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Production of Some Extracellular Enzymes by a Lignin Peroxidase-Producing Brown Rot Fungus, *Polyporus ostreiformis*, and Its Comparative Abilities for Lignin Degradation and Dye Decolorization

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Polyporus ostreiformis **produced Mn peroxidase, acid protease, α-amylase, and lignin peroxidase, with maximum activities of 40, 8,300, and 4,200 U liter^{-1} and 50 nkat liter^{-1}, respectively, in nitrogen-limited liquid media. The fungus removed only 18.6% lignin from rice straw in 3 weeks but effected 99% decolorization of Congo red dye in 9 days.**

The importance of lignin-degrading fungi and their ligninolytic enzymes has been well appreciated globally because of their potential use in the pretreatment of lignocellulosic materials for the production of liquid and gaseous fuels (1, 9). Recently, various other applications of such organisms and enzymes have been envisioned. The list includes decolorization of industrial effluents, biopulping, and biobleaching in paper industries, biological desulfurization of coal and petroleum, production of aromatics from lignin, production of improved cattle feed, removal of chlorinated organic compounds from wastewater, and biosorption of heavy metals from effluents (8, 11), etc. The white rot fungi and, more specifically, the strains of *Phanerochaete chrysosporium* have been well studied in this regard. Brown rot fungi are much less investigated in connection with lignin degradation. Also, very little is known about their enzymology of delignification, and the need for more research attention on this group has been emphasized by some investigators (8). Particularly, the usefulness of the brown rot fungi for decolorization of wastewater and the biosorption of heavy metals, etc., has not been probed at all.

It is, therefore, imperative to study the enzyme systems of the brown rot fungi in order to gain insight into their delignification mechanism and to explore the possibilities of utilizing these fungi for the above-mentioned purposes. With this end in view, we have been investigating the extracellular lignin-degrading enzyme systems of brown rot fungi. We first reported lignin peroxidase (LIP) production by *Polyporus ostreiformis* (4). In this paper we described the production of Mn peroxidase (MNP), proteases, and α-amylase and their abilities for rice straw delignification and dye decolorization.

P. ostreiformis BU, the brown rot (4, 13) fungal culture (received from B. Nandi, Mycology Laboratory, University of Burdwan), and *P. chrysosporium* NCIM 1197 were maintained on 2% malt extract agar slants, and for inoculum preparation they were grown on plates with the same medium for up to 9 days. Agar discs (2 nos; diameter, 1 cm each, with about 0.8 mg [dry weight] of mycelia) with a mycelial mat were inoculated in 250-ml Erlenmeyer flasks containing 20 ml of the nitrogen-limited liquid medium described by Tien and Kirk (16), with the initial pH set at 4.5, and were allowed to grow for 15 days at 38 ± 1°C, without intermittent oxygen or air flushing. The composition of the medium has been reported previously (4). The medium was supplemented with 1.0 mM benzyl alcohol, after 36 h of incubation, to stimulate lignin peroxidase production (4, 10). Culture filtrates were collected at 3-day intervals up to 15 days and were filtered (Whatman no. 1 filter) and centrifuged (10,000 rpm, 4°C, 10 min) for the assays of LIP, MNP, proteases, and α-amylase.

MNP was assayed by monitoring the oxidation of Mn(II) to Mn(III) by the enzyme (14). The increase in A_{238} was monitored at 37°C during the first 30 s of the reaction. One unit of MNP oxidizes 1 μmol of Mn(II) min^{-1}.

LIP was assayed by the veratryl alcohol oxidation method (16). The enzyme activity is expressed in nanokatals (1 nkat is the enzyme activity producing 1 nmol of veratraldehyde s^{-1}). One unit of enzyme activity is equivalent to 16.7 nkat.

α-Amylase was assayed as described by Bernfeld (2). One unit of activity is defined as the amount of reducing sugar (in milligrams) released from starch by 1 ml of culture filtrate at 40°C.

Acid, neutral, and alkaline protease activities of the culture

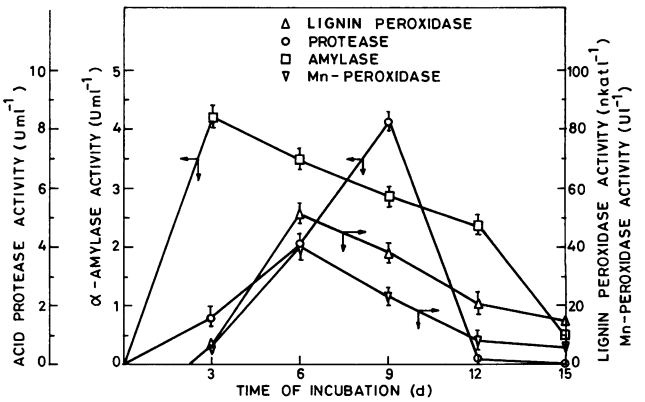

FIG. 1. Time courses of enzyme activities in liquid cultures of *P. ostreiformis*. The culture was grown at 38 ± 1°C in nitrogen-limited liquid medium (16) without shaking. Each point is the mean of three replicates, with standard deviations (error bars). The absence of an error bar indicates that the error was smaller than the symbol. d, days; Ul^{-1}, units liter^{-1}.

