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Production of Ligninolytic Enzymes for Dye Decolorization by Cocultivation of White-Rot Fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* Under Solid-State Fermentation

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Abstract

Lignocellulosic wastes such as neem hull, wheat bran, and sugarcane bagasse, available in abundance, are excellent substrates for the production of ligninolytic enzymes under solid-state fermentation by white-rot fungi. A ligninolytic enzyme system with high activity showing enhanced decomposition was obtained by cocultivation of *Pleurotus ostreatus* and *Phanerochaete chrysosporium* on combinations of lignocellulosic waste. Among the various substrate combinations examined, neem hull and wheat bran wastes gave the highest ligninolytic activity. A maximum production of laccase of 772 U/g and manganese peroxidase of 982 U/g was obtained on d 20 and lignin peroxidase of 656 U/g on d 25 at 28 ± 1°C under solid-state fermentation. All three enzymes thus obtained were partially purified by acetone fractionation and were exploited for decolorizing different types of acid and reactive dyes.

Index Entries: Neem hulls; white-rot fungi; solid-state fermentation; ligninolytic enzymes; lignocellulosic waste.

Introduction

Lignin is an integral part of virtually all higher plants and the second most abundant organic polymer in nature *(1)*. The importance of lignin in the atmospheric carbon cycle and interest in lignocellulosic material as a renewable feedstock have greatly accelerated research on lignin biodegradation *(2)*. Lignin is degraded by a narrow array of microbes than the other

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major biopolymers of which white-rot fungi are the most efficient *(3)*. Ligninolysis is associated with the production of the extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. These enzymes have varied substrate specificities, especially for phenolic compounds, and, therefore, these microorganisms or their enzymes are potentially useful to clean up environmental pollutants such as dyes *(4)*.

The efficiency of ligninolysis depends on the source of lignocellulosic substrates, their compositional structures, the process parameters, and enzyme production, which is influenced by carbon, nitrogen, and sulfur *(5)*. Moreover, most investigations are devoted only on biodegradation of modified forms of lignin, namely milled wood, kraft, lignosulfonates, or lignin-related aromatics, not native or protolignin *(6)*. In present study, we used natural readily available low-cost substrates: neem hull waste, sugarcane bagasse, and wheat bran and their combinations for their efficient degradation.

Much of the work on ligninolysis has been carried out using individual cultures *(7)*. However, different lignocellulosic substrates require different enzyme complexes for the most efficient hydrolysis, which may be achieved by cocultivation of different white-rot fungi.

The waste generated by textile and dyestuff industries is relatively recalcitrant *(8,9)*. Ligninolytic cultures as well as their enzymes have been reported to degrade and decolorize various dyes. Therefore, present work was carried out to investigate hydrolysis of lignocellulosic waste by *Pleurotus ostreatus* and *Phanerochaete chrysosporium* individually or under cocultivation mode by solid-state fermentation. Ligninolytic enzymes thus obtained were used to study degradation and decolorization of dyes with possible application in pollution control.

Materials and Methods

Chemicals

All chemicals used were of analytical grade. Neem hulls were obtained from National Tree Growers Co-operative Federation Limited, Anand, India. Wheat bran and sugarcane bagasse were obtained from a local market.

Microorganisms and Culture Condition

Two white-rot fungi, *P. ostreatus* and *P. chrysosporium*, were obtained as gift cultures from the Institute of Forstbotanik, Gottingen, Germany. They were grown on malt extract agar at 26 ± 1°C and stored at 4°C. Cultures were subcultured once every 2 mo.

