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Delignification of Palm-press Fibre by White-rot Fungi for Enzymic Saccharification of Cellulose

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ABSTRAK

Hampas kelapa sawit diinokulasi dengan sepuluh jenis kulat rot putih yang berbeza iaitu : Pleurotus sajor-caju *I, II dan III;* Pleurotus florida; Lentinula edodes *I, II, III, IV and V dan* Ganoderma lucidum *sebelum dieramkan selama tiga bulan. Daripada kulat yang dikaji, didapati bahawa* P. sajor-caju *I, III dan* P. florida *merupakan pengurai lignin yang terbaik merendahkan kandungan lignin sebanyak 35% dan meningkatkan kesenangan penghadamam sabut sebanyak 21%. Semasa pertumbuhan kulat tersebut, lignin adalah komponen sabut yang banyak terhapus manakala kehilangannya adalah sedikit bagi selulosa dan hemiselulosa untuk tempoh pengeraman dua bulan. Pemecahan hemiselulosa hanya berlaku selepas penghancuran lignin dan selulosa. Walaupun setengah daripada* L. edodes *yang dikaji menyerang hanya komponen lignin dan biarkan saja komponen selulosa dan hemiselulosa, tetapi kadar pemecahannya lebih rendah berbanding dengan* Pleurotus *spp.* G. lucidum *merupakan pengurai lignin yang lemah dan menggunakan hemiselulosa lebih dari selulosa untuk pertumbuhan.*

ABSTRACT

Palm-press fibres were inoculated with fungal mycelium of ten different isolates of white rot-fungi namely: Pleurotus sajor-caju *I, II and III;* Pleurotus florida; Lentinula edodes *I, II, III, IV and V and* Ganoderma lucidum. *The inoculated fibres were incubated for a period of up to three months. Of the fungi tested,* Pleurotus sajor-caju *I, III and* P. florida *were found to be the best lignin degraders, decreasing the lignin content by as much as 35%. This corresponded to an increase of 21% in the digestibility of the fibres. Lignin showed the largest proportionate loss during the growth of these fungi; cellulose and hemicellulose showed the lowest loss for incubation of up to two months. Degradation of hemicellulose seemed to take place later than lignin and cellulose. Some isolates of* L. edodes *preferably attacked the lignin component while leaving the cellulose and hemicellulose untouched; its rate of degradation however, was slower than* Pleurotus *spp.* G. lucidum *was a poor lignin degrader and under the present conditions preferred to utilise hemicellulose rather than cellulose for growth.*

Keywords : biological pretreatment, palm-press fibre, white-rot fungi

INTRODUCTION

Biological pretreatment techniques of lignocellulosic materials have not been developed as intensively as physical and chemical methods (Fan *et al.* 1982; Lee *et al.* 1983; Moo-young *et al.* 1985; Tong and Hamzah 1989; Adaskaveg *et al.* 1990). If the capacity of microorganisms is to be utilised more fully, a better understanding of microbial lignin degradation is necessary (Crawford 1981).

The most promising organisms for biological pretreatment of lignocellulose are the white-rot fungi (Hatakka 1983). It is possible to use these microorganisms to degrade the lignin component in lignocellulosic waste materials to make the cellulose and hemicellulose components more accessible for further biotechnological use.

Solid state fermentation of wheat straw with white-rot fungi of the genus *Pleurotus* has been shown to increase susceptibility to enzymatic saccharification (Detroy *et al.* 1980; Hatakka 1983; Mueller and Troesch 1986) as well as rumen digestibility (Zadrazil 1977, 1978 and 1980; Lindenfelser *et al.* 1979; McQueen and Reade 1983). Commercial growing of *Pleurotus* is increasing worldwide (Tong and Chen 1990). Thus, simultaneous production of mushrooms and highly digestible material either for enzymatic saccharification or as animal feed would be economically attractive (Bano and Rajaratnam 1982).

In the present work, several mushroom cultures were screened for selective lignin removal.

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The aim of this investigation was to use biologically treated palm-press fibres as a substrate in a further biotechnological process, i.e. enzymic sacharification of cellulose for the production of reducing sugars.

