliferative effect in lung carcinoma (A549) and glioma (C6) cells.

Here, we report for the first time a new aspect of *P. betulinus* biological activity. The ability to protect nervous cells against agents causing neurodegeneration can suggest a potential application of active compounds isolated from *P. betulinus* in treatment of neurological and neurodegenerative disorders.

---

# Genetic Variations in *Pleurotus* Species Collected from Central India

*Rupali Katekar, Alka Karwa, and Mahendra K. Rai*

Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati 444 602, India

*Pleurotus* has medicinal and nutritional importance and is a highly regarded edible mushroom throughout the world, considered second after *Agaricus*. It is commercially cultivated for its culinary delicacy and easy cultivation. The most desired among the genus, is the edible oyster mushroom species, *Pleurotus sajor-caju*. This mushroom was originally found in India, growing naturally on a succulent plant (*Euphorbia royleans*) in the foothills of the Himalayas. Different species of the oyster mushroom can be grown at moderate temperatures ranging from 22° to 28°C. Therefore, the climate in India is suitable to grow different species of *Pleurotus*. In central India, *P. florida* and *P. sajor-caju* are commercially cultivated and are also exported to other metro cities throughout India. Various agricultural residues are being utilized to

produce oyster mushrooms. A significant problem in oyster mushroom production using agricultural residues is the concern over sensory characteristics, nutrient content and medicinal-chemical composition of the mushroom when grown on different substrates. Though these variations are attributed to the type of substrate used or the varying cultivation conditions, other differences are also noted within the same species that are cultivated in different places. These variations include: change in color, size, biological efficiency, flavor, and sometimes texture. This may be due to the genetic variations among the species and within the species of *Pleurotus*. Research into these areas has been limited. The main aim of our study is to check the genetic variations among commercially cultivated species of *Pleurotus*. One of the tools for studying the genetic variations between isolates are the Rapid Amplification Polymorphic DNA analysis (RAPD) technique, which allows detection of polymorphisms in a rapid, direct, consistent, and low-cost manner. We have obtained pure cultures for *P. florida,* *P. sajor-caju,* and *P. djamor* (pink) from commercial mushroom cultivators within Maharashtra State and one wild member of *Pleurotus* was obtained from Satpuda mountain ranges during the rainy season in 2006. All four species were maintained in Potato Dextrose Agar (PDA). For mycelial culture these species were grown in Potato Dextrose Broth (PDB). A 10-day old mycelial mat was used for extraction of DNA. The DNA thus isolated was subjected to Rapid Amplification Polymorphic DNA analysis (RAPD) PCR. Primers used for amplification in this study were obtained from Operon Technologies. The observations and results obtained by selected primers show a variation among species, whereas different isolates of the same species showed phylogenetic relationships to some extent. The results will be discussed in detail during the presentation. The RAPD PCR technique is a good tool for the characterization of species variants. The study may lead to some new findings that can be helpful in improving the commercial strains being currently cultivated.

## Osteoclast-Forming Suppressing Substances from the Mushroom *Agrocybe chaxingu*

*Hirokazu Kawagishi,[1,2] Takuto Akachi,[2] Akihiro Ogawa,[2] Kikuko Masuda,[3] Kohji Yamaguchi,[3] and Kazunaga Yazawa[3]*

[1]Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan; [2]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan; [3]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo 108-8477, Japan

Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux. The hip fracture in senile patients is a very serious problem because it often limits the patient's quality of life. Therefore, the prevention of bone mass loss by food is very important. Osteoclasts are multinucleated, giant cells that are primarily responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and a clear zone. Osteoclast-like multinucleated cells can be differentiated *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factor 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) and prostaglandin E2 (PGE2). During the screening for osteoclast- formation suppressing effects of extracts of various mushrooms, we found very strong activity of the extract of *Agrocybe chaxingu.* Therefore, an attempt was made to isolate the active components from the mushroom and to determine their structures. This mushroom grows in dry and dead boles of broadleaf, such as grease tea plant and poplar, and exists only in mountainous areas in south China.

Powder of the dried fruiting bodies of *A. chaxingu* was extracted with $CHCl_3$, ethyl acetate, and then ethanol. The $CHCl_3$-soluble fraction only showed the

suppressing activity. After repeated chromatography of the fraction guided by the result of the assay, several compounds including a novel compound 1 and a known compound 2 were purified as active components (Fig. 1). The structure determination of the other compounds is now in progress.

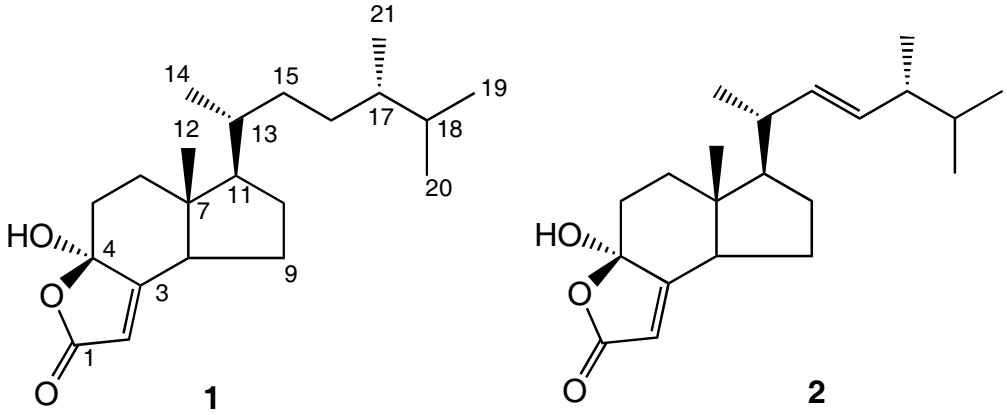



**FIGURE 1.** Active components isolated from *Agrocybe chaxingu* fruiting bodies.

## The Current Status of the "Culture Collection of Wild Mushroom Species" in Korea

*Sang Beom Kim,[1] Gun Woo Lee,[1] Hyun Su Rho,[2] Hyun Sook Lee,[2] Shim Mi Ja,[3] Hye Young Kim,[1] Seong Hwan Kim,[1] Kyung Rim Lee,[1] U Youn Lee,[1] Min Woong Lee,[4] and Tae Soo Lee[1]*

[1]Department of Biology, University of Incheon, Incheon, 402-749, Korea; [2]Department of Microbiology, Gyeongsang National University, Jinju 660-701, Korea; [3]Department of Life Science, University of Seoul, Seoul 130-743, Korea; [4]Department of Biology, Dongguk University, Seoul, 100-715, Korea

The "Culture Collection of Wild Mushroom Species (CCWM)" was founded in 2002 at the Department of Biology, University of Incheon, Korea. The Korean Ministry of Science and Technology (MOST) and The Korea Science and Engineering Foundation (KOSEF) provide all of the financial support for the operation of the culture collection. At present, most of the mushroom cultures preserved in CCWM originated from Korea. All of the mushroom cultures in CCWM were preserved in agar slant under 4°C and –80°C in a deep freezer. The mushroom cultures in agar slant were transferred at intervals of 6 months of storage. The white and brown rot fungi were stored for one and half years in sawdust media under room temperature. So far, the CCWM holds 3,100 cultures of 430 mushroom species, of which 70 and 20 species can be used for edible and medicinal purpose, respectively. If anyone is interested in mushroom cultures at CCWM, contact "http//www.wildmush.or.kr" or email "tslee@incheon.ac.kr".

# Inhibitory Effect of Mouse Sarcoma 180 by Crude β–D-Glucan Extracted from the Fruiting Body of *Lentinus giganteus*

*Sang Beom Kim,[1] Gun Woo Lee,[1] Mi Ja Shim,[2] Hyun-Su Rho,[3] Hyun Sook Lee,[3] Min Woong Lee,[4] U-Youn Lee,[1] and Tae Soo Lee[1]*

[1]Department of Biology, University of Incheon, Incheon 402-749, South Korea; [2]Department of Life Science, University of Seoul, Seoul 130-743, South Korea; [3]Department of Microbiology, Gyeongsang National University, Jinju 660-701, South Korea; [4]Department of Biology, Dongguk University, Seoul, 100-715, South Korea

*Lentinus giganteus*, one of the edible and medicinal mushrooms belonging to Tricholomataceae, has been known for outstanding curative effects on animal cancers.

This study was initiated to assess the antitumor effects of crude β–D-glucan extracted from the fruiting body of *L. giganteus*. Neutral salt-soluble (0.9% NaCl), hot-water soluble, and methanol soluble substances (hereinafter referred to as Fr. NaCl, Fr. HW, and Fr. MeOH, respectively) were isolated from the mushroom. *In vitro* cytotoxicity tests showed that Fr. HW and Fr. NaCl do not have cytotoxic effects against cancer cell lines such as Sarcoma 180, HepG2, and HT-29. Intraperitoneal injection with Fr. HW showed antitumor activity with life prolongation effect of 67.5% in ICR mice previously inoculated with Sarcoma 180. Fr. NaCl and Fr. MeOH also improved proliferation of spleen cells and the immunopotentiating activity of B lymphocyte by increasing the number of spleen cells and alkaline phosphatase activity, respectively. The weight of the spleen was increased slightly in the test group of ICR mice compared to the control group.

These experimental results suggested that the antitumor effect of the crude β-D-glucan against Sarcoma 180 of ICR mice was due to immuno-potentiation, and not by direct cytotoxic effects.

# Medicinal Agaricales s.l. of Israeli Mycobiota

*Annush Kosakyan and Eviatar Nevo*

Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel

The order Agaricales s.l. (Higher Basidiomycetes) includes about 6,000 species, which are distributed throughout almost all countries including edible, medical, and poisonous species. Mushrooms have been highly regarded as remedies for disease and as supporters of natural health for thousands of years. Mushrooms are an incredibly popular food in most countries. Fungi affect humans so profoundly and are such good sources of medicinally useful products. The potential ability of medicinal mushroom bioactive compounds to modulate so many important immune cells may be due to their structural diversity and variability. These species are known to demonstrate antitumor, immunoregulatory, genoprotective, cardiovascular, antimutagenic, antiviral, hepatoprotective, antiparasitic, antidiabetic, and other medical properties. Their nutritional value is very high containing different proteins and amino acids, carbohydrates and dietary fiber, fat, vitamins, minerals, trace elements, etc. For modern medicine, mushrooms represent an unlimited source of polysaccharides with antitumor and immunostimulating properties.

Based on the data concerning the distribution of Agaricales s.l. order in Israel, we found the presence of more than 500 species divided into 75 genera and 16 familes. Most of these species are therapeutically important and have been found in forests situated in the northern part of Israel, mainly in the Golan Heights, Carmel Mountains, and Upper Galilee. Due to the winter rainy season, biodiversity is much higher in this part of Israel. However, investigations on the production of fungal medicines and food supplements have

not been carried out in this region. Unfortunately, medicinal mushrooms, except for *Agaricus bisporus* (J.E. Lange) Imbach and *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm., are not cultivated for food or pharmaceutical purposes in Israel. Extensive observations and analyses have shown that most species from the following families existing in Israel have quite strong pharmacological potential, for example: from **Agaricaceae:** *Agaricus bisporus, A. bitorquis, A. campestris, Leucoagaricus leucothites, L. carneifolius, Macrolepiota procera, M. rechodes,* etc.; from **Boletaceae:** *Boletus edulis, B. erythropus*, *B. luridus, Xerocomus chrisenteron, Suillus granulatus,* etc.; from **Coprinaceae:** *Coprinus comatus, C. atramentarius,* etc.; from **Pleurotaceae:** *Pleurotus ostreatus, P. eryngii, P. pulmonarius,* etc.; from **Paxillaceae:** *Omplalotus olearius, Paxillus panuoides,* etc.; from **Tricholomataceae:** *Lepista nuda, Clitocybe geotropha, C. fragrans, C. nebularis, Flammulina velutipes, Laccaria laccata, L. ametistina, Mycena pura, Marasmius oreades,* etc.; from **Bolbitiaceae:** *Agrocybe dura, A. aegerita,* etc.; from **Amanitaceae:** *Amanita rubescens, A. ovoidea, A. vaginata,* etc.; from **Strophariaceae:** *Pholiota aurivella, P. flammans, Gymnopyllus spectabilis,* etc.; from **Crepidotaceae:** *Crepidotus mollis;* and from **Entolomataceae:** *Entoloma clypeatum.*

As far as we can see, these species show potential for further observations. For this reason, a special culture collection at the Institute of Evolution, University of Haifa (HAI), Israel, was established. HAI culture collection is an important source for carrying out fundamental studies and biotechnological applications of medicinal properties of mushrooms.

---

# Design of Effective Submerged Cultivation Methods for Xylotrophic Medicinal and Culinary-Medicinal Basidial Mushrooms

*Larissa M. Krasnopolskaya, Maria I. Leontieva, Anastasia V. Avtonomova, Igor V. Belitskii, Elena B. Isakova, and Vladimir M. Buchman*

Gause Institute of New Antibiotics, 11 Bolshaya Pirogovskaya St., Moscow 119021, Russia

It is widely recognized that submerged cultivation parameters, notably, the composition of cultivation medium, significantly affect the biological activity of the cultivated mushrooms.

The aim of the present study was to design an algorithm for developing effective methods for submerged cultivation of xylotrophic medicinal and culinary-medicinal basidial mushroom species. The effectiveness was evaluated according to the following three criteria: 1) air-dry biomass yield, 2) cultivation process duration, and 3) biological activity of the obtained biomass. More specifically, a cultivation method was considered effective if it allowed for a guaranteed yield of at least 20 g/L of air-dry (humidity ≤ 6%) biomass in 6 days (150 hours) of cultivation.

The chosen strains of *Flammulina velutipes, Ganoderma lucidum, Hericium erinaceus, Hypsizygus marmareus, H. ulmarius, Pleurotus djamor, P. ostreatus, P. pulmonarius, P. eryngii,* and *Trametes versicolor* were cultivated in two stages, including production of liquid inoculum and fermentation.

The optimal composition of liquid cultivation medium for each of the studied mushroom strains was determined in the course of a uniform three-stage selection process. At the first stage, we studied the dynamics of biomass accumulation for each strain on a specially designed universal medium, obtaining first estimates of the cultivation process length and biomass yield. The second stage was devoted to a qualitative selection of carbon, nitrogen, phosphorous, and microelements sources with an aim of choosing the best combinations of these ingredients. For many of the studied strains of xylotrophic Basidiomycetes, such combinations were successfully found. The third stage consisted of finding the optimal proportions of the previously selected nutrient sources in the cultivation media. The optimization was performed using full factorial design and steep ascension methods, which also helped to reveal some joint positive and negative effects of various nutrient sources on

mushroom growth, and point towards possible ways of strengthening positive and neutralizing negative interrelationships. Full factorial experiments were repeated with varying initial concentrations of nutrient sources, until the yield surface demonstrated a single global maximum at an internal point. Optimal media compositions were generally chosen on the basis of results of steep ascension experiments; in some cases, they were derived from the respective yield surface shapes.

The full factorial design was used for the development of the liquid inoculum growth method. Composition of cultivation medium, inoculum age, and inoculum quantity were selected as three experimental factors of full factorial design, with air-dry biomass yield at the fermentation stage as a response variable. Experiment results showed that, for most of the studied strains, maximal biomass yields were achieved with inoculum age of 3 days, introduced in doses of 0.2–0.5 g/L, air dry weight.

The developed final methods of submerged cultivation allowed the achievement of the following biomass yields: *Flammulina velutipes* – 24.0 g/L in 5 days, *Ganoderma lucidum* – 21.0 g/L in 4 days, *Hericium erinaceus* – 22.4 g/L in 6 days, *Hypsizygus marmareus* – 28.4 g/L in 6 days, *H. ulmarius* – 37.2 g/L in 3 days, *Pleurotus djamor* – 23.8 g/L in 4 days, *P. ostreatus* – 23.0 g/L in 3 days, *P. pulmonarius* – 23.4 g/L in 4 days, *P. eryngii* – 21.2 g/L in 5 days, *Trametes versicolor* – 23.5 g/L in 4 days.

Biological activity of the obtained *G. lucidum, H. erinaceus, H. marmareus, H. ulmarius, P. djamor, P. ostreatus, P. eryngii,* and *T. versicolor* biomass was assessed by the antitumor activity of water extracts of their mycelium and the water-soluble polysaccharide fractions. *In vivo* tests on BDF1 hybrid mice with inoculated P388 lymphatic leukemia showed significant antitumor activity of all of the studied substances, except *P. eryngii* mycelium water extract.

## The Effect of Initial pH on Biomass Production of *Ganoderma applanatum* and *G. lucidum* Strains

*Tatyana A. Krupodorowa*

M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev, GSP-1, 01601, Ukraine

The concentration of hydrogen ions is one of the main factors regulating mycelial growth of fungi. Ability of mycelium to grow at different values of the medium pH is connected with their property to change acidity of the culture medium. Optimum acidity of most Higher Basidiomycetes species is between a pH of 4.5 and 6.0.

The production of biomass of six strains of *Ganoderma applanatum* and 10 strains of *G. lucidum*, isolated from different climatic conditions and geographical regions (Belarus, Israel, Germany, Russia, Ukraine, and USA), was studied at different values of the medium pH. The medium consisted of (g/L): glucose - 25, $(NH_4)_2HPO_4$ - 4, $KH_2PO_4$ - 1, $K_2HPO_4$ - 1, $MgSO_4 \times 7\ H_2O$ – 0.5, 10 mL solution of microelements and $H_2O$ – 1 L. Before sterilization, the medium pH was adjusted to 3.2, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.3 by adding 1N HCl or 1N NaOH. The medium was inoculated by ca. 0.2 g/L biomass investigated strain and incubated at 28°C for 29 days.

The kinetics of biomass accumulation by different strains of *G. applanatum* and *G. lucidum* at different initial pH values show that for all strains of *G. applanatum*, in comparison to strains of *G. lucidum*, accumulation of biomass is greater. Optimal pH range for biomass accumulation by *G. applanatum* strains is narrower and yields 4.0; 5.0; 5.5; 6.5 and strains of *G. lucidum* - are wider and fall in the range of 4.0 to 7.0. The maximum biomass of *G. applanatum* (13.7 ± 0.1 g/L) and *G. lucidum* (7.7 ± 0.4 g/L) strains was noted at the initial pH of the cultural media (5.0 and 5.5, respectively). Our results demonstrate that strains of *G. applanatum* and *G. lucidum* may have 1 peak of optimal pH for accumulation biomass (50% strains of *G. lucidum* and 66% strains of *G. applanatum*), 2 peaks (30% strains of *G. lucidum* and 17% strains of *G. applanatum*) or plateau (20% strains of *G. lucidum* and 17% strains of *G. applanatum*). The given dates show that the index of optimal pH for biomass accumulation is specific for every investigated strain of *G. applanatum* and *G. lucidum*.

# *In Vitro* Formation of *Cordyceps bassiana* Fruiting Bodies from the *Beauveria bassiana* Isolate

*Je-O Lee, Bhushan Shrestha, Sang-Kuk Han, Won-Ho Lee, Beom-Suk Kim, and Jae-Mo Sung*

Entomopathogenic Fungal Culture Collection (EFCC), Department of Applied Biology, Kangwon National University, Chuncheon 200-701, Gangwon-Do, South Korea

*Beauveria* (Bals.) Vuill. (Hyphomycetes), an imperfect genus, is presumed to be related to the genus *Cordyceps* (Clavicipitaceae, Hypocreales, Ascomycota) by morphological and physiological characters. The teleomorph of *Beauveria brongniartii* (Sacc.) Petch was described as *Cordyceps brongniartii* by Shimazu et al. (1988). Similarly, the teleomorphic stage of another ubiquitous *Beauveria* sp., *B. bassiana* (Bals.) Vuill., was described as *Cordyceps bassiana* from China on a carpenterworm larva (Li et al., 2001).

In this study, we investigated the connection between *B. bassiana* and *C. bassiana* in culture. A specimen of *B. bassiana* (EFCC 13188), growing on an adult mantis, was collected from Yang-yang, Gangwon Province, Korea, in October 2006 and preserved in Entomopathogenic Fungal Culture Collection (EFCC), Kangwon National University, Chuncheon, Korea.

Eight single conidial isolates were obtained from the fresh specimen of *B. bassiana* (EFCC 13188) by dilution method and grown in half-strength SDAY agar plates for 3 weeks under a constant white florescence light at 25ºC. The isolates were numbered from EFCC 13188-1 to EFCC 13188-8. For artificial fruiting body induction, the single conidial isolates were inoculated in pairwise combinations in sterilized brown rice medium supplemented with silkworm pupa. The brown rice media were prepared in 1000 ml polypropylene bottles by mixing 60 gm of brown rice with 5 g of silkworm powder in 70 ml of distilled water. After inoculation, the brown rice media were incubated at 20 ± 1ºC under constant white fluorescence light and high humidity (80%) conditions. Mycelia started to grow inside and on the surface of brown rice in the first week and induced the primordia of stromata after 2–3 weeks of incubation. The stromata measured heights of 8–10 cm after 40–50 days of incubation. Perithecia were produced on stromata after 50–60 days, showing that the sexual stage occurred in *in vitro* conditions. On the one hand, not all the isolates/combinations produced perithecial stromata. Isolate EFCC 13188-4 produced perithecial stromata in single as well as in combination with most of the remaining isolates. On the other hand, isolate EFCC 13188-2 produced perithecial stromata in single, but not in combination with other isolates. Combinations EFFC 13188-3 × EFFC 13188-5 and EFFC 13188-5 × EFFC 13188-8 produced no stromata, but white mycelium only. We could not determine the mating compatibility among the isolates as perithecial stromata formation was not stable among the combinations of the isoaltes. The fruiting bodies were then compared to those of *C. bassiana* EFCC 783 produced *in vitro* (Sung et al., 2006). Also, we compared conidia and conidiophores of the two species by the slide culture method. Fruiting bodies produced from *B. bassiana* and *C. bassiana* were similar to each other. Perithecia, ascospores, and conidial structures were also similar.

The above research showed that some combinations of *B. bassiana* isolates produced artificial fruiting bodies in vitro. In this experiment, perithecial stromata of *Cordyceps bassiana* were produced from *B. bassiana* isolates for the first time. Further research is required to show the mating compatibility between *C. bassiana* and *B. bassiana* isolates through out-crossing and DNA sequences to confirm the connection between *B. bassiana* and *C. bassiana*.

