Biodegradation **3**: 189–205, 1992.

Utilization of lignocellulosic waste by the edible mushroom, *Pleurotus*

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Key words: lignin degradation, *Pleurotus*, lignocellulose utilization, solid state fermentation

Abstract

Lignocellulosic waste represents huge amounts of unutilized renewable resource. The use of the polysaccharides in the lignocellulosic complex is limited due to their high lignin content. White rot fungi are capable of selectively degrading lignin, thereby upgrading it. The focus of this article is on the potential utilization of edible mushrooms of the genus *Pleurotus*, via solid state fermentation, using cotton plant stalks as a substrate. This material poses agrotechnical problems since the stalks have a fibrous structure similar to that of hardwood. Potential uses for this material are as a fuel in rural areas, a substrate for mushrooms, an animal feed and substrate for paper making. In this study, degradation of cotton stalks by *Pleurotus* is described using chemical analyses and scanning electron microscopy. During four weeks of solid state fermentation, lignin content significantly decreased and *in vitro* digestibility was increased. The fermentation product was consumed by ruminants at a level of up to 40% of their diet.

Introduction

The lignocellulosic complex constitutes a major portion of the total carbon fixed by photosynthesis. However, only a small part of the cellulose, hemicellulose and lignin produced as agricultural or forestry by-products is utilized, while most of it is considered waste material. In Europe, for example, the amount of unexploited lignocellulosic by-products is immense. Cereal straw, the major by-product, shows a yearly surplus of $24 \cdot 10^6$ tons (Zadrazil & Reinger 1988). Most of the straw is either burned in the field creating environmental and ecological problems, or wasted by being ploughed back into the soil.

The direct use of lignocellulosic residue as animal feed, or as a component of such feeds, represents one of its oldest and most widespread applications and as such it plays an important role in the ruminant diet. However, lignocellulosic residues are not high value feeds: they are classified instead as low quality roughage, i.e. high in fiber, low in protein, vitamins and minerals.

The lignin component creates the barrier to efficient utilization, conversion or degradation of the polysaccharides in lignocellulose to useful products or high value animal feed (Buswell & Odier 1987). Lignin is defined as a polymeric product arising from an initial enzymatic step followed by chemically driven dehydrogenative polymerization of primary precursors possessing a p-hydroxycinnamyl alcohol structure (Monties 1989). Due to its complex and heterogeneous structure, lignin degradation is slow and limited to a relatively small number of microorganisms (Buswell & Odier 1987).

The white rot fungi can degrade all the major components of wood and are generally considered to be the main agents of lignin degradation in nature (Buswell & Odier 1987). The best studied organism of this group is *Phanerochaete chrysosporium* (Kirk & Farrell 1987; Schoemaker & Leisola

1990). Most of the knowledge regarding physiological, biochemical and genetic factors associated with lignin biodegradation as well as their practical uses, mainly in the pulp and paper industry, has been obtained from this model organism.

Another group of interesting white rot fungi that can utilize lignocellulose is comprised of the edible mushrooms. These saprophytic basidiomycetes have been successfully cultivated at a commercial level worldwide (Wood & Smith 1987). This paper focuses on the edible mushrooms of the genus *Pleurotus* and their potential in the utilization and upgrading of lignocellulosic wastes during solid state fermentation (SSF).

Cultivation of *Pleurotus* spp. on lignocellulosic waste

Pleurotus spp. are wood-degrading saprophytic fungi which are widely distributed and cultivated in many countries throughout the world. *Pleurotus* cultivation is gaining popularity in Europe, America and the Far East, and from the standpoint of annual production, it has become the third most important mushroom, after *Agaricus bisporus* and *Lentinus edodes* (Wood & Smith 1987). Commercial production techniques for this basidiomycete are well developed (Tautorus 1985; Wood & Smith 1987; Zadrazil 1978) and relatively simple as compared to those used with the most commonly cultivated mushroom, *A. bisporus*. *Pleurotus ostreatus*, popularly known as the "oyster mushroom" is the most common artificially cultivated species. The substrate is usually partially shredded, mixed with water (up to 70%) and placed in containers such as bags, trays or frames. However, unlike *A. bisporus*, no composting or casing layer is required. Because *P. ostreatus* is a wood-degrading fungus, it was first cultivated on logs (Falck 1917). Today it has become common practice to prepare *Pleurotus* substrate from shredded wheat straw, which can be supplemented with protein-rich materials such as alfalfa meal or soybean flour. Since *Pleurotus* spp. can decompose lignocellulose efficiently without chemical or biological pre-treatment, a large variety of lignocellulosic wastes can be utilized and recycled. Some examples of the agricultural wastes studied as substrates for *Pleurotus* spp. are presented here.

In coffee-growing regions, coffee pulp is considered to be one of the most abundant, as well as one of the hardest to handle agricultural wastes. To date, the majority of the by-product remains unused, causing contamination problems (Guzman & Martinez 1986). A most economical way of dealing with the excessive amount of pulp and of solving the environmental problem would be to use it for animal feed. However, chemical components such as lignin, phenolics and caffeine hamper its utilization by animals. Caffeine and phenolics are known to exert detrimental effects to both the rumen's microflora and the host animal. Biological pre-treatment could, however, improve the value of this waste product. The use of coffee by-products as a substrate for *Pleurotus* has been studied by Leon et al. (1983). Guzman & Martinez (1986) successfully cultivated *P. ostreatus* on coffee pulp on a semi-industrial scale, at a coffee farm in Mexico.

Sharma (1987) studied the utilization of flax shive, a woody by-product of little value. For every ton of flax fiber produced, 2.5 tons of shive are left over. Shive has a high lignin content (24.9%), preventing its direct utilization. *Pleurotus* grown on this substrate was able to reduce the lignin content of the shive to 12.4%.

Cassia spp. are tropical shrubs with medicinal, e.g. laxative, properties. The residue of extracted cassia plants was tested for cultivation of *Pleurotus* by Muller (1987). The utilization of *Pleurotus* in the recycling of medicinal plants could be of additional importance in cases where toxic components are present in the material. The reduction of such components by the fungus would simplify the problem of waste disposal (Muller 1987).

Oriaran et al. (1989) compared lignin degradation of hard and soft wood chips by *P. ostreatus*, L. *edodes* and *Phanerochaete chrysosporium*. All three white rot fungi selectively degraded the lignin on glucose-supplemented chips, an observation which could be useful in biopulping by the paper industry.

A large variety of other wastes, such as corn cobs

and leaves, sugar cane bagasse and leaves, citronella bagasse, rice hulls, water hyacinth leaves, cocoa shells and cotton gin waste, could easily be used as substrates for *Pleurotus* spp. The substrates used in each region would depend on the available agricultural wastes.

In our study, the use of cotton stalks as a substrate for SSF by *P. ostreatus* was analyzed. Platt et al. (1981) and Balasubramanya (1981) have successfully grown *P. ostreatus* and *P. sajor-caju* on this substrate.

Utilization of cotton stalks as a substrate for *Pleurotus* fermentation, as animal feed and in paper production

Cotton is Israel's main field crop and generates the largest proportion of local agricultural waste. Its worldwide importance is illustrated by the 10 million tons of cotton stalks reported for India yearly (Balasubramanya et al. 1989). Those remaining in the field, 5 tons/ha, are ploughed under the soil surface. In addition to wasting a potential agricultural resource, this treatment could lead to an increase in cotton diseases and pests, as well as to difficulties in cultivation due to slow decomposition in the soil (mainly in dry regions). The major obstacle to using cotton stalks as a mushroom substrate lies in its difficult preservation. This is due mainly to the high water content and to the high level of soluble carbohydrates (2–4%, as compared to 0.4–1.4% in wheat straw) (Silanikove & Levanon 1986). The substrate is rapidly overgrown by molds, resulting in spoilage and aerobic degradation. The anaerobic preservation of cotton stalks and the production of silage has been studied by Silanikove & Levanon (1986), Levanon et al. (1988) and Danai et al. (1989). Cotton stalks were harvested and chopped into 2–3 cm particles with a forage harvester originally designed to cut corn. The material was taken to a concrete silo with a storage capacity of 450 tons, pressed with a heavy tractor, and then covered with black plastic sheets. After one month of storage, the pH of the preserved cotton stalks stabilized at 5.5 and the material was successfully utilized for commercial *Pleurotus* cultivation, up to nine months after harvest (Danai et al. 1989; Levanon et al. 1988).