MATERIALS AND METHODS

Whatman No. 1 filter paper was obtained from Whatman Ltd. Palm-press fibres were kindly supplied by Sri Langat Palm Oil Mill, Ulu Langat, Selangor. Potato dextrose agar was a product of Difco Laboratories, Detroit USA. Cellulase enzyme from *Trichoderma viride* was purchased from Sigma Chemical Company, St. Louis, USA.

Fungi and Inocula

The following white-rot fungi were obtained from the culture collection of the Department of Biochemistry and Microbiology, Universiti Pertanian Malaysia: *Pleurotus sajor-caju* I, II and III; *Pleurotus florida; Lentinula edodes* I, II, III, IV and V; and *Ganoderma lucidum*. Stock cultures were maintained on potato dextrose agar slants at 4°C. Spawn was prepared from wheat grains. Bottles of sterilised grains were inoculated with actively growing mycelium from potato dextrose agar plates and incubated in the dark at room temperature (28°C) until the mycelium completely covered the grains. Incubation time varied from 16-33 days depending on the species.

Substrate Preparation

Palm-press fibres were soaked overnight in tap water and drained until moderately moist. $CaC0_3$ (3-5%) and rice bran (10%) were added and thoroughly mixed. Approximately 5 g (dry weight) of the fibres were then packed into a 250 ml wide-mouth bottle and autoclaved at 121°C, 15 psi for 1 h. One teaspoon of the spawn was added to the sterilised fibres and incubated in the dark at room temperature for a period of up to three months. At regular intervals of one month, samples were removed and again autoclaved as described above to kill the fungal mycelium. The treated fibres were then dried to a constant weight at 65°C for 24 hours and milled with a blender (National Model MK-C100N) to approximately 2-3 mm in length before analysis for chemical composition. The digestibility test was then carried out on the treated fibres as described below.

Chemical Analysis

The fibre components were estimated individually after a series of extraction procedures. The various components analysed included the lignin, cellulose, hemicellulose, and ash content. The method of Goering and Van Soest (1970) was adopted.

Digestibility Test

The effect of the biological pretreatment was evaluated by comparing the biodegradability of the untreated and treated fibres. Biodegradability of the treated fibres can be measured in an *in vitro* test system by determination of the amount of sugars liberated during incubation with cellulolytic enzymes. The reaction mixtures contained 25 mg fibres, 0.9 ml 0.1 M citrate-phosphate buffer, pH 5.0, 0.1 ml of enzyme solution of appropriate dilution and one drop (10µl) of toluene. After incubation at 37°C for 24 hours, 0.5 ml of the reaction mixture was withdrawn and assayed for reducing sugars.

The number of reducing sugar groups created by hydrolysis of the cellulosic substrate were measured spectrophotometrically by using the Nelson-Somogyi procedure (Nelson 1944; Somogyi 1952) as described earlier (Tong and Rajendra 1992).

RESULTS

The chemical composition of the starting untreated palm-press fibres was estimated to be 39.9% cellulose, 28.9% hemicellulose, 20.3% lignin and 3.6% ash content (Tong and Hamzah 1989). The digestibility test of these fibres showed that about 0.2 mg reducing sugars was released from the fibres under the conditions studied.

Palm-press fibres inoculated with the fungal mycelium were analysed for their chemical composition and digestibility at monthly intervals.

Lignin Content

All the microorganisms grew well on this substrate, but degraded it to a different extent *(Fig. 1a)*. *P. sajor-caju* II and *P. florida* were the best lignin degraders among the organisms tested, reducing as much as 27% of the lignin in the fibres by the end of the first month. This was followed by *P. sajor-caju* III, *L. edodes* II and III. All other organisms were unsuitable for biological delignification at this stage.

After two months of incubation, there was a general decline in the lignin content in all the treatments *(Fig. 1b)*. The rate of delignification was highest in fibres treated with *P. sajor-caju* I where the total amount of lignin removed was comparable to that achieved by *P. sajor-caju* II and III. However, it was *P. florida* that degraded the most lignin.