Analytical Procedure

Neem hull waste, wheat bran, and sugarcane bagasse were dried at 60°C and milled to get a mesh size of approx 50. Cellulose and lignin con-

Table 1
Composition of Ligninocellulosic Wastes

Composition (%)	Neem hull waste	Sugarcane bagasse[a]	Wheat bran[a]
Lignin	55.1 ± 4.1	25.8 ± 2.3	28.3 ± 2.5
Cellulose	32.1 ± 2.5	52.3 ± 2.9	36.0 ± 1.9
Hemicellulose	17.0 ± 2.5	16.0 ± 1.9	20.1 ± 1.5
Nitrogen	1.30 ± 0.21	0.41 ± 0.020	0.62 ± 0.1
Phosphorus	0.23 ± 0.01	0.07 ± 0.001	0.06 ± 0.001
Potassium	0.021 ± 0.001	0.21 ± 0.020	0.31 ± 0.010
Sulfur	0.141 ± 0.01	0.19 ± 0.020	0.21 ± 0.010
Micronutrient (ppm)			
Fe	46 ± 2.2	ND	ND
Zn	12 ± 1.9	ND	ND
Cu	11 ± 1.5	ND	ND
Mn	13 ± 1.8	ND	ND

[a]ND, not done.

tent were determined by the method of Goering and Van Soest *(10)*. Extracellular protein was measured by the method of Lowry et al. *(11)*. Nitrogen, phosphorus, potassium, sulfur, and minerals were estimated according to standard methods *(12)*. The composition of the different lignocellulosic substrates is given in Table 1.

Experimental Setup

Ten grams of each ligninocellulosic substrate was placed in a 500-mL Erlenmeyer flask containing 50 mL of medium. The medium contained the following ingredients: 1.0 g/L of KH_2PO_4, 0.5 g/L of KCl, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of yeast extract, and 0.325 g/L of L-aspargine. The medium along with the substrate was autoclaved at 121°C for 15 min. The pH of the medium was set at 5.0.

Pregrown cultures on malt extract agar plate (15 d old) were used for inoculation. For uniform inoculation, a piece of fungal growth (1.0 × 1.0 cm) was inoculated into each flask. The flasks were incubated at 28 ± 1°C with intermittent shaking for uniform mixing. Uninoculated control was also kept at the same temperature. All the experiments were performed in quadruplicate.

Extraction of Extracellular Enzymes

Fermentation was carried out for 8 wk and experiments were performed in quadruplicate. Flasks were removed at regular intervals from each set, to which 50 mL of 0.05 *M* acetate buffer (pH 5.0) was added. The mixture was kept on a shaker for uniform mixing and extraction of enzymes from the substrates into the buffer. The contents of the flasks were filtered using cheesecloth. The liquid extract obtained was further centrifuged, and the supernatant was used for enzyme purification.

Table 2
Summary of Ligninolytic Enzyme Purification by Acetone Fractionation

Purification step	Enzyme activity (U/g)	Protein (mg/g)	Specific activity (mg/protein)
Crude enzyme			
Laccase	772		864
LiP	656	0.893	759
MnP	982		1099
Acetone precipitation			
Laccase	805		1376
LiP	796	0.585	1361
MnP	1091		1865

Enzyme Purification

The culture fluid used for enzyme purification was filtered through Whatman no. 1 filter paper. Enzyme precipitation was performed using 2 vol of acetone. The precipitate was collected by centrifugation and dissolved in 50 m*M* acetate buffer (pH 5.0). Protamine sulfate was added to a final concentration of 1% (w/v) to remove brownish pigments. The precipitate was removed by centrifugation, and the supernatant was dialyzed overnight against the same buffer (Table 2). Such partially purified enzyme was used for dye decolorization.

Enzyme Assays

Laccase (EC 1.10.3.2) *p*-diphenol-oxidoreductase was measured according to Palmieri et al. *(13)*. LiP (EC 1.11.1.14) was determined according to Tien and Kirk *(14)*, and MnP (EC 1.11.1.13) was determined following a method by Katagiri et al. *(15)*.

Scanning Electron Microscopy

Samples were directly examined with a Philips XL30 ESEM (Environmental Scanning Electron Microscope) having a gaseous secondary-state detector. Samples were placed on aluminum and then into the chamber.

