



Review

Medicinal mushrooms: a rapidly developing area of biotechnology for cancer therapy and other bioactivities

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Abstract

Historically, medicinal mushrooms (basidiomycetes) have been shown to have profound health promoting benefits and recent studies, which are reviewed here, are now confirming their medical efficacy and identifying many of the bioactive molecules. Methods of large-scale cultivation by solid substrate and liquid culture fermentations are also briefly described.

Introduction

Many forms of chronic disease such as cancer and cardiovascular dysfunction can, in part, be attributed to diet and arising from the awareness of the relationship between diet and disease has evolved the concept of 'functional foods' (Sadler & Saltmarsh 1998). The US Academy of Science has defined functional foods as those that 'encompass potentially health products' including 'any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains' (Thomas & Earl 1994). 'Foods as medicine' underpins the paradigm of functional foods. Functional foods cannot claim to cure diseases but, increasingly, evidence is being produced that supports the role of some functional foods in disease prevention (Steinmetz & Potter 1991). The concept of foods as medicine does not fit easily within the current expertise in either pharmaceutical or food companies and the full creative development of functional foods may well require new alliances between these companies with respect to regulatory issues.

Fleshy mushrooms (members of the class Basidiomycetes) have long been valued as highly tasty and nutritional foods by many societies throughout the

world (Chang & Miles 1989). To the ancient Romans they were 'the foods of the Gods', to the early Egyptians they were 'a gift from the God Osiris' while, more appropriately, the Chinese considered them 'the elixir of life'. Several ancient societies dating back as far as the Palaeolithic period recognised the psychoactive, hallucinogenic properties of some mushrooms, especially *Amanita muscaria* and *Psilocybe* spp., and involved them in ancient religious beliefs and practices (Arora 1985, Samorini 2001).

Many cultures worldwide, but especially in the Orient, recognised that extracts from certain mushrooms could have profound health promoting benefits and, consequently, became essential components in many traditional Chinese medicines. There are at least 270 species of mushrooms that are known to possess various therapeutic properties (Ying *et al.* 1987) and the term 'medicinal mushroom' is now increasingly gaining worldwide recognition. Edible mushrooms which demonstrate medicinal or functional properties include species of *Lentinula*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus* and *Tremella* while others known only for their medicinal properties, viz. *Ganoderma lucidum* and *Trametes (Coriolus) versicolor*,

are decidedly non-edible because of their coarse texture and bitter taste.

Historically, most medicinal mushroom species were relatively scarce and were collected from the forests where they grew on dead or living trees and forest litter. They are predominantly lignocellulose degraders. For medicinal purposes, they were almost always prepared either as hot water extracts, concentrates or in powdered form. Nowadays almost all of the important medicinal mushrooms have been subjected to large-scale artificial cultivation by solid substrate or low moisture fermentation, thus removing the historical scarcity factor and allowing large commercial operations to develop. Mushroom cultivation is the only large-scale biotechnological process that creatively utilises lignocellulosics (Stamets 2000).

Medicinal properties

The practice of using medicinal mushrooms in Chinese traditional medicine dates back into antiquity and has been recorded in ancient Chinese manuscripts (Bensky & Gamble 1993). Increased scientific and medical research in recent years and published in peer-reviewed journals, especially in Japan, Korea and China, and more recently in the US, is increasingly confirming the medicinal efficacy and identifying the bioactive molecules (Wasser & Weis 1999, Ooi & Liu 2000, Hobbs 2000). Recent advances in chemical technology have allowed the isolation and purification of some of the relevant compounds especially polysaccharides which possess strong immunomodulation and anti-cancer activities. The bioactive polysaccharides isolated from mushroom fruit-bodies, submerged cultured mycelial biomass or liquid culture broths are either water-soluble β -D-glucans, β -D-glucans with heterosaccharide chains of xylose, mannose, galactose or uronic acid, or β -D-glucan-protein complexes i.e. proteoglycans (Mizuno 1999). While many are orally bioavailable, others are mainly effective only by intraperitoneal injection. The main medically important polysaccharides that have undergone extensive anti-cancer clinical trials include lentinan (*Lentinula edodes*), schizophyllan (*Schizophyllum commune*), PSK (polysaccharide-K, commercially sold as Krestin) and PSP (polysaccharopeptide) (*Trametes versicolor*), and Grifon-D (*Grifola frondosa*) (Kidd 2000). All of these compounds are currently produced by Asian pharmaceutical companies.

