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Influence of Media Nutrients on Synthesis of Lignin Peroxidase from *Aspergillus* sp.

Shamla Ahammed and Parukuttyamma Prema*

Biochemical Processing Division, Regional Research Laboratory, Trivandrum, India, 695 019, E-mail: prema@csrrltrd.ren.nic.in

Abstract

The effect of carbon and nitrogen sources, lignocellulosic substrates, and metal ions on lignin peroxidase (LiP) activity of *Aspergillus* sp., which was isolated from a mangrove area, was studied. Glucose (1%) was found to be the best carbon source. Among the various lignocellulosic substrates used, coir pith at 3% concentration increased LiP activity twofold on the second day of incubation. Peptone and KNO_3 completely inhibited the enzyme synthesis while $(NH_4)_2SO_4$ at 12.5 m*M* produced maximum activity. Since seawater contained all the requisite metal ions, any added ions had a negative effect on activity. Cu^{2+} had the most inhibiting effect while K^+ the least. When all the optimized conditions were provided, in nitrogen- and carbon-sufficient medium, a maximum LiP activity of 345 U/mL was obtained on the second day of incubation.

Index Entries: Lignin degradation; white rot; soft rot; lignin peroxidase; *Phanerochaete chrysosporium*; marine fungi; ligninase.

Introduction

Degradation of highly toxic environmental chemicals such as dioxins, polychlorinated biphenyls, various dyes, and polyaromatic hydrocarbons; decolorization of kraft bleach plant effluents; and biobleaching of paper pulps are few of the applications of lignin-degrading enzymes *(1)*. White rot fungi were used extensively for studying the physiologic requirements of ligninolysis. Lignin peroxidase (LiP), manganese peroxidase, laccase, and H_2O_2-producing oxidases are the extracellular enzymes involved in lignin and xenobiotic degradation by white rot fungi *(2,3)*. In liquid cultures, lignin was degraded only during secondary (idiophasic) metabolism, which was triggered by limitation of nitrogen *(4,5)*, carbon, or sulfur

*Author to whom all correspondence and reprint requests should be addressed.

(6). Ligninolytic peroxidase was suppressed and delayed by high concentrations of nitrogen *(4,5,7,8)*. However, lignin mineralization by some white rot fungi was not repressed by high nitrogen concentrations *(9)*. With some mutants of *Phanerochaete chrysosporium*, LiP production was even stimulated *(10)*.

Ascomycetes and Deuteromycetes fungi, especially from the marine environment, have been least studied *(11,12)*. During our earlier studies, we were able to isolate a halophilic ascomycete, *Aspergillus* sp., from decayed leaves from mangrove swamp capable of producing LiP. Here we report that in nitrogen- and carbon-sufficient medium, this fungus was able to produce the maximum LiP activity. We also studied the influence of various carbon and nitrogen sources on LiP production. In addition, we studied how secondary metabolic events are strongly influenced by trace metal nutrition *(13)*.

Materials and Methods

Culture and Culture Conditions

Aspergillus sp. was isolated from a mangrove area of Kochi, Kerala, and maintained on 2% malt agar slants. The fungus was grown in carbon-limited medium in seawater of 25 g/kg salinity that contained the following: 3.0 g/L of glucose, 5.0 g/L of KH_2PO_4, 1.0 g/L of NH_4NO_3, 1.0 g/L of $MgSO_4 \cdot 7H_2O$, 0.2 g/L of Tween-20, and 1 m*M* veratryl alcohol at pH 4.0 and 30°C. Spore suspension giving a spore count of 3×10^6 spores/mL was used as inoculum. Growth medium (25 mL) was placed in 100-mL Erlenmeyer flasks with rubber stoppers pierced with glass tubes for aeration. These were agitated at 80 rpm and also aerated for 4 h daily. Samples were taken every 24 h and analyzed for pH and biomass. The cells were separated by centrifugation (9800*g* for 10 min at 4°C), and the cell-free supernatant was used as the source for crude enzyme and for the estimation of reducing sugar and soluble protein.

LiP Assay

LiP activity was assayed by measuring the rate of H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde spectrophotometrically. The standard reaction mixture contained 0.8 m*M* veratryl alcohol and 150 m*M* H_2O_2 in 100 m*M* sodium tartrate buffer, pH 3.0, and 1 mL of cell-free culture supernatant was used as the enzyme source. The reaction was started by adding H_2O_2, and the linear increase in absorbance at 310 nm was monitored for 1 min at 30°C. One unit of LiP was defined as 1 µmol of veratraldehyde formed/min (extinction coefficient = 9300 $M^{-1}cm^{-1}$) under the assay conditions *(14)*.