Dye Decolorization by Ligninolytic Enzymes

Dye decolorization was started by adding an aliquot of enzyme to buffer solution, which contained dye (50 ppm). A control wherein enzyme being replaced by distilled water was conducted in parallel. The effect of dye and enzyme concentration on dye decolorization was investigated by using an initial rate method to avoid possible interference from decolorization intermediates, because a too fast decolorization rate caused a considerable error in the determination of the initial decolorization rate. Enzyme dose was optimized such that dye was degraded by <5% after a cuvet was placed in the spectrophotometer. The absorbance of the dye solution was

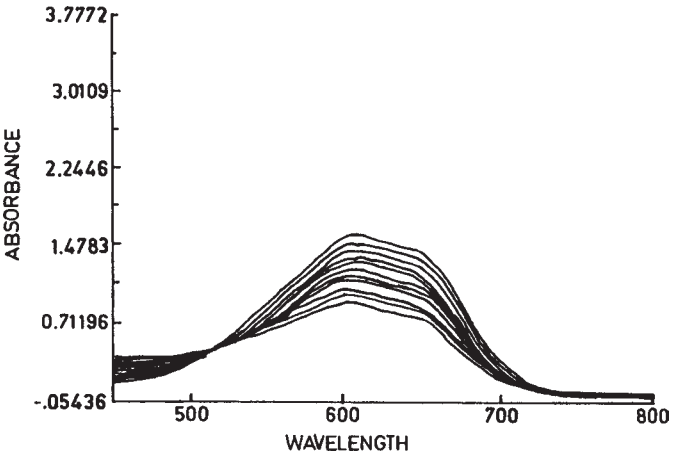

Fig. 1. Dye decolorization spectrum of Ranocid Fast Blue.

continuously monitored with time, as shown in Fig. 1, and the initial rate was calculated from the first 5–10% decline in absorbance.

Results and Discussion

The lignin-degrading white-rot fungi *P. ostreatus* and *P. chrysosporium* have been investigated extensively in the past decade for their versatile ability to degrade partially or completely recalcitrant organic pollutants, such as chlorophenols, nitrophenol polyaromatic hydrocarbons, and dyes, by its extracellular enzymes *(16,17)*. LiP laccase and MnP are the key enzymes for degradation of polycyclic aromatic compounds and could be exploited for dye decolorization. We are here reporting cocultivation of white-rot fungi *P. ostreatus* and *P. chrysosporium* for higher ligninolytic enzyme production on combinations of lignocellulosic wastes under solid-state fermentation. All three enzymes were detected in the cocultivation of *P. ostreatus* and *P. chrysosporium.* These enzymes thus obtained were exploited for dye decolorization.

Ligninolytic Enzyme Production by Individual Cultures on Different Substrates and Their Combinations

Our study compared solid-state fermentation of lignocellulosic substrates such as neem hull, sugarcane bagasse, and wheat bran alone and in combination in a 1:1 (w/w) proportion. Table 3 gives the results of the production of ligninolytic enzymes using neem hull waste, sugarcane bagasse, and wheat bran as lignocellulosic substrates inoculated with *P. ostreatus* and *P. chrysosporium* individually and in various combinations. *P. ostreatus* showed maximum laccase (156 U/g) and MnP (254 U/g) activities on neem hull waste on the d 20 of incubation. However, very low LiP activity was observed during neem hull degradation with maximum activ-

Table 3
Effect on Ligninolytic Enzyme Production by Individual Culture (*P. ostreatus* and *P. chrysosporium*) on Different Substrates and Their Combinations[a]