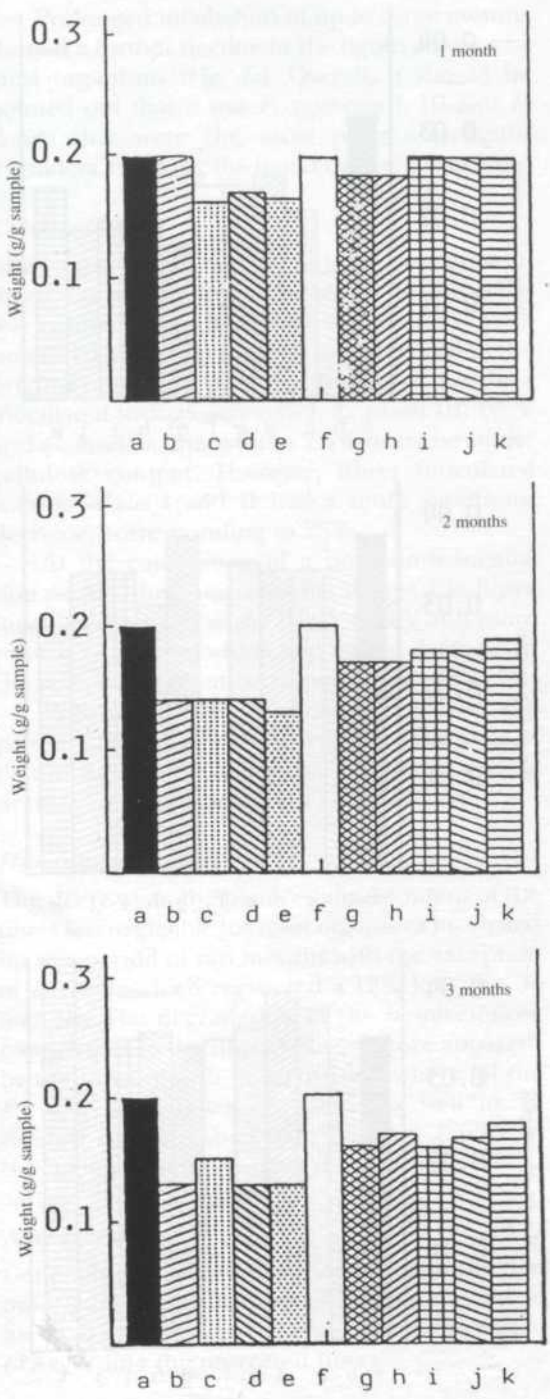

Fig.1: Effect of biological pretreatment by fungi on the lignin content of palm-press fibres. Results represent average of duplicates. The fungi tested were:

(a) *untreated fibres* *(b)* P. sajor-caju *I*
(c) P. sajor-caju *II* *(d)* P. sajor-caju *III*
(e) P. florida *(f)* L. edodes *I*
(g) L. edodes *II* *(h)* L. edodes *III*
(i) L. edodes *IV* *(j)* L. edodes *V*
(k) G. lucidum