# Factors Influencing the Production of Water-Soluble Endopolysaccharide and Exopolysaccharide from *Lentinus lepideus* and Their Effects on Immune Cytokine Production

*Wi Young Lee,[1] Jin Kwon Ahn,[1] and Kang Hyeon Ka[2]*

[1]Div. of Biotechnology, Korea Forest Research Institute, Suwon 441-350, Korea; [2]Div. of Wood Chemistry & Microbiology, Korea Forest Research Institute, Seoul 130-712, South Korea

*Lentinus lepideus* (Polyporaceae, higher Basidiomycetes), is an edible mushroom containing strong immunomodulating substances. The water-soluble glucan extracted from *L. lepideus* induces a variety of cytokines in the human peripheral lymphocyte system and hematopoiesis of granulocyte lineage *in vivo.* These results suggest the potential of the glucan as an effective biological response modifier (BRM), and its possible use as an immune enhancer in immunocompromised and immunosuppressed individuals. The biological activities are known to be associated with polysaccharides isolated from the fruiting bodies and mycelia.

Submerged cultures of edible fungi obviously have the potential for higher mycelial production to induce mass production of useful substances in compact spaces in a short time period with easy environmental control. This research deals with the production of water-soluble polysaccharides from *L. lepideus* by comparing the productivity endopolysaccharides (PPS) and exopolysaccharides (EPS) under various cultural conditions. In order to produce the immunostimulating water-soluble polysaccharides from the mycelia, the effects of several cultivating factors on the production of PPS and EPS were also studied. Immunostimulating activities were evaluated by measuring the production level of TNF-α and IL-10 under treatment of EPS and PPS.

The culture temperature, culture period, and carbon and nitrogen concentration in the media were major factors that influenced the productivity of EPS and PPS from *L. lepideus*. High-yield EPS required moderate temperature (25°C) and a longer culture period (16-20 days). In contrast, PPS production required higher culture temperature (35°C) and a shorter culture period (8 days). Carbohydrate concentration in the media also affected the production of polysaccharides.

The immune cytokine level in EPS treatment varied depending on mycelia cell lines, carbon sources, or culture periods. Highest amounts of immune cytokine were induced in the culture when glucose was used as a carbon source for longer cultivation than 10 days in the presence of EPS. Meanwhile, PPS appeared to produce almost the same level of cytokine, regardless of culturing factors used except for the culture period.

These results suggest that optimal culture conditions for the production of mycelial biomass, EPS, and PPS from *L. lepideus* mycelia. Immunomodulating activity of EPS was more affected by culture conditions than that of PPS in *L. lepideus.*

# Submerged Cultivation and Antitumor Activity of *Hypsizygus ulmarius*

*Maria I. Leontieva,[1] Anastasia V. Avtonomova,[1] Igor V. Belitskii,[1] Elena B. Isakova,[1] Anatolii I. Usov,[2] Vladimir M. Buchman,[1] and Larissa M. Krasnopolskaya[1]*

[1]Gause Institute of New Antibiotics, Russian Academy of Medical Sciences, 11 Bolshaya Pirogovskaya St., Moscow 119021, Russia; [2]Zelinsky Institute of Organic Chemistry, Russian Academy of Medical Sciences, 47 Leninsky Prospect, Moscow 117913, Russia

The identification of optimal ('perfect') objects for obtaining biologically active metabolites is one of the most significant problems of applied mycology. A 'perfect' object, in our opinion, is a species or strain that is characterized by both an accelerated process of ontogenesis of the culture and high yield of biomass and/or metabolites with high biological activity.

The present work is devoted to the elaboration of an effective method of producing *Hypsizygus ulmarius* vegetative biomass by submerged cultivation and studies the antitumor activity of the submerged culture's substances.

To determine the nutritive needs of the *H. ulmarius* strain a comparative analysis of 12 different nutrient media, representing various combinations of alternative carbon and nitrogen sources was performed. The maximum production of air-dried *H. ulmarius* biomass (water content below 5%) on medium containing vegetable oil and soybean meal was recorded on the 3rd day of submerged cultivation and reached 19.1 g/L.

The quantitative composition of nutrient media can easily be optimized using mathematically designed experiments. The optimal nutrient media for *H. ulmarius* were obtained in the course of experiments designed using full factorial and steepest ascent strategies. The air-dried mycelium yield was taken as the assessment criterion. The data obtained in full factorial experiments was used to estimate the regression coefficients. Comparing them with the confidence limit ε (significance level of 5%), we found the biomass yield of *H. ulmarius* to be positively responsive to two factors: oil and soybean meal, and also to the combination of these two. On the basis of the data obtained in the full factorial experiments for *H. ulmarius,* an experiment using the steepest ascent method was carried out. In this experiment we simultaneously increased concentrations of both significant factors according to the general steepest ascent algorithm using the regression coefficients previously obtained in the full factorial experiments. Air-dried biomass yield reached a maximum level of 32–33 g/L with the optimal period of *H. ulmarius* cultivation in about 72–76 hours. Optimal nutrient medium for *H. ulmarius* was identified using the yield surface constructed from the full factorial experiments and steepest ascent experiment results. We were able to achieve 37 g/L of air-dried biomass by the 3rd day of culti-vation on that medium.

*Hypsizygus ulmarius* submerged mycelium contained about 26%–27% of water-soluble carbohydrates. An analysis of neutral monosaccharide composition of the isolated polysaccharide fraction showed the presence of glucose, galactose, mannose, rhamnose, arabinose, and xylose in proportion of 23.5 : 9.5 : 7.0 : 2.0 : 1.5 : 1.0.

Antitumor activity of substances obtained from mycelium of *H. ulmarius* was studied *in vivo* on transplantable tumor T-cell lymphoma P388 in $B6D2F_1$ mice. Antitumor substances were administered by the oral-intragastric route. It has been shown that lyophilized submerged culture of *H. ulmarius,* hot water extract of submerged mycelium and total water-soluble polysaccharide fraction of mycelium possessed significant antitumor activity. Total water-soluble polysaccharide fraction of mycelium in a dose of 2 mg/kg/day, administrated orally, demonstrated higher antitumor activity compared to the other substances. Comparison of antitumor activity of total water-soluble polysaccharide fraction of mycelium in 2 mg/kg/day and 20 mg/kg/day doses were definitely in favor of the 2 mg/kg/day dose.

# Effect of Boron Compounds on Growth and Decay Abilities of Wood Decay Fungi

*Boštjan Lesar, Andreja Žagar, Boštjan Podlesnik, Jerneja Modic, Franc Pohleven, and Miha Humar*

University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

Wood, as natural material, is permanently exposed to biotic and abiotic factors that cause deterioration. Among them, wood decay fungi are the most important factors for failure of wood and wooden constructions in temperate zones. Therefore, if we want to use nondurable wood wet conditions, it has to be chemically protected. After introduction of Biocidal Products Directive (BPD, 1998) in the EU, most of the traditional biocides were removed from the market. Boron compounds (Borax and boric acid) are among the 39 remaining active ingredients for wood preservation. Boron compounds have several advantages; they are safe, cheap, and effective; unfortunately, these compounds leach from wood when exposed to weathering. Therefore, we have to combine them with other biocides to improve their properties.

In order to understand interactions between boron-based biocides and wood decay fungi several experiments were performed. Three different wood decay fungi were used: brown rot *Gloeophyllum trabeum* and *Antrodia vaillantii* as well as white rot fungi *Trametes versicolor.* In the first experiment, minimum inhibitory concentration of boron was determined in nutrient medium and on impregnated wood. Additionally, quaternary ammonium compounds were introduced to preservative solutions and the influence of this formulation on fixation, effectiveness against wood decay fungi and blue stain fungi (*Aureobasidium pullulans* and *Sclerophoma pityophilla)* were determined.

The results confirmed that boron is a very effective fungicide. It was even more affective than copper (II) compounds. Laboratory testing showed that significantly inhibited growth of mycelium *G. trabeum* and *A. vaillantii* were determined on medium with 0.0031% of boron. However, no fungal growth was detected on medium containing 0.0063% of boron. Brown rot fungi tested expressed higher resistance against boron than white rot fungus. Similar results were observed for copper as well. All wood samples impregnated with boron, even the ones impregnated with the solution of the lowest boron concentration ($c_B$ = 0.006%), were protected against wood decay fungi. Good fungicidal properties of boron compounds are especially important for protection of wood against copper tolerant fungal isolates, as boron itself ensures sufficient protection. However, boron compounds would make perfect combination for wood protection if we could achieve sufficient fixation; therefore, they are combined with quaternary ammonium compounds, which improves boron fixation and its performance against wood decay and staining fungi. Wood specimens impregnated with boron-quaternary ammonium compounds solution ($c_B$ = 0.1%; $c_{quat}$ = 0.1%) were resistant against tested wood decay fungi and blue stain fungi after artificial weathering, too.

# Research on the *in Vitro* Growth Inhibition Effect of *Inonotus obliquus* on Bacteria

*Xue Li, Yang Lin, and Qi Wang*

College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun 130118, China

*Inonotus obliquus* (Fr.) Pilat is usually known as chaga, has visible inhibitory action to tumor cell, and has active compounds for treating diabetes, AIDS, hypertension, hypersensitive skin, and so on. There are many reports on the active constituents and pharmacological action of *I. obliquus* fruiting body; but, there are only a few

about its mycelium and broth; there is no report about its bacteriostatic action. This paper discusses the bacteriostatic action of *I. obliquus* mycelium, the bacteriostatic action of *I. obliquus* hyphostroma to bacteria: *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium*; molds: *Aspergillus niger* Van Tiegh and *Trichoderma viride* Pers. : Fr. using the filter scrip and germ tablet methods. The main technique is the method of germ tablet: putting the germ tablet of *I. obliquus* on the surface of solid medium mixed with *S. aureus, E. coli, S. typhimurium, A. niger,* or *T. viride*. Cultivation took place at 37°C for 24 hours, the density of the three bacteria is as follows: *S. aureus*: $2 \times 10^6$ cfu/mL, *E. coli*: $2.256 \times 10^8$ cfu/mL, and *S. typhimurium*: $1.728 \times 10^8$ cfu/mL. The results show that the hyphostroma of *I. obliquus* has different bacteriostatic action. When the diameter of a germ tablet is 1.5 cm, the average diameter of the antibiotic circle on *S. aureus* is 2.5 cm; the average diameter of the antibiotic circle on *E. coli* is 2.6 cm; the average diameter of the antibiotic circle on *S. typhimurium* is 2.1 cm; the average diameter of the antibiotic circle on *S. typhimurium* is the most distinct. But the hyphostroma of *I. obliquus* has no bacteriostatic action on *A. niger* and *T. viride*. The result shows that *I. obliquus* hyphostroma contains some bacteriostatic constituent. The method of filter scrip consists of putting the extract of *I. obliquus* hyphostroma (using water, ethanol, or nbutanol) on the surface of the solid medium mixed with *S. aureus, E. coli, S. typhimurium, A. niger,* or *T. viride*. Cultivation took place at 37°C for 24 hours. The result shows that nbutano1 has bacteriostatic action on the three experimental bacteria and has the best bacteriostatic action on *S. typhimurium*. When the diameter using filter scrip is 0.8 cm, the average diameter of the antibiotic circle on *S. aureus* is 1.0 cm; the average diameter of the antibiotic circle on *E. coli* is 1.2 cm; the average diameter of the antibiotic circle on *S. typhimurium* is 1.3 cm; but the extractive of nbutanol has no bacteriostatic action on *the* two experimental molds. The results show that *I. obliquus* has a broad-spectrum of bacteriostatic action. However, the specific chemically active compounds that have bacteriostatic action and their stability will be further discussed.

---

# Inoculated Goat Willow (*Salix caprea*) Cuttings as a Model Plant Species for Phytostabilization at a Heavy Metal Polluted Site in Žerjav

*Matevž Likar and Marjana Regvar*

Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1001 Ljubljana, Slovenia

High-soil heavy metal pollution with maximal concentrations of up to 5490 mg $kg^{-1}$ of Zn, 390 mg $kg^{-1}$ of Cd, and 67,940 mg $kg^{-1}$ of Pb, demanding comprehensive remedial action, is the result of industrial activity in Žerjav, NE Slovenia. After screening for suitable plant species, *Salix caprea,* as the dominant willow woody species, was selected for further phytostabilization efforts. *Salix caprea* L. shrubs growing at the polluted site accumulated high concentrations of Cd and Pb, without any observed effects on their photosynthetic pigments, suggested high tolerance of suitability of the examined *S. caprea* shrubs to the elevated levels of heavy metals for phytostabilization at the polluted site.

Cuttings were used for the propagation of the goat willows, reaching a success rate of 12%–25%, due to the known poor rooting potential of this species. Additional treatment with auxins at the apex of the cuttings significantly increased the success rate to 40%, potentially providing enough plant material for phytoremediation on a larger scale.

As most terrestrial ecosystems are dominated by plants forming mycorrhizae or associations with dark septate endophytes (DSE), compatible fungal symbionts are vital for plant establishment and persistence, especially in extreme environments, such as in heavy metal polluted sites. Ectomycorrhizal (EM) and arbuscular mycorrhizal (AM) fungi have been shown to contribute to the metal tolerance of host plants by providing a metal exclusion barrier and improving plant nutritional status. The ability of willows to form associations with AM and EM fungi as well as DSE

facilitates their incorporation into a turf dominated by AM grasses and herbs, while EM and DSE fungi are advantageous when progressively increasing the proportion of soil N, present in an organic form.

Analysis of the fungal endophyte community using temporal gradient gel electrophoresis (TGGE) and sequencing showed that ectomycorrhizal fungi (EM), arbuscular mycorrhizal fungi (AM), and dark septate endophytes (DSE) are symbionts of *S. caprea* at the heavy metal polluted site. EM was the dominant form of symbiosis, followed by DSE and AM fungi. The majority of the DSE fungi belonged to genus *Phialophora*, while ectomycorrhizal fungi were mainly represented by the Basidiomycetes family Thelephoraceae and the Ascomycetes Sordariaceae.

Isolation of fungal root endophytes yielded in 20 different fungal isolates, as indicated by PCR-RFLP patterns of the ITS rDNA region and sequencing. Among these, the three most frequent colonizers of goat willow shrubs at the polluted site were chosen for further metal tolerance and inoculation experiments. Inoculation of *S. caprea* cuttings with inoculum, composed of indigenous fungi from the polluted site, significantly improved the growth of the cuttings in the polluted soil, confirming the functional importance of the indigenous fungi in the alleviation of metal stress. For further standardization of the remediation technique using inoculated *S. caprea* cuttings, the selected fungal isolates will be tested in inoculation experiments for selection of the optimal fungal partner(s) for willow cuttings that can than be used for contemporary management of the polluted site.

Development of a characterized inoculum with optimal matching between *S. caprea* and its fungal partner(s) can be used as a model for phytoremediation of the polluted site. However, more work should be done to significantly contribute to the progress in the field of mycorrhizal biotechnology, using inoculated plants for the stabilization of the contaminated soils.

## ACKNOWLEDGMENTS

The work was supported by MSZS L1-5146-0481 Tolerance of organisms in stressed ecosystems, P1-0212 Biology of plants, COST 8.38 Managing AMF for Improving Soil Quality and Plant Health in Agriculture, MPI Rudnik Mežica and Mobitel d.d.

---

# Effects of Red Pepper as a Substrate Additive on the Growth and Metabolic Activity of *Pleurotus ostreatus*

*Jong-Hyun Lim, Min-Jin Song, Kyung-Yun Kim, Eun-Suk Son, Joong-Ho Joh, Young-Sub Cho, and Chang-Soo Lee*

Department of Applied Biochemistry, College of Biomedical and Health Science, Konkuk University, Chung-Ju 380-701, South Korea

*Pleurotus ostreatus* is economically highly praised for its nutritional and pharmacological values. Its fruit bodies are rich in carbohydrates, dietary fiber, proteins, essential amino acids, vitamins, and minerals. Biologically active substances, especially polysaccharides and lectins, isolated from *P. ostreatus* have hypocholesterolemic, antitumor, immunomodulating, antiviral, and antibacterial effects. Mushroom can be cultivated using many agricultural and industrial by-products as substrates such as all cereal straws, wood shavings, sawdust, nutshells, vegetable wastes, coffee pulp, and tea wastes. The sawdust- and straw-based materials are commonly used as substrates. We studied the effects of red pepper as a substrate additive on the formation of primordia or fruiting bodies and the metabolic activity of *P. ostreatus*. Red pepper is an important vegetable used as a food and as a spice. Also, its biological activities including pharmacological, neurological, and dietetic effectiveness are well known.

To measure the effect of red pepper as a substrate additive for mushroom growth, mycelia were routinely grown on mushroom complete media (MCM) at 28°C for 5–10 days, and the fruiting process was performed in substrates at 18°C for 25 days. At this time, red pepper was mixed with sawdust- and straw-

based substrates in different ratios (up to 20%). As a result, the negative effects on mushroom growth and production by the addition of red pepper were not shown, but rather positive effects were observed on mushroom yield and the number of cultivating days of primordial formation and fruiting.

To assay the changes of metabolites by the addition of hot pepper, mushrooms were extracted with methanol and several other solvents, and their extracts were analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). The high sensitivity of MS combined with HPLC makes the technique an extremely powerful tool for the analysis of large populations of metabolites. By combining MS with liquid chromatography, molecular identification and quantification of polar, less-polar, and neutral metabolites can be achieved, even when they are present at relatively low-concentration levels and in a complex matrix. Mushrooms were dried, powdered, and extracted with a mixture of methanol : water (70:30,v/v) for 3 h at 80°C. Methanol extracts were further fractionated with several organic solvents of hexane, chloroform, ethyl acetate as non-polar solvents, and butanol as the polar solvent. The analysis of mushroom metabolites was carried out on an HPLC system consisting of a photodiode array detector. UV-vis spectra were recorded in the range of 200–800 nm. Mass spectral data was obtained using an ion-trap mass spectrometer with an electrospray interface (ESI-IT-MS). HPLC separations were performed using a Reverse Phase analytical column, and peaks were detected in both full scan and selective ion monitoring mode, respectively. On the one hand, several major peaks, referring to the mushroom metabolites, were detected in extracts of both non-polar solvents and polar solvents. The areas of these major peaks were increased or decreased by the addition of red pepper. On the other hand, the appearance of new peaks was observed in mushrooms cultivated with red pepper as a substrate additive.

## The Study on *in Vitro* Bacteriostasis Testing of Puffballs' *Hyphostroma*

*Yang Lin, Qi Wang, Xue Li, and Huocai Zhang*

College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun, 130118, China

*Lasiosphaera fenzlii* Reich is a traditional Chinese medicinal mushroom used extensively and known as the puffball mushroom in China. *L. fenzlii* has been recorded by the Pharmacopoeia of the People's Republic of China, and has the following functions: detumescence, deintoxication, hematischesis, and is clinically, mainly used for treating tonsillitis, decubital ulcer, frostbite, bleeding wounds, etc. *Calvatia saccata* (Vahl: Fr.) Morg, *Bovista nigrescens* Pers., *Bovistella sinensis* Lloyd, and *Secotium agaricoides* (Czern.) Hollos are also used extensively as folk medicines in Chinese daily life, but have not been recorded by the Pharmacopoeia of the People's Republic of China. According to related literature, the immature *C. saccata* is edible; the spore of *B. nigrescens* can be used for treating bleeding wounds; *B. sinensis* and *S. agaricoide* have the same medicinal effectiveness as *L. fenzlii*, especially *B. sinensis* which is used extensively as the puffball for treating hemoptysis, tonsillitis, and bleeding wounds in major Chinese medicine clinics in many provinces such as Jilin, Jiangsu, Hubei, Jiangxi, and Fujian. At present, there are several related reports about the bacteriostatic action of the extract of several kinds of puffball fruiting bodies, but not many reports on the bacteriostatic action of the puffballs' *Hyphostroma*. Therefore, this study investigated the bacteriostasis of the puffballs' *Hyphostroma* on *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Aspergillus niger,* and *Trichoderma viride* using the method of viable organism activity mensuration and five kinds of the above-mentioned puffballs harvested from the Jilin province of China as the experimental material. The results show that the five kinds of puffballs' *Hyphostroma* have different bacteriostatic action, but all of them do not have the obvious bacteriostatic action to the experimental molds; *L. fenzlii* has significant bacteriostatic action to *E. coli* and *S. aureus* and their average diameters of antibiotic circles are 21 mm and 26 mm, but *L. fenzlii* does not have the

effect on *S. typhimurium; C. saccata* has extremely significant bacteriostatic action on *E. coli,* and its average diameter of the antibiotic circle is 37 mm; the average diameters of antibiotic circles on *S.aureus* and *S. typhimurium* both are 12 mm; *S.agaricoides* has significant bacteriostatic action to *E. coli,* and its average diameter of the antibiotic circle is 23 mm, but the average diameters of antibiotic circles on *S. aureus* and *S. typhimurium* are only 14 mm and 13 mm; both *B. nigrescens* and *B. sinensis* do not have the obvious bacteriostatic action to the experimental bacteria; *C. saccata* and *S. agaricoides* can inhibit all of the experimental bacteria with different effects. The bacteriostatic effects of *S. agaricoides* and *C. saccata* on *S. aureus* are less than that of *L. fenzlii,* but their bacteriostatic effects on *E. coli* and *S. typhimurium* are better than the latter; therefore, these experimental results are consistent with regular use of *S. agaricoides* and *C. saccata* in folk medicine, but the specifically active compositions of puffballs' *hyphostroma* having significant bacteriostatic action need to be further researched.

# Antioxidative and Antibacterial Activity of Some Lignicolous Basidiomycetous Fungi from Serbia

*Karaman A. Maja,[1] Mimica-Dukic M. Neda,[2] Petar N. Knežević,[1] and Matavulj N. Milan[1]*

[1]Department of Biology and Ecology, [2]Department of Chemistry; Faculty of Sciences, University of Novi Sad, Trg D. Obradovica 2, Novi Sad 21000, Serbia

In the last decades, different genera of basidiomycetous fungi became important as sources of natural bioactive metabolites of pharmacological interest, with various medical effects: antitumor, immunomodulating, cardiovascular, antimicrobial, antiparasitic, hepatoprotective, antidiabetic, and others. Biological activity and chemical composition of fungal species vary broadly and depend on the strains and the substrates (place) of growth. With regard to the production of oxygen radicals causing pathological changes in organisms, which can be diminished by the antioxidative activities of selected mushroom extracts, wild growing lignicolous fungi from northern Serbia (Fruska Gora Mountain) were analyzed for their antioxidative properties. At the same time, these extracts were screened for their antibacterial potential also.

Organic extracts (methanol and chloroform) of six fungal species were examined: *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst., *G. applanatum* (Pers.: Wallr.) Pat., *Trametes versicolor* (L.: Fr.) Quél., *Meripilus giganteus* (Pers.: Fr.) P. Karst., *Laetiporus sulphureus* (Bull.: Fr.) Murrill, and *Piptoporus betulinus* (Bull.: Fr.) P. Karst.