Chemical treatment for the upgrading of cotton stalks and their subsequent utilization as animal feed has also been investigated (Ben-Ghedalia et al. 1983; Ben-Ghedalia et al. 1980). Apparent digestibility of organic matter in ozone-treated cotton stalks used in a sheep diet was 75%, as compared to 30 and 39% in control and NaOH-treated substrates. Cotton stalks could also potentially be used as raw material for the manufacture of some grades of paper (Balasubramanya et al. 1989; Pandy & Shaikh 1987).

The use of *Pleurotus* to improve digestibility of cotton stalks in ruminants

The lignocellulose complex in straw and other plant residues is degraded very slowly by ruminants because of the physical barrier imposed by lignin polymers, which prevent free access of hydrolytic enzymes such as cellulases and hemicellulases to their substrates. Normally, the rates of decay of plant debris are proportional to their lignin content. Biological delignification of straw seems to be the most promising way of improving its digestibility (Kamra & Zadrazil 1986; Streeter et al. 1982; Zadrazil & Reinger 1988). Several authors have examined this possibility, using mainly wheat straw and *Pleurotus* spp. under different conditions and substrate pre-treatments (Kamra & Zadrazil 1986; Lindenfelser et al. 1979; Streeter et al. 1982; Zadrazil 1980). A significant increase in *in vitro* dry matter digestibility of wheat straw by *Pleurotus* was reported by Lindenfelser et al. (1979), who used commercial cellulase to compare the release of glucose from untreated straw to that of straw following *Pleurotus* fermentation. They found that lignin content decreased by 51% during the incubation period (90 days). Similar results were reported by Zadrazil (1985) showing an increase of 23–32% in the *in vitro* digestibility following fermentation by *P. eryngii*, *P. sajor-caju*, *P. ostreatus* and *P. serotinus*.

These results encouraged us to examine the effect of *Pleurotus* on cotton stalks during SSF. SSF

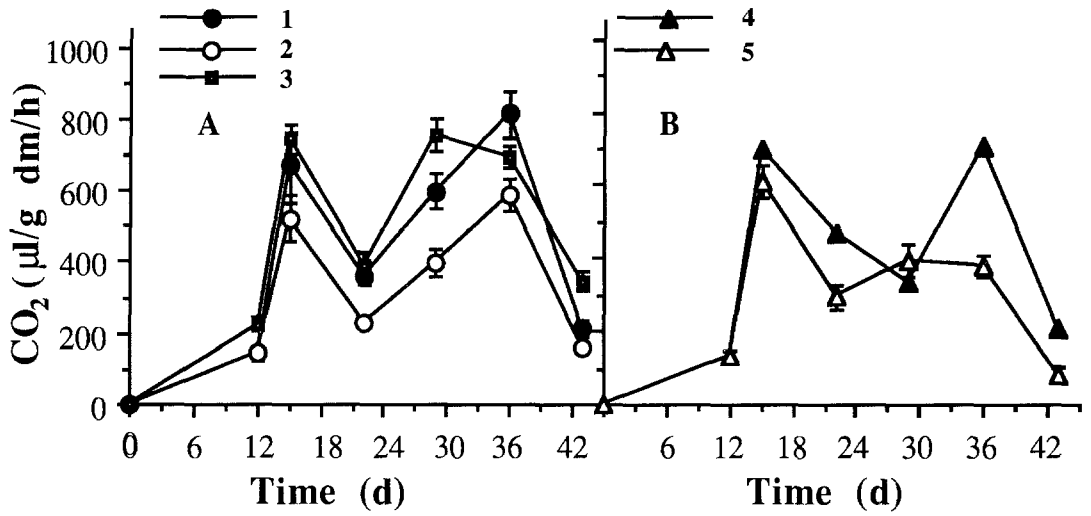


Fig. 1. Pleurotus respiration during SSF on ground (A) and chopped (B) cotton stalks. 1–5 are the treatments as described in the text. Bars represent standard errors.

seems to be the most appropriate method, both for conducting basic research and for applied, large-scale processes involving selective lignin biodegradation. SSF was carried out in the laboratory to define optimal conditions for increasing the *in vitro* digestibility of cotton stalks and improving the SSF process. Five pre-treatments were compared:

1. ground cotton stalks (particle size 2–5 mm), 70% water content;
2. ground cotton stalks, 70% water content plus the following nutrients: (g/Kg dry cotton stalk) 6.0 K_2HPO_4; 2.0 $MgSO_4 \cdot 7H2O$; 4.0 $CaCO_3$; 0.008 thiamine·HCl; 4.0 Bacto-peptone, pH 5.6;
3. ground cotton stalks, 75% water content;
4. chopped cotton stalks (particle size 2–7 cm), 70% water content;
5. chopped cotton stalks, 70% water content plus the nutrients as in treatment 2.

The chopped cotton stalks were harvested in the

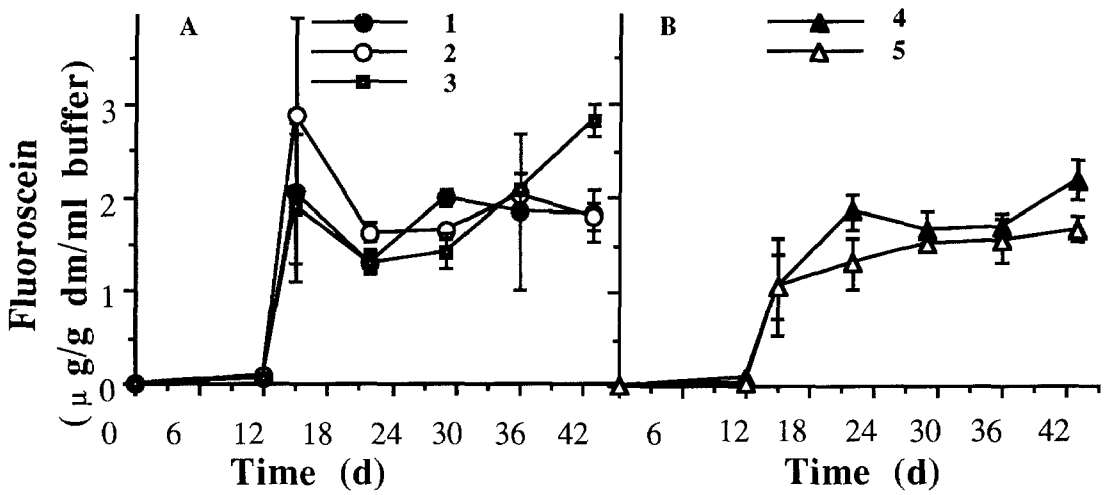


Fig. 2. Fluoroscein diacetate hydrolysis during SSF of *Pleurotus* on ground (A) and chopped (B) cotton stalks. 1–5 are the treatments as described in the text. Bars represent standard errors.

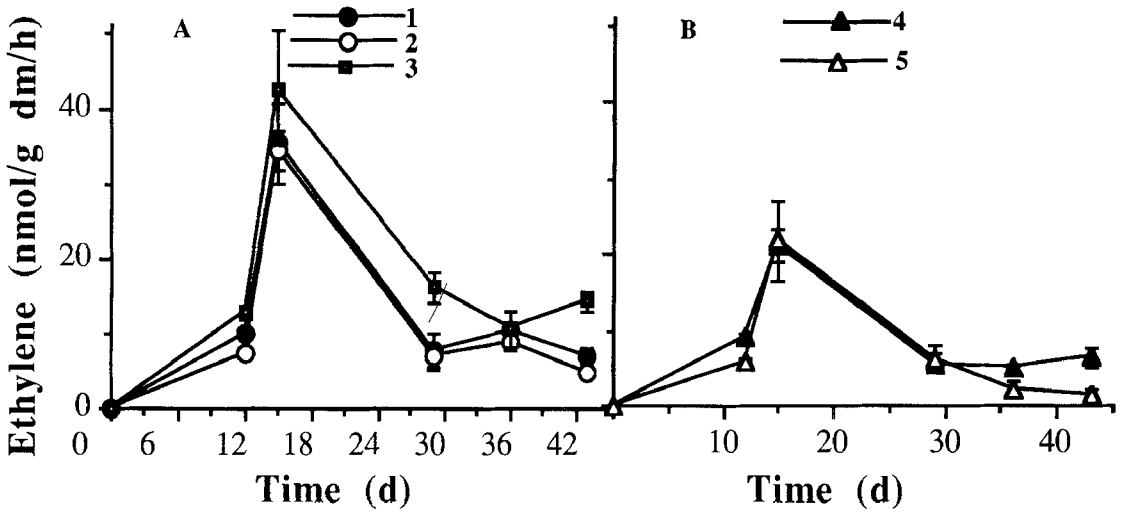


Fig. 3. Ethylene generation from KTBA during SSF of *Pleurotus* on ground (A) and chopped (B) cotton stalks. 1–5 are the treatments as described in the text. Bars represent standard errors.

field using agricultural equipment, with no further treatment.