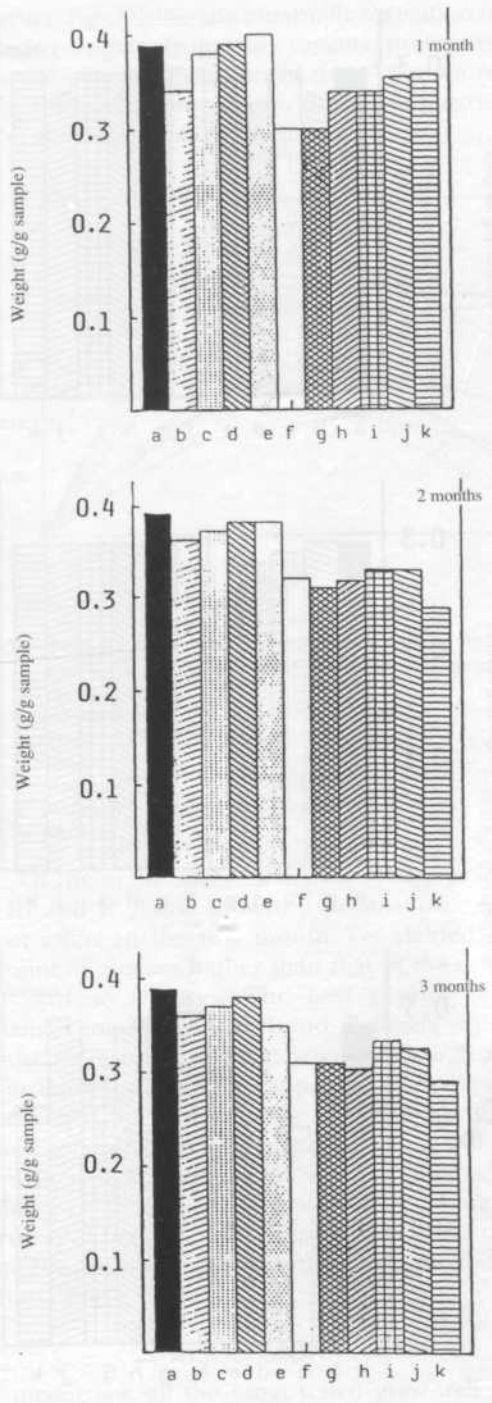

Fig.2: Effect of biological pretreatment by fungi on the cellulose content of palm-press fibres. Results represent average of duplicates. The fungi tested were:

(a) *untreated fibres* *(b)* P. sajor-caju *I*
(c) P. sajor-caju *II* *(d)* P. sajor-caju *III*
(e) P. florida *(f)* L. edodes *I*
(g) L. edodes *II* *(h)* L. edodes *III*
(i) L. edodes *IV* *(j)* L. edodes *V*
(k) G. lucidum

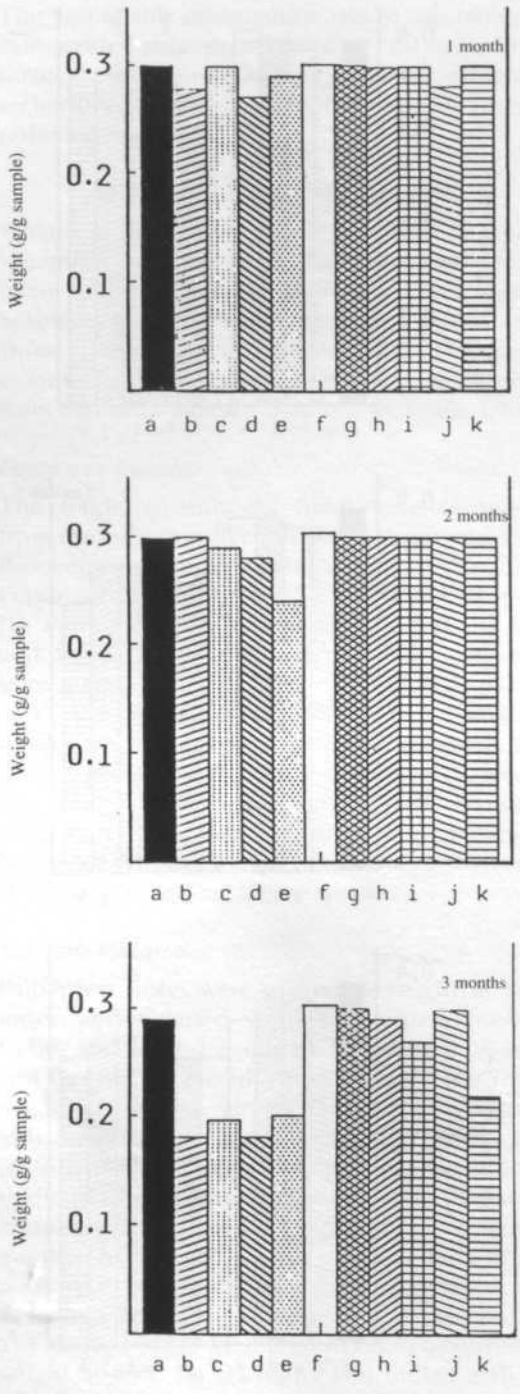

Fig. 3: Effect of biological pretreatment by fungi on the hemicellulose content of palm-press fibres. Results represent average of duplicates. The fungi tested were:

