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Bio-technologies of Recycling Agro-industrial Wastes for the Production of Commercially Important Fungal Polysaccharides and Mushrooms

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Introduction

The activities of mankind, and indeed those of all living systems, involve the production of wastes, which represent surplus materials that have not been effectively utilized. In a series of processes in agricultural production, some wastes from one process in a chain may constitute the input of the next one. Ultimately, the final waste products emerge that are so low in energy they appear only useful to certain microbes. The wastage from the food industry in some developed countries exceeds 20%, while losses of edible materials in the developing countries may, in some cases, be in excess of 80% (Buckle, 1993).

If wastes from the agro-food industry could be reduced by 30–50%, the available food supply could be increased by up to 15%, with no need to bring new land into production. Agricultural processing wastes, whether solid or liquid, have high *biological oxygen demand* (BOD) values and cause problems from several points of view, including collection costs, treatment and disposal, and the loss of valuable raw material (Pimental and Pimental, 1983). This, together with the existence of increasingly restrictive anti-pollution laws, has led to the development of recycling technologies, reprocessing and utilizing wastes, rather than simply discharging them untreated into the environment (Loehr, 1977). The utilization of agro-industrial wastes can be considered as 'successful' if it leads to a beneficial use of the waste and

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Abbreviations: BE, biological efficiency; GI, gastrointestinal; I.V., intravenous.

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sometimes, but not always, to a marketable and profit-making product. Many wastes are such a burden to the environment that processes would be considered very satisfactory if they would only cause the overall cost of waste management to be less than other alternatives.

Lignocellulose residues are the most abundant renewable biomass on Earth. Their utilization by fungi through biotechnological approaches could allow self-sustainable processes and products, such as mushroom production (Chang, 1993; Philippoussis *et al*., 2000), enzyme production (Buswell and Chang, 1993; Giovanozzi Sermanni *et al*., 1994), improvement of digestibility for feeding animals (Moyson and Verachtert, 1991; Zadrazil *et al*., 1996), energy production (alcohol, methane, etc.), and bioremediation of toxic molecules (Barr and Aust, 1994).

The cultivation of edible mushrooms represents the only current, large-scale, controlled application of microbial technology for the profitable conversion of waste lignocellulose residues from agriculture or forestry. From each kilogram of cellulose and/or lignin containing dry waste, we can obtain one kg fresh mushrooms. Moreover, 330 kg dry waste becomes 1000 kg after moistening, and on this prepared substrate, we obtain mostly 200 to 300 kg mushrooms, i.e. bioconversion efficiency 20–30% (Zervakis and Philippoussis, 2000).

Mushroom cultivation not only reduces disposal problems caused by residue accumulation, but also provides an economically acceptable alternative for the production of high quality food and fodder, which may contribute significantly to the increase of a farmer's income. Moreover, the by-product, namely spent compost, can be used as animal feed and crop fertilizer.

Among mushroom fungi, *Lentinula* and *Pleurotus* species reveal high efficiency in lignocellulolytic degradation of a wide range of plant litter, such as wheat straw, cotton wastes, including stem-leaf residues and gin trush, corn cobs, wood chips, sawdust, peanut shells, grape vines, olive oil mill wastes, and others, into fungal protein suitable for human consumption (Ragunathan *et al*., 1996; Campell and Racjan, 1999; Philippoussis *et al*., 2000, 2001a,b; Poppe, 2000). Their mycelium can produce extensive enzymes, which can degrade lignocellulosic wastes and use them as nutrients for their growth (Buswell *et al*., 1996).

The utilization of waste materials from agricultural production in many current practices are operations which facilitate the reduction of some waste management problems. Examples may include fruit and vegetable wastes, which are being utilized as animal feed. Tomato skins and seeds after dehydration are used part as animal feed, as are lignocellulosic by-products like straw, corn stalk husks, and cobs (Israilides *et al*., 1981). Solid wastes from the food canning industry, such as peas, apricots, corn, etc., as well as citrus wastes after the extraction of juice and further de-oiling, have been converted into a dried cattle feed. Processes, such as the extraction and purification of pectin from citrus peel and apple pomace, have been well established, as are the conversion of animal blood and bones to animal feeds and fertilizers (Buckle, 1993).