These medicinal polysaccharides are primarily modifiers of biological response where these polymers interact with the immune system to up-regulate or down-regulate specific aspects of the response of the host and this may result in various therapeutic effects (Bohn & BeMiller 1995). Their ability to enhance or suppress immune responses can depend on a number of factors including dosage, route of administration, timing and frequency of administration, mechanism of action or the site of activity. Several of these compounds have been shown to potentiate the host's innate (non-specific) and acquired (specific) immune responses and to activate many kinds of immune cells that are important for the maintenance of homeostasis, e.g. host cells (such as cytotoxic macrophages, monocytes, neutrophils, natural killer cells, dendritic cells) and chemical messengers (cytokines such as interleukins, interferon, colony stimulating factors) that trigger complement and acute phase responses (Li 1999, Ooi & Liu 2000). They can also be considered as multi-cytokine inducers capable of modulating gene expression of various immunomodulatory cytokines via specific cell membrane receptors (Maeda et al. 1994, 1996). Lymphocytes governing antibody production (β -cells) and cell-mediated cytotoxicity (T-cells) are also stimulated.

Anti-cancer properties

There have been extensive *in vivo* studies demonstrating the anti-cancer activity of the extracted, purified glucan polysaccharides and polysaccharide peptides in animal models (Wasser & Weis 1999). These studies strongly suggest an immunomodulating mode of action. However, in *in vitro* studies on various cancer cell lines, there is evidence for direct cytotoxic effects on the cancer cells for some, but not all, of the polysaccharides (Borchers *et al.* 1999). Many of the proprietary mushroom polysaccharides have proceeded through Phase I, II and III clinical trials mainly in Japan and China while some are now occurring in the US (Kidd 2000). In almost all cases the polysaccharides are used as adjuvant treatments with conventional chemotherapy/radiotherapy with many forms of cancer. Highly encouraging results have been forthcoming together with the observations that their incorporation into treatment regimes significantly reduced the side-effects so often encountered by patients. Several of the purified mushroom polysaccharides have been in clinical use in Japan, China and Korea for

many years, with no reports of any significant short-term or long-term adverse effects. These compounds are not miracle drugs but can increase the quality of life of cancer patients and may offer increased survival rates for some types of cancer (Kidd 2000).

There is also increasing experimental evidence that regular incorporation of certain powdered medicinal mushrooms in the diets of animals or topical application of extracts can have a cancer prevention effect and restriction of tumour metastasis (Ikekawa 2001, Shon & Nam 2002). A survey conducted among mushroom workers in the Nagano Prefecture in Japan implied that regular eating of medicinal mushrooms (mainly *Flammulina velutipes*) was associated with a much lower death rate from cancer than for other people in the Prefecture (Ikekawa 2001).

Extracts of medicinal mushrooms are also extensively used in Chinese traditional medicine to treat viral and other microbial infections, for cardiovascular diseases, diabetes and also for hepatoprotection. Commercial preparations are available as tablets, elixirs or capsules, and are extensively on sale in most Oriental countries and increasingly in the USA and Europe as nutraceuticals in natural food/medicine markets. *Ganoderma lucidum* or Reishi, is the premier medicinal mushroom and the annual value of extracts of this mushroom alone sold worldwide is estimated at over US\$ 1.6 billion (Chang & Buswell 1999) and mostly used as a tonic for the immune system.