The antioxidant activity was evaluated by the determination of free radicals ($DPPH^{\cdot}$ and $OH^{\cdot}$) scavenging activity and the effect on $Fe^{2+}$/ascorbate-induced lipid peroxidation (LP) in lecithin liposomes. The contents of total phenols were determined spectrophotometrically in all crude extracts.

The antibacterial activity was examined against 18 strains of both Gram-positive and Gram-negative bacteria, including reference strains (*Staphylococcus aureus* ATCC 25923; *Sarcina lutea* ATCC 9341; *Escherichia coli* ATCC 25922; *Salmonella enteritidis* ATCC 13076; and *Proteus mirabilis* ATCC 35659), strains isolated from humans (*Staphylococcus aureus*, *Enterobacter* sp., *Shigella flexneri, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa,* and *Salmonella enteritidis*), and animal pathological material (*Rhodococcus equi, Listeria iwanovii,* and *Staphylococcus aureus*) as well as animal commensals (*Escherichia coli, Bacillus* sp., and *Micrococcus luteus*). Preliminary tests were carried out by the agar well diffusion method. The susceptibility of strains to commonly used antibiotics was also tested using the disk diffusion method. The level of antimicrobial effects was established using an *in vitro* broth dilution susceptibility test in microtitre plates, and minimum inhibitory concentration (MIC) was determined. The minimum bactericidal concentration (MBC) was estimated by inoculation of Müeller Hinton agar plates with samples from wells with no turbidity.

MeOH extracts expressed higher activity than $CHCl_3$ extracts, both in antioxidative and antibacterial assays. The most effective antioxidants were extracts of the examined *Ganoderma* species and *L. sulphureus*. The best results were achieved with the $DPPH^{\cdot}$ assay, with very good scavenger activity of MeOH extracts of *G. applanatum* (12.5 μg/ml, 82.80%) and $CHCl_3$ extract of *G. lucidum* (510.2 μg/ml, 69.12%). The highest LP inhibition was obtained with the methanol extracts (500.0 μg/ml) of *G. applanatum* (91.83%) and *G. lucidum* (85.09%). The highest scavenging effect on $OH^{\cdot}$ generated by the Fenton reaction (68.47% and 57.06%) was reached with MeOH extracts (400.0 μg/ml) of *G. applanatum* and *L. sulphureus*. Antioxidant activity was in correlation with the total phenols content, which ranged from 0.14 to 1.17 mg/g.

The examined fungal extracts inhibited mostly Gram-positive bacteria (*Bacillus* sp., *Rhodococcus equi,* and *Staphylococcus aureus*), including multiple resistant animal commensals and isolates from pathological material resistant to β lactames, cephalosporins, older tetracyclines, and a combination of trimetoprim and sulfametoxazole. The two examined organic extracts of *P. betulinus* (both MeOH and $CHCl_3$) showed the best inhibitory effect on all tested Gram-positive bacteria. Also, notably effective were the extracts of *Ganoderma* species and *T. versicolor*. Extracts of *M. giganteus* and *L. sulphureus* expressed moderate antibacterial properties. MIC ranged between 0.02–10 mg/ml.

The obtained results suggest that analyzed fungi are of potential interest as sources of strong natural antioxidants, but can be also recommended for their antibacterial prophylactic and therapeutic characteristic for use in veterinary and human medicine against Gram-positive bacteria.

## Changes in Electrophoretic Patterns of Laccase and HR-Peroxidase Fungal Isozymes in the Presence of Very Low Doses of Phenol

*Elzbieta Malarczyk, Grzegorz Nowak, and Janina Kochmanska-Rdest*

Biochemistry Departament, M.Curie-Sklodowska University, Lublin, Poland

Based on our earlier works, it was assumed that the Basidiomycetes fungi, cultivated in the lab, are very sensitive for studies on the effect of low and very low doses of phenol on extra cellular laccase and HR-peroxidase activities. Up to now these changes were observed mainly by spectral methods; the isoelectrophoresis in PAGE techniques was used for comparison of both isozyme patterns in cultures of *Trametes versicolor*, growing 14 days in the presence of high phenol dilutions.

The results showed the quantitative and qualitative differences in protein patterns, colored specifically for enzymatic activities. The differences in the activity correlated well with our earlier data obtained in spectral and luminescence analysis. In conclusion, it should be noted that mycelia cells changed their characteristic patterns for enzymatic proteins, making them more or less active according to the rate of dilution. These results are in agreement with the hormetic effect and promise some profits for the future of nanobiotechnology.

# Application of the RAPD Method for the Identification of *Lentinus edodes* Strains

*Natalya Matjushkova,[1] Uldis Malinovskis,[1] Indra Petrauska,[1] and Nina A. Bisko[2]*

[1]Faculty of Biology, University of Latvia, 4 Kronvalda Blvd., LV 1586, Riga, Latvia; [2]M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev 01601, GSP, Ukraine

The widening of geography of *Lentinus edodes* cultivation, which grows in nature only in countries of Southeast Asia, resulted in the appearance of strains-duplicates, having the same genetic origin. The method of vegetative incompatibility is sufficiently simple, but it often does not give reliable results about genetic identity of investigated *L. edodes strains.*

The random amplified polymorphic DNA (PAPD) method was used for the identification of *L. edodes* strains. Various polymerase chain reaction (PCR) primers were analyzed and a comparative analysis was made for the tested strains' vegetative incompatibility.

The following strains, received from various sources, were investigated: DSMZ3565, DSMZ 3343, DSMZ 1899, and DSMZ 2989 (German Collection of Microorganism and Cell Cultures), 395, 714, 361, 355, 386, 353, and 365 (The Culture Collection of Mushrooms, M.G. Kholodny Institute of Botany, Kiev, Ukraine), R4b-2c, R6b-2c, RA1, and RF1 (Latvia). The last strains were obtained from non-identified fruit bodies of mushroom shiitake, successfully grown in the farms of Latvia.

The following primers were used in this study: MP13-20, OPA-1, CT-8, CA-8, GAC-5, GTG-5, and TGTC-5. All DNA samples were compared on the same gel for each primer. Electrophoresis of DNA profiles of fragments (amplicons) for strains differed only in the following primers: MP 13-20, OPA-1, and CT-8. Each primer of mushroom strain showed distinct or similar DNA profiles. They were selected for strain identification. In contrast, by using the following primers, CA-8, GAC-5, and TGTC-5, there were no visual distinctions in electrophoresis of DNA profiles of fragments, and they cannot be used for shiitake strain identification.

All shiitake strains were tested for mutual incompatibility. Most of them were incompatible. Non-compatible mycelia were recognized by distinctive lines of demarcation, which is formed between two contacting mycelial fronts. Some of the studied shiitake strains did not form a zone of incompatibility at joint cultivation, so we were able to presume that strains are identical: RA1 and 714, R4b-2c and R6b-2c, DSMZ3565 and 353, and RF1 and DSMZ2989. Only one pair of strains (RA1 and 714) from compatible four pairs of strains was identical. All other strains could be considered as closely related.

Our investigations suggest that presented RAPD method could be successfully used for *L. edodes* strain identification. Thus, the presented method of RAPD helped to distinguish closely related strains that were revealed as compatible in the tests of vegetative incompatibility. For the successful study of distinction among different strains, we suggest various primers to be used in PCR analysis.

# Extraction of Polysaccharides from Selected Malaysian Mushroom Species: Yield and Antioxidant Properties

*Shaiful Azuar Mohamad, Mat Rasol Awang, and Tengku Ahbrizal Farizal Tengku Ahmad*

Agrotechnology and Bioscience Division, Malaysian Nuclear Agency, Bangi, Kajang 43000, Malaysia

Numerous polysaccharides and protein bound polysaccharide complexes have been detected from mushrooms and can potentially be used as sources of therapeutic agents.

A modified version of Mizuno method was used to extract polysaccharides from selected mushroom species available in Malaysia. Using this method, a mushroom hot-water extract was precipitated by the

addition of ethanol, followed by a reduction in volume and lyophilization, to obtain a shiny to brown colored solid crystal and amorphous polysaccharide. The laboratory scale extraction process of up to 1 kg/batch and a larger scale of 10–35 kg/batch were compared. The results showed a consistent yield in polysaccharides obtained. The yield from the lab was between 0.5% and 5%, whereas the yield from the larger scale was in the range of 1%–4%. An assay for mushroom antioxidant activities has also been performed using the protocol of Total Antioxidant Assay Kit. Antioxidant activities have been detected in hot-water extracts for both strains of *Pleurotus sajor-caju* (Gray Oyster mushroom) and *P. tuberregium* (Tiger Milk mushroom). Using the Total Antioxidant Assay Kit, the solution turned green in the reaction with the extracts. A solution of 0.1% extract gave rise to 1 mM and 0.6 mM antioxidants in *P. sajor-caju* and *P. tuberregium,* respectively. Two additional protocols were employed for analysis. Ferric Thiocynate (FTC) and Thiobarbiturate (TBA) analysis protocols were found to be more sensitive in detecting antioxidant activities. Sensitivity increased between 10- to 20-fold for TBA and FTC, respectively.

## Immunomodulating Activity of *Agaricus brasiliensis* KA21 in Human Volunteers

*Masuro Motoi,[5] Ying Liu,[1] Yasushi Fukuwatari,[1] Ko Okumura,[2] Kazuyoshi Takeda,[2] Ken-ichi Ishibashi,[3] Naohito Ohno,[3] Kazu Mori,[4] and Ming Gao[4]*

[1]Mibyou Medical Research Center (Institute of Preventive Medicine), Japan; [2]Department of Immunology, School of Medicine, Juntendo University School of Medicine, Japan; [3]Laboratory for Immunopharmacology of Medical Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Japan; [4]Department of Acupuncture and Moxibustion, Suzuka University of Medical Science, Japan; [5]Toei Pharmaceutical Co., Ltd., Japan

*Agaricus brasiliensis* S. Wasser et al. (=*Agaricus blazei* sensu Heinem.) is a health food that has received recent attention. *A. brasiliensis* KA21 used in this study is a fruit body cultivated outdoors in Brazil. Fruit bodies were air dried by a ventilator with a blowing temperature lower than 60°C to maintain their enzyme activities. KA21 also has high protein and fiber content. It also has high levels of vitamins B1, B2, B6, niacin, pantothenic acid, folic acid, and biotin. It contains many minerals including large amounts of iron, potassium, phosphorus, magnesium, zinc and copper, and certain amounts of manganese and selenium. In addition, it has high vitamin D content as it is cultivated under the sunlight.

*Agaricus brasiliensis* has been reported to improve symptoms of lifestyle-related diseases and to have antitumor, cancer inhibitory, and immunoenhancing effects. However, many reports were either animal studies or clinical studies with several cases. We have recently examined the structure and antitumor activity of polysaccharide fractions of the fruit body and concluded significant contribution of the highly branched 1,3-beta-glucan moiety on the activity.

To successfully achieve and maintain food safely and effectively, analysis at the molecular level for basic research and proving their effects by clinical research are important. In the present study, we demonstrated the safety and immunomodulating effect of *A. brasiliensis* KA21 using healthy human subjects.

Research was performed on 31 healthy subjects who were not taking any medication prior to or at the time of the study. We explained the study to them in writing and obtained consent to use the test results.

Before determining the safety of *A. brasiliensis* KA21, a normal dose was administered for 3 months to 13 subjects as a preliminary experiment and measured changes of general clinical parameters. Mean body weight, size of waist, percentage body fat, and BMI did not show any clinical sign of illness by taking the normal dose. Thus, to precisely determine the safety of *A. brasiliensis* KA21, a dose of three times higher than the normal dose was administered for 6 months to 11 subjects, and subjective changes in conditions, liver function, renal function, and nutritional conditions were measured and analyzed. After measuring the biochemical parameters, we confirmed

no statistically significant difference before and after administration, and no side effects were caused by long-term administration.

In order to evaluate the effect of *A. brasiliensis* KA21 on immune function, NK cell number and function were examined by eight subjects in a double-blinded experimental protocol. The normal dose or placebo was administered to eight subjects for 7 days, and NK cell number and activity in peripheral blood was compared. A comparison of NK cell count before and after administration, and a comparison between the *A.brasiliensis* group and placebo group were made; no statistically significant differences were observed. Before administration, no significant differences of NK cell activity were observed between the *A. brasiliensis* group and placebo group. After administration, there were significant differences between the two groups. NK cell activity was increased significantly in *A. brasiliensis* groups. When individual cases were examined, almost all cases showed increasing NK cell activity with the administration of *A. brasiliensis*, although there were differences in the degree of increase. It is possible that polymorphism may be related to individual differences observed in the effects of *A. brasiliensis*. Research into receptors for mushroom components is not extensive. Dectin-1 was recently determined to be the receptor for cell wall beta-glucan, a major component of mushrooms. The effects of *A. brasiliensis* and receptor gene polymorphism may be related. Further analysis is necessary in the future. Through clinical research on human volunteers, we found that *A. brasiliensis* is safe and activates the immune function in mibyou patients (people with poor health).

## Slovenian Fungal Database: Boletus Informaticus

*Nikica Ogris,[1] Andrej Piltaver,[2] and Dušan Jurc[1]*

[1]Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia; [2]Institute for Systematics of Higher Fungi, Zofke Kvedrove 24, SI-1000 Ljubljana, Slovenia

Databases of fungi are of utmost importance for assessing biodiversity, identifying threatened species of fungi, and in determining a state of their populations and habitats.

The Database of fungi in Slovenia (Boletus informaticus) was established in 2004. The database included 154,935 records until the end of January 2007. In Slovenia 2,452 species of fungi are registered. The collected data on the species and distribution of fungi in Slovenia are mainly from the archives of the Mycological Association of Slovenia and the personal archives of its members, as well as, in part, from existing collections and literature sources (mostly macromycetes). Besides this, the data originate also from research work in numerous domestic and international projects and from reporting, prognostic and diagnostic services for forests, which is performed by Slovenian Forestry Institute and Slovenia Forest Service (mostly micromycetes).

The database is aimed at systematically recording species of fungi, their distribution, and information regarding their habitat. In addition, this program allows various ways of processing materials, various means of data retrieval, and cartographical presentations of finds within the grid of the Central European mapping of flora and the UTM grid. Boletus informaticus is designed within the Microsoft Access. The basic structure of Boletus informaticus was taken from the MycoRec program, created by Jerry Cooper, from the British Mycological Society. This has allowed the Boletus informaticus to remain compatible with the database Index Fungorum, which is the world's largest collection of names of fungi. Data sources are numerous. It should be mentioned that we have a long tradition of mushroom picking and, accordingly, there is also mass interest in fungi of Slovenia. The Mycological Association of Slovenia organizes excursions and traditional exhibitions of fresh fungi every autumn. Today, the Association has around 1,000 members who are organized into 20 local mycological societies. Other data sources are: taxonomic and private collections, professional literature, and local registers of fungi.

The procedure for data entry into the database follows five steps: (1) from different data sources (2) the data is entered (3) into personal database Boletus informaticus (4) that is occasionally forwarded through the subsystem of data verification (5) to the central

database, Boletus informaticus. A personal database is represented by the local database *Boletus informaticus* that is installed on the user's personal computer. Data verification is done on two levels. On the first level the data verification is done automatically by logical tests. On the second level the data verification is done by experts that are responsible for certain taxonomic groups of fungi. When the data verification is done, the verified records are stored into the central database *Boletus informaticus,* administered by the Slovenian Forestry Institute. This function is compatible with its mission for managing mycotheca on the state level.

Recently, it has come to be known as a depository for culture collections as well. Snapshot of the database on 1st October 2004 is available through the web site for the Slovenian Forestry Institute (http://www.gozdis.si/). A list of fungi and their distribution maps are available on the website. More detailed descriptions of distribution maps are found in the monograph "Fungi of Slovenia: Species and distribution," which was published in 2005 by Silva Slovenica.

---

# Cultivation and Determination of the Bromatological and Chemical Composition of the Colombian Wild Fungus, *Pleurotus cornucopiae*

*Oscar Eliseo Mejía Orozco,[1] José Henry Toro López,[1] Héctor Jairo Osorio Zuluaga,[2] Augusto Rivera Umaña,[3] Maryeimi Varón López,[4] and Susana Hernández Niño[2]*

[1]Facultad de Ciencias Agropecuarias, Universidad de Caldas, Manizales, Caldas, Colombia; [2]Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Caldas, Colombia; [3]Departamento de Química, Universidad Nacional de Colombia Sede Bogotá, Bogotá, D.C., Colombia; [4]Centro Agropecuario, Servicio Nacional de Aprendizaje SENA, Manizales, Caldas, Colombia

Despite its relatively small size, Colombia is the second most biologically diverse country on earth, home to about 10 percent of the world's species. This biological diversity results from Colombia's varied ecosystems: from the rich tropical rainforest, to the coastal cloud forests as well as to the open savannas. Colombia´s biodiversity holds great scenic and cultural value but more importantly has potential medicinal value. Although scientific research concerning the biodiversity of Colombia has a very short history, considerable knowledge has been achieved in the last 10 years, particularly in regard to species inventories and ecosystem characterizations. However, scientific knowledge concerning the potential medicinal value of this biological diversity, including the mycoflora, warrants further exploration.

In the present work we adapted the growth and development of the Colombian wild fungus, *Pleurotus cornucopiae,* to laboratory conditions on substrates of agroindustrial waste from coffee, and determined the bromatological and chemical composition of its fruiting body. The commercial *P. pulmonarius* was used as the reference strain during the cultivation cycle and for both the bromatological and chemical analyses. The main goal of this work is to evaluate the potential nutritional and medicinal values of this Colombian wild fungus.

Our results showed a biological efficiency of 28% for the wild strain, *P. cornucopiae,* compared to 65% obtained for the commercial *P. pulmonarius*. The fruiting bodies of both *Pleurotus* strains were harvested and used for bromatological analysis. A protein content of 40.30% was found in *P. cornucopiae,* compared to 34.01% determined in *P. pulmonarius*. In this study gas chromatography-mass spectrometry, after ethanol extraction of the fruiting body tissue, was used for chemical analysis. Gas chromatography-mass spectrometry revealed the presence of caffeine, palmitic acid, linoleic acid, and ergosterol as the most abundant chemical compounds in the wild strain, *P. cornucopiae*. A notable similarity in the fruiting bodies chemical composition for both cultured *Pleurotus* species was also established by gas chromatography-mass spectrometry.

# Development of New Antithrombotic Substance from Mushrooms

*Jeong Sik Park,[1] Won Sik Kong,[1] Kab Yeul Jang,[1] Chang-Hyun You,[2] and Jong Soo Lee[3]*

[1]Applied Microbiology Division, Agricultural Sciences and Technology Institute, R.D.A. Suweon 441-707, South Korea; [2]Forest Mushroom Research Center, National Forestry Cooperatives Federation, Yeoju 469-803, South Korea; [3]Department of Genetic Engineering and Bio-medicinal Resource Research Center, Paichai Univ. Daejeon 302-735, South Korea

Nowadays, mushrooms have become an attractive source of physiologically active compounds. They are typically used as food and food flavoring substances, and also as traditional oriental medicines. Their extracts have been reported to exert hematological, antiviral, antitumor, hypotensive, and hepatoprotective effects. Mushrooms also constitute an important source of thrombolytic agents. Korean traditional anecdotes suggest that mushrooms can be, and have been, used in the treatment and prevention of thrombosis. Some reports have described the fibrinolytic activity of certain edible mushrooms including *Flammulina velutipes*, *Pleurotus ostreatus*, *Grifola frondosa,* and *Armillaria mellea*.

We have attempted to find fibrinolytic substances in mushrooms. In this study we describe the selection and characterization of a fibrinolytic substance from 55 mushroom strains. Several extracts of mushrooms were prepared and fibrinolytic activity and platelet aggregation inhibitory activity were investigated. We used mushroom mycelia from the Culture Collection of National Institute of Agricultural Science and Technology. Fruiting bodies were used in this study, except for *Sparassis crispa* ASI 150006, 150010, 150011, and 150016, *Inonotus obliquus* ASI 74006–74013, and *Agaricus blazei* ASI 1174, which were used as mycelia. Platelet aggregation inhibitor from the selected mushroom was purified by solvent extraction, ultrafiltration, Sephadex G-10 filtration, and RP-HPLC, and then characteristics were investigated. Among 52 sample mushrooms, the ethanol extracts of *I. obliquus* ASI 74006 mycelia had potential plate inhibitory activity of 81.2%. After the purification of the plate aggregation inhibitor, an active fraction with 91.6% inhibitory activity was obtained. The purified plate aggregation inhibitor was a novel peptide with 314 Da of molecular weight. An antithrombotic mushroom drink was also prepared and characterized. A formula for an antihypertensive drink made by extracts of *I. obliquus* contained mushroom mycelia extract (300 mg), *Schisandra chinensis* extract (500 mg), fructose (4,000 mg), sucrose (7,000 mg), and small quantities of vitamins in 50 ml volume. Physiochemical properties of *I. obliquus* drink showed that the pH was 4.1, Bx 16.5°, turbidity at 660 nm absorbance was 81.3, and color at 430 nm absorbance was 1.17. Sensory evaluation of antithrombotic drink made by *I. obliquus* showed good acceptability and mushroom flavor. In conclusion, the fibrinolytic substance we obtained from the medicinal mushroom *I. obliquus* exhibited profound fibrinolytic activity and can be used for making commercial drink products. It may be useful for thrombolytic therapy to develop other similar potent fibrinolytic substances, such as nattokinase and earthworm enzyme. These substances could provide an adjunct to the costly fibrinolytic substances that are currently used for managing heart disease since large quantities can be conveniently and efficiently produced. Therefore, *I. obliquus* may become a new source of fibrinolytic substances for future applications. Lastly, further studies are necessary to elucidate its molecular biological characteristics as well as its medicinal applications.

# Comparison of the *Pleurotus ostreatus* Growth Rates on Beech and Aspen Stumps

*Martin Pavlík*

Department of Forest Protection and Game Management, Technical University in Zvolen, Zvolen, Slovak Republic

The oyster mushroom *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. is a native, commonly widespread mushroom in Slovak forests, and is important as a decomposer of hard and soft wood of various deciduous trees.

The growth of this mushroom has not had a long tradition in Slovakia. The great expansion in the growth of oyster mushrooms started in the 70s of the last century and various kinds of wood fractions and waste biological substrates from agricultural production were used. In the second part of the eighties, it was the boom of oyster mushroom growing, but in the nineties disappeared nearly all mushroom producing facilities. Since the beginning of the 21st century interest in oyster mushrooms and its cultivation has returned.

Growing of oyster mushroom on woody stumps does aim neither fast production nor continual production of fruit bodies. However, it could be a perspective mushroom for cultivation in forest management conditions. Plenty of wooden waste materials left from the forestry production, and it is not possible to use them effectively. Thin branches, broken pieces of trunks, and tree stumps are leaving after cutting, representing a source of organic matter in the soil. This process of decomposition is very slow and such wooden pieces are often a barrier for another forest growing.