Analytical Procedures

Growth was determined by dry weight method, soluble protein by Lowry's method *(15)*, and reducing sugar by dinitrosalicylic acid method

(16). In the case of medium containing lignocellulosic substrate, growth was determined as the difference between the initial weight of the medium before inoculation and the final weight of the medium after the completion of growth.

Effect of Carbon Sources and Lignocellulosic Substrates on Enzyme Production

Different carbon sources—fructose, sucrose (sugars), glycerol, starch (polysaccharide)—were replaced in the basal medium for glucose; various lignocellulosic wastes such as wheat bran, coir pith, rice bran, sawdust, bagasse, pigeon pea hull, and rice straw taken at 1% level (w/v) were incorporated into the medium in the absence of 1 m*M* veratryl alcohol. Varying concentrations of glucose (0.1–1.5%) and coir pith (0.25–3%) were added to determine the optimum concentration for LiP activity.

Effect of Nitrogen Sources on Enzyme Production

NH_4NO_3 in the basal medium, which was optimized for carbon and lignocellulosic substrate, was replaced by different inorganic nitrogen sources ammonium tartarate, ammonium sulfate, ammonium chloride, sodium nitrate, and potassium nitrate at 12.5 m*M* and organic nitrogen sources (peptone and yeast extract at 0.1% concentration) in the absence of 1 m*M* veratryl alcohol. The effect of $(NH_4)_2SO_4$ concentration ranging from 2 to 20 m*M* on enzyme activity was also determined.

Effect of Metal Ions on Enzyme Production

Seawater of different salinities (0–35 g/kg) was used for the preparation of the basal medium. In addition, the effect of metal ions on the enzyme production was studied by adding 1 m*M* each of KCl, $MnSO_4$, $CaCl_2$, $CuCl_2$, $FeSO_4$, $ZnCl_2$ and $NiCl_2$ to the growth medium.

Results and Discussion

Effect of Carbon Sources and Lignocellulosic Substrates

Among the various carbon sources tested, 1% glucose gave maximum LiP activity on the seventh day of incubation, glucose being a necessary energy source for enzyme activity. Carbohydrate, especially glucose limitation, resulted in early onset of ligninolytic activity and also increased enzyme production. The importance of source of carbohydrate on enhancing LiP production has been reported by others *(6,14)*. The easily oxidizable nature of glucose in comparison to the other substrates studied makes it more favorable for growth and LiP production by *Aspergillus* sp. In the absence of glucose, little growth and LiP activity were shown. Results are given in Figs. 1 and 2. Coir pith turned was found to be the best lignocellulosic substrate, giving maximum activity on the second day when glucose became exhausted, suggesting that lignin-degrading enzymes are

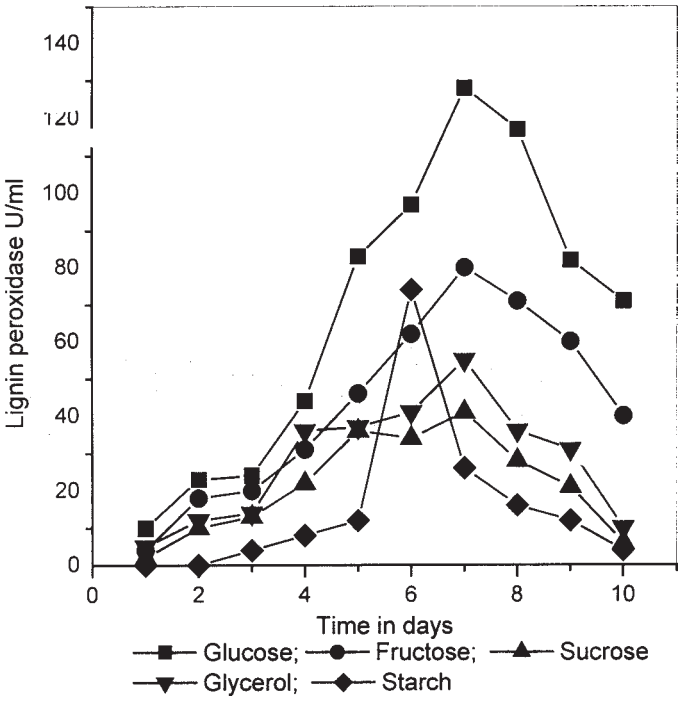

Fig. 1. Effect of supplementation of carbon sources on production of LiP by *Aspergillus* spp.