Technology of medicinal mushroom cultivation

There are at least 12 000 species of fungi that can be considered as mushrooms with at least 2000 species showing various degrees of edibility (Chang 1999a). To date, only about 35 mushroom species have been cultivated commercially and, of these, about 20 are currently cultivated on an industrial scale. The majority of these cultivated species are both edible and possess certain medicinal properties. Overall, the world production of cultivated edible and/or medicinal mushrooms was recorded as 4909×10^3 tons in 1994, increasing to 6158×10^3 in 1997, with an estimated value in excess of 14 billion US dollars (Chang 1999b). Mushroom cultivation is a worldwide practice. Output yield of the leading 10 species cultivated made up about 92% of total world production and of these, six species, viz.: *Agaricus bisporus* – the white button mushroom (31.8%); *Lentinula edodes* (25.4%); *Pleurotus* spp. (14.2%); *Auricularia auricula*

(7.9%); *Flammulina velutipes* (4.6%) and *Volvariella volvacea* (7.9%) made up 87% of the total production. By late 1994, of these species, only *Agaricus* and *Pleurotus* were cultivated worldwide to be joined in 1997 by *Lentinula*. The other three of the major six species are grown almost exclusively in Asia (Chang 1999b). World production of mushrooms over the last two decades has shown a phenomenal pattern of growth, with a 5 times increase in tonnage. While *Agaricus bisporus* (with few specific medicinal claims) still retains the highest overall world production, its relative contribution is decreasing due to the dramatic increase in the other species, viz: *Lentinula* and *Pleurotus* in particular. In 1981, *Agaricus* production represented 72% of world production but by 1997 this had dropped to 32%. Overall world production of mushrooms is increasingly being dominated by species that are both edible and have medicinal properties or are only medicinal (*Ganoderma* and *Trametes*).

Medicinal mushrooms can be cultivated through a variety of methods. Some methods are extremely simple and demand little or no technical expertise, while cultivations which require aspects of sterile handling technology are much more technically demanding (Stamets 2000). For production of the fleshy mushroom fruit-bodies, various forms of solid substrate or low moisture fermentations are employed whereas, for mycelial biomass production, liquid tank fermentations are now becoming increasingly important especially for nutraceutical and pharmaceutical productions.

Solid substrate fermentation

All medicinal mushrooms are lignocellulose degraders and can use wood as substrate for mycelial growth and fruit-body production. The historical method of cultivation, and still practiced mainly in Asia, is on logs of hardwood trees. This process occurs over several years and yields two crops of mushrooms each year and continues until the log physically collapses due to wood degradation. This is primarily an outdoor process, producing quality mushrooms, but is not economically suitable for worldwide production (Stamets 2000).

The rapid worldwide cultivation of medicinal mushrooms is due largely to the use of specially designed 1–2 l polypropylene bags or containers with microfilter windows for air exchange. The bags contain the substrate of sawdust and selected nutrients,

and after autoclaving, are inoculated with the mushroom mycelium with the entire growing process being carried out under controlled environmental conditions over a reduced time scale (1–3 months). Almost all edible medicinal mushroom fruit-bodies are now produced worldwide by modifications of this method (Stamets 2000).

The batch solid substrate fermentation process occurring within the polypropylene bag (bioreactor) can be viewed as a dynamic operation comprising three phases in which the inoculum will grow and undergo morphological development, viz.:

1. The solid phase involving a lignocellulosic substrate (sawdust or wood chips) with other essential nutrients.
2. An aqueous phase intimately associated with the solid surfaces and in various states of sorption.
3. A gaseous phase continuous with the external environment.

This is essentially a low moisture fermentation with no visible free water.

Strain selection of individual mushroom species is essential before starting fruit-body production. Individual strains can show differences in growth rate, yield, temperature requirements, degree of required aeration and physical features of the fruit-body such as colour and shape. A mushroom species can consist of many strains each with a distinct genotype and phenotype (Stamets 2000). Strain stability and identification can be problematic.