The oyster mushroom fruit bodies grown on original substrate – wood are usually of a very good quality and taste. The ecologically clear and natural production is interesting especially for forest and wood owners.

On the basis of previous results, the present work is oriented to the observation of production rate of oyster mushrooms on the most suitable wood species – beech (*Fagus sylvatica)* and aspen *(Populus tremula)*. The production of fruit bodies on wood stumps is compared according to the biological efficiency. The actual results of the 3-year-growth are very good with regard to the economic profits of this kind of oyster mushroom growing.

# Some Medicinal and Other Cultivated Mushrooms in Thailand

*Ahchara Payapanon,[1] Leka Manoch,[2] Satit Thaitatkul,[3] Pramote Thaitatkul,[3] Boonchok Thaitatkul,[3] Onuma Jeamjitt,[2] and Tida Dethoup[2]*

[1]Department of Agriculture, Ministry of Agriculture & Co-operative, Bangkok 10900, Thailand; [2]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand; [3]Arunyik Mushroom Research Center Co., Ltd, 3/1 Moo 8, Phutthamonthon 4 Road, Kratumlom, Sampran, Nakornpathom 73220, Thailand

The purpose of this research was to study the diversity, distribution, and techniques for cultivation of some economically important tropical mushrooms. Thailand is a tropical country in Southeast Asia where several species of economically cultural mushrooms have been produced. *Ganoderma lucidum* is the most popular and expensive medicinal mushroom followed by *Macrolepiota gracilenta, Lentinula edodes, Hericium erinaceus, Schizophyllum commune, Lentinula polychrous,* and *L. squarrosulus*. Other cultivated mushrooms in Thailand include *Agaricus bisporus, Agrocybe cylindracea, Auricularia polytricha, Coprinus cinereus, Flammulina velutipes*, *Ganoderma lucidum, Hericium erinaceus, Lentinula sterigosus, Macrolepiota gracilenta, Macrocybe crassum, Pleurotus cystidiosus, P. eryngii, P. ostreatus, P. sapidus, P. sajor-caju,* and *Volvariella volvacea*. The success of mushroom cultivation in Thailand is due to

high-consumer demand domestically as well as to an increase in industrial use. In addition, there are sufficient available varieties of agricultural wastes suitable as mushroom growing substrates such as rice straw, pararubber sawdust, water hyacinth, mungbean stem and pod, sorghum seed, tapioca waste, oil palm waste, etc. Mushroom cultivation technology has been continuously developed and improved through multidisciplinary research and development activities. Local people consume various kinds of mushrooms for their meals, and cultivated mushrooms are popular as a healthy, nutritive food.

One interesting mushroom, *Oudemansiella canarii,* was first found on a log of pararubber at Pikulthong Research Centre in Narathivas province in Southern Thailand. Later on, it was found in Tak province in the north. This mushroom belongs to the Class Hymenomycetes, Order Agaricales, Family Tricholomataceae. It can be found in the temperate and tropical regions, but is more commonly found in the tropic. *O. canarii* from Narathivas province produces a cap 2–8 cm in diam, brownish black in the center and pale brown at the margin, slimy surface; stipe white, 6–8 cm long; gill white at maturity; spore hyaline, globose with refractive guttules and hilum, smooth, wall with thickening, 15–18 × 14–16 μm; pleurocystidia 100–212 × 7–44 μm; hypha with clamp connection, edible. There are slight differences between the two strains. For the Narathivas strain, the cap is more reticulate, wrinkled, darker color at the center; stipe straight, harder, white whereas the strain from Tak produces non-reticulate, dark cap with white scale; stipe shorter, stout, grey to dark color. *O. radicata* from Loei province, northeastern Thailand was found from soil under the decay log. The cap is pale brown with thin margin, slightly wrinkled; gill white to cream with brown or dark brown margin; stipe long, blackish brown; spore white, oval, smooth, thick wall, with one to several oil guttules, 10–20 × 9–12 μm; spore print white; hypha with clamp connection, edible.

For isolation technique of *O. canarii*, the inner tissues from the cap and stipe were transferred onto potato dextrose agar (PDA) and incubated for 4 days at 28–35°C. The colonies produced white mycelium with basidiocarps formation on agar media. For inoculum production, the mycelium was transferred to autoclaved sorghum seeds in a bottle and incubated for 2 weeks. The mycelia were transferred to 200 gm autoclaved sawdust media with nutrition in a 250 ml Erlenmeyer flask and incubated for 2 weeks. White mycelia and basidiocarps were produced.

For mass production, the mycelium from 15–20 sorghum seeds was transferred to 800 gm sawdust media with nutrition in a plastic bag and incubated at 28–30°C for 3–4 weeks. White mycelium was formed throughout the media, and the basidiocarps were developed. Cultivation of other edible mushrooms as well as the maintenance for pure cultures of mushroom strains in the culture collection has been reported.

---

# Inhibitory Effects of *Marasmius oreades* Extract Fractions on NF-κB Activation Pathway

*Roumyana D. Petrova,[1,2,3] Nili Ruimi,[2] Sherbel Sussan,[2] Solomon P. Wasser,[1] Eviatar Nevo,[1] Abraham Z. Reznick,[3] and Jamal A. Mahajna[2]*

[1]International Center for Biotechnology and Biodiversity of Fungi, Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel; [2]Migal-Galilee Technology Center, South Industrial Zone, Kiryat Shmona, Israel; [3]Faculty of Medicine, Technion, Haifa, Israel

*Marasmius oreades* (Bolton: Fr.) Fr. is a common edible mushroom known to produce biologically active metabolites, such as agrocybin and drimane sesquiterpenes. Other representatives of the same genus, i.e., *Marasmius conigenus, M. ramealis,* and *M. scorodonius* were found to exhibit antimicrobial, antifungal, phytotoxic, and mutagenic activities, which are based on the production of sesquiterpenes and other bioactive compounds. However, no data on the biological and medicinal properties of *M. oreades* was reported.

Our study demonstrated that this species possesses a high medicinal potential due to the modulation of the nuclear factor-kappa B (NF-κB) activation pathway. NF-κB is a nuclear transcription factor with a major role in tumorogenesis, realized through several mechanisms such as the promotion of cell proliferation, inhibition of apoptosis, and increasing tumor metastasis and angiogenesis.

Previously, we found out and reported that a crude ethyl acetate extract of *M. oreades* culture liquid strongly inhibited the NF-κB reporter activity in MCF7 breast cancer cell line, carrying a luciferase reporter gene under the control of NF-κB-responsive promoter. Also, the crude extract showed an anti-proliferative potential and affected the phosphorylation of the inhibitory protein kappa B (IκBα). IκBα is a crucial protein that maintains NF-κB in an inactive form in the cytoplasm. Only after the phosphorylation of IκBα, NF-κB is free to enter the nucleus where transcriptional activation of target genes begins. In an attempt to isolate active fractions of *M. oreades* we followed the ability of fractions to inhibit expression of some NF-κB responsive genes, such as iNOS. The chemical fractionations were carried out by liquid chromatography. The effects of these fractions on IκBα phosphorylation were evaluated using Western blotting. It was found that two out of four fractions strongly inhibited the phosphorylation of IκBα in a dose-dependent manner. In addition, effects of fractions on iNOS expression were also evaluated. The two active fractions were combined and subjected to an additional fractionation in order to receive a pure active fraction.

As a result, five secondary fractions were obtained and also tested for their inhibitory effects on IκBa phosphorylation. In addition, the effects of these fractions on the expression of NF-κB responsive gene, iNOS, were evaluated. The present study demonstrates that *M. oreades* is a promising source of natural bioactive metabolites that can modulate the NF-κB activation pathway. This species possesses valuable medicinal properties and can be possibly applied in cancer treatment by modulating NF-κB activation.

---

# Recent Advances in the Applied Research, Human Health-Related Properties, and Commercial Cultivation of Selected Medicinal Mushrooms Grown in Greece

*Antonios Philippoussis,[1] George Zervakis,[2] Panagiota Diamantopoulou,[1] Panagiotis Katsaris,[2] Demetrios Kletsas,[3] Eleftherios Lahouvaris,[4] Athanasios Mastrogiannis,[4] and Cleathes Israilides[5]*

[1]National Agricultural Research Foundation, Institute of Agricultural Engineering, Laboratory of Edible Fungi, 61 Democratias St., 13561 Ag. Anargyri, Athens, Greece; [2]National Agricultural Research Foundation, Institute of Kalamata, 85 Lakonikis St., 241 00 Kalamata, Greece; [3]National Center of Scientific Research "Demokritos", Institute of Biology, Laboratory of Cell Proliferation & Ageing N.C.S.R. "Demokritos", 153 Athens, Greece; [4]Hellenic Mushroom Substrate Co., Kathenoi Dirfys, 34 0 18 Euboea, Greece; [5]National Agricultural Research Foundation, Institute of Technology of Agricultural Products, Biotechnology Laboratory, 1 Sof. Venizelou St., 14123 Lycovrissi, Athens, Greece

For more than 15 years the authors have been involved in applied research work and cultivation of various edible mushrooms, especially those belonging to the genera *Agaricus*, *Pleurotus*, *Agrocybe*, *Volvariella*, *Auricularia*, etc.

Recently, research activities have focused on the cultivation of the medicinal mushrooms *Lentinus edodes* and *Panaeolus cyanescens*, both with no prior history of cultivation in Greece. Experiments mainly dealt with the valorization of several low-value lignocellulosic residues as growing substrates, and with the understanding of their impact on mushroom productivity and quality.

Concerning *P. cyanescens*, the effects of $CaCO_3$ supplementation to the wheat straw substrate, as well as of the casing additives on basidiomata production were studied. The comparison of growth rates on wheat straw substrate supplemented or not with $CaCO_3$ showed faster colonization in the latter case. Moreover, the casing layer, consisting of peat, beton-

ite, and $CaCO_3$, promoted fructification. In the case of *Lentinus edodes*, experiments were conducted to evaluate the influence of substrate type (wheat straw, corn cobs, cotton wastes, etc., examined in comparison to standard oak-wood sawdust) on mycelium colonization rate and mushroom yield. Mycelium growth measurements conducted in glass tubes demonstrated faster colonization of oak sawdust and wheat straw substrates (4.8 and 4.3 mm/day, respectively). Furthermore, wheat straw appeared to promote early fructification (52–55 days after inoculation) and mushroom quality, while corn cobs favored productivity (80.64 BE%). Correlation analysis showed that carbon and nitrogen content of substrates affected both earliness of fructi-fication and productivity. In fact, there was a strong negative correlation between mushroom yield (mushroom number and BE%) and C/N ratio of the substrates. Finally, the impact of different substrates on the quality and nitrogen content of *L. edodes* basidiomata used as a nutritional/pharmaceutical and dietary supplement was established.

In addition, *in vitro* evaluation of the cytostatic and immunomodulatory properties of *L. edodes*, demonstrated the cytostatic effects of mycelia and mushroom extracts on MCF-7 human breast cancer cell line. As a result of these research activities, along with the efforts of pioneer mushroom growers, *L. edodes* commercial cultivation on hard-wood logs and bag-logs was recently introduced in Greece, laying the foundations of a developing medicinal mushroom industry.

## Antiproliferative and Adhesion Effects of Lectins from Mushroom *Clitocybe nebularis* on Human Cancer Cell Lines

*Jure Pohleven,[1,2] Nataša Obermajer,[2] Bogdan Kralj,[3] Janko Kos,[2] Borut Štrukelj,[1,2] and Jože Brzin[1]*

[1]Department of Biotechnology, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia; [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Ljubljana, Slovenia; [3]Mass Spectrometry Centre, Department of Environmental Chemistry, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

*Clitocybe nebularis* (Clouded agaric) is a mycorrhizal basidiomycete fungus shown to have strong insecticidal effects on the Colorado potato beetle (*Leptinotarsa decemlineata*) and fruit fly (*Drosophila melanogaster*). It was suggested that the effects occurred due to the activity of proteins, presumably lectins. In this study we isolated and biochemically characterized several lectins from fruiting bodies of fungus *C. nebularis* and studied their effects on proliferation and adhesion of human cancer cell lines.

Lectins are a diverse group of non-homologous proteins found in humans, animals, plants, fungi, and microorganisms. These proteins bind specifically to different types of carbohydrates (mono-, di-, and oligosaccharides). Most lectins are multivalent, containing more than one carbohydrate-binding site; therefore, they can cross-link cell surface carbohydrates and agglutinate cells. Their biological activity is exerted by binding to glycoconjugates on cell surfaces, extracellular matrix, and cytoplasmic or nuclear glycoproteins. Lectins elicit many physiological effects in different organisms and are suggested as having many biological functions, which are still not fully understood.

Galectins are a family of homologous lectins that are related in amino acid sequence and bind specifically to β-galactoside carbohydrates, such as lactose. Galectins have been found in humans, animals, and also in fungi. The evolutionary conservation of galectins probably reflects the roles of galectins in cellular processes essential for the development and function of multi-cellular organisms including adhesion, migration, differentiation, proliferation, and apoptosis.

After homogenization of *C. nebularis* fruiting bodies, the crude extract was applied to affinity chromatography columns with glucose, galactose,

lactose, and sucrose, respectively, immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Isolated lectins were purified using high performance liquid chromatography (HPLC) and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular masses were determined by mass spectrometry. The lectins were shown to be oligomeric by gel filtration fast performance liquid chromatography (FPLC) and native gel electrophoresis. N-terminal sequences were determined by Procise Protein Sequencing System 492 (PE Applied Biosystems, Foster City, CA, USA) and show no similarities to any protein in the databases. Effects on proliferation and adhesion were studied on four human cell lines – human breast epithelial tumor cell line (MCF-10A NeoT), human T leukaemia cell line (Mo-T), and differentiated and undifferentiated human monocytic lymphoma cell line (U-937).

Lactose-specific lectins had an antiproliferative effect on the human T leukaemia cell line and galactose-specific lectins stimulated adhesion of differentiated human monocytic lymphoma cells. Because of their antiproliferative effect on leukaemia cells, these lactose-specific lectins, from mushroom *C. nebularis*, have potential use in treating hematopoietic malignancies. In further studies we aim to determine full nucleotide and amino acid sequences of the lactose-specific lectin.

---

## Lipid Profile and Effect of *Agaricus bisporus* as Protective Factors in Experimental Hypercholesterolemia

*Goundappan K. Poongkodi*

Medwin Hospitals Academic Block, Medwin Hospitals, Nampally, Hyderabad 500 001, Andra Pradesh, South India

The objective of relevance of mushroom use in the diet proved to be very effective in bringing down the lipid levels in experimental animals. Clinical intervention studies have demonstrated the therapeutic importance of correcting hypercholesterolemia. The initial step in lowering cholesterol is a special diet low in fat and saturated fatty acids and rich in crude fibers. The role and function of *Agaricus bisporus* in the atherodiet inducing hypercholesterolemia is discussed.

Male albino rats of the Wistar strain, with an initial body weight of about 265–275 gm, were used for this study. The animals were divided into four groups consisting of six rats per group. Group I served as controls to obtain the base line data on biochemical parameters (100 gm). Group II received mushroom powder of 5% by oral feeding (fruit bodies and stipes 3:1 ratio). This 5% is based on 5 g mushroom powder and 95 g normal feed. Group III consisted of hypercholesterolemic rats in which hypercholesterolemia was induced by the following diet, to be referred to as the atherogenic diet (atherodiet) and was fed to the animals. It was prepared by mixing the commercial pelleted feed with the following ingredients: cholesterol (5%), sucrose (20%), hydrogenated vegetable oil (20%), lactose (2%), choline chloride (0.2%), sodium cholate (0.4%), 2 - thiouracil (0.15%) [47.75%], and remaining normal pelleted feed (52.25%). Group IV consisted of HFD and 5% Dry Mushroom Powder Fed Rats (47.75% + 5.0% + 47.25%) as above.

At the initial stage, 5th, 10th, and at the end of the 15th week, the rats were anaesthetized by ether inhalation and cardiac puncture subsequent to blood collection from the sinuous venosus since whole blood samples with EDTA as the anticoagulant and centrifuged for 10 min for separation of plasma. Brain, aorta, heart, liver, and small intestine were dissected out. The faecal matter collected was dried and powdered for experimental purpose and the biochemical parameters were analyzed.

Soon after sacrificing a portion of the tissues, e.g., aorta, heart, liver, kidney, small intestine, spleen, and brain were kept in formalin for about 2 weeks for fixing of the tissues. The sections were viewed under Polarised Photo Microscope for histopathological changes in the tissues.

Adult albino rats with plasma cholesterol about 129 mg/dL were used to induce experimental hypercholesterolemia by altering the dietary pattern. The dietary alterations included addition of cholesterol, saturated fats, bile salts, cholic acid, and thyroid suppressant thiouracil. The animals developed atherosclerosis in 105 days, which can be seen by the development of fatty streaks,

fibrous plaque, and less lipid accumulation in the intima and media. MR was administered along with the high-fat diet and the plasma cholesterol, triglyceride and free fatty acid, free cholesterol, and ester cholesterol levels were monitored. In animals receiving cholesterol, fat and other supplements along with MR and the plasma lipid profile were lowered significantly.

The activity of lipoprotein lipase in plasma was decreased in all experimental groups when compared with their healthy counterparts. Further activities of VLDL CH, VLDL TG, LDL CH, LDL TG, and HDL TG in plasma were marginally elevated in the atherosclerotic animals, whereas only HDL CH levels registered a significant decline. These elevated levels were brought down significantly in MR treated groups and a slight increase was noticed in HDL CH of the MR treated group. A mild reduction was observed in MR treated animals.

The concentration of lipid profile such as cholesterol, triglyceride, and phospholipid in RBC membrane were significantly increased in atherosclerotic rats. As a consequence of mushroom therapy for 105 days, a significant reduction in these levels was observed. Therefore, it is clear that the lipoprotein intrinsic factors that regulate the transfer of MR from lipoproteins to cells reduced the lipid profiles in RBC. In tissues such as brain, aorta, liver, and intestine the lipid profiles were increased in an HF diet fed animals. One of the mechanisms by which the plasma lipid lowering action of MR was effected was identified as the capacity to stimulate greater elimination of faecal cholesterol, and cholic and deoxycholic acid. The possibility of the treatment interfering with the enterohepatic recirculation of cholesterol and bile acids is also discussed. At the end of the experiments, histological evaluation of all animals revealed infiltration of the intima and proliferation of the endothelial cells with sporadic vacuolization of the cytoplasm (aorta), infiltration of the branches of coronary arteries and centrolobular degeneration of hepatocytes, and small and large drop fatty degeneration of the liver. The degree and range of these changes were smaller in rats fed with the mushroom diet than in rats fed with the control diet. Considering the results of the histological studies on brain, aorta, heart, liver, kidney, small intestine, and spleen, it is evident that the treatment with MR on HFD fed rats expresses their individual remedial impact.

---

# The Activity of Certain Medicinal Mushrooms after Light Influences

*Natalya L. Poyedinok,[1] Asya S. Buchalo,[1] Oksana B. Mykchaylova,[1] and Anatoliy M. Negriyko[2,4]*

[1]M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev, GSP-1, 01601, Ukraine; [2]Institute of Physics, National Academy of Sciences of Ukraine, 46 Nauki Prosp., 01022, Ukraine

It was proved that light is one of the main morphogenetic factors for growth and development of many cultivated mushrooms. Up to now, no practical uses were found for light in the biotechnologies of industrial cultivation of medicinal macromycetes and the mechanism of photoreception in mycelium and fruiting bodies has not been sufficiently studied.

As our studies and works of other scientists show, mushroom reaction to the influence of light is not the same. It was discovered that mushrooms have a regulatory system, called mycochrome by analogy with higher plants' phytochrome system. Today, mycochrome systems have been found in representatives of different taxonomic groups: Ascomycetes, Basidiomycetes, and Deuteromycetes. The overwhelming majority of mushrooms in which the presence of mycochrome systems was ascertained have pigments of carotinoid and melanin nature.

We research the influence of low-intensive visible light of different wavelengths (coherent and noncoherent) on the growth and biosynthetic activity of different species of higher basidial mushrooms, possessing medicinal characteristics. Among them, particular attention belongs to pigment-containing strains of *Inonotus obliquus* and *Laetiporus sulphureus.* We investigated the effect of light irradiation

in the visual part of the spectrum on *Ganolerma lucidum, Inonotus îbliquus, Pleurotus ostereatus, Lentinus edodes,* and *Hericium erinaceus* production, and determined that light treatment accelerated mycelial growth, shortened the phases of mushroom development, produced more vigorous mycelium, and increased the fruit body yields (36%–51%). The activation of sowing mycelium by means of red and blue light irradiation makes it possible to reduce the dose necessary for substrate inoculation twofold. Nowadays, researchers' and elaborators' efforts are aimed at searching for better scientific and technical solutions in considering higher economic indicators when obtaining medicinal mushroom biomass and at creating competitive pharmacological preparations and remedies. The method of submerged cultivation of medicinal mushrooms is the essence of this task. It was shown that light irradiation also stimulated the mycelial growth in submerged culture and enlarged the accumulation of biomass twofold. When analyzing the dynamics of biomass accumulation, we discovered that irradiation of sowing mycelium leads to the reduction of the lag phase and to the increase in the speed of culture growth. At the present stage of research, one cannot unequivocally affirm the advantages of coherent or non-coherent light. One may only assert that there is positive influence of light on the growth and biomass accumulation of studied mushrooms in red and blue parts of the spectrum. However, the results of our experiments allow us to state that pulsed light is biologically more active. The research of the influence of light with different wavelengths on the biosynthesis of polysaccharides, melanin, and carotene pigments was carried out. Irradiation of *Ganodrma lucidum, Lentinus edodes,* and *Hericium erinaceus* sowing mycelium with blue and red light increased polysaccharide accumulation by 40%–64%. The maximum melanin accumulation by *Inonotus îbliquus* was observed under the influence of blue light – 10.5 g/l (in control – 6.05 g/l), and the maximum of biomass increase and carotene pigments accumulation by *Laetiporus sulphureus* – under red light irradiation. The influence of low-intensity laser light with a wavelength of 633 HM (He-Ne laser) on antibiotic activity of *Pleurotus ostreatus* under submerged cultivation was investigated. Antibiotic activities of extracts obtained from mycelium and cultural liquid, against *Micrococcus luteus, Staphyloccocus aureus,* and *Bacillus mycoides* increased by 10%–20%.

The obtained results prove the prospects of the use of light to increase biological activity of investigated mushrooms. The next stage of research may consist of the study of light activity mechanisms, selection of effective wavelengths, and optimization of irradiation regimes, to determine its influence on synthesis and composition of synthesized substances.