(a)	*untreated fibres*	*(b)*	P. sajor-caju *I*
(c)	P. sajor-caju *II*	*(d)*	P. sajor-caju *III*
(e)	P. florida	*(f)*	L. edodes *I*
(g)	L. edodes *II*	*(h)*	L. edodes *III*
(i)	L. edodes *IV*	*(j)*	L. edodes *V*
(k)	G. lucidum		

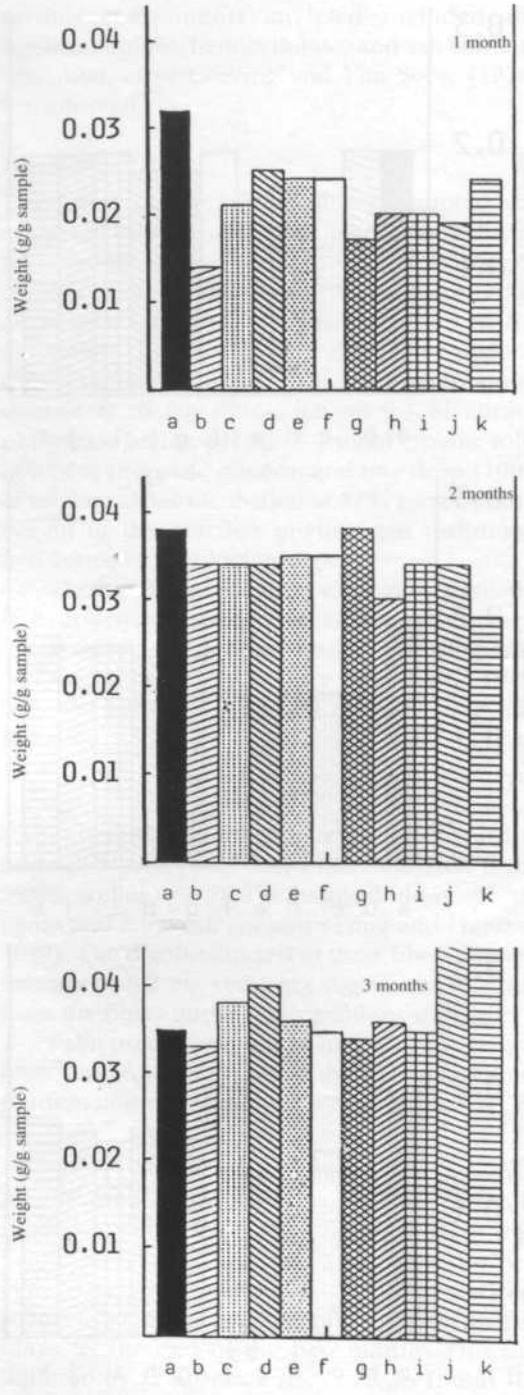

Fig. 4: Effect of biological pretreatment by fungi on the ash content of palm-press fibres. Results represent average of duplicates. The fungi tested were:

(a)	*untreated fibres*	*(b)*	P. sajor-caju *I*
(c)	P. sajor-caju *II*	*(d)*	P. sajor-caju *III*
(e)	P. florida	*(f)*	L. edodes *I*
(g)	L. edodes *II*	*(h)*	L. edodes *III*
(i)	L. edodes *IV*	*(j)*	L. edodes *V*
(k)	G. lucidum		

Prolonged incubation of up to three months showed a further decline in the lignin content by most organisms *(Fig. 1c)*. Overall, it should be pointed out that it was *P. sajor-caju* I, III and *P. florida* that were the most promising lignin degraders, reducing the lignin by as much as 35%.