Polystyrene bags containing 95 g (d.w.) of cotton stalks were either wetted with water or supplemented with nutrient solution and sterilized once for one hour, then again 24 h later. At various stages after inoculation with *P. ostreatus*, bags from each treatment were sampled for enzymatic and chemical analyses. Methods used in this study have been described previously (Kerem et al. 1992). Fungal activities and chemical composition of the fermentation product were followed.

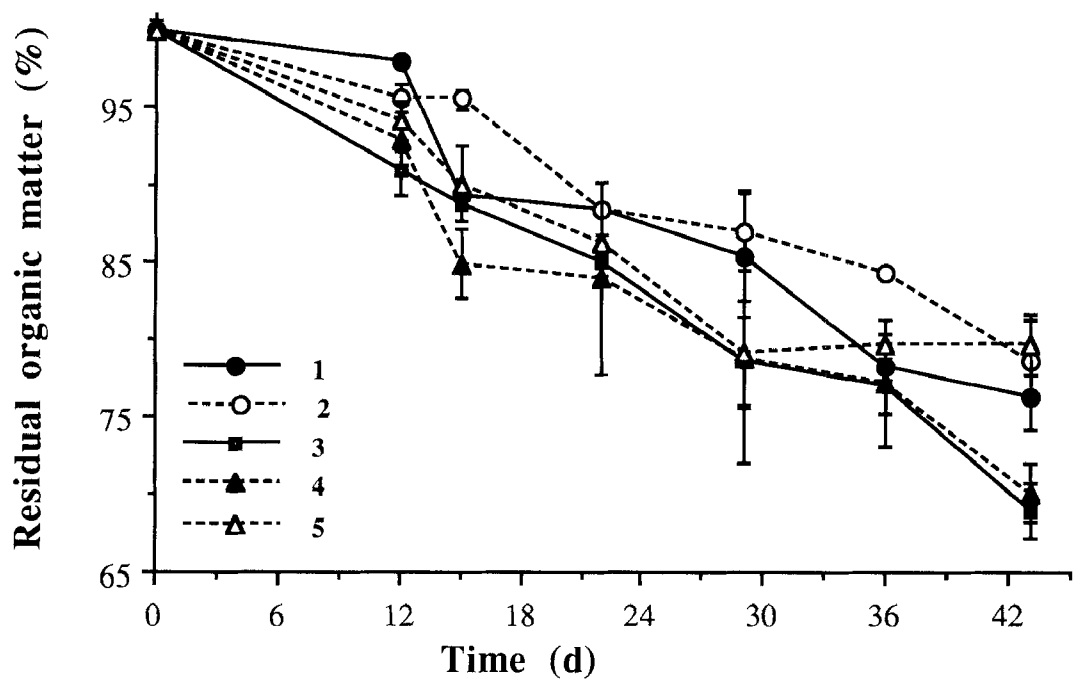


Fig. 4. Mineralization of organic matter during SSF of *Pleurotus* on cotton stalks. 1–5 are the treatments as described in the text. Bars represent standard errors.

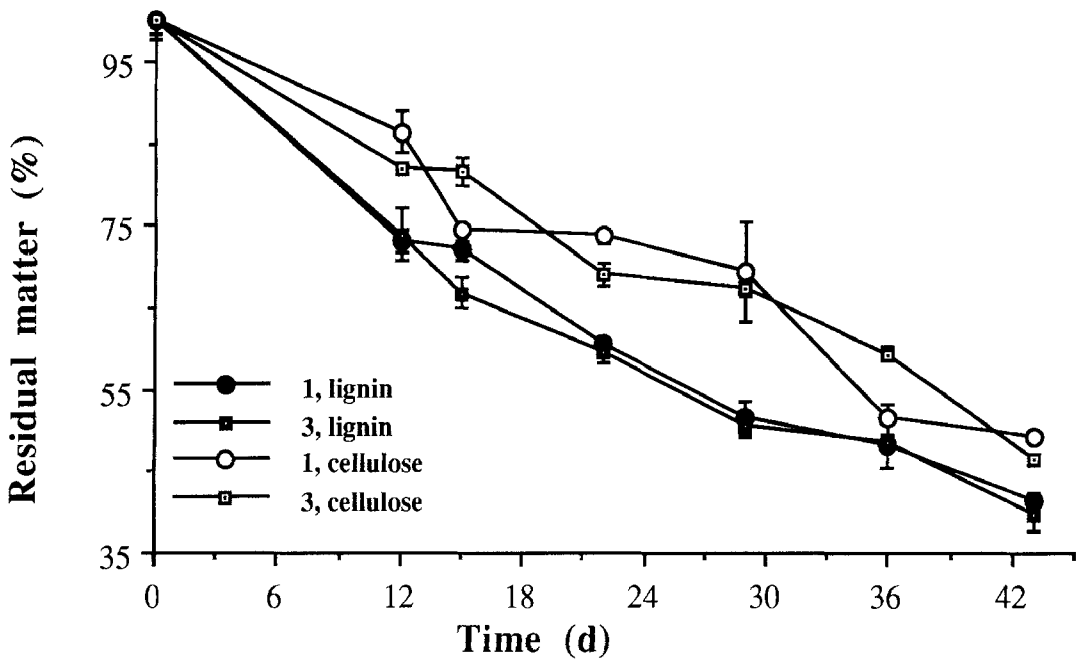


Fig. 5. Lignin and cellulose degradation by *Pleurotus* during SSF of ground cotton stalks with 70% (1) and 75% (3) water content. Bars represent standard errors.

Fungal activities related to colonization: Fungal respiration was recorded as CO_2 evolution per hour, as determined by gas chromatograph (Fig. 1). Respiration rates rose for the first 15 days, then stabilized. On day 18, the contents of the bags were mixed to disperse the colonized bulks and on day 22, respiration rates had decreased. This could have been due to dispersion of the already colonized bulk. However, mixing showed no effects on the biodegradation process. Treatment 5 showed no resumption of fungal activity after mixing. Fungal activity defined by extracellular hydrolysis of fluorescein diacetate (Schnurer & Rosswall 1982) showed a similar trend for all treatments (Fig. 2). There was a slow rise in the extracellular activity towards day 12, a definite increase towards day 15 and stabilization towards the end of the experiment (day 42). Treatment 3 showed a second peak towards the end of the experiment.

Ethylene generation from KTBA (α-keto-γ-thio-ethyl butyric acid): KTBA is a water-soluble molecule which, in the presence of free (·OH centered) radicals, is cleaved to release gaseous ethylene (Kelley 1988). Cleavage activity was observed from day 12, reaching a maximum on day 15 and continuing at a low level from day 29 to the end of the experiment (day 42) (Fig. 3). Treatment 3 showed the highest activity level. Treatments 1 and 2 produced slightly lower activities than treatment 3 and treatments 4 and 5 were found to have the lowest levels of activity.

Organic matter degradation: Mineralization of organic matter was calculated from changes in the ash content of the substrate throughout the experiment. In treatments 2, 3 and 5, a steady mineralization rate was observed, with treatment 3 producing the highest rate (Fig. 4). In treatments 1 and 4 an acceleration was observed between days 12 and 15.