---

# A Newly Recorded *Amauroderma subresinosum* from National Park of Cat Tien, Vietnam

*Dang Ngoc Quang,[1] Le Xuan Tham,[2] Le Thi Hoang Yen,[3] and Duong Van Hop[3]*

[1]Faculty of Chemistry, Hanoi University of Education, 136-XuanThuy road, Cau Giay, Hanoi, Vietnam; [2]Vietnam Atomic Energy Commission (VAEC), Center for Nuclear Techniques, HoChiMinh City, Vietnam; [3]Center of Biotechnology, National University of Hanoi

Based on morphological characters of fruit bodies, there are controversial arguments on the treatments of taxonomy of a rare Black Lingzhi fungus, either *Ganoderma subresinosum*, *Magoderna subresinosum*, *Trachyderma subresinosum,* or *Amauroderma subresinosum*, based on *Fomes subresinosum.* We determined that the latter point of view is sufficiently reasonable with authentic specimens just collected in National Park of Cat tien (~80.000 ha), South Vietnam.

In the present study, characteristics in basidiospore structures are clearly shown, typically intermediate between amaurodermoid and ganodermoid forms: subglobose with minimized or non-aperture (germpore) and thin double wall, but with a hillar on the bottom (attachment to sterigma on basidia). Basidiocarps annual to perennial, sessile, corky to woody, sometimes substipitate, up to 3.5–16.5 × 1–3 cm. Upper surface black, sublaccate, the context quite white, whitish later becoming faintly pinkish brown,

and the layer of tubes quite whitish yellow or cinnamon. Pore surface is whitish and becoming whitish grey. Small spores (very rare) are colorless, unitunicate, and quite subglobose. This interesting species was shown as an intermediate form between genera *Ganoderma* and *Amauroderma* with some transient taxa significant in phylogeny, found in South China, India, Sri Lanka, Southeast Asian countries, Papua New Guinea, and East Africa.

Our investigations on chemical compositions of the fruit bodies of *A. subresinosum* has led to the isolation and structural elucidation of five fatty acids, pentadecanoic acid (4%)(**1**), 14-methyl pentadecanoic acid (24%)(**2**), 9,12-octadecadienoic acid (19%)(**3**), 9-octadecenoic acid (42%)(**4**), and octadecanoic acid (10%)(**5**), together with a mixture of three sterol esters (**6–8**), sugars and deficiency of triterpenoids (see Fig. 1). These results support a new and further taxonomy of the Ganodermataceae family, in which *A. subresinosum* is designated in the distinct genus *Amauroderma*, isolated from *Ganoderma*, due to mainly lacking derivatives of triterpenoids, which are commonly elaborated from *Ganoderma* sp., particularly in *G. lucidum*.

Molecular examinations of rDNA with D1, D2 regions (26S) have revealed that this taxon is separated from the *Ganoderma* sp. The sequences of rDNA 26S showed similarity of 98% (628/641) to that of *G. lucidum* and *Fomes fomentarius* (630/641). Thus this taxon should be one of species placed in the genus *Amauroderma*.

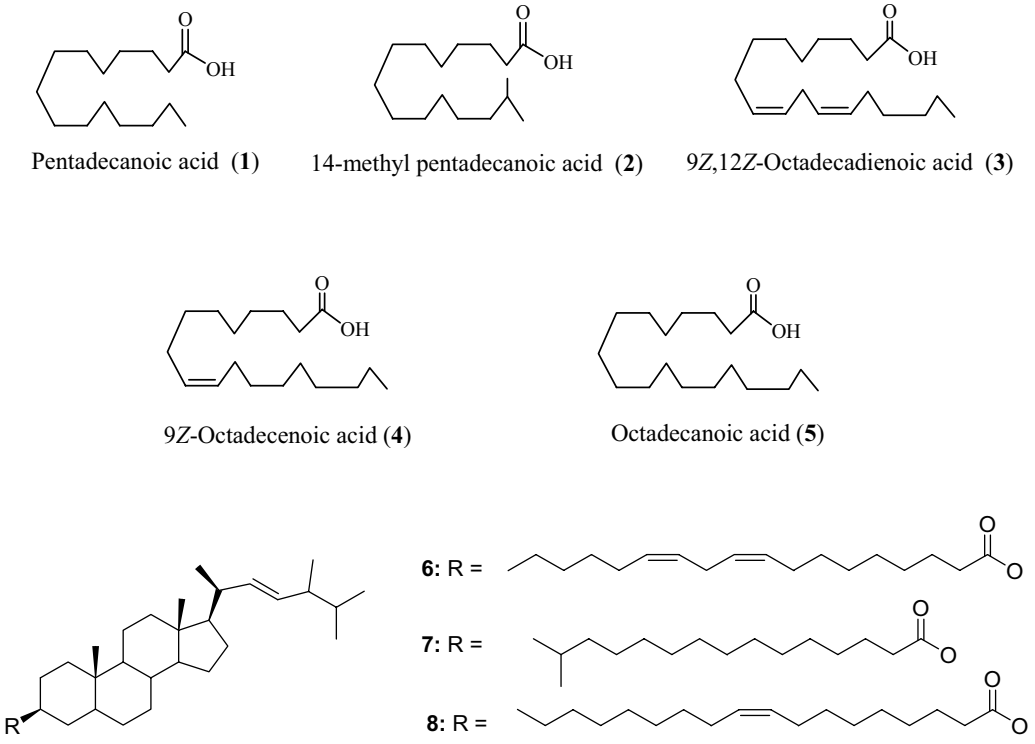



**FIGURE 1.** Fatty acids isolated from the fruit bodies of *A. subresinosum.*

# Anti-Insect Activity of *Ganoderma lucidum* Extract Against *Tribolium castaneum*

*Hossein Riahi and Fatema Sargazi*

Department of Biology, Shahid Beheshti University, Evin, Tehran

The protection of agricultural products in storage against attack by pests is essential but application of chemical pesticides may not be sufficiently affective since insects develop resistance to them. Some fungi have been tested to control insects.

Since ancient times, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Polyporaceae, Basidiomycetes), has been considered "the King of Herbs". *Ganoderma lucidum*, as one of the most famous traditional Chinese medicinal herbs, is used as a health food and medicine in the Far East for more than 2000 years.

Mushroom culture used in this study was obtained from edible fungi in the Institute of Shanghai in China. Sawdust, wheat bran, and $CaCO_3$ were used for the preparation of substrates.

Fresh fruiting bodies of this mushroom were washed, disintegrated, and dried in room temperature, then extracted with ethanol and water-ethanol (50:50 v/v) at room temperature for 14 days. The extract was dried by rotary evaporation at 40ºC, and the residue was weighed.

Larvae, pupaes, and adults of *T. castaneum* were obtained from cultures of the same age and kept on artificial diets of white wheat flour and beer yeast (95: 5) at a constant temperature of 30ºC, 70% relative humidity, and in darkness (24 h) in the laboratory.

Ethanol extract from *Ganoderma lucidum* was screened for anti-insect activity using adults and larvaes of *T. castaneum* Herbst. (Tenebrionidae).

In a test using the filter paper diffusion method at 3.5 mg/cm², ethanol extract of this mushroom produced 10% mortality in adults (7 days old) and larvae (3 days old) of *T. castaneum* within 5 days.

After drying, the water-ethanol extract of *G. lucidum* was sprayed in different concentrations (10, 25, 50 mg of drying extract/ml of water), to all of them (3-day-old larvae and 7-day-old adults). The total mortality was observed in different hours.

After exposure, there was a significant difference ($p < 0.01$) in insecticidal activity between different concentrations of the mushroom extract. A concentration of 50 mg/mL was more effective on the mortality of the larvae and adults of *T. castaneum*.

# Production of Cytokines and Cytotoxic Mediators by Peritoneal Macrophages in Mice on a *Ganoderma lucidum* Supplemented Diet

*Rosália Rubel,[1] Herta Sutz Dalla Santa,[1,2] Sergio Ricardo Bello,[3] Sandro Bonatto,[3] Luiz Cláudio Fernandes,[3] Raffaello Di Bernardi,[4] Juliana Gern,[1] Adenise L. Woiciechoski,[1] Fan Leifa,[1,5] and Carlos Ricardo Soccol[1]*

[1]Bioprocess Engineering and Biotechnology Division, University Federal of Paraná (UFPR), Paraná, Brazil; [2]Department of Food Engineering, Unicentro, Guarapuava, Paraná, Brazil; [3]Department of Physiology, UFPR, Paraná, Brazil; [4]Department of Cellular Biology, UFPR, Paraná, Brazil; [5]Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

The present work investigates some alterations in the production of cytokines and cytotoxic mediators from peritoneal macrophages obtained from mice put on a diet supplemented with *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. mycelium produced by solid-state cultivation.

Activated macrophages play a key role in host defense against intracellular parasitic microorgan-

isms and tumor cells, producing more than one hundred different substances such as cytokines, reactive oxygen species (superoxide anion, hydrogen peroxide), and several other chemical mediators (e.g., nitric oxide). *G. lucidum* was cultivated by solid-state fermentation in wheat grains at 30ºC, 90% air relative humidity, during 18 days. After this, the fermented material was dried out (55ºC), milled (particles <2 mm), and supplemented with lipids (soy oil) and proteins (egg albumin) to provide for their nutritional needs. Samples of the fermented material were analyzed by ergosterol content for fungi biomass estimation. The obtained product was used in the preparation of the G10 diet, which contained 10% of *G. lucidum* mycelium. Fourteen male Swiss mice (*Mus musculus*) - healthy, 35 days old (± 3), weighing 18.85 g (± 1.25) - were divided into two groups (7 per group) and labeled G10 and Control, according to the diet they were on. The control group received only the ordinary diet, without *G. lucidum* mycelium. The mice were kept in polycarbonate cages under controlled conditions of temperature (24 ± 2ºC) and relative humidity (55 ± 10%), with a 12 h/12 h light-dark cycle. During 12 weeks, the animals were fed the respective diets and received water *ad libitum*. At the end of the 12th week, the mice were killed through ethereal inhalation and resident macrophages were obtained by intraperitoneal lavage with 5 mL of sterile phosphate buffered saline (PBS, pH 7.2). A pool of macrophages was obtained from 4 or 5 animals. This peritoneal cells suspension was centrifuged (1000 rpm for 5 min, at 4ºC), the pellet was resuspended in PBS or RPMI medium, and the counting of viable cells was performed using Trypan blue solution (1%). The macrophages were isolated from other peritoneal cells by incubating them in tissue culture plates for 2 h (37ºC); subsequently, the non-adherent cells were removed by washing with PBS. In the supernatant of the culture medium, the following cytokines were investigated: IL-12p70, MCP-1 (Monocyte chemoattractant protein), TNF-α, IL-10, IL-6, and IFN-γ concentration, using a flow cytometry FACScalibur and CELL Quest software (BD Biosciences). Then, the production of the following cytotoxic mediators was assessed: superoxide anion, hydrogen peroxide, nitric oxide (NO), lysosomal volume, and phagocytosis. The production of phagocytosis was measured by the reaction of the macrophages with neutral-red stained zymosan; the lysosomal volume through uptake of the cationic dye neutral red. The superoxide anion production was estimated by the nitroblue tetrazolium (NBT) reduction assay, the hydrogen peroxide was measured by reaction with horseradish peroxidase (HRPO), and NO production was calculated by nitrite concentration, which was measured by Griess's reaction. Peritoneal macrophages from mice on the G. *lucidum* mycelium supplemented diet showed alterations in the cytokines release. There was an increase of IFN-γ concentration ($p < 0.05$ *vs.* control group), and a decrease of TNF-α concentration ($p < 0.05$ *vs.* control group). The other cytokines analyzed (MCP-1, IL-12p70, IL-10, and IL-6) remained unchanged in relation to controls ($p > 0.05$). NO production was significantly reduced by peritoneal macrophages from mice on a *G. lucidum* mycelium supplemented diet, either with or without lipopolysaccharide (LPS), when compared to controls ($p <0.01$ *vs.* control). Superoxide anion, hydrogen peroxide, lysosomal volume, and phagocytosis remained unchanged ($p > 0.05$ *vs*. control).

In the present study *G. lucidum* showed both a pro-inflammatory effect (arising IFN-γ production) and an anti-inflammatory effect (inhibiting the TNF-α and NO production) on peritoneal macrophage from mice. Complementary studies are necessary in order to understand how this immunomodulating effect, triggered by *G. lucidum* consumption, could contribute to the maintenance of homeostasis.

## ACKNOWLEDGMENTS

The authors thank CNPq (Process Nº475798/2003-1), FUNDAÇÃO ARAUCÁRIA, and the UFPR for their financial support.

# Immunological Profile in Mice Fed on a *Ganoderma lucidum* Supplemented Diet

*Rosália Rubel,[1] Herta Sutz Dalla Santa,[1,2] Raffaello Di Bernardi,[4] Juliana Gern,[1] Kamille S. Pereira,[1] Andressa Bisol,[1] Luciana P. S. Vandenberghe,[1] Fan Leifa,[1,3] and Carlos Ricardo Soccol[1]*

[1]Bioprocess Engineering and Biotechnology Division, University Federal of Paraná (UFPR), Paraná, Brazil; [2]Department of Food Engineering, Unicentro, Guarapuava, Paraná, Brazil; [3]Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou, China; [4]Department of Cellular Biology, UFPR, Paraná, Brazil

The present work investigated the effect of a *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Basidiomycetes) supplemented diet on the immune system of mice.

The used *G. lucidum* strain, CG 144 originating from Fuzhou, China, was provided by EMBRAPA (The Brazilian Agency for Farming and Cattle Raising Research). The strain was kept by subcultivation in potato dextrose agar (PDA), once in every 2 months, at 30ºC for 10 days and subsequently stored at 4ºC. This mushroom was cultivated in Erlenmeyer flasks (1 L) containing 500 mL of the following medium (g $L^{-1}$): glucose - 35; peptone - 5; yeast extract - 2.5; $KH_2PO_4$ - 0.883; and $MgSO_4.7H_2O$ - 0.5, pH 5.5. The flasks were incubated under agitation (120 rpm) at 30ºC, for 10 days. The inoculum was prepared with the resulting biomass, which was separated by centrifugation and diluted in 200 ml of sterile de-ionized water. Trays containing wheat grain were previously autoclaved at 120ºC, 1.2 Torr, for 45 min. The inoculum was sown onto the wheat up to a rate of 40% to 50% (v/w), and the trays were incubated at 30ºC, 90% relative humidity of air for 20–30 days. The material was dried at 50ºC with circulating air, and then grounded (0.4–1.7 mm particles). The dry fermented *G. lucidum* shows the following composition: 73.3g%, 15.0g%, 1.7g%, 8.0g%, and 2.0g% of carbohydrates, proteins, lipids, humidity, and ashes, respectively. The mycelia growth in the fermented wheat was estimated at 0.628 mg/g, according to the ergosterol content, by high performance liquid chromatography (HPLC). The obtained product was used in the preparation of the G10 diet, with 10% *G. lucidum* mycelium. Fourteen male Swiss mice (*Mus musculus*) - healthy, 35-days-old (± 3), and weighing 18.85 g (± 1.25) - were used. The animals were divided into two groups (seven per group), labeled G10 and control – according to the diet they were on. The control group received only the ordinary diet, without *G. lucidum* mycelium. The mice were kept in polycarbonate cages under controlled conditions of temperature (24 ± 2ºC) and relative humidity (55 ± 10%), with a 12 h/12 h light-dark cycle. For 12 weeks the animals were fed the respective diets and received water *ad libitum*. At the end of the 12th week, under ether anesthesia, blood samples were collected from the mice by cardiac puncture and the plasma concentration of IL-12p70, MCP-1 (Monocyte chemo-attractant protein), TNF-α, IL-10, IL-6, and IFN-γ were measured by flow cytometry (FACS-Calibur - Becton Dickinson), using BD Biosciences Pharmigen reagents. Subsequently, the mice were killed through ethereal inhalation. Splenic tissues were removed in order to measure the $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$cells population by flow cytometry. Mice fed the G10 diet showed a decrease in $CD3^+$ and $CD8^+$ spleen cells population ($p < 0.05$ *vs*. control). An elevation in the IFN-γ concentrations was also observed in the G10 group ($p < 0.05$ *vs*. control). The other cell populations and cytokines were not altered ($p > 0.05$ *vs*. control). IFN-γ is a pro-inflammatory cytokine with a potent anti-viral activity, which is involved in cell-mediated immunity. The direct cytotoxic activity of T-cells ($CD8^+$) represents a fundamental step in the conclusion of the immune response of the host against tumor development. By the way, as the over expression of T-cells is implicated in the pathogenesis of autoimmune diseases, the suppression of T cells can be desirable in the treatment of these diseases or in tissue transplants.

What is most intriguing in this work is that the diet supplemented with *G. lucidum* mycelium triggered not only an immunostimulatory effect, raising the IFN-γ concentration, but also an immunosuppressive effect,

diminishing CD8+ cells populations. It's possible that the appropriate balance between these activities could be responsible for the maintenance of homeostasis. However, complementary studies must be conduced in order to elucidate this issue.


## ACKNOWLEDGMENTS

The authors thank CNPq (Process Nº475798/2003-1), FUNDAÇÃO ARAUCÁRIA, and the UFPR for their financial support.


# Molecular and Biochemical Characterization of a New Italian *Ganoderma lucidum* Strain


*Roberta Saltarelli,[1] Paola Ceccaroli,[1] Mirco Iotti,[2] Alessandra Zambonelli,[2] Michele Buffalini,[3] Lucia Casadei,[4] Luciana Vallorani,[1] and Vilberto Stocchi[3]*

[1]Istituto di Chimica Biologica "G. Fornaini", Università degli Studi di Urbino "*Carlo Bo*", Urbino (PU), Italy; [2]Dipartimento di Protezione e Valorizzazione Agroalimentare, via Fanin 46, Bologna 40127, Italy; [3]Centro di Biochimica delle Proteine, Università degli Studi di Urbino "*Carlo Bo*", Urbino (PU), Italy; [4]Istituto di Ricerca sull'Attività Motoria, Università degli Studi di Urbino "*Carlo Bo*", Urbino (PU), Italy


Species of *Ganoderma* are important wood-decaying fungi, occurring on conifers and hardwoods throughout the world. The macro-micromorphological characters of *Ganoderma* are extensively variable, and more than 250 species have been described. Their morphological identification is very difficult and several phylogenetic studies were set up to distinguish between *Ganoderma* species from different countries. These studies revealed that some species, such as *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst., have high-intraspecific variability.

*Ganoderma lucidum* has been used as traditional medicine in Asia and, in the literature, is reported that the components extracted from this fungus have several medicinal effects such as immunomodulating, antifibrotic, anti-tumor activity, and free-radical scavenging.

In this study, a new Italian *G. lucidum* strain has been isolated from a fruit body harvested in Central Italy. The mycelium was molecular and biochemically characterized and compared with *G. lucidum* strain from China. ITS1-5.8S-ITS2 sequences of the Italian and Chinese *G. lucidum* strains showed about 95% similarity (identity = 605/639), confirming the differences between European and Chinese *G. lucidum* strains.

Evaluation of some enzymes of carbohydrate and nitrogen metabolism has showed that there are significant differences among the two strains examined. In particular, the specific activity of the Italian *G. lucidum* hexokinase, a key enzyme of the glycolytic pathway, was two times higher than the Chinese one, whereas the phosphofructokinase activity was about ten times higher in the Chinese *G. lucidum*. On the contrary, no significant differences were found in the activity levels of pentose phosphate pathway enzymes, such as glucose-6-phosphate and 6-phosphogluconate dehydrogenase. Furthermore, the mannitol dehydrogenase involved in the mannitol synthesis showed very low activity in both strains.

The differences observed in the enzyme activity of two mycelia strains were also evidenced by the proteomic analysis performed by 2D-PAGE. The comparison of the electropherograms revealed that the two strains differed in a qualitative and quantitative protein expression. In particular, a substantial number of high *Mr* proteins were present only in the Chinese *G. lucidum*.

Also, the carbohydrate composition was analyzed and the HPLC chromatograms revealed that the Italian *G. lucidum* presented carbohydrate content three times higher than the Chinese strain.

This comparative study revealed that there are significant biochemical and molecular differences between our strain and the Chinese *G. lucidum*, already extensively studied. The increased interest to identify natural molecules with a therapeutic use suggests future investigations to use the Italian *G. lucidum* as a potential medicinal candidate.

# Strain Improvement of *Agaricus bisporus* through Protoplast Fusion

*Sardul Singh Sandhu,[1] Inder Singh Rana,[1] and Mahendra K. Rai[2]*

[1]Department of Biological Sciences, R.D. University, Jabalpur, India; [2]Department of Biotechnology, SGB Amravati, University, Amravati, India

*Agaricus bisporus* (J. Lange) Imbach is economically the most important mushroom worldwide. It is commonly known as the white button mushroom. It is the most common cultivated mushroom in Europe, North America, China, and Australia. *Agaricus bisporus*, the cultivated button mushroom, has a mostly secondarily homothallic life cycle. This mode of sexual reproduction could limit outcrossing and recombination among homokaryons in natural populations and also creates difficulties in mushroom breeding.

Strain improvement of *A. bisporus* thus far has been based on conventional breeding methods. The latest increase in *A. bisporus* production was gained mainly by improving culture conditions rather than by obtaining better strains. Although conventional breeding techniques have been applied successfully for the production of novel strains, this procedure, however, is complicated by the aberrant life-cycle of *A. bisporus* due to the generation of usually heterokaryotic spores with two parental nuclei, which show almost no genetic exchange. Strain selection alone based on single spores, multispores, or tissue cultures may improve in the short-term, but it is unlikely to be as affective as methods involving controlled crossing. However, breeding of improved strains by conventional methods is a labor intensive and time-consuming procedure.

Protoplasts have proved to be valuable tools in fungal genetics. In recent years, many efforts have been made to define the conditions for the formation, regeneration, and fusion of protoplasts in higher Basidiomycetes.

Mushroom cultures were procured form IMTECH Chandighar, IARI New Delhi, and Department of Biological Sciences, R.D. University, Jabalpur, India. Fungal strains employed in the present work were *A. bisporus* MTCC 1792, *A. bisporus* ITCC 1924, *A. bisporus* FGCC M 01, *A. bisporus* FGCC M 02, *A. bitorquis* MTCC 1794, and *A. bitorquis* ITCC 3619.

Protoplasts were isolated and regenerated for isolation of homokaryons. Only slow growing colonies were separated from protoplast regenerated colonies. These slow growing isolates were assumed as homokaryons (putative homokaryons) and were subjected to growth rate (mm/day), colony morphology, and spawn run studies on non-composted substrates.