Straw can also be converted into soil conditioner, admixed with other agricultural wastes through composting, and with horse manure as a medium for growing mushrooms. This material also provides a source for the production of organic chemicals, such as furfural. Other possibilities that have been studied include the production of tartarate from wine grape residue, monosodium glutamate from bagasse, and soap, leather glue, and animal feed from meat packing waste products. High added value products, such as hormones, vitamins, and enzymes, have also been produced from meat packinghouse residues. In the olive oil producing countries, a fuel material is produced from the olive stone wooden residue, although high-quality soil conditioner seems to be a better alternative (Israilides *et al.*, 2000).

It is a fact that these methods of waste utilization very seldom solve the entire problem of agro-industrial wastes, since they only utilize or remove a small component from the waste, and usually leave large quantities of residues behind for further management. However, the efforts for agro-industrial waste utilization should be directed toward effective and economical solutions, as well as toward the development of adequate markets for the usable by-products. Without a sufficient market, the material produced may be of little economical value. Biotechnology may help to reduce the amount of waste produced, or increase the degree of treatment or utilization, in three major areas:

1) modification of plants or animals so that less waste occurs during processing;
2) modification of microorganisms or enzyme systems to recover and utilize more waste materials;
3) improvement in current treatment methods for liquid and/ or solid waste disposal.

The various utilization processes, which have appeared applicable to a variety of agro-industrial wastes, may be indicated as: composting, drying and dehydration, by-product development, such as biomass, production of food ingredients and enzymes, methane generation, and water reclamation. In this paper, the production of two industrially important polysaccharides from agro-industrial wastes, namely *pullulan* and *lentinan*, will be reviewed. In this case study, the capital investment for the production of these two polysaccharides can be fully justified.

For many agro-industrial wastes, there has been a continuous effort to find ways of ecological and economical utilization in order to avoid the current practice, which for the vast quantity of agricultural wastes, continues to be land disposal in a crop production cycle, with or without any prior treatment of the waste.

Pullulan

Pullulan is an exocellular homopolysaccharide produced by the yeast-like fungus *Aureobasidium pullulans*. It is composed of maltotriosyl units linked through α(1,6) glycosidic bonds (le Duy *et al.*, 1983; Shin *et al.*, 1989). Its industrial applications have been thoroughly reviewed by Desphpande *et al.* (1992). Pullulan has many industrial applications in the food, pharmaceutical, and other fields. In the food industry, it is mainly used as a starch substitute in low calorie foods, and as a bulking, binding agent in tablet manufacturing in the pharmaceutical industry.

Other applications are as a packaging material as biodegradable plastic, since it is edible, resistant to oils and grease, impermeable to oxygen, nontoxic, and heat resistant (Yuen, 1974). The use of agro-industrial wastes as substrates for pullulan has been reported by many researchers (Han *et al.*, 1976; Biely *et al.*, 1978; Zajic *et al.*, 1979; le Duy and Boa, 1983; le Duy *et al.*, 1983; Shin *et al.*, 1989; Hayashibara Biochemical Labs, Inc., 1990; Bambalov and Jordanov, 1993; Israilides *et al.*, 1993;

Roukas and Biliaderis, 1995; Barnett *et al.*, 1999; Roukas and Liakopoulou-Kyriakides, 1999; Arapoglou *et al.*, 2002). The market price of food and pharmaceutical grade pullulan is about 88 Euros per kg. The current world supply (circa 10 000 tones p.a.) is produced almost exclusively by the Japanese company, Hayashibara Co. Ltd., Okayama, Japan.