The inoculum or *spawn* should be a vigorous mycelial growth of a pure strain on a chosen vehicle (liquid medium, grain or sawdust) and this living, vegetative propagation stage will be used to inoculate the sterilised sawdust substrate in the polypropylene bags, e.g.

Liquid spawn: mycelial spawn produced in flasks or small fermenters and is a rapid method of multiple point inoculation.

Grain spawn: a variety of mycelium-coated grains such as millet, rye, wheat, sorghum and milo, is widely used commercially.

Sawdust spawn: supplemented mycelium impregnated sawdust-bran substrate is less often used.

Mushroom growers rarely produce their own spawn and normally obtain guaranteed cultures from a wide range of specialist inoculant companies.

For the cultivation of most medicinal mushrooms the basic substrate is hardwood sawdust (a mixture of fine and coarse sawdust to ensure good aeration), 75–80%, supplemented with wheat bran (coarse) – 20%,

gypsum (calcium sulphate) – 1%, \pm sucrose – 1%, moisture content – 60–65% and pH – 5.5–6.5 (Chen 1999a,b, Stamets 2000). The bags are sealed and autoclaved to create a sterile environment and on cooling, inoculated aseptically and resealed. Total asepsis is required until the substrate is colonised. Only after colonisation are the bags opened to facilitate fruit-body formation. Each mushroom species will require specific growth parameter requirements and attention must be given to regulate temperature, relative humidity, light and air flow. Each of these conditions will vary with the stage of development, viz.: spawn run, primordium initiation and fruit-body development. The overall process is extremely complex and will span 1–3 months depending on type of mushroom being cultivated. Any deviation in parameter control can have disastrous consequences. [For fuller details reference should be made to Stamets (2000) and Chen (1999a,b)].

Mushroom fruit-bodies are complex structures, both morphologically and physiologically with undoubted variations in chemical composition from batch to batch. The chemical make-up of a mushroom fruit-body will mirror the composition of the basic substrate and supplementary ingredients which can vary considerably since the basic raw materials are derived from lignocellulosics from agriculture or forestry. Also, within any batch of mushrooms, there will be some degree of variation in size and age which will, undoubtedly, influence specific biochemical composition (Gunde-Cimerman 1999, Wasser *et al.* 2000). While this is not critical when producing fruit-bodies for the fresh market it could create problems and preclude standardisation of the extracted products (nutraceuticals/pharmaceuticals) without extensive and costly purification.

The way ahead for mushroom nutraceuticals and pharmaceuticals must be an increasing dependency on pure culture mycelial cultivation strategies. By adopting modern fermentation practices it will generate many production and safety advantages, e.g. speed of growth with major reduction in production time, optimisation of medium composition and physicochemical conditions to allow regulation of mushroom metabolism, improved yield of specific products and designed variation of product types. Furthermore, since the final product(s) can be better controlled, a more consistent and reproducible production can be achieved which will be an important factor in the current climate of Good Manufacturing Practice (GMP) required by the regulatory authorities.

Liquid submerged fermentation

Submerged pure culture fermentation techniques have been widely developed for most of the main medicinal mushrooms and used in the propagation of mycelium for three main applications, viz.: (1) liquid spawn for solid substrate fruit-body production; (2) biomass that can be used for food and dietary supplements; and (3) biomass and/or extruded metabolites especially exo-polysaccharides as raw materials for pharmaceutical studies. In all cases the underlying principle in each approach is to use mycelium in the active physiological state and of known purity. As in any fermentation study, the factors which can affect mycelial growth rate, yield of biomass and metabolic production, include inoculum size, pH, composition of nutrients, aeration and temperature. While many studies have been restricted to shake flask cultures, others have used laboratory and pilot-scale liquid cultivation technology, with dry mass yields of 16–18 g l⁻¹ during 4–5 days of cultivation for several medicinal mushroom species (Solomko 2001, Puchkova *et al.* 2001, Reshetnikov *et al.* 2001).