Resistance to fungicides was checked against two dominant selectable markers viz., benomyl (Benlate 50%) and bavistin. Antifungal markers used in the present study were Ketocanazole, Clotrimazole, Flucanazole, and Griseofulvin. Out of the six markers tested, only griseofulvin and clotrimazole were used for the development of the hybrid, by protoplast fusion between *A. bisporus* ITCC and *A. bitorquis* ITCC, as there was a very close difference in the sensitivity of all the strains with respect to other markers or the contrasting markers were not found.

Protoplast fusion was done between the homokaryon isolate H – 13 of *A. bisporus* ITCC and isolate H – 15 of *A. bitorquis* ITCC. Only 9 colonies were observed on regeneration medium after protoplast fusion. Three fast growing fusant colonies were subcultured and screened for biochemical studies. The fusants showed increased amylase, protease, and cellulase enzymatic activity over the parental heterokaryons.

# *Agaricus brasiliensis* Promote Alteration in the Cytotoxic Mediators by Peritoneal Macrophages *in Vitro*

*Herta Sutz Dalla Santa,[1,2] Rosália Rubel,[1] Luiz Cláudio Fernandes,[3] Sandro Bonatto,[3] Sergio Ricardo Bello,[3] Raffaello Di Bernardi,[4] Kamille S. Pereira,[1] Juliana Gern,[1] Luciana P. S. Vandenberghe,[1] Adenise L. Woiciechoski,[1] Fan Leifa,[1,5] and Carlos Ricardo Soccol[1]*

[1]Bioprocess Engineering and Biotechnology Division–University Federal of Parana (UFPR), Paraná, Brazil; [2]Department of Food Engineering, UNICENTRO, Guarapuava, Paraná, Brazil; [3]Department of Physiology, UFPR, Paraná, Brazil; [4]Department of Cellular Biology, Paraná, Brazil; [5]Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

*Agaricus brasiliensis* S. Wasser et al. (=*A. blazei* sensu Heinem.), known as "Cogumelo do Sol", "Cogumelo de Deus", "Himematsutake", is native from Piedade, a small town in the mountains of São Paulo, Brazil.

In this study we investigated alterations in the production of cytotoxic mediators from peritoneal macrophages, obtained from mice after long intake of a supplemented diet with *A. brasiliensis* mycelium produced by solid state cultivation on wheat grains. The strain *A. brasiliensis*–LPB-03 was cultivated in Petri dishes with PDA (Potato dextrose agar), during 10 days at 30ºC. The steps of pre-inoculum and inoculum production by submerged culture was done in medium containing ($g/L^{-1}$): glucose (35), yeast extract (2.5), peptone (5), $KH_2PO_4$ (0.88), $MgSO_4.7H_2O$ (0.5), pH 5.5 during seven days at 120 rpm at 30ºC. A mycelium suspension was obtained and used for solid state cultivation. The inoculation of 0.653g of mycelium per Kg of wheat grains was done in sterile conditions and the initial humidity of inoculate substrate was adjusted to 40% (± 2). The incubation was carried out at 30ºC, 90% relative humidity of air, during 18 days. The cultivated material was dried out (55ºC), milled (particles <2 mm), and supplemented with lipids (soy oil) and proteins (egg albumin) to fill up the nutrition needs. Samples of the cultivated material were analyzed by ergosterol content for fungi biomass estimation. The regular diet of the treated mice was mixed with 10% of this cultivated material and then given to the animals. Fourteen swiss female mice (*Mus musculus*) aged 30–35 days weighing 17–24 g were divided into two groups. The control group received a normal diet (C). For 12 weeks another set of mice was fed a normal diet supplemented with *A. brasiliensis* 0.289 mg of mycelium/g of cultivated material (*Ab* group). At the end of the 12th week resident macrophages from the mice were obtained by intraperitoneal (i.p.) lavage. After this, the cells were centrifuged, (290 *g,* 4ºC, for 5 min), washed, and resuspended in PBS or RPMI medium after counting in a Neubauer chamber by optical microscopy by using a Trypan blue solution (1%) and viability of 95%. A pool of macrophages was obtained from 4 or 5 animals. Macrophages were further purified by incubating peritoneal cells in tissue culture plates for 2 h and then washing three times with PBS to remove the non-adherent cells. The production of phagocytosis was measured by the reaction of the macrophages with neutral-red stained zymosan, the lysosomal volume through uptake of the cationic dye neutral red. Superoxide anion production was estimated by the nitroblue tetrazolium (NBT) reduction assay, the hydrogen peroxide was measured by reaction with HRPO and nitric oxide production was calculated by nitrite concentration, which was measured by Griess's reaction. Phagocytosis and lysosomal volume by peritoneal macrophages from mice fed a regular diet (C) and in those supplemented with *A. brasiliensis* (*Ab*) were not different ($P > 0.05$). Superoxide anion production was significantly reduced by peritoneal macrophages from mice supplemented with *A. brasiliensis,* with 0.0738 (± 0.0165) $mg^{-1}$ protein when compared to C group 0.0734 (± 0.02303) $mg^{-1}$ protein ($P < 0.05$). There was a tendency to decrease the production of hydrogen peroxide but this was not statistically different ($P > 0.05$ *vs*. C). Nitric oxide production under non-stimulated condition was higher by peritoneal macrophages from mice fed a regular diet (C) when compared with that from mice fed a regular diet and supplemented with *A. brasiliensis* (*Ab*) ($P < 0.001$). The NO production was reduced by peritoneal macrophages; the stimulation with LPS induced an increase of NO production in both groups: C+LPS and

*Ab*+LPS, when compared to their respective groups without LPS. However, the results obtained for *Ab* groups, with and without LPS, resulted in lower NO production when compared to their respective Control groups ($P < 0.001$ *vs.* C). Peritoneal macrophages from mice supplemented with cultivated wheat grains by *A. brasiliensis* did not increase phagocytosis and lysosomal volume but presented lower production of species reactive oxygen and nitrite production. This can suggest an anti-inflammatory effect in the mice fed with the mushroom *A. brasiliensis*. Macrophages and neutrophils must generate reactive oxygen species in order to kill some types of bacteria that they engulf by phagocytosis. It is reported that mushroom products can stimulate macrophages in many ways to release inflammatory cytokines, or the extract can inhibit the production of TNF by LPS-stimulated macrophages.

In conclusion, the long supplementation of *A. brasiliensis* mycelium promote, in mice, lower species reactive oxygen and nitrite production, without differences in the phagocytosis and lysosomal volume from the peritoneal macrophages cultivated *in vitro*.

## ACKNOWLEDGMENTS

The authors thank CNPq (Process Nº475798/2003-1), FUNDAÇÃO ARAUCÁRIA, UNICENTRO, and UFPR for their financial support.

# Development of Natural Multifunctional Cosmetic Ingredients Using a Biotechnological Approach

*Madhuri Shamanna,[1,2] Tajalli Keshavarz,[2] and Danka Tamburic[1]*

[1]London College of Fashion; University of Arts London, 20 John Princes St., W1M 0BJ, London, UK; [2]School of Biosciences, University of Westminster, 115 New Cavendish St., W1W 6UW, London, UK

Higher Basidiomycetes are known to have immunobeneficiary properties since ancient times. *Grifola frondosa* is one of the edible mushrooms that have been evaluated for its nutritional as well as pharmaceutical properties. Polysaccharides extracted from *G. frondosa* have been studied for their pharmacological use and they have been characterized as β-glucans. Beta glucans have been reported as good thickeners and stabilizing agents, as well as effective anti-aging ingredients. Use of polysaccharides from *G. frondosa* for commercial production is generally overlooked due to the costs and time involved. The aim of this project is to test potential uses of *G. frondosa* polysaccharides as cosmetic raw materials and multifunctional ingredients in cosmetic preparations.

*Grifola frondosa* was grown by submerged liquid fermentation and extracellular polysaccharides were extracted using ethanol precipitation. Intracellular polysaccharides were extracted using hot-water extraction from mycelial biomass. Biochemical assays showed that the polysaccharides contained low levels of uronic acids and proteins. The effect of these polysaccharides on reactive oxygen species production by white blood cells was tested using a fluorescent dye and the results indicated that the extracts have antioxidant properties. Purification, structural characterization of the polysaccharides, and testing the activity of these polysaccharide fractions on the collagen production by the fibroblasts are part of the project's aims.

Polysaccharides will be analyzed for their functionality in cosmetic formulations. Effect on rheology/viscosity, antimicrobial, antioxidant, and anti-aging properties will be studied along with *in vivo* tests on human volunteers.

# Effect of Cultural Conditions on Mycelial Growth and Fruiting of *Agrocybe aegerita*

*Elvira F. Solomko and Margarita L. Lomberg*

M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska St., Kiev 01601, Ukraine

The Black Poplar Mushroom - *Agrocybe aegerita* (Brig.) Singer (=*Agrocybe cylindracea* (DC.: Fr.) Maire, *Pholiota cylindracea* (DC.: Fr.) P. Kumm.) growing as lignotroph on stumps of hardwoods in southern Europe and in similar climatic zones of the Far East and the United States. This species is not known to occur in the Ukraine. This edible mushroom and closely related species produce antitumor and immunostimulating polysaccharides and unique antibiotics. *A. aegerita* (6 strains) from the Culture Collection of Mushrooms, N.G. Kholodny Institute of Botany NASU (acronym IBK), were used for the investigation of mycelium growth rate, cultural characteristics, and fruiting body production under different conditions. Stock cultures were maintained at 4–8ºC in test tubes on beer wort agar media.

When cultures were incubated in Petri dishes at 26ºC on beer wort agar the radial growth rate was from 1.9 to 5.3 mm/day depending on the strain. The maximal growth rate of mycelium (from 3.2 to 6.4 mm/day) was observed on wheat and oatmeal agar media (pH 6.0) for all strains. At first, the investigated cultures are white and cottony. As the mycelial colony become older, the spotted brown pigmentation was more pronounced on rich organic nutrition media, especially with peptone (MPA, MYPA). For all investigated strains, the optimum temperature for mycelial growth was near 28ºC. The growth rate was significantly lower at 18 and 24ºC. At 34 and 37ºC mycelia growth was not observed during 14 days of incubation. The cultures of *A. aegerita*, however, did not die. After lowering the temperature to 28ºC, active growth of mycelia began 2 days later.

The highest biomass production in submerged culture (1.7 g/L/day) was obtained for a selected strain of *A. aegerita,* IBK-960, on complex liquid medium with glucose and peptone. The mycelial suspension of submerged cultures was injected into sterilized wheat grain, and after 10 days this grain spawn was ready and used for the next experiments. The strain was tested for fruiting body production on different sterilized substrates. The shortest duration of colonization (20 days) was observed on beech wood sawdust supplemented with corn meal or wheat bran (15%). Slowly, the spawn developed on beech wood sawdust without any supplements (the duration was 44 days). On other substrates (cornstalks, husks of sunflower seed (HSS), mishmash supplemented sawdust and HSS) duration of spawn run was 24–25 days. Primordia formation was observed beginning on day 40 from the inoculation date first on HSS-substrates, then on all others. Two flushes of fruiting body development were obtained on a small-scale cultivation. The yield of fresh mushrooms was around 20% from the wet mass substrates, except for non-supplemented sawdust (only 5%) and for complex mishmash substrates (25%).

# Ingestion of *Agaricus bisporus* Lowers Blood Triglyceride and Total and Low-Density Lipoprotein Cholesterol Levels in Hyperlipidemic Rats

*Chi-Hyun Song,[1] Byung-Keun Yang,[1] Sang-Chul Jeong,[2] Yip Cho,[2] Sundar Rao Koyyalamudi,[2] Gerald Pang,[2] and Percy Wong[2]*

[1]Department of Biotechnology, Daegu University, Gyungsan, Gyungbuk 712-714, South Korea; [2]School of Natural Sciences, University of Western Sydney, Locked Bag 1797, Penrith South DC NSW 1797, Australia

The nutritional value of the button mushroom, *Agaricus bisporus* (J. Lange) Imbach, is well-documented from the point of view of its contents of vitamins, minerals, trace elements, fiber, and phenolic compounds. However, no studies have been done to determine the health benefits of *A. bisporus* in terms of its effects on cardiovascular disease risk factors, such as blood triglyceride and total and low-density lipoprotein (LDL) cholesterol. The aim of this study was to evaluate the effects of *A. bisporus* on blood total and LDL cholesterol and triglyceride levels of rats fed on a high-fat diet.

In a model of hyperlipidemia, rats (n = 8) rendered lipidemic on a high-fat diet for 6 weeks were fed *A. bisporus* in a powdered form (200 mg/kg body wt.) in saline, administered daily by oral intubation for 4 weeks. Control rats (n = 8) were fed saline alone and normal rats (n = 8) were fed a conventional rat diet. At the end of 4 weeks, the diet was withheld from the rats for 14 hrs, after which time arterial blood was collected under anaesthesia before being sacrificed. Plasma total cholesterol (LDL + HDL), triglycerides, and body weight were measured.

Rats fed a high-fat diet gained body weight (6.13 ± 0.28 gm per day) compared with normal rats (4.96 ± 0.25 gm per day) on a conventional diet but this was significant. However, lipidemic rats fed *A. bisporus* powder had a reduction in body weight, but this was not significant compared with controls (5.67 ± 0.19 vs. 6.13 ± 0.28 g per day). There was a significant reduction in total cholesterol (97.32 ± 6.61 mg/dl) ($P < 0.05$) and LDL (66.78 ± 4.82 mg/dl) ($P < 0.05$) in lipidemic rats fed *A. bisporus* compared to control rats (124.77 ± 4.68 mg/dl and 98.23 ± 5.18 mg/dl, respectively). In contrast, there was an increase in HDL (22.06 ± 1.66, $P < 0.05$) in lipidemic rats fed *A. bisporus* compared with control rats (16.55 ± 1.28 mg/dl). No significant difference in plasma triglyceride levels was detected between normal, control, and lipidemic rats fed *A. bisporus*. There was no significant difference in total cholesterol, LDL, and HDL levels between normal and lipidemic rats fed *A. bisporus*. A decrease in atherogenic index (total cholesterol- HDL/HDL) was noted in lipidemic rats fed *A. bisporus* compared with control rats (6.54 ± 0.31 vs. 3.41 ± 0.27, $P < 0.01$). A decrease in cardiac risk factor was also noted (7.54 ± 0.41 vs. 4.41 ± 0.35, $P < 0.01$). A decrease in liver weight (4.56 ± 0.10 g/100 gm BW) ($P < 0.05$), total cholesterol (3.68 ± 0.26 mg/g) ($P < 0.01$), and triglyceride (6.41 ± 0.47 mg/g)($P < 0.05$) in lipidemic rats fed *Agaricus bisporus* compared with controls (5.02 ± 0.15, 5.77 ± 0.71 and 8.09 ± 0.45, respectively) was observed.

The results indicated that *A. bisporus* ingestion lowers lipidemia and beneficially affects cholesterol metabolism and may therefore lead to a reduction in the risk of coronary artery disease.

# Higher Basidiomycetes Laccase and Manganese Peroxidase Activity in Submerged Fermentation of Food Industry Wastes

*George G. Songulashvili,[1,2] Vladimir I. Elisashvili,[2] Eviatar Nevo,[1] and Yitzhak Hadar[3]*

[1]Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel; [2]Durmishidze Institute of Biochemistry and Biotechnology, Academy of Sciences of Georgia, 10 km Agmashenebeli kheivani, Tbilisi 0159, Georgia; [3]Dept. of Plant Pathology and Microbiology, Faculty of Agricultural and Environmental Quality Sciences, The Hebrew

The evaluation of 18 strains of higher Basidiomycetes laccase and manganese peroxidase (MnP) activity in submerged fermentation of mandarin peelings and ethanol production waste (wheat) showed that the expression of enzyme activity is species- and strain- dependent. The laccase activity among species of the genus *Ganoderma* varied from 192 U $l^{-1}$ to 61488 U $l^{-1}$. *Phellinus robustus* 250 appeared to be a promising producer of MnP, accumulating more than 4000 U $l^{-1}$ of enzyme activity. Laccase and MnP production proved to be very much dependent on the lignocellulosic growth substrate. Of eight complex substrates examined in submerged fermentation by *G. lucidum* 447, wheat bran and soy bran gave the highest laccase activity with a maximum value of 93000–97000 U $l^{-1}$. Proof that both the titre and time of maximal enzyme activity are influenced by nutrient nitrogen is presented.

*Ph. robustus* 250 produced both laccase (700-4000 U $l^{-1}$) and MnP (1000–11300 U $l^{-1}$) in fermentation of nine food wastes, whereas *G. adspersum* 845 produced only laccase (600–34000 U $l^{-1}$). Glucose provided high laccase and MnP activity of *Ph. robustus* 250 but repressed enzyme production by *G. adspersum* 845. Ammonium sulphate and ammonium tartrate increased the *Ph. robustus* 250 laccase yield (3-fold), whereas the accumulation of MnP was not enhanced by additional nitrogen.

In subsequent experiments with *Ph. robustus* 250 the synthetic and wheat bran media were supplemented with copper and manganese since this fungus is a good producer of MnP. The data represented show that *Ph. robustus* 250, like *G. lucidum* 447, is not sensible to the tested concentrations of microelements since the biomass yield reached at least the same level as in the control medium. The evaluation of fungus enzyme activity showed close dependence of laccase accumulation on the nature of microelement. The supplementation of medium with $Cu^{2+}$ at the time of inoculation or during the culture growth sharply stimulated laccase production. The most pronounced stimulating effect of 300 μM $Cu^{2+}$ was revealed when it was added to the medium at the time of inoculation or after 3 days of fungus cultivation. In this case, the laccase activity increased from 20 U $l^{-1}$ by control medium to 3000 U $l^{-1}$ in medium with $Cu^{2+}$. The later $Cu^{2+}$ was added to the culture the weaker the stimulating effect of microelement was on laccase production. However, the later $Cu^{2+}$ was added to the culture the faster the enzyme accumulation in culture liquid preceded.

Subsequently, the effects of microelements on *Ph. robustus* 250 growth and enzyme activity were studied in submerged fermentation of wheat bran. Both $Cu^{2+}$ and $Mn^{2+}$ added to the medium at the time of inoculation enhanced laccase production two-fold compared to the enzyme activity in the control medium. The concentration of $Cu^{2+}$ and the time of its supplementation to the medium are important factors determining the level of extracellular laccase. The addition of 1 mM and 3 mM $Cu^{2+}$ at the time of medium inoculation increased *G. lucidum* 447 laccase activity 20-fold and 143-fold, respectively, compared with the control medium. The same laccase yield was achieved when 1 mM $Cu^{2+}$ was added to growing culture on day 3. The data showed that the addition of 3 mM $Cu^{2+}$ at the same time inhibited the enzyme secretion during subsequent cultivation. Moreover, the medium supplementation by 1 and 3 mM $Cu^{2+}$ after 5 or 7 days of fungus cultivation completely suppressed the laccase accumulation. More data was revealed when medium containing wheat bran was supplemented with copper. In this case no inhibition of *G. lucidum* 447 growth was observed. The control medium ensured a high level of laccase accumulation. The addition of $Cu^{2+}$ to the medium elevated the yield of enzyme in all test culture conditions.

However, the highest stimulation of laccase stimulation was achieved when a microelement was added

to the medium at time of inoculation. Especially high laccase activity (a twofold increase compared with the control medium) was reached with the addition of copper in concentration of 3 mM. This concentration of the microelement appeared to be optimal to stimulate enzyme secretion.

## Effect of Different Nitrogen Sources and Concentrations, and Medium pH on the Production of Laccase and Mn-Oxidizing Peroxidases by *Ganoderma lucidum*

*Mirjana Stajić, Jelena Vukojević, and Sonja Duletić-Laušević*

Institute of Botany, Faculty of Biology, University of Belgrade, Takovska 43, Belgrade 11000, Serbia

*Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. is an medicinal species that belongs to a group of white-rot fungi due to its ability to produce extracellular ligninolytic enzymes: laccase, Mn-oxidizing peroxidases, and lignin peroxidase, and modify and degrade lignin and different aromatic compounds. However, knowledge on the ligninolytic system of *G. lucidum* has not been completed yet. The aim of this study was to analyze the effect of different nitrogen sources and concentrations as well as medium pH on the production of laccase and Mn-oxidizing peroxidases by *G. lucidum*.

*Ganoderma lucidum* HAI 447, originated from Tel Aviv (Israel), from *Quercus* sp., was used for the study. *G. lucidum* culture is preserved in the Culture Collection of the Institute of Evolution, University of Haifa (HAI). The solid-state fermentation was carried out in 100 ml flasks containing 2 g of wheat straw as the carbon source and 10 ml of a synthetic medium with one of three nitrogen sources: ammonium nitrate and ammonium sulfate, at nitrogen concentrations of 10, 15, 20, 25, 30, and 40 mM, and peptone, at concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0%, for seven days. Wheat straw and distilled water were used as controls. The analyzed medium pH were: 3.5, 4.0, 4.5, 5.0, and 6.0. Laccase and Mn-oxidizing peroxidases activities were determined using ABTS and phenol red, respectively, as the substrates. An UV-160A Spectrophotometer (Shimaden) was used for these assays.

The studied *G. lucidum* strain was a good producer of laccase and Mn-oxidizing peroxidases under conditions of solid-state fermentation of wheat straw in the presence of all analyzed nitrogen sources, as well as in the control medium. The highest level of laccase activity was found in the medium with ammonium nitrate as the nitrogen source, at a concentration of 10 mM, at pH 5.0 (181.4 $Ul^{-1}$), which was more than two times higher compared to the control (76.1 $Ul^{-1}$). The best nitrogen source for phenol red oxidation in the presence of $Mn^{2+}$ was ammonium nitrate (112.8 $Ul^{-1}$), at a concentration of 20 mM, at pH 5.0, while the level of phenol red oxidation in the absence of $Mn^{2+}$ was the highest (35.1 $Ul^{-1}$) in the medium with peptone as the nitrogen source, at the concentration of 0.25% and at pH 6.0. The production of Mn-oxidizing enzymes was significantly lower in the control medium, 24.5 $Ul^{-1}$, in the presence of $Mn^{2+}$, and 16.9 $Ul^{-1}$, in the absence of $Mn^{2+}$.