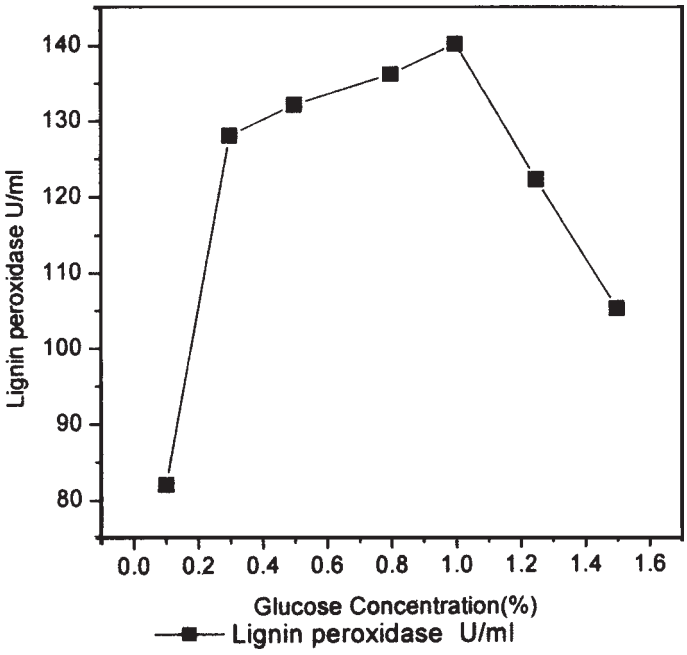

Fig. 2. Effect of supplementation of different concentrations of glucose on production of LiP by *Aspergillus* spp.

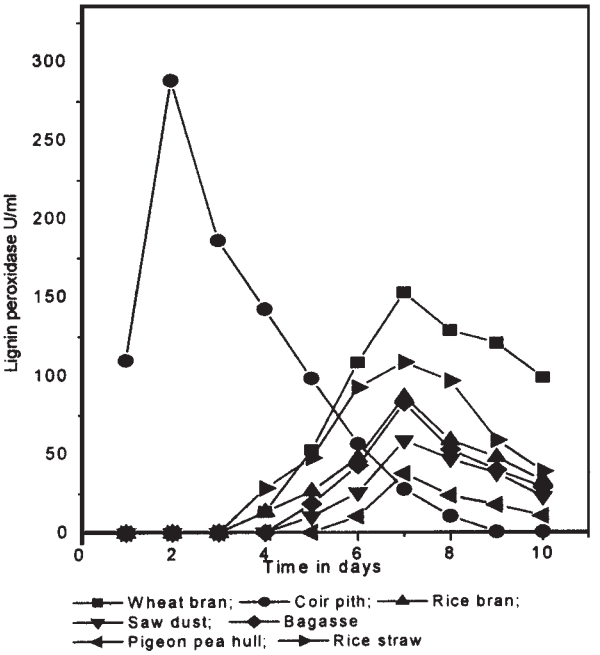

Fig. 3. Effect of supplementation of different lignocelluloses on production of LiP by *Aspergillus* spp.

secondary metabolites synthesized after the cessation of cell growth and complete utilization of glucose. Coir pith contained approx 43% lignin with nitrogen and potash, which may be the reason for the induction of enzyme activity on the second day *(17)*. In addition, there was a gradual increase in activity with increasing concentration of coir pith. Results are given in Figs. 3 and 4. The addition of other substrates had only a meager effect on the enzyme activities.