By the end of the experiment (day 42), 35% of the organic matter had been mineralized in treatments 3 and 4, as compared to 30% in treatment 1 and 20% in treatments 2 and 5. A correlation was evident between mineralization patterns and respiration rates. Differentiation of fiber composition and *in vitro* digestion: Cellulose and lignin, the major fiber components of the substrate, were analyzed. Since chemical analysis of these components

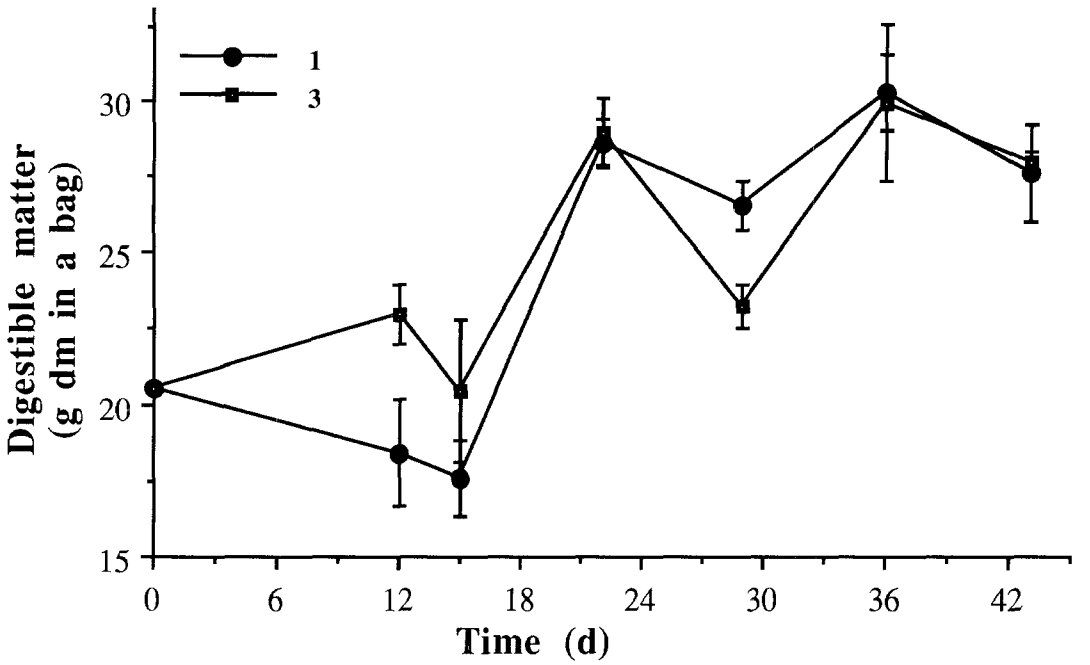


Fig. 6. In vitro digestibility of ground cotton stalks with 70% (1) and 75% (3) water content during SSF of *Pleurotus*. Bars represent standard errors.

is time-consuming, only treatments 1 and 3 were studied in detail. Results are shown as residual lignin and cellulose (Fig. 5). Lignin degradation patterns were similar in both treatments, as were those of cellulose. During the first 15 days, lignin degradation rates were highest, whereas cellulose degradation accelerated from day 22. Thus, after 21 days of fermentation, the amount of degraded lignin was higher than that of cellulose. *In vitro* digestibility of the substrate was measured for treatments 1 and 3 (Fig. 6). Results are presented as dry weight of digested matter per bag. Digested matter in treatment 1 (70% water) decreased for the first 15 days, increased thereafter until day 22 and then stabilized until the end of the experiment. The level of digested matter in both treatments peaked on days 22 and 36. On those days, the amounts of digested matter per bag were found to be similar in both treatments. It was concluded that fermentation for 22 to 36 days produces the highest levels of digestibility, rendering the fermentation product suitable for use as animal feed. Day 22 represents the lowest percentage of digested matter, while day 36 represents the longer fermentation period and a higher ash content due to overall organic matter degradation by the fungus.

Chemical analyses of the substrates from the five treatments were therefore conducted on days 22 and 36. Acid insoluble fibers, cellulose, lignin and ash contents, are presented in Table 1, along with *in vitro* digestibility and the amount of residual dry substrate per bag. Since treatments 2 and 5 were supplemented with nutrients, their initial composition differed from that of the other three treatments, leading to higher ash and nitrogen contents in the fermentation products.

During the first 22 days, a 5% decrease of the fibers was observed in treatments 1–4 and a 10% decrease in treatment 5. An additional 4% decrease in treatment 1 and 1% in treatment 2 were observed during a further 15 days of fermentation. Cellulose content decreased slightly in the five treatments throughout the fermentation period, at a rate similar to that of overall organic matter degradation. A decrease of 5–6% in substrate lignin content was observed in treatments 1, 3, 4 and 5, 3% in treatment 2, during the first 22 days. On day 36, lignin content reached levels of between 15.7

and 17.5%, with the lowest level being recorded in treatment 4. *In vitro* digestibility was increased by 12–15% in all treatments during the first period, and by an additional 5% during the following two weeks.

Protein content and composition were analyzed on days 22 and 36 in the five treatments (Table 2). Crude protein was determined by the Kjeldahl method. An increase in its nitrogen content shows that no crude protein was lost from the system. Furthermore, the content of the true protein fraction, i.e. the amino acid fraction (Table 2) increased beyond the increase in crude protein content, indicating the fungus' ability to decompose nonprotein nitrogen, probably from lignoprotein compounds, and to incorporate the released nitrogen into its own true protein. A source of nonprotein nitrogen in the final product could be chitin, a major component of the fungal cell wall.

Digestibility of the five treated substrates was similar. Treatment 4, requiring the least pre-treatment, was therefore chosen for experiments on a larger scale. One ton of chopped cotton stalks was fermented in an industrial mushroom production plant. The fermentation product was fed to ruminants - goats and heifers, at levels of up to 40% of their diets. No side effects were observed in animals consuming this diet. The *in vivo* nutritional value of this feed remains to be evaluated.

Cotton stalks, harvested using commercial equipment, preserved using silage technology and fermented with *P. ostreatus*, exhibited increased digestibility and could therefore be utilized by ruminants as a dietary supplement.

Table 1. Ash, ADF, cellulose and lignin contents, *in vitro* digestibility and residual dry weight in treated cotton stalks after 0, 22, and 36 days of fermentation by *P. ostreatus*.

Treatment	Ash (%)	ADF (%)	Cellulose (%)	Lignin (%)	*In-vitro* digestibility (%)	Residual dry weight (g/bag)
A – 0 days	6.4	68.2	46.5	22.1	21.8	100
B – 22 days						
1. Ground, 70% water content	8.4	63.4	46.4	17.2	34.4	83
2. Ground, 70% water content + nutrients	9.8	59.3	40.3	19.1	35.1	83
3. Ground, 75% water content	7.7	63.2	45.3	17.6	36.2	80
4. Chopped, 70% water content	8.5	59.4	42.4	16.9	35.4	78
5. Chopped, 70% water content + nutrients	10.4	58.8	42.2	16.3	37.0	81
C – 36 days						
1. Ground, 70% water content	8.9	59.4	41.5	17.5	41.1	73
2. Ground, 70% water content + nutrients	10.3	57.7	40.5	17.4	38.8	80
3. Ground, 75% water content	9.4	62.4	45.2	16.6	41.4	72
4. Chopped, 70% water content	8.3	59.8	44.2	15.7	38.1	73
5. Chopped, 70% water content + nutrients	10.8	57.5	40.6	16.8	40.7	75

Ultrastructural changes in cotton stalks during *Pleurotus* growth

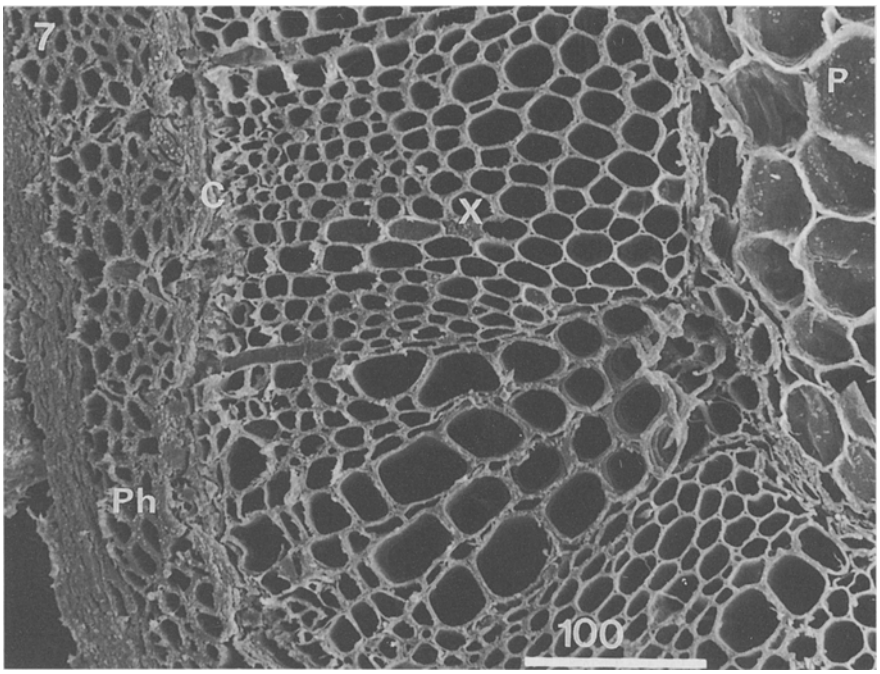
Pleurotus spp. can degrade lignin in a variety of lignocellulosic waste materials (Kamra & Zadrazil 1986; Kerem et al. 1992; Platt et al. 1984). In addition, *Pleurotus* has been found to degrade ^{14}C-labeled DHP to $^{14}CO_2$ (Platt et al. 1983b; Trojanowski & Huttermann 1987) and to decolorize the polymeric dye Poly-B411 (Platt et al. 1985). Even though lignin degradation by white rot fungi has received a great deal of attention over the last two decades, a direct method for following this degradation is not available. One of the techniques which is increasingly being used in studies of the micromorphological changes taking place during the delignification process is scanning electron microscopy (SEM) (Blanchette 1991). Otjen and Blanchette (1986) reviewed variations in wood decay by observing its microscopic appearance. They demonstrated that white rot fungi cause several recognizable patterns of cell wall decomposition, influenced by the host cell type and the fungal species. With the aid of SEM they distinguished between three decay patterns: a white pocket rot, a mottled decay pattern and a uniform white rot. Blanchette (1984) identified selective lignin degradation in wood in his study of 26 white rot fungi. SEM was also used by Agosin et al. (1990) to characterize ultrastructural changes in the Palo Podrido. Ultrastructural changes during lignin degradation by various white rot fungi in different lignocellulosic materials such as sugar cane bagasse and wheat straw, have also been reported (Agosin et al. 1990; Agosin et al. 1987; Johnsrud et al. 1987).