Among the many agro-industrial wastes reported as suitable for pullulan production have been: straw hydrolystate (Han *et al.*, 1976); hemicelluloses in waste water from the production of viscose fibres (Biely *et al.*, 1978); spent sulphite liquor, a by-product of the timber industry (Zajic *et al.*, 1979); peat hydrolysate, which is the liquid resulting from the heat treatment of raw peat in an acid solution (le Duy and Boa, 1983); lactose, which is the main sugar in whey, a by-product of the cheese industry (le Duy *et al.*, 1983); inulin from Jerusalem artichokes, which is an inexpensive carbon source (Shin *et al.*, 1989); starch wastes and corn glucose syrup (Hayashibara Inc., 1990; Barnett *et al.*, 1999). Recently, *jaggery*, a concentrated sugar cane juice produced by cottage industries in India, was used as an inexpensive substrate for pullulan production (Vijayendra *et al.*, 2001). Finally, olive oil wastes, grape skin pulp extract (Bambalov and Jordanov, 1993; Israilides *et al.*, 1993; Arapoglou *et al.*, 2002), carob pod extract and molasses (Roukas and Biliaderis, 1995; Roukas and Liakopoulou-Kyriakides, 1999), were also used experimentally as substrates.

Although, due to the many other factors involved, it is difficult to make a fair comparison of the effectiveness of each substrate in pullulan production, it can nonetheless be concluded that agro-industrial wastes can serve as similar, or better, substrates compared to conventional or defined ones. Nevertheless, it has also been shown that the composition of the crude precipitated substance from the fermentation broth may vary widely, depending on the substrate and fermentation conditions. Additionally, the 'pullulans' produced often vary in purity and molecular weights (Israilides *et al.*, 1994b), and therefore it is strongly recommended to test for the presence of actual pullulan in the agglutinating substance each time, as a large amount of other material – usually high molecular weight – precipitates together with pullulan during separation. *A. pullulans* is capable of producing other polysaccharides on most carbon sources, depending on the carbon source and strain.

A coupled enzyme assay has been developed to access pullulan from the crude agglutinating substance in pullulan fermentations (Israilides *et al.*, 1994a). The method is rapid, inexpensive, and reliable, and the results are in good agreement with other, more complex and laborious methods, like detection of maltoriose units after hydrolysis by HPLC or capillary electrophoresis.

In general, pullulan biosynthesis is dependent on cultural conditions and morphological type. These factors have been reviewed in a previous paper (Israilides *et al.*, 1999). Among the many agro-industrial wastes being tested for pullulan production, grape skin pulp and starch wastes have been the best substrates, producing the highest yields and the most pure pullulan, as assessed by various methods (Israilides *et al.*, 1994b).

In conclusion, the production of pullulan from agro-industrial wastes is both feasible and ecologically sound. Therefore, it would be prudent to search for the available and suitable substrate and select the proper strain of the pullulan-producing organism to produce a valuable product, while protecting the environment from pollution.

Cultivation of *Lentinula edodes* mushroom for nutritional and medicinal purposes

The cultivation of *Lentinula edodes* (Berk.) Pegler [=*Lentinus edodes* (Berk.) Singer], also known by the common names shiitake, snake butter, pasania fungus, or forest mushroom, is a prime example of the bioconversion of many types of low-value, agro-industrial, lignocellulosic wastes into higher-value products.

Lentinula edodes, valued as a luxury food for its texture, flavour, and medicinal attributes (antitumour, antiviral, anticholesterol and antithrombotic effects), is the third most widely cultivated edible mushroom worldwide, surpassed only by *Agaricus* spp. and *Pleurotus* spp. (Breene, 1990; Campbell and Racjan, 1999). The shiitake mushroom has become increasingly popular with the constantly rising demand for healthy diet and new mushroom flavours (Mizuno, 1995). However, its production in western countries is still limited, and the market demands are being met mainly by imported dried mushrooms originating from the Far East (Royse, 1995; Sabota, 1996).