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TABLE 1. Dye decolorization by *P. chrysosporium* and *P. ostreiformis*[a]

Fungus	Dye	Day 6			Day 9		
		A^b	% Residual color	% Color removed/day	A	% Residual color	% Color removed/day
P. chrysosporium	Congo red	0.713	42.8	9.5	0.434	26.0	8.2
	Methylene blue	0.838	44.8	9.2	0.718	38.4	6.8
P. ostreiformis	Congo red	0.372	22.3	13.0	0.017	1.0	11.0
	Methylene blue	0.608	32.5	11.3	0.419	22.4	8.6

[a] Values are the means for triplicate cultures. Standard deviations were in the range of 2 to 6% of the means.
[b] A values for the control sets were 1.666 for Congo red and 1.872 for methylene blue.

filtrate were estimated by the casein hydrolysis method as outlined by Walter (17). One unit of activity is expressed in terms of the amount of peptides (in milligrams) liberated from casein by 1 ml of filtrate.

For delignification, rice straw (ball milled; −60 mesh; 5 g [dry weight]) was soaked in 15 ml of Tien and Kirk medium (16). Glucose [20% (wt/wt)] was added to the straw. The straw was sterilized by autoclaving (121°C, 1.25 h) in cotton-plugged polypropylene packets. Two malt extract agar discs (1-cm diameter) with growing mycelia of *P. chrysosporium* and *P. ostreiformis* (6 days old) were inoculated into the straw and were incubated for 3 weeks at 38 ± 1°C at 90% relative humidity. A control set without organisms was maintained under identical conditions. The biodelignified and control straw were washed and centrifuged repeatedly in hot (100°C) deionized distilled water to remove soluble interfering materials such as sugars, salts, and pigments, etc. The residues were lyophilized, and the lignin contents were determined by the acetyl bromide method (12).

To study decolorization, the azo dye Congo red and the thiazine dye methylene blue were added at 0.003 and 0.001% concentrations, respectively, to growing liquid cultures of *P. chrysosporium* and *P. ostreiformis* after 3 days of incubation (day 4). A control without organisms was maintained under identical conditions. Absorbances ($A_{494.8}$ for Congo red and $A_{663.8}$ for methylene blue) were recorded on days 6 and 9.

Time courses of the enzyme activities presented in Fig. 1 show that α-amylase and acid protease attained maximum levels on days 3 and 9, respectively. Both LIP and MNP attained peaks on day 6. From Fig. 1, it is apparent that with the increasing level of acid protease, the activities of the other three enzymes declined, suggesting thereby a detrimental effect of acid protease on the other three enzymes. The data were reproducible, and similar results were reported for white rot fungi (5, 6). The maximum recorded activities of LIP, MNP, α-amylase, and acid protease were 50 nkat liter^{-1} and 40, 4,200, and 8,300 U liter^{-1}, respectively. On the other hand, the white rot fungi *Lentinula edodes*, *Phanerochaete magnoliae*, and *Phellinus pini* produced about 40, 46, and 25 U of MNP liter^{-1}, respectively (3). The liquefying α-amylase activities of the strains IFO 4928 and IFO 6505 of *Schizophyllum commune* and strain BU MH 75164 of *P. ostreiformis* are reported to be 5,800, 1,700, and 2,900 U liter^{-1}, respectively (13, 15). The acid protease activities of strains NCIM 1197, ATCC 24725, and ATCC 32629 of *P. chrysosporium* were about 6,900 (caseinolytic), 8.0 (A_{520}), and 260 (A_{520}) U liter^{-1}, respectively (5, 6, 7). The time courses and levels of the neutral and alkaline proteases (maximum activities [± standard deviations] on day 9 were 7,000 ± 350 and 9,000 ± 250 U liter^{-1}, respectively) were more or less similar to those of acid protease.

P. chrysosporium is about three times more efficient than *P. ostreiformis* in lignin removal. Of the 775 mg of lignin in 5 g of rice straw, the former degraded 423 ± 8.4 mg (i.e., about 54.6%) and the latter degraded 144 ± 3.7 mg (i.e., about 18.6%) in 3 weeks. Data are the means of triplicate cultures (± standard deviations) and are reproducible.

Table 1 shows that the dye decolorization efficiency of *P. ostreiformis* is much higher than that of *P. chrysosporium*. Almost total decolorization of Congo red is effected by *P. ostreiformis*. Color removal between days 4 and 6 was much higher than that for the next 3 days (i.e., days 7 to 9). Incidentally, day 6 was the day of maximum titers in the culture filtrates for LIP and MNP. The higher decolorization efficiency of *P. ostreiformis* than of *P. chrysosporium* NCIM 1197 may be due to its reasonably high MNP activity. Michel et al. (11) reported that MNP plays a more important role than LIP in bleach plant effluent decolorization by *P. chrysosporium*.

In conclusion, the results indicate that *P. ostreiformis* produces two important enzymes, namely LIP and MNP, implicated in ligninolysis. Two other brown rot fungi, namely *Poria monticola* NCIM 1090 and *Lenzites trabea* NTCC 1314, failed to produce these two enzymes (4). Reports on the production of LIP and MNP by any other brown rot fungus seem to be nonexistent. The significant dye decolorization property of this organism reveals its usefulness in water pollution control. It will be worthwhile, perhaps, to try to use this and other brown rot fungi for that purpose. Even mixed cultures of white rot and brown rot fungi may be utilized for evolving efficient processes for biological decolorization because the reported (9) efficient chain-splitting and demeth(ox)ylating capability of brown rot fungi may complement the ring cleavage activity of the white rot group, thereby resulting in faster dye degradation.

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