We followed the pattern of lignin degradation in cotton stalks during SSF. Sterile cotton stalks were inoculated with *P. ostreatus* and incubated for six months. Each week a sample was prepared as follows: radial and tangential sections of the cotton stalks were mounted on brass stubs and fixed by vapors of osmium oxide (OsO_5) and glutaraldehyde.

A radial section of sterile cotton stalk which had not been incubated with *P. ostreatus* (control) is shown in Fig. 7. There are no signs of lignin degradation in the domains where lignin is abundant. In cotton stems lignin is distributed throughout the cell wall layers. Greater quantities of lignin are found in the tracheal walls, the middle lamella and between the pith and the xylem. The xylem layers (Fig. 7-X) are intact to the pith (Fig. 7-P) and there is no degradation of the middle lamella. A tangential section after one week of incubation with *P. ostreatus* clearly shows fungal hyphae inhabiting the meristematic cells (Fig. 8). A whole radial section of a cotton stalk, incubated with *P. ostreatus* for two weeks, can be seen in Fig. 9. The pith is detached from the xylem and many holes appear due to lignin degradation, since this is a tissue

Table 2. Amino acid and crude protein content of cotton stalks inoculated with *P. ostreatus* after 0, 22 and 36 days of fermentation.

Treatment	Total amino acids (mg/g substrate)	Essential amino acids (mg/g substrate)	Crude protein (mg/g substrate)
A – 0 days	22.7	10.6	64.5
B – 22 days			
1. Ground, 70% water content	26.9	13.6	79.9
2. Ground, 70% water content + nutrients	33.0	16.4	87.1
3. Ground, 75% water content	28.4	13.5	77.7
4. Chopped, 70% water content	31.7	15.7	66.8
5. Chopped, 70% water content + nutrients	34.6	17.2	83.2
C – 36 days			
1. Ground, 70% water content	34.2	17.3	85.6
2. Ground, 70% water content + nutrients	31.9	15.5	88.7
3. Ground, 75% water content	27.4	13.7	95.1
4. Chopped, 70% water content	25.4	12.4	70.8
5. Chopped, 70% water content + nutrient	35.0	17.9	85.0



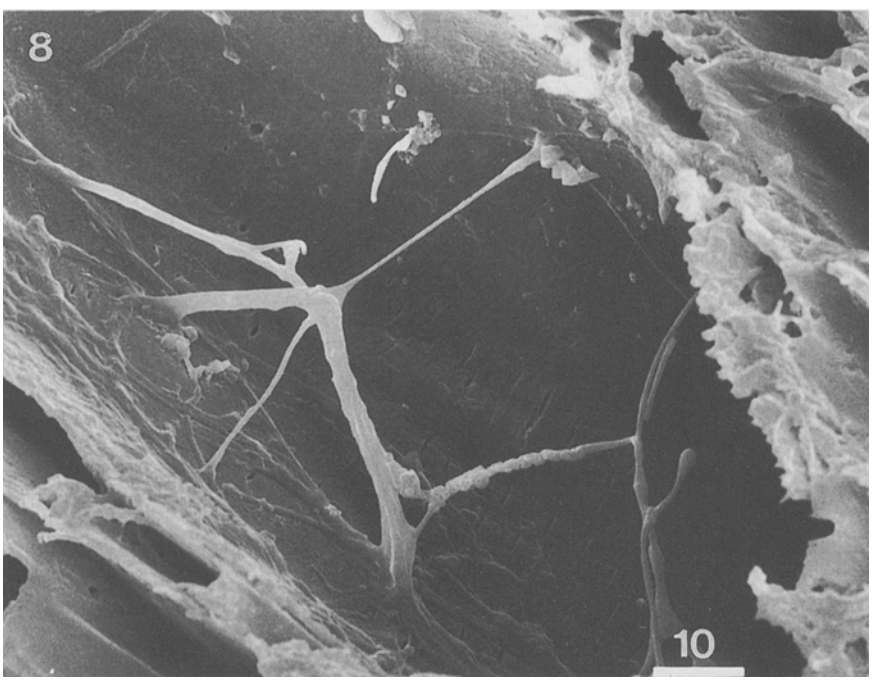


Fig. 7. Radial section of a control cotton stalk demonstrating the different areas, which have not been degraded. P – pith ; X – xylem ; C – cambium ; Ph – phloem.

Fig. 8. Tangential section of a cotton stalk , after one week of incubation with *P. ostreatus*. The fungal hyphae populate the cells and grow through them.

which is rich in lignin. Figure 10 shows radial sections in which areas of delignification in the middle lamella can be observed. Cells are separated from each other and degradation of the cell walls is apparent. Another domain of lignin deposition is in the tracheal cell walls. A tangential section of the trachea cells, after a three-week incubation with *P. ostreatus* (Fig. 11), shows areas of complete decay. Fungal hyphae populate the cells and are destroying the tracheal cell wall (Fig. 11-DT). Figure 12 is a radial section of a cotton stalk after six months of incubation with *P. ostreatus*. Most of the lignin has been degraded and the cells have lost their shape and collapsed.

These observations provide us with a record of the sequence of events occurring during *P. ostreatus* colonization on cotton stalks. A section of a uninoculated cotton stem shows a complete structure with no signs of delignification. After one week of incubation, fungal hyphae were colonizing the cells and lignin degradation was taking place. After the third week delignification had proceeded to the lignin-rich tissues. The pith was detached from the xylem and the trachea cells were degraded. The same phenomenon was also observed in the middle lamella. These observations are in agreement with results of chemical analyses of fermented cotton stalks and with the rate of [^{14}C]lignin mineralization (Kerem et al. 1992).

Enzymes involved in lignin degradation by *Pleurotus* spp.

Extracellular enzyme activity related to lignin degradation has been reported by Platt et al. (1984) and Kerem et al. (1992) during SSF of cotton stalks by *Pleurotus*. The major activity was that of laccase, peaking between days 6 and 8. Similar trends were observed by Sharma (1987), who followed *Pleurotus* activities on flax shive and by Kannan et al. (1990), growing *P. sajor-caju* on paper-mill sludge. Sannia et al. (1986) purified and partially characterized laccase from *P. ostreatus*. Lignin peroxidase, the most widely studied enzyme in relation to lignin degradation, was not detected in *Pleurotus* cultures. This observation is in agreement with others who were unable to detect lignin peroxidase in *Pleurotus* at the protein level using antibodies (Waldner et al. 1988) or by using DNA probes (Kimura et al. 1990). The importance of laccase in cleaving phenolic β-0-4 lignin substructure model compounds has been demonstrated by Higuchi (1990) who suggested that the same chemical principle, i.e. a phenoxy radical as intermediate, is involved in the degradation of phenolic substructure model compounds by both lignin peroxidase and laccase. Platt et al. (1984) suggested that some correlation exists between the level of laccase activity and lignin degradation. *P. ostreatus* 'florida' activities were compared to those of *P. ostreatus* 'P3' (Table 3). The results demonstrated a possible relationship between lack of laccase activity and the rate and mode of lignin degradation, DHP mineralization or Poly-B411 decolorization. Nevertheless, laccase is not the only enzyme responsible for lignin degradation in the genus *Pleurotus* since both strains were capable of degrading lignin.