The traditional cultivation of *L. edodes* on oak or other broad-leaved hardwood logs is a well-established technique in southeastern Asia, where land, substrate availability, and time for a complete crop cycle are usually not restricting parameters (Przybylowicz and Donoghue, 1990). This extensive cultivation system utilizes freshly-cut hardwood logs, which, once inoculated, are stacked in the woodland during the spawn run period, and fruited under semi-controlled conditions in polyethylene tunnels or farm buildings. Mycelium growth for spawn preparation takes 4–6 months, the spawn run phase (after inoculating spawn into holes on logs) and the appearance of the first mushroom flush takes 1–2 years, and up to 6 years for completion of the cropping (Campbell and Racjan, 1999).

Intensive shiitake cultivation, through the use of sterilized or pasteurized supplemented sawdust or agricultural wastes, is a rapidly developing technology in Western countries (Israilides and Henderson, 1981; Kirchhoff and Lelley, 1991; Zadrazil, 1993; Olivier, 1994; Kalberer, 1995, 2000). With this procedure, the shiitake mycelium colonizes the substrate within a 2-month period, the first mushrooms usually develop after 3–4 weeks, while subsequent flushes may be completed within 6 months. Moreover, there is a significant difference in the biological efficiency (BE: percent fresh weight of mushrooms per dry weight of substrate) of the natural and artificial methods. For the natural log method, the maximum BE usually averages 20–30%, while the BE of an artificial log is much over 50% (Leonard and Volk, 1993; Royse and Sanchez-Vasquez, 2001). Short fruiting cycle, large harvests, and ease of labour are the main benefits of the heat-treated substrate in artificial logs (Tan and Moore, 1992).

The cultivation process involves: 1) preparation and heat treatment of substrate; 2) inoculation of spawn; 3) spawn run; and 4) fruiting. This rather expensive cultivation system involves special handling, hygiene, accurate growth conditions control, maintenance of 'sterile' substrate throughout incubation, and depends largely on the genotype of the strain employed (Royse and Bahler, 1986; Donoghue *et al.*, 1996; Chen *et al.*, 2000). Sawdust is the most popular basal ingredient worldwide for use in synthetic formulations of substrate. Starch-based supplements (20–30% d.w.), such as wheat bran, rice bran, millet, rye, oatmeal, or cornflour, are added to the mix, serving as sugar, nitrogen, vitamin and mineral supplements to provide a more optimum growth medium (Royse *et al.*, 1990; Kalberer, 2000). Although hardwood sawdust is the most popular basal ingredient used in synthetic formulations of substrates for the cultivation of shiitake, its non-availability in many other regions has necessitated finding cheap alternatives (Salmones *et al.*, 1999; Pire *et al.*, 2001). In view of this, wheat straw and different agricultural by-products, such as waste after processing coffee, cacao, cotton, sunflower, grape, etc., have been tried (Przybylowicz and Donoghue, 1990; Levanon *et al.*, 1993; Bis'ko and Bilay, 1996).

A recent experimental study, in which selected *L. edodes* strains were grown on oak-wood sawdust, wheat straw, and corncobs substrates, has revealed that the type of wastes, as well as the strain used, exercised a considerable influence on mycelium growth and on sporophore production characteristics (Philippoussis *et al.*, 2001b, 2002). Mycelial growth measurements conducted in 'race tubes' (*Figure 11.1a*) demonstrated faster colonization on oak-wood sawdust and on wheat straw, than on corncobs. This was attributed to the lower C/N ratio of the supplemented oak-wood sawdust and wheat straw substrates. Moreover, a negative correlation was found between final salt content of the substrates and mycelium extension rates. However, subjecting fully colonized substrates to cold-shock treatment resulted in fruiting 58–65 days after inoculation in the tubes, with wheat straw and corncobs substrates promoting earlier sporophore initiation than oak-wood sawdust. When the same *L. edodes* strains were cultivated in bag-logs (*Figure 11.1b*), earliness was promoted on the wheat straw and oak-wood sawdust substrates since basidiomata formation was achieved sooner than on corncobs. Apart from earliness, wheat straw appeared to promote crop productivity (BE 105%), presented shorter cropping period (90 days), and equal yield distribution among flushes. However, corncobs supported the highest cumulative BE (122%), but within the longer cropping period (120 days).