The limited availability of the caterpillar fungus, *Cordyceps militaris*, used in Chinese traditional medicine for hypoglycaemic activity, prompted the development of mycelial fermentations in liquid culture (Yang *et al.* 2000, Park *et al.* 2002). These fermentation studies have been highly successful with product development closely similar to that obtained from the whole fungus/insect combination. Similarly, the hypoglycaemic polysaccharide from *Phellinus linteus* has been successfully achieved by submerged mycelial culture (Kim *et al.* 2002). Ganoderic acid has been successfully produced by liquid fermentation of a mycelial culture of *Ganoderma lucidum* (Fang *et al.* 2002). A glycoprotein with strong immunosuppressive activity has been extracted from cultured mycelium of *Ganoderma lucidum* (Tsumoo *et al.* 1994). The two most widely used anti-cancer polysaccharides, PSK and PSP from *Trametes (Coriolus) versicolor* produced respectively in Japan and China, have always been obtained by liquid tank fermentations and now new generation products are being developed with new strains and novel cultivation techniques. The exact details of these fermentations are not available.

While standard stirred-tank liquid fermentations have been the most widely used mycelial techniques, several studies have used air-lift fermenters for exopolysaccharide production from mycelia of *Ganoderma lucidum* (Lee *et al.* 1999a,b). In contrast

to the normal pellet growth obtained in stirred fermenters, air-life fermenters normally produce filamentous growths.

A recurring problem with the use of Basidiomycete fungi in liquid fermentation conditions has been the low rate of mycelial growth as compared with other microorganisms such as bacteria, yeasts, and filamentous fungi. The vegetative mycelial state of most medicinal mushrooms will be the dikaryon, the binucleate cell containing the opposite sexual nuclei. The vegetative propagation of such cells involves complex clamp connections which may be an impediment to rapid mycelia propagation. The dikaryon is the stable, long-living stage of the Basidiomycete life-cycle whereas the monokaryotic stage is normally short-lived. However, a technique has been developed to convert the dikaryotic stage of *Trametes versicolor* into a long-living monokaryotic form with ensuing extracellular product formation. When the dikaryotic stage was subjected to a mechanical treatment such as grinding or shearing in a liquid submerged medium, a monokaryotic mycelium was produced which was characterised by extremely high propagation rate and polysaccharide production (Yoshikumo *et al.* 1979). It is not known if this technique is widely used commercially with other Basidiomycetes that occur in the dikaryotic vegetative stage.

Another growth promoting effect for Basidiomycetes in both liquid culture and solid substrate culture has been obtained by adding to the culture medium a straight chain saturated aliphatic alcohol with a carbon number within a specific range of 26 to 36 carbon atoms (Takita *et al.* 1983).

While mycelial cultures dominate most Basidiomycete fermentations yeast-like cultures have also been used commercially. *Tremella mesenterica* fruit-bodies contain a polysaccharide, glucuronoxylomanan, with reputed hypocholesterolemic effects. However, such production from fruit-bodies can be highly variable and new methods have been developed to standardise production by liquid fermentation methods, for the nutraceutical market. This fungus has a very complex life cycle in contrast to other Basidiomycetes. A single basidiospore can germinate on nutrient medium to hyphal growth or by yeast-like budding. The haploid yeast budding culture has been developed and shown to be the best form of growth for submerged liquid culture and for producing large amounts of the desired polysaccharide (Reshetnikov *et al.* 2001, Wasser & Reshetnikov 2002). The dynamics of this submerged culture can be divided into

two stages. In the first phase or trophophase, unicell biomass accumulation is favoured in conditions of balanced nutrient uptake while the second phase occurs in the presence of excess carbon source, but limited nitrogen assimilation, and is characterised by glucuronoxylomannan accumulation.

While solid substrate fermentations will remain the chosen method of production for whole mushrooms for food and nutraceutical purposes, there will be a continued increase in the development of submerged liquid culture to produce a more uniform and reproducible biomass for dietary supplements and pharmaceutical products. Western biotechnology companies have yet to recognise the potential of this area of medical bioscience.

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