Acidic peroxidase, neutral peroxidase laccase and veratryl alcohol oxidase activities were investigated in cultures of *P. sajor-caju* (Fukuzumi 1987). The crude extracellular enzymes reduced the brown color of chlorinated oxi-lignin.

Bourbonnais and Paice (1988) found two veratryl alcohol oxidizing enzymes in the culture medium of *P. sajor-caju*. 4-Methoxybenzyl alcohol was oxidized the most rapidly, followed by veratryl alcohol. Not all aromatic alcohols were oxidized. Oxidases may play a role in biodegradation by producing H_2O_2 during the oxidation of lignin fragments (Bourbonnais & Paice 1988). These findings are supported by those of Guillen et al. (1990) who found H_2O_2 production by aryl alcohol oxidase from *P. eryngii*. The most rapidly oxidized substrates were anisyl alcohol and veratryl alcohol. It seems that the enzymes described in these two reports have similar substrate preferences.

A similar enzyme was discovered in cultures of *P. ostreatus* (Sannia et al. 1991) and purified. The enzyme is a glycoprotein containing FAD as a prosthetic group. Cinnamyl alcohol was the most rapidly oxidized substrate. Sannia et al. (1991) also suggested that veratryl alcohol oxidase plays a central role in the biodegradation of lignin by *Pleuro-*

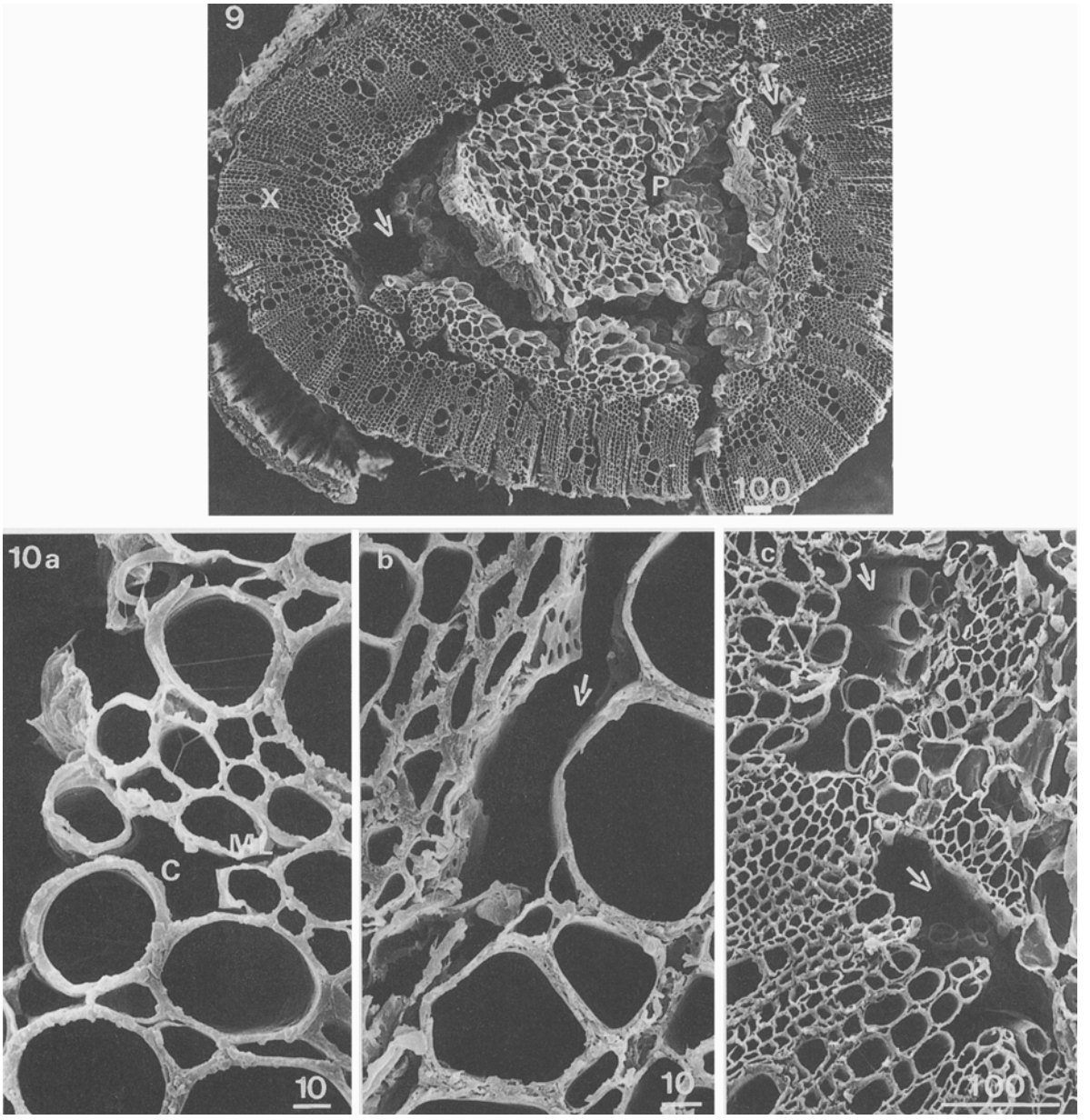


Fig. 9. Radial section of a cotton stalk, two weeks after inoculation, shows clear signs of degradation. The pith is detached from the xylem layers. P – pith ; X – xylem ; → – areas of degraded lignin.
Fig. 10. Radial sections of a cotton stalk, three weeks after inoculation. In all three sections there are clear domains of degraded lignin in the middle lamella. ML – degraded areas of the middle lamella ; C – complete degradation of the cell wall ; → – degraded areas.

tus. Kim et al. (1986) demonstrated the activity of polyphenol oxidase in *P. ostreatus*, which is able to oxidize dihydroxyphenylalanine (DOPA). Enzyme activity was evident only after induction with phenolic compounds, such as ferulic and gallic acids.

The mechanism of lignin degradation by *Pleurotus* has been studied significantly less than those of *Phanerochaete chrysosporium* and some other