The differences noted in mushroom yields among flushes confirmed the fact that the nature of substrate also influences yield distribution. Substrate analyses (Philippoussis *et al.*, 2002) revealed that wheat straw possessed significantly higher N and lower lignin content in comparison to corncobs. It is well known that lignin is particularly difficult to biodegrade, and reduces the bioavailability of the other cell wall constituents (Rayner and Boddy, 1988; Eriksson *et al.*, 1990). Moreover, it is known that *L. edodes* mycelia demonstrate higher lignilolytic enzyme activities in response to N-sufficient conditions, and that nutrient nitrogen has been indicated as a growth-limiting factor (Kaal *et al.*, 1995). As far as qualitative properties of basidiomata are concerned, although heavier sporophores were produced by wheat straw, corncobs furnished basidiomata with higher protein and total carbohydrate content (Philippoussis, unpublished data). Hence, the potential for the commercial production of *L. edodes* seems promising on wheat straw and on supplemented corncobs, in the view of the intensification of this medicinal fungus cultivation.

Lentinan and antitumour activity

Lentinan is a polysaccharide derived from the fruiting bodies or the mycelium of the edible mushroom *Lentinula edodes* (Chihara *et al.*, 1987; Chihara, 1993). Lentinan is a β-1,3 glucan with many β-1,6 glucopyranosidic chains, and molecular weight of about one million (Maeda *et al.*, 1971). The isolation and purification of lentinan was

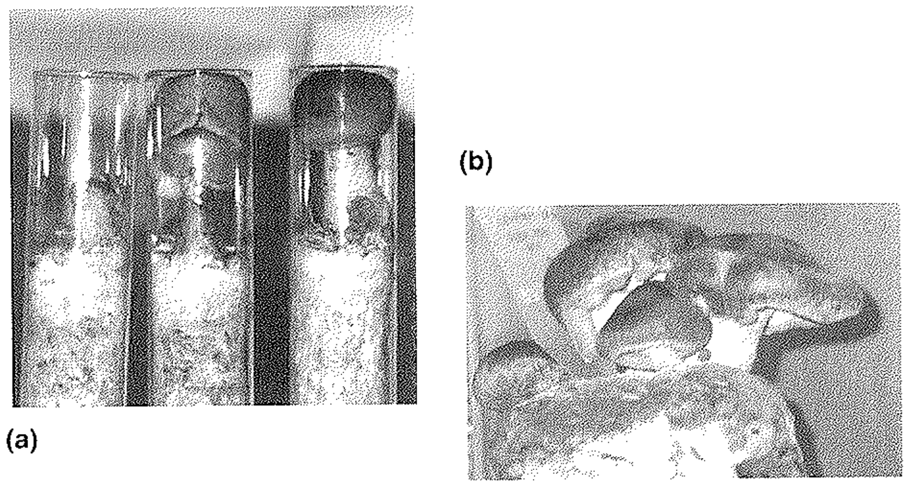

Figure 11.1. Fructification of *L. edodes* on lignocellulosic substrates. (a) in glass tubes. (b) in bag-logs.

achieved by a series of extraction and fractionation steps, starting from fresh fruiting bodies of *L. edodes* to a white powder-like polysaccharide with an overall yield of 0.016% (Chihara *et al.*, 1970).

Lentinan has been studied in detail, and has been recognized for its antitumour activity for almost 30 years. The antitumour properties of lentinan have been reported by many researchers (Shibata *et al.*, 1968; Hamuro and Chihara, 1985; Flynn, 1991). The antitumour effect of lentinan was originally confirmed in Swiss albino mice transplanted with Sarcoma 180 cancer cells, with a simultaneous injection of lyophilysed hot water extracts of mushroom. There was a 97.3% regression of the solid type of tumours, while in six out of nine mice there was complete regression when the dose was 200 mg/kg/day (Ikekawa *et al.*, 1969). Polysaccharides from other basidiomycetes, as well as lichens, have exhibited similar antitumour activities, with the stronger one from lentinan (Wasser and Weis, 1999).