Organism and substrate	Enzyme activity (U/g)		
	Laccase	LiP	MnP
P. chrysosporium			
Neem hull waste	28.2 ± 1.2	192 ± 4.2	54.1 ± 1.5
Sugarcane bagasse	25.2 ± 1.5	119 ± 1.3	45.1 ± 1.1
Wheat bran	21.1 ± 0.92	167 ± 1.9	34.2 ± 0.8
Neem hull waste and wheat bran (1:1)	34.2 ± 1.9	243 ± 4.1	54.2 ± 1.7
Neem hull waste and sugarcane bagasse (1:1)	22.1 ± 1.5	145 ± 1.9	65.2 ± 1.4
Wheat bran and sugarcane bagasse (1:1)	31.2 ± 0.7	198 ± 1.5	46.3 ± 1.9
P. ostreatus			
Neem hull waste	156 ± 2.4	20.2 ± 1.1	254 ± 2.9
Sugarcane bagasse	112 ± 2.8	21.2 ± 2.1	212 ± 2.7
Wheat bran	142 ± 3.1	19.2 ± 0.2	217 ± 1.6
Neem hull waste and wheat bran (1:1)	198 ± 3.7	32.1 ± 1.7	243 ± 3.6
Neem hull waste and sugarcane bagasse (1:1)	145 ± 2.7	34.2 ± 1.2	194.2 ± 3.1
Wheat bran and sugarcane bagasse (1:1)	171 ± 2.8	23.2 ± 0.8	211 ± 2.8

[a]Data represent the day on which maximum production was observed.

ity (20 U/g) on d 25 of incubation. In comparison with *P ostreatus*, *P. chrysosporium* produced higher amounts of LiP as compared to MnP and laccase during degradation of neem hull waste. Maximum LiP (192 U/g) activity appeared on d 25, whereas highest production of laccase (142 U/g) and MnP (112 U/g) was observed on d 20 of incubation. *P. chrysosporium* produced significantly lower amounts of LiP during solid-state fermentation of wheat bran (167 U/g) or sugarcane bagasse (119 U/g) compared with that on neem hull waste. Higher production of ligninolytic enzymes by *P. ostreatus* and *P. chrysosporium* using neem hull wastes compared with the other two substrates may be owing to some unidentified compounds of neem hull waste that have a stimulatory effect on the ligninolytic activity of *P. ostreatus* and *P. chrysosporium.*

Different combinations of lignocellulosic substrates were investigated in order to design a blend of substrates for maximum production of ligninolytic enzymes. Enhanced production of laccase and MnP was observed when *P. ostreatus* was cultivated on a 1:1 mixture of wheat bran and neem hull waste compared with that on individual substrates alone (Table 3). Similarly, higher amounts of LiP and MnP were produced in

Table 4
Effect on Ligninolytic Enzyme Production by Combination of White-Rot Fungi (*P. ostreatus* and *P. chrysosporium*) on Different Substrates and their Mixture[a]

Organism and substrate	Enzyme activity (U/g)		
	Laccase	LiP	MnP
P. chrysosporium + *P. ostreatus*			
Neem hull waste	467 ± 5.1	398 ± 4.6	542 ± 4.3
Sugarcane bagasse	286 ± 2.9	254 ± 3.2	426 ± 3.9
Wheat bran	398 ± 3.2	286 ± 3.9	423 ± 4.3
Neem hull waste and wheat bran	772 ± 4.5	656 ± 2.7	982 ± 5.2
Neem hull waste and sugarcane bagasse	672 ± 3.5	543 ± 4.2	782 ± 3.8
Wheat bran and sugarcane bagasse	687 ± 4.1	512 ± 4.9	791 ± 3.7

[a]Data represent the day on which maximum production was observed.

presence of both wheat bran and neem hull by *P. chrysosporium* compared with those in presence of either substrates alone. The effects of various lignocellulosic substrates on production of ligninolytic enzymes indicate large inherent variability with respect to their nutritional requirements *(18)*. Hutterman et al. *(19)* and Harrs et al. *(20)* have demonstrated a role of C:N ratio and stimulating agents on the production of ligninolytic enzymes by *P. ostreatus* and other fungi.