# Effect of Ostreolysin on Cell-Sized Lipid Vesicles

*Vid Šuštar,[1] Janja Majhenc,[1] Kristina Sepčić,[2] Sasa Svetina,[1,3] and Peter Maček[2]*

[1]Institute of Biophysics, Faculty of Medicine, University of Ljubljana, 2 Lipičeva, Ljubljana 1000, Slovenia; [2]Department of Biology, Biotechnical Faculty, University of Ljubljana, 111 Večna pot, Ljubljana 1000, Slovenia; [3]"Jožef Stefan" Institute, 39 Jamova, Ljubljana 1000, Slovenia

During the period of fast growth of primordia and young fruiting bodies, the edible mushroom *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. synthesizes large amounts of the cytolytic protein Ostreolysin (Oly). Its exact biological role is not yet known, but it is assumed that it might be involved in the initial phases of fruiting body formation, hyphae aggregation, and apoptosis. Together with some other bacterial and fungal proteins with similar amino acid sequences, thermolability, low-isoelectric points, and molecular weights of about 16 kDa, Oly belongs to the aegerolysin protein family. Oly causes colloid-osmotic lysis of natural and artificial lipid membranes, with formation of pores with functional, inner diameters of 4 nm. It specifically binds to cell membrane domains enriched with cholesterol and sphingomyelin. Combined cholesterol and sphingomyelin tend to form liquid ordered ($l_o$) domains. Liquid ordered domains in cell membranes are also called lipid rafts because of their relatively greater thickness in comparison to liquid disordered and crystalline domains, and consequential inclusion of special raft proteins with signaling and other significant functions. Oly promises to be an excellent marker of physiologically important and interesting lipid rafts.

Cells are complex, not fully understood structures; therefore, many different, simpler models for studying lipid rafts, with known and constant lipid composition, such as giant unilamellar vesicles (GUVs) are used. GUVs are especially suitable because their size is comparable to the cells, and easy direct observation and manipulation under the phase contrast and fluorescent microscope. Our experiments on the effect of Oly on permeability of GUVs were done with a computer-assisted phase contrast microscope. GUVs were made by the electro-formation method; the inner solution of sucrose was isomolar, but optically denser in comparison to the surrounding solution of glucose, allowing the visualization of GUVs under the phase contrast microscope. We determined GUVs lipid composition by the fractions of constituent lipids prior to electro-formation. One vesicle at a time was singled out and transferred into the isomolar glucose solution containing Oly, using the micropipette technique. After the transfer of a vesicle into the solution with Oly, the pore formation induced the flow of glucose into the vesicle and also the flow of sucrose out of the vesicle. Concomitantly, as a consequence of the membrane permeabilization by Oly, the bright halo around the vesicle observed with the phase contrast microscopy began to fade.

Created GUVs were of different sizes, so we could also study the dependence of GUV size on the speed of their permeabilization by Oly. We also examined the influence of GUVs and Oly aging on the rate of permeabilization. The rate of GUVs permeabilization was dependent on Oly concentration, excluding the possible permeabilization effects by other factors. GUVs composed of equimolar ratio of cholesterol and sphingomyelin were the most susceptible to Oly concentrations, while GUVs containing 30 or less molar % of cholesterol were resistant to the protein. On the one hand, the rate of GUVs permeabilization decreased with the aging of Oly, possibly because of the loss of its bioactivity and degradation. On the other hand, permeabilization increased with the aging of GUVs. The rate of permeabilization was not particularly dependent on GUVs size. We also examined possible phase separation of different lipid domains in GUVs containing cholesterol and sphingomyelin, using the fluorescent microscopy technique. Phase separation that was large enough to be detected by optic fluorescence microscopy was observed in GUVs supplemented with palmitoyl-oleoyl-phosphatidylcholine. On the contrary, GUVs made of an equimolar cholesterol-sphingomyelin mixture did not show phase separation with different domains larger than 1 μm.

# Biologically Active Polysaccharides of *Agaricus brasiliensis*: Isolation and Structural Characterization

*Andriy Synytsya,[1] Petra Blafková,[1] Kateřina Míčková,[1] Ivan Jablonský,[1] Jiři Spěváček,[2] and Jana Čopíková[1]*

[1]Institute of Chemical Technology, Department of Carbohydrate Chemistry and Technology, Technická 1905, 166 28 Prague 6, Czech Republic; [2]Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, 162 06 Prague 6, Czech Republic

The higher Basidiomycetes mushroom *Agaricus brasiliensis* S. Wasser et al. (=*A. blazei* sensu Heinem.), a species native to Brazil, has been widely used as health food in traditional medicine for the prevention of cancer, diabetes, artherosclerosis, and other diseases due to the presence of biologically active compounds in fruit bodies. Among such bioactive compounds, specific β-glucans and their complexes with proteins and other polysaccharides could be important as immunomodulating agents. Branched glucans from *A. brasiliensis* are supposed to be responsible for some healthy properties of these mushrooms including anticarcinogenic and antimutagenic ones.

This work was devoted to the preparation and characterization of polysaccharides from this fungus. Hot water and alkali soluble and alkali insoluble fractions were isolated from fruit bodies of *A. brasiliensis* by a two-step extraction procedure. Contents of dietary fibers, α- and β-glucans were determined by Megazyme enzymatic kits. The structure of obtained products was analyzed by spectroscopic methods (FTIR, Raman, and NMR). Protein contents were estimated by organic elemental analysis and specific photometric assays. Enzymatic analyses confirmed significant differences in contents of dietary fibers and glucans among the strains, harvest time, and topology (pilei or stems). In most cases, stems contained more glucans, more total and insoluble but less soluble dietary fibers than pilei. Therefore, low-food quality stems of *A. brasiliensis* could be a valuable source of β-glucans for the preparation of food supplements. According to spectroscopic analysis, water soluble fraction consists of protein and glucan components, while alkali soluble fractions are mainly polysaccharides. Alkali insoluble fractions are β-glucans associated with significant amounts of chitin forming a chitin-glucan complex. Treatment of hot-water extracts with phenol reagent led to the separation of protein-rich phenolic and glucan-rich aqueous phases. Spectroscopic methods confirmed that the main component of polysaccharide fractions obtained from hot water extracts was identified as α-1,4-glucan similar to amylose, while alkali soluble polysaccharide fractions consisted mainly of β-glucans.

## ACKNOWLEDGMENT

This work was supported by the Grant Agency of the Czech Republic (project 525/05/0273).

# Two Forms of Tropical Red Lingzhi *Ganoderma tropicum* Newly Recorded from South Vietnam: A Precious Source of Materia Medica

*Le Xuan Tham,[1] Le Thi Hoang Yen,[2] Duong Van Hop,[2] Dang Ngoc Quang,[3] Bui Quang Trung,[4] and Le Tien Son[4]*

[1]Center for Nuclear Techniques, HoChiMinh City, Vietnam Atomic Energy Commission (VAEC); [2]Center of Biotechnology, National University of Hanoi; [3]Faculty of Chemistry, Hanoi University of Education, 136-XuanThuy, CauGiay, HaNoi, [4]CongThanh Biotechnology Co. Ltd., Long Khanh, Dong Nai, Vietnam

For the first time Junghuhn described *Polyporus tropicus* (1838) with type specimens from Java, Indonesia, and Bresadola, amended as *Ganoderma tropicum* (Jungh.) Bres. (1910), which were found in Munoz, Philippines, in 2000, and in Ho Chi Minh City, South Vietnam, since 2002. This species sometimes would be confused with *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. and with several others, considered as belonging to the group "Red Lingzhi"—precious materia medica in folklore medicine in Oriental Asia (China, Taiwan, Japan, Korea, and Vietnam).

## I. Systematics Based on Morphology

Collections of specimens: HCMC 003 – III – 2003, HCMC 004 – 6 – 2004, HCMC 005 – XI – 2004, HCMC 006 – VII – 2005; Nhatrang 2006; Cattien 2006.

*Ganoderma tropicum* (Jungh.) Bres., *Polyporus tropicus* Jungh. In Verh. Bataviaash Genootsch. 17 (II): 63. 1838 ["1839"], *Fomes tropicus* (Jungh.) Sacc., Syll. Fung. VI: 183. 1888, *Ganoderma tropicum* (Jungh.) Bres. *In:* Annls mycol. 8: 586. 1910, *Ganoderma oroleucum* Pat. et Har. *In:* Bull. Trimmest. Mycol. Soc. Fr. 22: 118. 1906, *Ganoderma lucidum* [non Karst.] Sawada, Trans. Nat. Hist. Soc. Form. XXIV: 302, 1934.

Corner (1983) considered *Ganoderma tropicum* —one of the Red Lingzhi—to be a complex, probably pantropical, of many races and divided it into 3 varieties, according to their spore structures. The type collections were from Java, Indonesia, China incl. Taiwan and Hainan island (Wu et al., 1998), Japan, and distributed widely through all of Asia and Australia (Smith & Sivasithamparam, 2000). Zhao (1989), Zhao & Zhang (2000) didn't agree with Corner's interpretation of this species. However, Imazeki (1939), clearly described two forms: varieties stipitate, long, and very short—substipitate or sessile.

We have completed the procedure of cultivations for both *Ganoderma tropicum* and *G. lucidum* in Cong Thanh Biotechnology Co. Ltd., Long Khanh, Dong Nai Province.

On mixed substrates based on rubber tree sawdusts (1.5 kg/bag), during 70–80 days, perfect fruit bodies of both forms obtained maturity with discharged basidiospores, BE reached up to 20%–25%, higher than *G. lucidum*.

## II. Phylogenetic Systematics—Speciations in rDNA

Molecular analysis of D2 (rDNA 26S) was conducted in Center of Biotechnology, National University, Hanoi, and the results of sequences shown below were quite similar to ones obtained with D1, D2, ITS1, IT2 (rDNA 5.8S) by Moncalvo et al. (1995), Gottlieb et al. (1998, 2000), and Smith and Sivasithamparam (2000).

### Sessile Form

AATAAGCGGAGGAAAAGAAACTAACAAGGA
TTCCCCTAGTAACTGCGAGTGAAGCGGGAA
AAGCTCAAATTTAAAATCTGGCGGTCTTTGG
CCGTCCGAGTTGTAGTCTGGAGAAGTGCCCG
CGCTGGACCGTGTATAAGTCTCTTGGAACAG
AGCGTCATAGAGGGTGAGAATCCCGTCTTTG
ACACGGACTACCAGTGCTTTGTGATGCGCTC
TCAAAGAGTCGAGTTGTTTGGGAATGCAGCC
AAAATGGGTGGTGAATTCCATCTAAAGCT
AAATATTGGCGAGAGACCGATAGCGAAC
AAGTACCGTGAGGGAAAGATGAAAAGCACT
TTGGAAAGAGAGTTAAACAGTACGTGAAAT
TGCTGAAAGGGAAACGCTTGAAGTCAGTCG
GTCGTCCGGAACTCAGCCTTGCTTTCGCTT
GTGCACTTTCCGGATGACGGGTCAGCATGAT
TTTGACCGTCGGAAAAGGGCTAGAGTAATG

TGGCACCTCCGGGTGTGTTATAGACTCTGG
TCGCATACGGCGGTTGGGATCGAGGAACGC
AGCGCGCCGCAAGGCAGGGGTTCGCCCAC
TTTCGCGCTTAGGATGCTGGCATAATGGCTTT
AAACGACCCGTCTTGAAACACGGAC

### Stipitate Form

CAATAAGCGGAGGAAAAGAAACTAACAAG
GATTCCCCTAGTAACTGCGAGTGAAGCGG
GAAAAGCTCAAATTTAAAATCTGGCGGTCT
TTGGCCGTCCGAGTTGTAGTCTGGAGAAGTG
CTTTCCGCGCTGGACCGTGTATAAGTCTCTT
GGAACAGAGCGTCATAGAGGGTGAGAAT
CCCGTCTTTGACACGGACTACCAGTGCTTTG
TGATGCGCTCTCAAAGAGTCGAGTTGTTTGG
GAATGCAGCTCAAAATGGGTGGTGAATTCC
ATCTAAAGCTAAATATTGGCGAGAGACCGA
TAGCGAACAAGTACCGTGAGGGAAAGATGA
AAAGCACTTTGGAAAGAGAGTTAAACAGTAC
GTGAAATTGCTGAAAGGGAAACGCTTGAAG
TCAGTCGCGTCGTCCGGAACTCAGCCTTG
CTTTCGCTTGGTGCACTTTCCGGATGACGG
GTCAGCATCGATTTTGACCGTCGGAAAAGGG
CTAGAGTAATGTGGCACCTCCGGGTGTGTT
ATAGACTCTGGTCGCATACGGCGGTTGGGA
TCGAGGAACGCAGCGCGCCGCAAGGCAG
GGTTCGCCCACTTTCGCGCTTAGGATGCTGG
CATAATGGCTTTAAACGACCCGTCTTGAAAC
ACGGACCAA

The sequence of both these forms is similar to *G. lucidum* by 99% (590/591bp) and (639/641bp), respectively.

### III. Composition of Bioactive Ingredients

The procedure of primary determinations of the main bioactive ingredients was conducted in the Faculty of Chemistry, Hanoi University of Education. The results of analysis are well supported by Aryantha et al. in Indonesia (2002). The authors have shown the similarity to *G. lucidum*, particularly the spectra of triterpenoid derivatives. Further studies are underway with emphasis on bioactive compositions of novel fatty acids.

---

# Secondary Metabolites from Submerged Cultures of Higher Basidiomycetes and Their Effect on the Growth of Ovarian Cancer Cells

*Amal Toubi (Rouhana), Solomon P. Wasser, and Fuad Fares*

Faculty of Science and Science Education, Department of Evolutionary and Environmental Biology, University of Haifa, Mount Carmel, Haifa 31905, Israel

Higher Basidiomycetes mushrooms have been used in folk medicine throughout the world since ancient times, and they represent a major potential for pharmaceutical products. The anti-tumor property of mushrooms has been reported in many species. Clinical trials using medicinal mushrooms products for the treatment of various types of cancers have achieved some success in extending survival of cancer patients and/or improving the quality of life in patients with advanced cancer. There are two main groups of substances produced by higher Basidiomycetes mushrooms, capable of exhibiting anti-tumor activity: high-molecular-weight compounds such as polysaccharides, proteins, lipopolysaccharides, and glucoproteins; and low-molecular- weight compounds such as triterpenoids, lanostanoids, ergo sterols, caffeic acid, etc.

Among the different anticancer activities of higher Basidiomycetes mushrooms products is the potential activity against ovarian cancer. Epithelial ovarian cancer is the most lethal of all gynecological cancers and accounts for approximately one-quarter of all genital tract cancer in women. The estimated total number of cases worldwide is 192,000 per year. Chemotherapy has made a significant advance in the treatment of cancer patients; however, chemotherapeutic agents cause a variety of severe and life-threatening side effects, such as severe immunosuppression and bone

marrow depression. Our hypothesis is that among the different kinds of LMW substances produced by HBM, some can inhibit the growth of ovarian cancer. LMW substances were extracted from biomass powder of mushroom extracts and there anticancer activity was tested using human ovarian cancer cells. In order to find the mushroom species with the highest activity we have cultivated 25 different medicinal mushroom species. The mushrooms were grown on malt extract agar, for periods of 5–10 days, at a temperature of 27°C. Inoculums of each sample were inserted into 100 mL fermentation medium, composing: glucose, soy peptone, yeast extract, and potassium phosphate salts, and grown at 27°C, on a shaker of 120 rpm and for periods of 7–20 days, until maximum biomass was obtained. The mediums with biomass were homogenated twice and inserted into 1000 ml of fermentation medium. The yield of biomass was screened through a 200 μm fabric, washed with distilled water, squeezed, and then dried at a temperature of up to 45°C, for 2 days. The dried biomass were ground to a powder and saved at room temperature in a closed container. In order to obtain specifically low-molecular-weight substances we used organic solvents for the extraction. Each powdered sample was extracted with three different pure solvents: ethyl acetate (aprotic dipolar), ethanol (protic polar), chloroform (non polar) to cover large area of hydrophilic/lipophilic substances. One gram of powder was extracted three times, each time with 10 ml, for one hour, on a shaker of 150 rpm. The extractions were merged and placed on filter paper and then left to dry at room temperature. Dimethyl sulfoxide was added to each vile until the concentration of 100 mg/ml and the samples were stored at –70°C. The activity of the mushroom extracts will be first determined by an *in vitro* model system, using human ovarian cancer cell lines, at 3 stages: well differentiated, moderately differentiated, and poorly differentiated.

## Medicinal Aphyllophorales Mushrooms of Israeli Mycobiota

*Daniel Ţura and Eviatar Nevo*

Institute of Evolution, University of Haifa, Mt. Carmel, Haifa 31905, Israel

Aphyllophorales (higher Basidiomycetes) species play a major role in natural forest ecosystems; wood inhabiting species are especially well known as important wood decomposers.

Israeli forests are composed mainly of *Pinus hallepensis* and *Quercus calliprinos,* located in the northern and central region of Israel, where the climate provides enough rain for good growth of vegetation cover. Most of medicinal mushroom species were found growing especially in shady areas of the Golan Heights, Upper Galilee, and Carmel Mountains where botanical biodiversity is higher than in other regions of the country.

The order Aphyllophorales, with more than 200 species in Israel, includes therapeutically important species of great interest, especially in Oriental traditional medicine. Nowadays, modern medicine uses these mushroom species as great sources for pharmaceutical products.

Despite Israel's dry climate, most of the popular medicinal mushrooms in the Far East have been found inhabiting Israeli forests as well. Species such as: *Tremella mesenterica* Retz., *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst., *Laetiporus sulphureus* (Bull.) Murrill, *Schizophyllum commune* Fr.: Fr., *Auricularia auricula-judae* (Fr.) Quél., *Trametes versicolor* (L.: Fr.) Lloyd., *Fomes fomentarius* (L.) J.J. Kickx, *Ganoderma applanatum* (Pers.) Pat., *Gloeophyllum trabeum* (Pers.) Murrill, and *Phellinus contiguus* (Pers.) Pat., are a few of them.

*Ganoderma lucidum* in Israel occurs on various broad-leaved substrata such as: *Quercus calliprinos, Nerium oleander, Populus alba,* and *Cupressus* sp. in the Golan Heights, on the Carmel Mountains, and on the Philistean Plain. Current research made on *Ganoderma lucidum* has shown that its fruit body and mycelium contain approximately 400 different bioactive compounds, which mainly include steroids, lactones, alkaloids, polysaccharides, and triterpenes. The key active constituents of this mushroom have shown antitumor, immunostimulating, anti-allergenic, immunomodulating, cholesterol lowering and blood pressure reducing effects.

*Trametes versicolor* hot water extracts have been used in traditional Chinese medicine from historical

times. Modern studies have identified two important compounds, PSK or "Krestin", a water-soluble, protein-bound polysaccharide and PSP, a polysaccharide-peptide derived from mycelial cultures. These compounds have shown significant anti-tumor, antiviral, and immunomodulating activities. In Israel, *T. versicolor* was found inhabiting *Pinus hallepensis* forests in the Hula Plain and Upper Galilee.

*Tremella mesenterica* can be found in places where moisture can occur such as in shady oak forests of the Upper Galilee and the Golan Heights. According to traditional Chinese medicine, its healing capacity is well known. It nourishes the lung, stomach, kidney, strengthens bones, helps maintain ideal weight, and provides proper moisture to the skin. Nowadays, research has shown that *T. mesenterica* healing power is found in polysaccharide glucuronoxylomannan, which stimulates vascular endothelial cells, has antitumor activity, possesses pronounced antiradiating effects, stimulates hematogenesis, has a hepatoprotective effect as well as anti-allergic, anti-inflamatory, and hypocholesterolemic activities. Glucuronoxylomannan is used also to produce skin products; some properties exhibit excellent skin moisture retention, skin protection, flexibility, and flattening effects.

From data accumulated from mycological research made on *T. mesenterica* at the Institute of Evolution, University of Haifa, Israel, a new and distinct variety of mycelium grown in submerged culture was developed. The Israeli strain CBS 101939 attained 25–27 g/l of dry biomass in 4–5 days, and its amount of protein, amino acids, polysaccharides, and vitamins (by weight) was significantly greater than the regular levels in fruiting bodies of *T. mesenterica* (Wasser et al., 2004).

In view of the rapidly growing popularity of mushroom-based products, including numerous products of Aphyllophorales species, further elucidation of active principles, mechanisms of action, and their possible adverse effects as well as the quest for other biological-response modifiers by means of screening programs, is crucial in implementing safety measures for public health. Approximatively two hundred strains of Aphyllophorales, present in the culture collection of Higher Basidiomycetes Mushrooms of the Institute of Evolution, University of Haifa (HAI), will be screened for antiproliferative, antibacterial, and antioxidative activities.

The screening will involve hot-water, methanol, and ethanol extracts prepared from biomass grown in submerged cultures. Antiproliferative activity will be evaluated against a number of cancer cell lines including K562 (human chronic myclogenous leukemia cell line), Jurkat (human T lymphoblast cells), HT 29 (human colon adenocarinoma cells), and HBAE (adult bovine aortic endothelial cells) using XTT proliferation assay. Antibacterial activity will be checked on *Staphylococcus aereus*, *Escherichia coli*, and *Enterococeus faecium.*

## Liquid Medium Selection for Fungal Biodegradation of Lindane and Sodium Pentachlorophenolate

*Iztok Vidic,[1] Kristina Sepčić,[2] and Franc Pohleven[1]*

[1]Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Rožna dolina, Cesta VIII/34, Ljubljana 1000, Slovenia; [2]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana 1000, Slovenia

Polychlorinated aromatic compounds have been used for wood preservation and also for a wide range of domestic, agricultural, and industrial purposes for more than 60 years. They are presently widespread in the environment.

Lindane (γ-hexachlorocyclohexane, γ-HCH) has been an extensively used insecticide worldwide for the preservation of wood and in the control of agricultural and medical pests. It is known for its persistence in the environment, its tendency to bioaccumulate, and its toxicity to non-target organisms. Sodium pentachlorophenolate (Na-PCP) was frequently applied as a fungicide for the protection of wood, and it is still used in many countries to treat wood against blue stain.

Among Basidiomycetes, degradation of chlorophenols has only been shown for the wood decaying fungi under cometabolic conditions, i.e., growth on a complex medium. Among wood decaying fungi,

white-rot fungi use a variety of oxidative processes for the depolymerization and degradation of lignin. White-rot fungi are able to tolerate considerably higher concentrations of a toxic pollutant than organisms, which need to absorb the toxic substance in order to decompose it.

The aim of our research was to select the liquid medium for the growth of mycelium of wood-decaying fungi and to identify their potential to degrade lindane and sodium pentachlorobenzolate for further bioremediation.

Brown-rot fungus *Gleophyllum trabeum* (strain Gt2) and three white-rot fungi *Pleurotus ostreatus* (strain Plo5)*, Trametes versicolor* (strain Tv6), and *Hypoxylon fragiforme* (strain Hf) were obtained from the ZIM collection at the Biotechnical Faculty, Ljubljana.

Liquid mediums used in this study were potato dextrose broth (PDB), medium according to Shemakhanova (containing $KH_2PO_4$, $NH_4Cl$, $Ca(NO_3)_2$, $MgSO_4$, $FeCl_3$, malt extract, and glucose) and medium defined by Hadar (containing glucose, asparagine, yeast extract, $MgSO_4$, $KH_2PO_4$, and KCl). Before being autoclaved, pH was adjusted to 5.5 and the mediums were supplemented with lindane and Na-PCP, respectively, to give a final concentration 30 μmol $l^{-1}$ in all cases.