The mechanism of antitumour action of lentinan is considered to be immunomodulatory, by activating the natural killer cells (NK cells), T cells, B cells, and increasing macrophage activity (Hamuro and Chihara, 1985; Chihara *et al.*, 1987; Borchers *et al.*, 1999). Therefore, it can be used as an 'adjuvant' in various therapies in cancer patients undergoing conventional treatment.

Therapeutic effects of lentinan as an agent for post-operative adjuvant therapy have been reported with good results in cancers of the GI track (Hazama *et al.*, 1995). According to a study in Japan, there was an increase in survival of people with recurrent stomach cancer after receiving a highly purified form of lentinan (i.v.), particularly when used in combination with chemotherapy (Taguchi, 1987). Positive results with the use of lentinan have also been reported in one small clinical trial with people suffering from pancreatic cancer (Matsuoka *et al.*, 1997), and in gynaecological cancers of breast type (Kosaka *et al.*, 1982; Taguchi, 1983), as well as cervical cancer patients (Shimizu, 1989). In another study, there was a complete remission of cancer of the ovaries and high interleukin-2 production in a 60-year-old woman who had been treated with lentinan, together with another immunostimulant substance, S-Fu (Shimizu, 1989). Interestingly enough, the early studies on the anticancer effects of lentinan failed to show any positive results. The reason for this was elucidated later, and was due to the very high dose used in the experiments (Aoki *et al.*, 1984). Large doses of lentinan were shown to be not only ineffective, but sometimes harmful. Aoki *et al.* (1984) suggested that a dose of 1 mg lentinan given intravenously twice a week would have the greatest effect. Lentinan can also be taken orally, but would require about five times the i.v. dose to produce the same results.

Lentinan is 'heat stable' (Aoki *et al.*, 1984), which probably means it can withstand normal cooking. Therefore, given the fact that low doses of lentinan are effective, traditional ways of using the mushroom may be the best available way to receive the beneficial effects of the polysaccharide.

Cardiovascular and cholesterol-lowering effect

There are also studies which report on other beneficial health effects of lentinan. For example, in studies with rats and humans who have taken diets with *L. edodes*, there was a reduction of cholesterol levels (Sugiyama and Yamakawa, 1996), while there was a lowering of the blood pressure due to high ratio of K/Na. There is also evidence that *L. edodes* acts as an anticlotting agent (Subbarao *et al.*, 1979).

Antimicrobial and antiviral activity

It was reported that lentinan exhibited a potent anti-HIV activity *in vitro* when used in combination with azidothymidine (AZT) than AZT did alone (Tochikura *et al.*, 1987). In one study with an AIDS patient, one mg of lentinan per day was given for five months. Four months after the treatment, the patient was negative for AIDS antibodies, and two years later she was reported being healthy (Aoki *et al.*, 1984).

In trials with people with chronic hepatitis and viral hepatitis B, administration of lentinan has shown favourable results (Amagase, 1987; Jones, 1988). Also, *L. edodes* possesses potent antibacterial substances and was effective against various kinds of bacterial and parasitic infections (Cochran, 1978). The substances that were extracted by chloroform or ethylacetate from dried shiitake mushrooms had efficient anti-bacterial activities against *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *Porhyromonas* spp., of oral origin (Hirasawa *et al.*, 1999).

Conclusions

Among the primary goals of the European Union is the development of its agriculture and industry without negative environmental impact. Unfortunately, in many European countries many agro-industrial by-products are usually disposed of as wastes, and create serious environmental problems. Many of these, if they had been properly utilized and recycled, could have been a new economical source, with a simultaneous protection of the environment. The bioconversion of selective agro-industrial wastes into high commercial value mushrooms and polysaccharides, like pullulan and lentinan, is ecologically sound and economically feasible. Using 'waste' by-products, which are usually of low, or even negative costs, as substrates for the production of these value-added products, will eventually lower the per unit cost of production, and also give rise to new jobs, besides protecting the environment.

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