Effect of Nitrogen Sources

Nitrogen and carbon sources have a coordinating influence on LiP production. In the case of *Aspergillus* sp., KNO_3 and peptone completely inhibited the enzyme, while $(NH_4)_2SO_4$ at 12.5 m*M* gave maximum activity, with lower and higher concentrations reducing the yield. NH_4NO_3 and NH_4Cl gave comparable activity (Figs. 5 and 6). It was found that nitrogen in the form of nitrates and peptides is not utilized by this fungus and favored NH_4^+-N for LiP production. Low amounts of nitrogen decreased LiP activity. The importance of nitrogen stimulation in nature is not clear, because the nitrogen content of most wood is very low. Certain conditions under which the fungi could have access to high levels of nitrogen could occur naturally. Some nitrogen-fixing bacteria are associated with major decay fungi of wood. Cowling and Merrill *(18)* suggested that nitrogen from *in situ* nitrogen fixation might supplement the existing and meager nitrogen resources in wood available to fungi. It was demonstrated in *Bjerkandera*

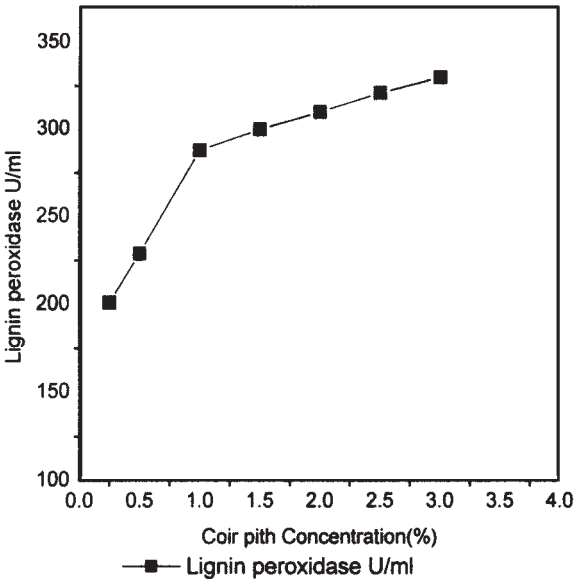

Fig. 4. Effect of supplementation of different concentrations of coir pith on production of LiP by *Aspergillus* spp.

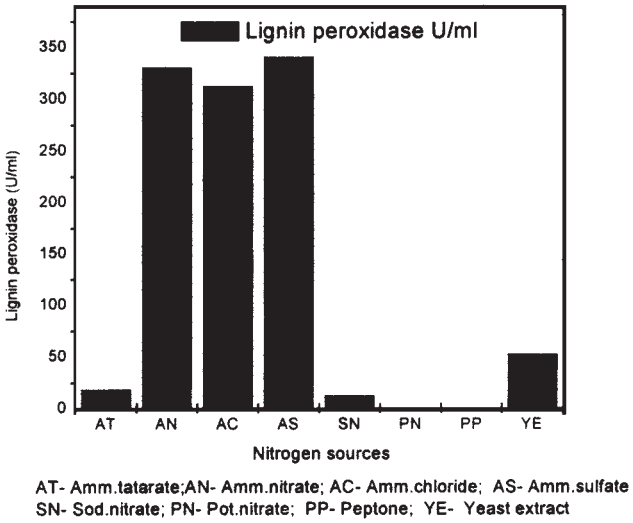

Fig. 5. Effect of supplementation of different nitrogen sources on production of LiP by *Aspergillus* spp. AT, ammonium tartrate; AN, ammonium nitrate; AC, ammonium chloride; AS, ammonium sulfate; SN, sodium nitrate; PN, potassium nitrate; PP, peptone; YE, yeast extract.

sp. strain BOS55 that endogenous production of veratryl alcohol was much higher in nitrogen-sufficient medium than in nitrogen-limited medium *(19)*. The nitrogen supplements could have a role in imitating the conditions that the fungus encounters during carbon limitation. Overproduction

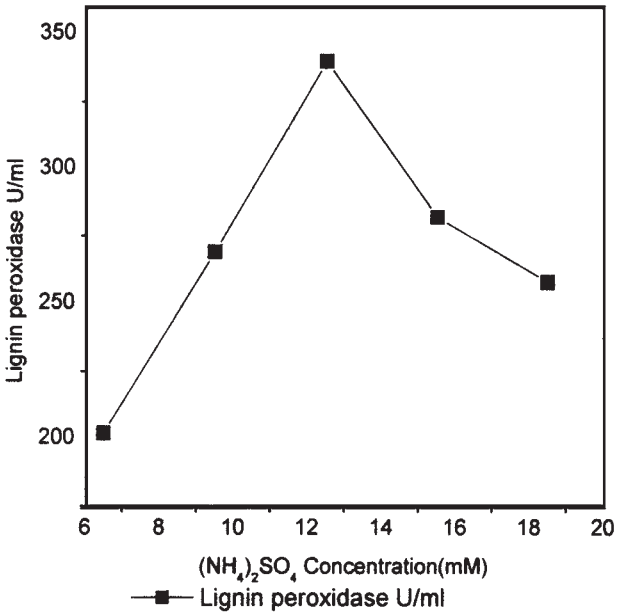

Fig. 6. Effect of supplementation of different concentrations of ammonium sulfate on production of LiP by *Aspergillus* spp.

of LiP in the presence of sufficient nitrogen level and excess nitrogen levels seemed to occur as a response to carbon starvation after rapid glucose depletion. The NH_4^+ in the extracellular fluid reappeared as soon as glucose was depleted, suggesting that an alternative energy source was generated by self-proteolysis of cell proteins *(3)*.