The chemical composition of all three lignocellulosic substrates used is shown in Table 3. A mixture of neem hull waste and wheat bran had a complementary effect on ligninolytic enzyme production by white-rot fungi, and it seems that both these substrates contribute to stimulatory agents for better production of these enzymes.

Enhanced ligninolytic enzyme production on combinations of substrates could be result of an enzyme induction mechanism caused by small amounts of water-soluble compounds (e.g., phenol, low molecular mass compounds, and macromolecule-containing phenolic groups) from lignin and lignocellulosic fraction *(20)*. MnP activity in *P. chrysosporium* is induced under carbon and nitrogen starvation *(21)*. Moreover, neem hull waste contains micronutrients such as manganese, which may be responsible for higher production of MnP activity *(22,23)*.

Ligninolytic Enzyme Production by Combination of White-Rot Fungi on Different Substrates and Their Mixture

Table 4 gives the result of enzyme production on all three lignocellulosic substrates by cocultivation of *P. ostreatus* and *P. chrysosporium*. Cocultivation of these fungi resulted in higher production of ligninolytic enzymes on all substrates tested compared to individual cultures alone. Compared to pure cultures of *P. ostreatus*, its cocultivation with *P. chrysosporium* resulted in a 10- to 15-fold increase in LiP and a 2- to 3-fold increase in MnP and laccase production. Whereas cocultivation of *P. ostreatus* and

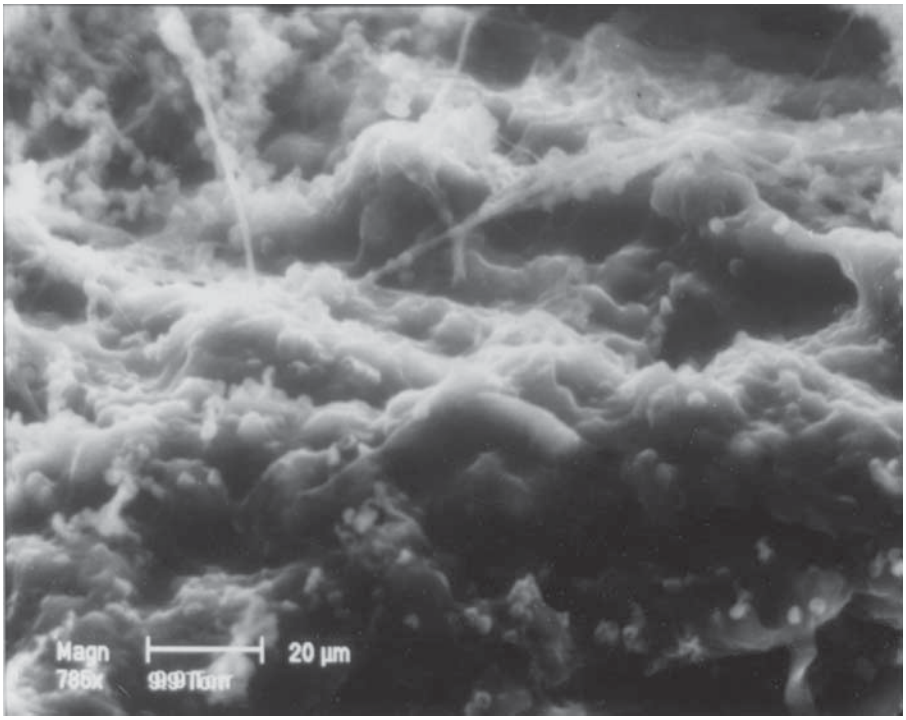

Fig. 2. Scanning electron micrograph of cocultivation of *P. ostreatus* and *P. chrysosporium* on neem hull waste.