Cellulose Content

After one month of biological pretreatment, there was no significant difference in the cellulose content of the fibres inoculated with *P. sajor-caju* II, III and *P. florida (Fig. 2a)* compared to the original untreated fibres. In the case of the fibres inoculated with *P. sajor-caju* I, *L. edodes* III, IV, V and *G. lucidum*, there was a 7.6% decrease in the cellulose content. However, fibres inoculated with *L. edodes* I and II had a more significant decrease, corresponding to 25%.

At the completion of a two-month incubation period, there was a further decrease in fibres inoculated with *L. edodes* III, IV and V and more so with *G. lucidum* which had a total decrease in the cellulose content corresponding to 28%.

With a three-month incubation period, the pattern of cellulose content remained the same as that of the second month but registered a slight decrease in fibres inoculated with *P. florida*.

Hemicellulose Content

The decrease in the hemicellulose content of the fibres was negligible for most organisms incubated up to a period of two months with the exception of *P. florida* which registered a 12% loss *(Fig. 3a and 3b)*. The degradation of the hemicellulose component in the fibres became more apparent by the third month of incubation where all the *P. sajor-caju* isolates, *P. florida* as well as *G. lucidum* caused substantial decrease (30%) in the hemicellulose content *(Fig. 3c)*.

Ash Content

Generally, the ash content decreased in the first month *(Fig. 4)* of incubation but continued to increase with prolonged incubation to the extent of exceeding the untreated fibres.

Digestibility Test

With regard to the use of lignocellulosic waste for bioconversion purposes, the enzymatic hydrolysis of cellulose was the most important limiting step (Mueller and Troesch 1986). Biodegradability of the treated fibres was determined *in vitro* by measuring the amount of glucose liberated during incubation with cellulolytic enzymes. *Fig. 5* shows the enzymatic formation of reducing sugars from the various pretreated fibre samples as a function of time. Untreated fibres released approximately 0.2 mg of glucose under the conditions studied.

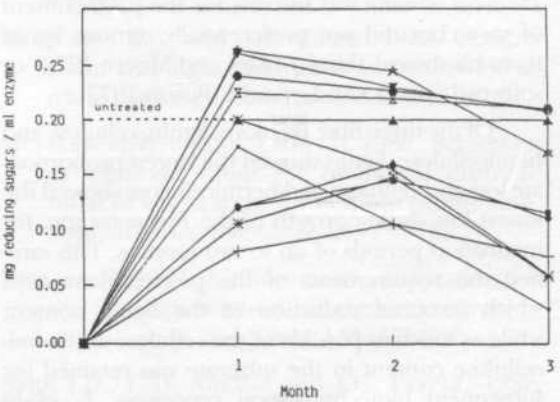

Fig. 5. Reducing sugars released from palm-press fibres treated with different organisms. Each point represents the average of triplicates.

(a)	*untreated fibres*	*(b)*	P. sajor-caju *I*
(c)	P. sajor-caju *II*	*(d)*	P. sajor-caju *III*
(e)	P. florida	*(f)*	L. edodes *I*
(g)	L. edodes *II*	*(h)*	L. edodes *III*
(i)	L. edodes *IV*	*(j)*	L. edodes *V*
(k)	G. lucidum		

Of the organisms tested, only *P. sajor-caju* I, II, III and *P. florida* showed a positive pretreatment effect in the first month, i.e. yielded an amount of glucose higher than that of the original untreated fibres. The best results were obtained for *P. sajor-caju* I and *P. florida* which yielded reducing sugars which amounted to 0.26 mg, an increase of about 30% compared to the control. The actual amount of sugars liberated would have been much higher for reasons which will be explained in the discussion section. Biodegradability of the fibres dropped with prolonged incubation periods. All other organisms did not have the promotary effect on the biodegradability of the fibres.

DISCUSSION

In most cases, all the fungi tested grew well on palm-press fibres, but they increased the digestibility of this substrate to different extents. All components of the lignocellulose could be degraded by white-rot fungi but only those fungi which degraded lignin preferentially can be used for biological pretreatment. Although most of the organisms tested degraded lignin it was *P. sajor-caju* I, III and *P. florida* that were the most promising

lignin degraders, reducing the lignin by as much as 35%. Mueller and Troesch (1986), working with wheat straw treated with *P. florida*, reported a decrease of only 4% in the lignin content. This is understandable because selectivity of attack may depend on the type of lignocellulose materials. *Pleurotus ostreatus* was suitable for the pretreatment of straw but did not preferentially remove lignin from hardwood (birch) (Kirk and Moore 1972) or softword (pine) (Ander and Eriksson 1977).