Effect of Metal Ions

In comparison with earlier studied microorganisms, *Aspergillus* sp., LiP production was considerably influenced by the salinity of the medium. Seawater of salinities of 25 and 35 g/kg gave comparable enzyme activities while at lower salinities, owing to unavailability of adequate mineral nutrients, little activity was shown, thus confirming its halophilic nature. The results are given in Fig. 7 and Table 1. Among the metal ions, studied individually, Cu^{2+} inhibited LiP markedly while Ni^{2+} and K^+ had the least effect. Bonnarme and Jeffries *(20)* have reported that unlike the effect of carbon and nitrogen, Mn^{2+} affects the cell in a relatively specific manner, in which the production of mycelial dry weight and extracellular protein is not affected nor are the rates of consumption of carbon and nitrogen sources.

The growth profile of *Aspergillus* sp. under optimized conditions is shown in Fig. 8. The pH of the medium was reduced to 3.0 by the first day, which may be owing to the production of acid metabolites by the fungus on utilization of glucose. Maximum growth was reached by the second day, when glucose became exhausted, leading to nutrient limitation and the appearance of secondary metabolism. The soluble protein increased with

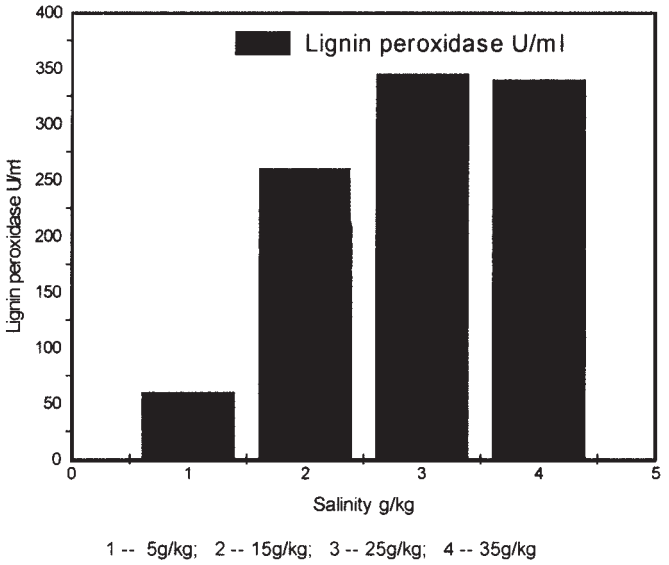

Fig. 7. Effect of supplementation of different levels of salinities (g/kg) on production of LiP by *Aspergillus* spp. 1 (5 g/kg); 2 (15 g/kg); 3 (25 g/kg); 4 (35 g/kg).

Table 1
Percentage Inhibition
of Different Metal Ions
on LiP Production

Metal ions	Inhibition (%)
KCl	6.21
$NiCl_2$	10.46
$CoCl_2$	15.91
$ZnCl_2$	23.35
$MnSO_4$	26.40
CaCl2	34.08
$FeSO_4$	44.65
$CuCl_2$	86.04

peak enzyme activity, while the decrease in enzyme activity after the second day may be owing to the appearance of extracellular protease activity *(21)*.

In contrast to *P. chrysosporium*, carbon- and nitrogen-sufficient medium gave maximal activity for *Aspergillus* sp. Even in this carbon- and nitrogen-sufficient medium, glucose became exhausted by the second day, correlating with peak LiP activity as well as growth. When all the optimized conditions were provided, an LiP activity of 345 U/mL was obtained on the second day of incubation. Early onset of LiP activity from this halophilic fungus makes it and its enzyme suitable candidates for effluent treatment from paper pulp industry as well as for biobleaching of paper pulp.

Fig. 8. Growth profile of *Aspergillus* spp. in modified medium.

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