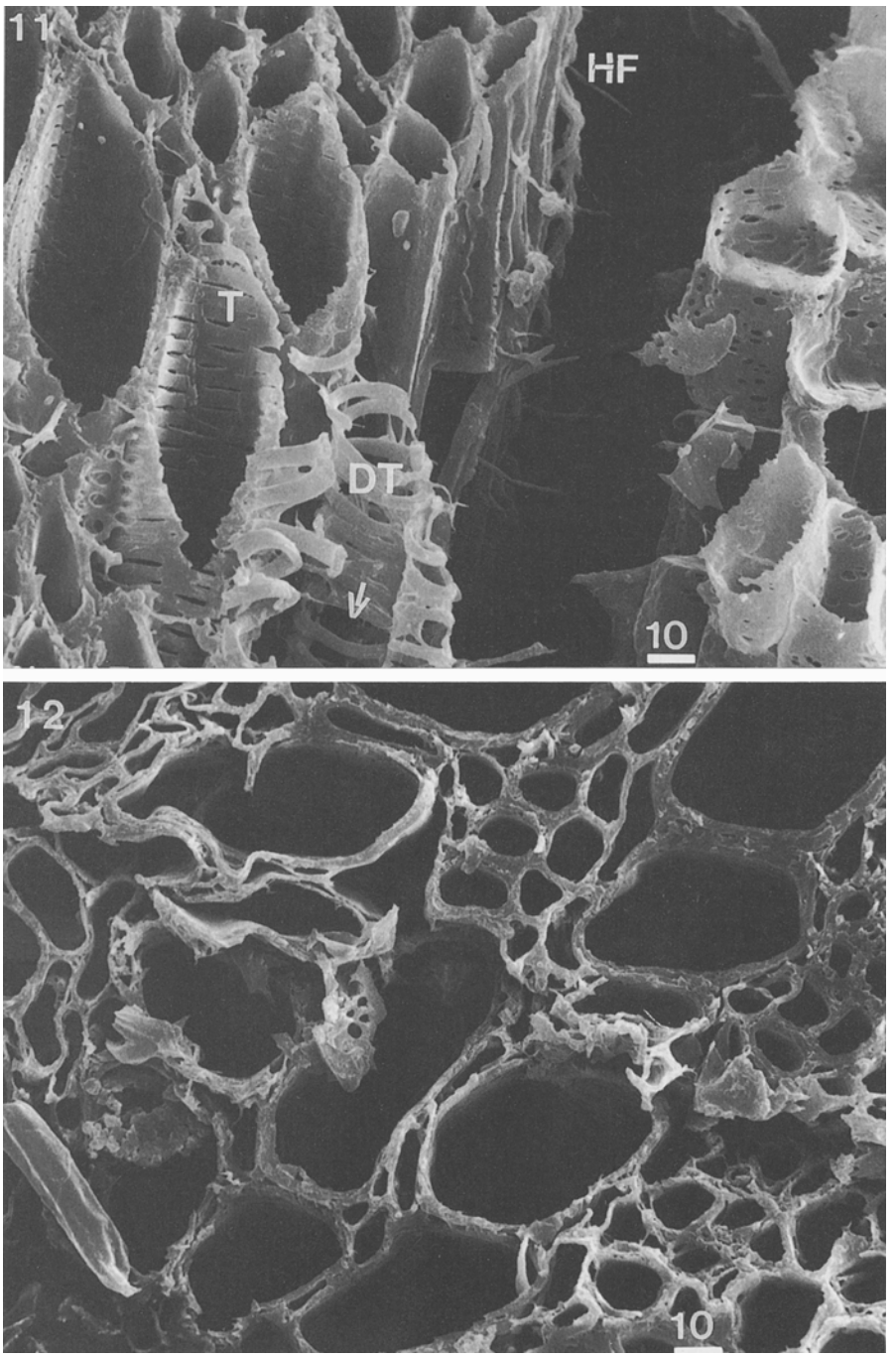


Fig. 11. Tangential section of trachea cells in a cotton stalk, three weeks after inoculation. HF – fungal hyphae which are seen in a degraded area ; → – degraded areas ; T – undegraded trachea cell wall ; DT – degraded trachea cell wall.
Fig. 12. Radial section of a cotton stalk after six months of incubation with *P. ostreatus*, revealing complete destruction of the cells' shape. The cell walls have collapsed, having no lignin to support them.

white rot fungi. From studies conducted to date however, a different enzymatic system seems to be responsible for lignin degradation in *Pleurotus*.

The effect of cotton stalk extract on *Pleurotus* growth and activity

Growth and some activities of some *Pleurotus* strains have been found to be enhanced by the phenolic constituents found in crude water extracts of some plants. Zadrazil (1975) reported the metabolism of flavonoid-type phenolic compounds existing in straw substrates by *Pleurotus* spp. in the early stages of degradation. Sharma (1987) reported that flax shive extract enhanced degradation of flax shive by four strains of *Pleurotus*. The extract also induced a significant increase in the number of primordia produced by these fungi. The infrared spectrum of a chromatogram of flax shive extract showed the presence of a flavonoid-type compound in the aqueous extract of the flax shive.

Platt et al. (1981, 1983a) showed that *P. ostreatus* 'florida' grows faster on cotton straw than on other substrates. After 21 days, 17.1% of the initial dry weight was reduced by the fungi, as compared to a 10.8% reduction in the dry weight of wheat straw. The addition of crude aqueous cotton stalk extract to wheat straw increased its degradation by 33%. When the fungus was grown on water-extracted wheat straw, degradation of both lignin and straw was slower than in the native straw. The crude extract also induced high laccase activity, similar to activity found after induction with known phenolic compounds (Platt et al. 1984). The active fraction in the extract was characterized as a flavonone or a dihydroxyflavonol-type flavonoid (Platt et al. 1983a).

When cotton stalk extract was incorporated into a solid synthetic growth medium, the linear growth rate of the fungal colonies increased, by 14% for *P. ostreatus* 'florida' F6, 20% for *P. ostreatus* IMI 341688, 14.3% for *P. salmoneo stramineos* and 17.6% for *P. pulmonarius* P3014. Other white rot fungi were also studied: Growth rate of *Ganoderma applanatum* increased by 17%, and *Phlebia tremellosus* by 12%. Other fungi, such as *Trametes versicolor* and *Gleophyllum striatum*, were not affected, whereas in the case of *L. edodes*, a 9% inhibition of growth was observed. A similar trend was noted when fungal biomass grown in stationary liquid cultures was monitored. Laccase activity was studied in liquid medium amended with cotton stalk extract. In preliminary studies, all strains tested showed different levels of laccase induction, ranging from a three to fivefold increase in activity

Table 3. Fungal activities and lignin degradation by *P. ostreatus*.

Activity	*P. ostreatus* 'florida' F6	*P. ostreatus* P3	Reference
Cotton degradation (%)	17	9	Platt et al. (1984)
Lignin degradation (%)	56	10	Platt et al. (1984)
Laccase activity (OD/ml/min)	0.86	0	Platt et al. (1984)
Poly-B411 decolorization: absorbance (486nm:553nm) ratio change in 7 days (initial ratio = 3)	0.95	2.9	Platt et al. (1985)
Release of $^{14}CO_2$ from ^{14}C-ring DHP (% of initial)	7	0.7	Platt et al. (1983b)
	7.5	4	Trojanowski & Hüttermann (1987)
Release of $^{14}CO_2$ from DHP-O-$^{14}CH_3$ (% of initial)	21	9	Platt et al.(1983b)
	18.1	7.5	Trojanowski & Hüttermann (1987)
$^{14}CO_2$ release from ^{14}C organosolve lignin (% of initial)	9.4	5	Trojanowski & Hüttermann (1987)
^{14}C-water solubles in culture (% of initial)	35.3	11.5	Trojanowski & Hüttermann (1987)

level within 6 h. *T. versicolor* and *G. applanatum* showed a similar increase, while other laccase-producing fungi, such as *Rhizoctonia solani*, were not affected by the added extract (Ardon & Hadar, unpublished).

Mineralization of [^{14}C]lignin by *P. ostreatus* in the presence of cotton stalk extract was studied. A twofold increase was observed after two weeks of growth, changing only slightly thereafter until the end of the experimental period (75 days). Forty-one and 47% of the total radioactivity was released as $^{14}CO_2$, from the control and the treated substrate, respectively. These results suggest a change in fungal lignin metabolism, affected by the cotton stalk extract. The role of plant extracts in lignin degradation by *Pleurotus* remains to be elucidated.

Conclusions

The utilization of lignocellulosic agricultural wastes as well as forest products via SSF by white rot fungi could be applied in several industries (Buswell & Odier 1987). Its major potential advantages are the upgrading of under utilized resources (agricultural waste) and the introduction of environmentally sound biotechnologies to the pulp and paper industry.

Such technologies could be introduced in several areas:

1. delignification of wood, straw and bagasse to increase digestibility by ruminants, and for enzymatic hydrolysis of cellulose;
2. delignification of wood to reduce energy consumption and the utilization of harmful chemicals in pulping (biopulping);
3. delignification, bleaching and modification of pulp fibers (biobleaching);
4. modification of lignin to produce useful chemicals;
5. treatment of waste bleach water to reduce color and toxicity;
6. treatment of soil or waste water to remove toxic pollutants such as PCB and DDT.

Pleurotus degrades lignin efficiently and selectively, as evidenced by electron microscopy and chemical analyses. Our study on its SSF of cotton stalks suggests the usefulness of the fermentation product as a supplement to ruminant diets. Since *Pleurotus* has been cultivated commercially for human consumption for decades, it can be considered safe for animals as well. This conclusion is in line with findings on SSF of *Pleurotus* on wheat straw (Zadrazil & Reinger 1988) and other wastes.

The mechanism of lignin degradation by *Pleurotus* should be targeted for future research. The enzymes involved seem to be laccase and aryl alcohol oxidases. However, related activities need to be investigated before any definitive conclusions can be drawn.