P. chrysosporium resulted in an 8- to 12-fold increase in the production of laccase and MnP, only about 1.5-fold higher amounts of LiP were produced as compared to *P. chrysosporium* alone. We also observed that delignification was greater in the consortium than in the individual cultures (data not shown). Table 3 shows that of various substrate combinations (1:1) inoculated with *P. chrysosporium* and *P. ostreatus*, neem hull waste and wheat bran resulted in a remarkable increase in ligninolytic enzymes. Production of ligninolytic enzymes increased by two- to threefold when solid-state fermentation was carried out using different combinations of substrates as compared to individual substrates alone by cocultivation of *P. chrysosporium* and *P. ostreatus*. Enhanced ligninolytic enzyme production during cocultivation of *P. ostreatus* and *P. chrysosporium* indicated the role of synergism in degradation of lignocellulosic waste *(23)*.

Scanning electron microscopes were used to study growth of *P. ostreatus* and *P. chrysosporium* under cocultivation mode on neem hull waste after 35 d of incubation (Fig. 2). The results indicate that both fungi grow well across the surface and throughout the samples. Thin thread-like mycelia without sporulation represent the growth of *P. ostreatus*, while thicker hyphae with spherical spores on its surface indicate the growth of *P. chrysosporium.*

Table 5
Dye Decolorization by Ligninolytic Enzyme[a]

Dye	λ_{max} (nm)	Type of dye	Decolorization in absence of H_2O_2 (%)	Decolorization in presence of 0.2 m*M* H_2O_2 (%)
Porocion Brilliant Blue HGR	604	Reactive	85.3 ± 2.1	97.2 ± 1.2
Porocion Red Brown H4R	530	Reactive	90.5 ± 3.5	94.5 ± 2.5
Porocion Navy Blue H3R	580	Reactive	78.6 ± 3.2	93.4 ± 2.9
Porocion Blue H5G	620	Reactive	84.8 ± 2.4	92.4 ± 3.1
Orange 3R	486	Reactive	75.4 ± 4.3	88.3 ± 1.8
Orange 2R	486	Reactive	78.5 ± 2.5	89.7 ± 2.4
Ranocid Fast Blue	538	Acid	90.5 ± 3.4	93.2 ± 3.1
Acid Red 119	520	Acid	85.1 ± 3.4	93.9 ± 1.5
Navidol Fast Black MSRL	570	Acid	73.4 ± 2.2	85.2 ± 1.8

[a]Reaction mixture contained 50 m*M* acetate buffer, 50 ppm dye concentration, and 0.2 mL of purified enzyme, and decolorization was observed in the absence of H_2O_2 and in presence of 0.2 m*M* H_2O_2.

Dye Decolorization by Ligninolytic Enzymes

Extracellular ligninolytic enzyme complex was obtained by cocultivation of *P. osteratus* and *P. chrysosporium* on combination of neem hull waste and wheat bran under solid-state fermentation for 25 d. Enzyme was partially purified by acetone fractionation, and this partially purified enzyme was used for textile dye decolorization. We have used Porocion series reactive and acid dyes, mainly Porocion Brilliant Blue HGR, Porocion Red Brown H4R, Porocion Navy Blue H3R, Porocion Blue H5G, Orange 3R, Orange 2R, Navidol Fast Black MSRL, Ranocid Fast Blue, and Acid Red 119. The enzymatic dye decolorization is shown in Table 5. When we performed dye decolorization in liquid basal salt medium by the individual cultures, it took longer for dye decolorization to occur because not only does it require enzyme production but hydrogen peroxide, coordinately in the flask *(24)*. We observed that dye decolorization is influenced by hydrogen peroxide. In our experiment, by adding 0.2 m*M* hydrogen peroxide, dye decolorization increased remarkably for Porocion Brilliant blue HGR and Porocion Red Brown H4R, Porocion Navy Blue H3R, Porocion Blue H5G, Acid Red 119, Navidol Fast Black MSRL, Orange 3R, and Orange 2R.

Our study demonstrates successful optimization of lignocellulosic waste decomposition and production of ligninolytic enzymes, which could be further exploited for decolorization of synthetic dyes.

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