Cultivation was carried out in 250-ml flasks containing 50 ml of respective medium. Each flask was inoculated with three mycelial discs (diameter 8 mm) obtained from the periphery of 7-day-old inoculum plates. Cultures were sealed and kept agitated at 130 rpm and 25ºC in the dark for 22 days. Fungal biomass was then separated from liquid medium by filtration. The toxicity of both chlorophenols in liquid cultures was estimated by comparing the weight of dry fungal biomass to the control variants without lindane or Na-PCP, respectively. All experiments were carried out in five replicates, and the results are the mean of the five.

Comparison of the mycelial dry mass for individual fungi showed the highest yields in the PDB (0.324 g for *P. ostreatus*, 0.228 g for *T. versicolor*, and 0.0923 g for *H. fragiforme*). *G. trabeum* was an exception, with the highest yield obtained in Hadar's medium (0.169 g).

In the second set of experiments, we tested only the growth of selected fungal species in the liquid medium defined by Hadar et al. (Appl Environment Microbiol, 1986, 51(6):1352–1354), with the addition of lindane and Na-PCP, respectively. In the case of *P. ostreatus*, statistically, significantly higher dry mass yields were obtained in the liquid medium with lindane compared to the control. The statistical analysis for *T. versicolor* and *H. fragiforme* growing in the presence of lindane showed no statistically significant differences to the controls. Only *G. trabeum* yielded statistically, significantly lower dry mass in comparison with the control group.

When testing the growth in the presence of Na-PCP in the Hadar's medium, the obtained dry mass yields were statistically, significantly lower for all four tested fungal species, compared to the controls growing in the medium without Na-PCP.

Our study demonstrated that, among the tested liquid mediums, only Hadar's was appropriate for our ongoing biodegradation studies of polychlorinated aromatic hydrocarbons. The screening test for the most convenient liquid medium was based on the comparison of the obtained mycelial dry masses. The dry mass yields for *P. ostreatus*, *H. fragiforme,* and *T. versicolor* grown on Hadar's medium were slightly lower in comparison to the PDB, with an exception of brown-rot fungus *G. trabeum*. In contrast to PDB, Hadar's medium is chemically defined and enables us to do future analyses of the degradation products of polychlorinated hydrocarbons during the process of mycoremediation using gas chromatography/mass spectrometry techniques.

*Gleophyllum trabeum* is a brown-rot fungus that uses different enzyme systems of degradation compared to the white-rot fungi; therefore, when testing the growth of selected fungal species in the presence of lindane, only *G. trabeum* yielded statistically, significantly lower dry mass in comparison with the controls. The obtained dry mass yields for all fungi were considerably lower in the case of Na-PCP, which is the consequence of its fungicidal activity, in contrast to lindane that has an insecticidal efficacy.

The long persistence of the organochlorine compounds in the environment could be attributed to their resistance to or slow degradability, by microorganisms. Therefore, the search for new bioremediation agents for these compounds remains a challenge.

# Investigation of the Effect of the *Cordyceps militaris* Extract on Human and Canine Mammary Tumor Cell Apoptosis

*Yu-Chern Wei, Yu-Shen Lai, Su-Der Chen, and Yeong-Hsiang Cheng*

Institute of Biotechnology, National I-Lan University, I-Lan, Taiwan

The objectives of this study were to produce cordycepin from *Cordyceps militaris* solid fermentation and to investigate the effect cordycepin has on tumor cell apoptosis. *Cordyceps militaris* is a rare and exotic medicinal mushroom known in China for centuries. In traditional Chinese medicine, *Cordyceps* has been used to treat a wide range of conditions including respiration, pulmonary diseases, renal, liver, cardiovascular diseases, hyposexuality, and hyperlipidemia. It is also regularly used in all types of immune disorders and as an adjunct in cancer therapy. The first and secondary metabolites, including adenosine and cordycepin, of *Cordyceps militaris,* showed anti-tumor activities. The different nitrogen resources (yeast extract, yeast powder, and peptone) were applied in wheat media for the *C. militaris* solid-phase fermentation system, and the production of cordycepin and adenosine was monitored by HPLC during fermentation. The results showed that yeast powder, as the sole nitrogen resource, revealed on day 21 the highest production of cordycepin with a total yield of 4087.6 μg/g. The highest adenosine production appeared on the 17th day of fermentation with a total of 221.4 μg/g. The hot-water extracts from *C. militaris* were co-cultured with canine mammary cell lines, DE-E, DTK-SM2, and DTK-SME-like; and human mammary cell lines, HCC 1937 and HCC 1500; the results revealed that 10 μg/mL cordycepin added in a co-culture for 12-18 hrs could induce canine and human mammary cell line apoptosis under microscope observation. DNA smear phenomenon was also found in electrophoresis. The results showed that *C. militaris* hot-water extract could induce apoptosis in human and canine mammary carcinoma cell lines; the mechanism of apoptosis pathway was confirmed by caspase 3 analysis.

# Fas Upregulation is Involved in *Trametes versicolor* Polysaccharopeptide-Induced Apoptosis in Leukemic Molt-4 Cells

*Xiaotong Yang, Mei Li Li, Huiqing Feng, Ke Mi, and Qingyao Yang*

Institute of Microbiology and Immunology, Shanghai Normal University, 8th floor, Building No. 14, 100 Guilin Road, Shanghai 200234, China

The polysaccharopeptide of *Trametes versicolor* (L.: Fr.) Lloyd (PSP, comercial name, YunZhi Essence or I'm-Yunity), is a non-toxic adjuvant used in treatment of cancer patients. It can restore suppressed immunity and antagonize the side effects of chemo- and radiotherapy. It has also been used to boost the immunity in healthy people and for cancer prevention.

Recent studies demonstrated that PSP could also induce apoptosis in certain types of cancer cells, and the mechanisms were related to the activation of caspase-3, -9, and -8, downregulation of Bcl-2 expression level and Bcl/Bax ratio, and the loss of mitochondria transmembrane potential. These results suggest that the mechanisms of PSP-induced apoptosis are related to the mitochondrial signaling pathway. However, to inhibit the activation of caspase-9 alone only resulted in a weaker apoptosis blockade effect compared to that of inactivating caspase-8 and -9, both simultaneously. It is known that caspase-8 is also a key signaling molecule in the death receptor

pathway. Therefore, we hypothesize that, in addition to mitochondrial pathway, the death receptor pathway may also be involved in PSP-induced apoptosis.

In this study, we detected apoptotic events in leukemia Molt-4 cells and Fas/TRAIL expression after PSP treatment. Morphological observation revealed that PSP at a concentration over 100 μg/ml could significantly alter the appearance of Molt-4 cells dose-dependently, resulting in cell shrinkage, cell membrane blebbing, forming apoptotic body and nuclei cleavage, which were all typical apoptotic characteristics. The apoptosis events were further confirmed by using the Annexin V assay and the sub G1 peak analysis using flow cytometry. Western blotting results showed that caspase-8 was activated and Fas expression was upregulated dose-dependently. The flow cytometry assay further confirmed the increase of Fas expression in molt-4 cells after PSP treatment, and the unchanged expression level of TRAIL.

These data reveal that not only the mitochondrial pathway but also the death receptor pathway are activated during PSP-induced apoptosis. Fas but not TRAIL is the key molecule in PSP triggered death receptor pathway signaling cascade.

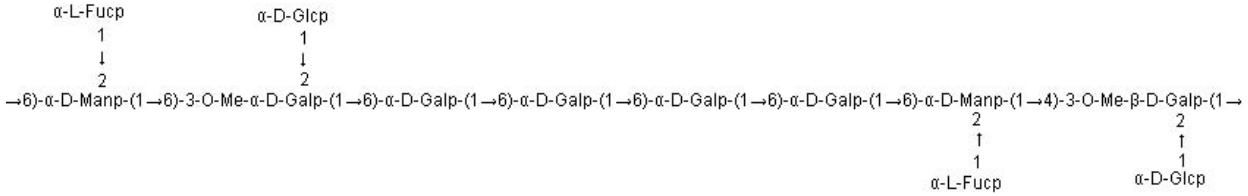
# Isolation, Purification, Structural Elucidation, and Anti-Tumor Activity of PIP60-1, a Polysaccharide from Fruit Bodies of *Phellinus igniarius*

*Yan Yang, Jingsong Zhang, Qingjiu Tang, Yanfang Liu, and Wei Jia*

Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, Shanghai 201106, China

*Phellinus igniarius* (L.: Fr.) Quél. (Hymenochaetaceae, higher Basidiomycetes) is a medicinal species well known for many centuries in traditional Chinese medicine as "Sanghuang (yellow polyporus)". Crude polysaccharide preparations from *Ph. igniarius* have been reported to possess antitumor activity. However, there are no reports related to the chemical structure and the biological activities of pure polysaccharides isolated from this mushroom.

Therefore, in this study, we have purified and structurally characterized a novel neutral polysaccharide, PIP60-1, from fruit bodies of *Ph. igniarius* and assessed it for antitumor activity using a mouse model. Pure PIP60-1 was isolated from aqueous extracts of *Ph. igniarius* by repeated chromatographic separation using Fast Flow DEAE-Sepharose and High Resolution Sephacryl S-400. UV spectrophotometry revealed no absorption at either 280 nm or 260 nm indicating that PIP60-1 contained no protein or nucleic acid. PIP60-1 appeared as a single symmetrical peak on HPLC and had a total carbohydrate of 99.47% according to the phenol-sulfuric acid method. Based on high performance anion exchange chromatography (HPAEC) and GC/MC data, PIP60-1 had an estimated molecular weight of $1.71 \times 10^4$ Da and was composed of fucose, glucose, mannose, galactose, and 3-*O*-Me-galactose in the ratio of 1:1:1:2:1. Structural determination of PIP60-1 using sugar and methylation analysis combined with $^1H$ and $^{13}C$ NMR spectroscopy, including COSY, TOCSY, NOESY, HSQC, and HMBC experiments, established that the repeating unit of the polysaccharide had the following structure (see below):



The effect of PIP60-1 on tumor growth was examined using subcutaneously transplanted H22 and Lewis Lung Carcinoma (LLC) tumor mouse models. In addition to normal feeding, control mice were administered physiological saline and the treated mice with different doses of PIP60-1 or with other polysaccharides isolated from *Ph. igniarius*. Cyclophosphamide or *Trametes versicolor* polysaccharopeptide served as positive controls in evaluating tumor response. Results showed that PIP60-1 at the most effective dose of 100 mg/kg inhibited the growth of H22 and LLC by 48% and 37%, respectively.

## Study on Germplasm Characteristics of *Pleurotus nebrodensis* in China

*Jinxia Zhang and Chenyang Huang*

Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing 100081, China

*Pleurotus nebrodensis* (Inrenga) Quél. is distributed on wasteland in Xinjiang, China. The fruit bodies were usually found from early March to middle May in spring. *P. nebrodensis* was cultivated successfully at first in the mid-1980s. Currently, its cultivation has spread to many areas throughout China because of its successful domestication. Its distribution was closely related to *Ferula singkiangnesis* in Xinjiang, China, and the host was different from the report (Venturella, 2000) investigated in Sicily, Italy. It grew on the roots or basic stems of dead or half dead *F. sinkiangensis* in China, but Venturella reported that *Cachrys ferulacea* was the only host of *P. nebrodensis* in Italy. The ITS sequence differs by 3% between *P. nebrodensis* from China and from Italy. Twenty-six samples from Xinjiang were examined in this study. It suggested that germplasm characteristics appeared to be abundant in diversity for *P. nebrodensis* populations in China. A vegetative incompatibility phenomenon was found among the population, and, therefore, they were divided into six vegetative incompatibility groups.

The mycelium could grow in a large range of temperatures, from 10 to 35°C. The suitable range for growth was in 20–30°C for every group. However, the optimum temperature varied with each group: 25°C for the most and 30°C for some. The fruiting temperature also varied, 8–18°C for some groups, but 8–22°C for others. The common optimum fruiting temperature was 12–15°C for all of them. Fruit bodies were almost equally tolerant to low temperature, but obviously different to high temperature for all. The tolerance ability of fruit bodies to high temperature could be examined at 20°C as a critical parameter for the identification of agronomical characteristics. Moreover, the responses of fruiting to temperature shock were different. The temperature shock was essential to the fruiting bodies for some but was not for others.

There was abundant diversity in cultivated characteristics in the population. The running period varied with groups, from 40 to 70 days under controlled conditions. Some group could form fruit bodies immediately after the running period finished, but some could not because they needed a period of transformation for fruiting after the running period finished, which was long or short, from 10 to 90 days. The period was 50–150 days from inoculation to fructification. There were various flush characteristics in the population. Some lasted only 20 days but some took 60 days for one flush. There were different water demands during cultivation. More water was demanded by substrates with a satisfactory yield, and low water would make the fruit body loose for the short-term group.

There was abundant diversity in fruit body morphology, mussel-like, flat funnel, palmated in shape; pure white, ivory-white, and rice-yellow in color. The tightness was deferent, usually it was tightest to palmated, general to mussel-like, and loose to flat funnel. Gills were ivory-white, cream, yellow, and pale flesh in color. Stipe was central, lateral, and eccentric in the population and was defferent in length, 1.8 cm for the palmated type, 3.7 cm for mussel-like and 8 cm for flat funnel. The stipe varied in shape, anti-triangle, columned, endlong, or bended on base.

Differences were found in micro-morphology in stages of development for the population. The size of the basidiospores varied with groups from 10.6 × 5.3 μm to 13.8 × 6.6 μm. The size of monokaryon was different from the dikaryon in diameter and usually smaller than the latter. Moreover, the monokaryon changed with the mating type in diameter, 2.7–5.8 μm (4.0 μm), 2.9–4.9 μm (3.6 μm), 1.7–4.2 μm (2.5 μm), and 2.5–3.5 μm (2.9 μm), respectively, for every type ($A_1B_1$, $A_1B_2$, $A_2B_1$, $A_2B_2$,). The size of dikaryon was 3.8–8.3 μm (6.2 μm) in diameter.

# Modulation of Neutrophilic Inflammation *in Vivo* by *Coprinus comatus* Proteoglucans

*Jelena Živanović,[1] Marina Soković,[1] Jasmina Glamočlija,[1] Srdja Drakulić,[1] Leo J.L.D. van Griensven,[3] and Milena Kataranovski[1,2]*

[1]Institute for Biological Research »Siniša Stanković«, Bulevar despota Stefana 142, Belgrade, Serbia; [2]Faculty of Biology, University of Belgrade, Studentski Trg 16, Belgrade, Serbia; [3]Plant Research International, Wageningen University and Research, P.O. Box 166700 AA, Wageningen, The Netherlands

Extracts of medicinal mushrooms are intensively investigated with regard to their immunological activities. The majority of available data were obtained in *in vitro* experimental systems, while *in vivo* approaches are less frequently used.

In this work we describe the effect of extracts from *Coprinus comatus* on the neutrophil inflammatory response by using sterile inflammation (polyvinyl sponge implantation model) and *Aspergillus fumigatus* infection in rats.

Female Albino Oxford (AO) rats were used in our experiments. Full thickness dorsal skin incisions (1 cm) were made and sterile polyvinyl sponges were placed subcutaneously. Sponges were soaked in nonpyrogenic physiological saline (F group), sterile *C. comatus* extract (20 mg/sponge, C group), *Aspergillus fumigatus* conidia ($10^6$ in saline, A group) and *A. fumigatus* conidia + *C. comatus* extract (C+A group). Migrating cells (mostly neutrophils) were isolated from sponges 18–21 hours after application. Quantitative (total and differential counts) and qualitative measurements were conducted in order to determine metabolical cell viability (MTT reduction assay) and spontaneous and PMA–stimulated cell activation (NBT reduction assay).

Subcutaneous application of sponges, which contained only physiological saline, (F) resulted in neutrophil migration and activation. Significant increases of total leukocyte and granulocyte numbers were noted in cells recovered from sponges of C and A groups, with further increase when C+A were applied simultaneously. Increased levels of MTT reduction revealed increased metabolic cell activity in both C, A and C+A cells, with the highest levels in A group. Application of *C. comatus* extract and *A. fumigatus* conidia resulted in increase in both spontaneous neutrophil activities as well as in responsiveness to exogenous stimulation by phorbol myristate acetate (PMA) compared to neutrophils from control sponges.

Our data demonstrated proinflammatory and immunomodulatory activity of *C. comatus* extract in the sponge/infection model of inflammation. Obtained results pave the way for future investigations of the biological significance of these activities.

## ACKNOWLEDGMENT

Supported by Ministry of Science and Environmental Protection, grants # 143038 & #143041.

# To Reveal Natural Groups within the Genus of the Medicinal Fungi *Trametes* (Polyporales, Basidiomycota)

*Ivan V. Zmitrovich[1] and Wjacheslav A. Spirin[2]*

[1]Laboratory of Systematics and Geography of Fungi, Komarov Botanical Institute, Russian Academy of Sciences, 2 Prof. Popov St., St. Petersburg 197376, Russia; [2]University of Humanities, 15 Fuchika St., St. Petersburg 192238, Russia

The genus *Trametes* s.l. seems to be a highly perspective object for biotechnological use, because a range of species are important medicinal mushrooms (e.g., *Trametes suaveolens*, *T. versicolor*, *Pycnoporus cinnabarinus)*. It is obvious that an adequate taxonomical arrangement could play an important role in the elaboration of a strategy for searching bioactive producers, considering that the genus stays highly heterogeneous till now.

The morphological scope does not give a satisfactory resolution of the *Trametes* heterogeneity and, due to the variability of character combinations in different species it confirms the larger concept of the genus (Corner, 1990). The best results are given by the analysis using the sequence of LSU and ITS regions of nuclear ribosomal DNA (Tomšovský et al., 2006), although a few species were involved in these studies.

Our analysis has combined the results of large-scale rearrangement and original data on fine hyphal morphology and peculiarities of the hymenial elements in some tropical and boreal species. On this base, we can make some preliminary conclusions.

1. The genus *Trametes* s.l. is strongly merged into *Polyporus*, so LSUrDNA phylogenies can not give a normal resolution of branching patterns of trametoid taxa. The genera *Datronia, Dichomitus,* and *Fomes*, apparently, during a time will enter into *Polyporus* – on the manner of core-*Lentinus* species (Krüger, Gargas, 2004).
2. The area '*Trametes* s. mes.' tends to look like much clearer due to the *Lenzites* separation (*Lenzites gibbosa, L. elegans, L. betulina,* and *L. warnieri*).
3. Even in this circumscription, the *Trametes* remains heterogeneous. Its boundary area linked with 'large *Polyporus*' is represented by genera *Funalia* and *Trametes* s. str. both characterized by extended spores and clear trimitic structure. Their differences are fine details of basidiome structure: *Funalia* (incl. *Donkioporia, Fuscocerrena*, and *Cerrena*) has colored, more or less duplex context, whereas *Trametes* s.str. has white highly homogeneous context. On the other hand, *Trametes* has a border area with minute polypores, *Antrodiella* and *Irpex*. This boundary area can be designated as an enlarged *Pycnoporus* (incl. *Coriolus, Microporus,* and *Diplomitoporus*) which is characterized by small cylindrical (as a rule asymmetrical) spores, the absence of pegs and non-thickened pilei. Within this group, however, the species of *Coriolus, Microporus,* and *Pycnoporus* have clearly trimitic hyphal system and CB– skeletals, whereas the rest of *Diplomitoporus* are rather dimitic and have CB+ skeletals (there are some exclusive species).
4. The general evolutionary trend of *Trametes* can be established from stipitate forms, via forms reminiscent of *Polyporus udus*, to tyromycetoid and trametoid ones. It seems that the real area of radiation (= natural genera) are correspond only to two items – the basal one, *Polyporus* (incl. *Trametes–Funalia–Lenzites*) and the derived one, *Irpex* (incl. *Pycnoporus–Antrodiella*).

# Mechanisms of Toxicity of Ostreolysin, a Cytolytic Protein from the *Pleurotus ostreatus*, in Rodents

*Monika C. Žužek,[1] Katja Rebolj,[2] Urska Batista,[3] Kristina Sepčić,[2] Peter Maček,[2] Vojteh Cestnik,[1] and Robert Frangež[1]*

[1]Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, University of Ljubljana, Ljubljana 1000, Slovenia; [2]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana 1000, Slovenia, [3]Institute of Biophysics, Faculty of Medicine, University of Ljubljana, 2 Lipičeva, Ljubljana 1000, Slovenia

The *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. (oyster mushroom) is a highly appreciated edible mushroom with important medicinal, biotechnological, and environmental applications. However, sporadic local intoxications following human and animal ingestion of large quantities of the fresh mushroom were recorded, and it was suggested that the toxicity is associated with thermolabile proteinaceous molecules. Recently, purification and isolation of a novel 16 kDa haemolytic and cytolytic protein ostreolysin from *P. ostreatus* has been described. In this work, ostreolysin putative toxic and lethal effects were studied on rodents to evaluate its potential involvement in the observed adverse effects of the mushroom.

Lethality of ostreolysin was determined by the intravenous administration of 800–1400 μg/kg ostreolysin on male Balb/C mice, which were observed for 24 h for signs of intoxication and lethality. The signs of intoxication were dose-dependent; cyanosis, cessation of movement, and hair bristling were observed. The *i.v.* $LD_{50}$ of ostreolysin was determined to be 1170 μg/kg mouse body weight. Cardiorespiratory effects were studied after intravenous injection of one mouse $LD_{50}$ to male Wistar albino rats. A few seconds after ostreolysin administration, a transient increase in arterial blood pressure was recorded, followed by a progressive fall to mid circulatory pressure accompanied by bradicardia, myocardial ischaemia, and ventricular extrasystoles. Similar changes produced by ostreolysin were observed in vagotomised and artificially respirated animals, indicating that vagotomy and hypoxia do not play a primary role in toxicity. Further investigation of ostreolysin effects showed that the protein was able to induce a concentration-dependent increase in aortic ring tension in the range from 5 to 30 μg/ml.

Ostreolysin induced lysis of rat erythrocytes *in vitro*, and probably also *in vivo* as indicated by the increase in serum potassium. The protein was also shown to be cytotoxic on human umbilical vein endothelial cells and Chinese hamster lung fibroblasts. Cytolytic effects of the protein may be responsible for the observed hyperkalaemia and may significantly contribute to its previously observed cardiotoxicity, which may derive from the injury of endothelial cells, or the mechanism responsible for ACh endothelium-mediated relaxation.

Since many of the observed signs of ostreolysin intoxication resemble those previously reported on the crude mushroom extract, it is suggested that the sporadic recorded local intoxications after ingestion of the oyster mushroom may derive from the activity of ostreolysin.