References

Agosin E, Rouau X & Brillouet JM (1987) Fermentation of wheat straw xylan by the white rot fungus *Dichomitus squalens*. Can. J. Microbiol. 33: 1050–1054

Agosin E, Blanchette RA, Silva H, Lapierre C, Cease KR, Ibach RE, Abad AR & Muga P (1990) Characterization of Palo Podrido, a natural process of delignification in wood. Appl. Environ. Microbiol. 56: 65–74

Balasubramanya RH (1981) An edible mushroom crop on cotton stalks. Indian Soc. Cotton Imp. J. 6: 104–106

Balasubramanya RH, Pai DY, Saikh AJ & Khandeparker VJ (1989) Biological softening of spent cotton-plant stalks for the preparation of pulp. Biol. Wastes 30: 317–320

Ben-Ghedalia D, Shefet G & Miron J (1980) Effect of ozone and ammonium hydroxide treatments on the composition and in vitro digestibility of cotton straw. J. Sci. Food Agric. 31: 1337–1342

Ben-Ghedalia D, Shefet G & Dror Y (1983) Chemical treatments for increasing the digestibility of cotton straw. J. Agric. Sci. 100: 393–400

Blanchette RA (1984) Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. 48: 647–653

Blanchette RA (1991) Delignification by wood decay fungi. Annu. Rev. Phytopathol. 29: 381–398

Bourbonnais R & Paice MG (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem. J. 255: 445–450

Buswell JA & Odier O (1987) Lignin biodegradation. Crit. Rev. Biotechnol. 6: 1–60

Danai O, Levanon D & Silanikov N (1989) Cotton straw silage as a substrate for *Pleurotus* sp. cultivation. Mush. Sci. 12: 81–90

Falck R (1917) Uber die Waldkultur des Austernpilzes (*Agar-*

icus ostreatus) auf laubholzstubben. Zeitschrift fur Forst-und Jagdwesen 49: 159–165

Fukuzumi T (1987) Ligninolytic enzymes of *Pleurotus sajor-caju*. Coll. INRA 40: 137–142

Guillen F, Martinez AT & Martinez AJ (1990) Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. Appl. Microbiol. Biotechnol. 32: 465–469

Guzman G & Martinez D (1986) *Pleurotus* growing on coffee pulp in semi-industrial plant - a new promising mushroom cultivation technology in the subtropics of Mexico. Mushroom Newsletter for the Tropics 6: 7–10

Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technol. 24: 23–63

Johnsrud SC, Fernandez P, Lopez I, Gutierrez A, Saez A & Eriksson KE (1987) Properties of fungal pretreated high yield bagasse pulps. Nord. Pulp Paper Res. J. 2: 47–52

Kamra DN & Zadrazil F (1986) Influence of gaseous phase, light and substrate pre-treatment on fruit-body formation, lignin degradation and in vitro digestibility of wheat straw fermented with *Pleurotus* spp. Agric. Wastes 18: 1–17

Kannan K, Oblisami G & Loganathan BG (1990) Enzymology of ligno-cellulose degradation by *Pleurotus sajor-caju* during growth on paper-mill sludge. Biol. Wastes 28: 1–8

Kelley RL (1988) Ligninolytic activity of *Phanerochaete chrysosporium* measured as ethylene production from α-keto-γ-methylthiolbutyric acid. In: Wood AW & Kellog ST (Eds) Biomass: Lignin, Pectin and Chitin, Vol 161b (pp 79–82). Academic Press, San Diego, CA

Kerem Z, Friesem D & Hadar Y (1992) Lignocellulose degradation during solid-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58: 1121–1127

Kim KJ, Shin KS & Hong SW (1986) Induction of extracellular polyphenol oxidase from two white rot fungi. Korean J. Mycol. 14: 43–47

Kimura Y, Asada Y & Kuwahara M (1990) Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. 32: 436–442

Kirk TK & Farrell RL (1987) Enzymatic 'combustion': the microbial degradation of lignin. Ann. Rev. Microbiol. 41: 465–505

Leon RD, Morales E, Agreda LD & Rolz C (1983) Coffee byproducts and citronella bagasse as substrates for *Pleurotus* production. Mushroom Newsletter for the Tropics 4: 13–16

Levanon D, Danai O & Masaphy S (1988) Chemical and physical parameters in recycling organic wastes for mushroom production. Biol. Wastes 26: 341–348

Lindenfelser LA, Detroy RW, Ramstack JM & Worden KA (1979) Biological modification of the lignin and cellulose components of wheat straw by *Pleurotus ostreatus*. Dev. Indust. Microbiol. 20: 541–551

Monties B (1989) Lignins. In: Harborne JB (Ed) Plant Phenolics, Vol 1 (pp 113–157). Academic Press Ltd., London

Muller J (1987) Cultivation of the oyster mushroom, *Pleurotus ostreatus* (Jack. ex Fr.) Kummer, on cassia-substrate. Mush. J. Tropics 7: 89–95

Oriaran TP, Laboski P Jr. & Royse DJ (1989) Lignin degradation capabilities of *Pleurotus ostreatus*, *Lentinula edodes* and *Phanerochaete chrysosporium*. Wood Fiber Sci. 21: 183–192

Otjen L & Blanchette RA (1986) A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. Can. J. Bot. 64: 905–911

Pandy SN & Shaikh AJ (1987) Utilization of cotton plant stalk for production of pulp and paper. Biol. Waste 21: 63–70

Platt MW, Chet I & Henis Y (1981) Lignocellulose degradation during growth of the mushroom *Pleurotus* sp. 'florida' on cotton straw. Eur. J. Appl. Microbiol. Biotechnol. 13: 194–195

Platt MW, Hadar Y & Chet I (1984) Fungal activities involved in lignocellulose degradation by *Pleurotus*. Appl. Microbiol. Biotechnol. 20: 150–154

Platt MW, Hadar Y & Chet I (1985) The decolorization of the polymeric dye Poly-Blue (polyvinylamine sulfonate-anthroquinone) by lignin degrading fungi. Appl. Microbiol. Biotechnol. 21: 394–396

Platt MW, Hadar Y, Henis Y & Chet I (1983a) Increased degradation of straw by *Pleurotus ostreatus* sp. 'florida'. Eur. J. Appl. Microbiol. Biotechnol. 17: 140–142

Platt MW, Trojanovski J, Chet I & Huttermann A (1983b) Differences in the degradation of specifically ^{14}C-labelled lignin model compounds within the *Pleurotus* genus group. Microbiol. Lett. 23: 19–21

Sannia G, Giardina P, Luna M, Rossi M & Buonocore V (1986) Laccase from *Pleurotus ostreatus*. Biotechnol. Lett. 8: 797–800

Sannia G, Limongi P, Cocca E, Buonocore F, Nitti G & Giardina P (1991) Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim. Biophys. Acta 1073: 114–119

Schnurer J & Rosswall T (1982) Fluoroscein diacetate hydrolysis as a measure of total microbial activity in soil and in litter. Appl. Environ. Microbiol. 43: 1256–1261

Schoemaker HE & Leisola MSA (1990) Degradation of lignin by *Phanerochaete chrysosporium*. J. Biotechnol. 13: 101–109

Sharma HSS (1987) Comparative study of the degradation of flax shive by strains of *Pleurotus*. Appl. Microbiol. Biotechnol. 25: 542–546

Silanikove N & Levanon D (1986) Cotton straw: composition, variability and effect of anaerobic preservation. Biomass 9: 101–112

Streeter CL, Conway KE, Horn GW & Mader TL (1982) Nutritional evaluation of wheat straw incubated with the edible mushroom *Pleurotus ostreatus*. J. Anim. Sci. 54: 183–188

Tautorus TE (1985) Mushroom fermentation. Adv. Biotechnol. Proc. 5: 227–273

Trojanowski J & Huttermann A (1987) Screening of wood inhabiting fungi for their capacity to degrade and to solubilize ^{14}C-labeled lignin. Microbios. 50: 91–97

Waldner R, Leisola MSA & Fiechter A (1988) Comparison of

ligninolytic activities of selected white-rot fungi. Appl. Environ. Microbiol. 29: 400–407

Wood DA & Smith JF (1987) The cultivation of mushrooms. In: Norris JR & Pettipher GL (Eds) Essays in Agricultural and Food Microbiology (pp 309–343). John Wiley & Sons Ltd, New York

Zadrazil F (1975) Die Zersetzung des Stroh-zellulose-Lignin-Komplexes mit *Pleurotus* florida und dessen Nutzung. Z. Pflantzenernahr Bodenk 138: 263–278

Zadrazil F (1978) The cultivation of *Pleurotus*. In: Chang S & Hayes W (Eds) The Biology and Cultivation of Edible Mushrooms (pp 521–524). Academic Press, New York

Zadrazil F (1980) The conversion of different plant wastes into feed by basidiomycetes. Eur. J. Appl. Microbiol. Biotechnol 9: 243–248

Zadrazil F (1985) Screening of fungi for lignin decomposition and conversion of straw into feed. Angew. Botanik. 59: 433–452

Zadrazil F & Reinger P (1988) Treatments of Lignocellulosics with White Rot Fungi. Elsevier Applied Science Publishers Ltd, London