Of the three fibre fractions, lignin, cellulose and hemicellulose, lignin showed the largest proportionate loss and cellulose and hermicellulose showed the lowest loss during growth of the *Pleaurotus* spp. for incubation periods of up to two months. This satisfied the requirements of the pretreatment steps which favoured reduction of the lignin content while as much as possible of the cellulose and hemicellulose content in the substrate was retained for subsequent biotechnological processess. *L. edodes* (with the exception of *L. edodes* I) seemed to attack lignin preferably while leaving the hemicellulose almost completely untouched but degraded a small amount of the cellulose. Unfortunately, its rate of lignin degradation was slower than the *Pleurotus* spp. Degradation of hemicellulose took place later than cellulose and lignin. *G. lucidum* was a poor lignin degrader and preferred to utilise hemicellulose rather than cellulose for growth.

None of the basidiomycetes tested was able to remove only the lignin component in lignocellulosic material throughout the entire three-month incubation period. Nevertheless, some fungi degraded lignin preferentially over the cellulose and hemicellulose component within the first two months. *P. sajor-caju* I, III and *P. florida* would be ideal for biological pretreatment of fibres because of their higher affinity for lignin.

Good delignification is not in all cases equivalent to good digestibility. Therefore, a digestibility test with cellulases is important in estimating the usefulness of a fungus for biological pretreatment of lignocellulosics. Of the organisms tested, it was only *P. sajor-caju* I, II, III and *P. florida* that showed increase in digestibility of the fibres after biological pretreatment. However, the cellulase digestibility of the fibres declined somewhat after the first month although the lignin content continued to drop. Similarly, all other organisms seemed to have a negative pretreatment effect even though delignification took place. Most probably a new physical barrier was built up by the thick growth of the fungal mycelium covering the fibres which hindered the hydrolysis effect of the cellulase enzymes. Thus the sugar yields following enzymic hydrolysis were lower in this case where washing to remove the mycelial barrier was not carried out. Such a decrease in cellulase digestibility was also reported by Tsang *et al.* (1987). In addition, water-soluble lignin degradation products may have repressed the action of cellulolytic and hemicellulolytic enzymes (Reid *et al.* 1982; Hatakka 1983).

Cultivated mushrooms are normally harvested over a period of several months in the farm. Based on the present results, such a lengthy growth period would presumably have the advantage of increasing the total amount of lignin lost since lignin content continued to decrease with prolonged incubation. However, its effect on the cellulose and hemicellulose components of the fibres would have to be ascertained under mushroom-growing conditions.

To help minimise the cost of any biotechnological process that utilises biological pretreatment, it may be feasible to couple the needs of that process with the methodology and by-products of another industry such as the mushroom industry. Many exotic mushrooms are now produced commercially on supplemented sawdust or palm-press fibres in Malaysia (Tong and Chen 1990). Having harvested the mushrooms, the spent substrate, which was normally considered as waste by the mushroom industry, could then be utilised for subsequent biotechnological processes. In addition, the luxurious growth of the fungal mycelium in the spent substrate could serve as a rich protein source for animal feed. Thus, the simultaneous production of mushrooms and a highly digestible substrate for further biotechnological processess would be economically attractive.

Rapid progress in lignin biodegradation (Crawford 1981) resulting from optimization of cultivation conditions (Kirk *et al.* 1978) has, in recent years, made it possible to accelerate lignin degradation in natural substrates. Therefore, it may be possible in the near future to improve on the selectivity of attack on lignin by white-rot fungi by choosing suitable conditions which stimulate lignin degradation, and which at the same time repress degradation of polysaccharides. This procedure, involving microbial delignification and production of useful products, offers the possibility of utilising and removing the waste palm-press fibres in a completely biological way.

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