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OPTIMIZATION OF INDUSTRIALLY IMPORTANT ENZYMES LACCASE AND PEROXIDASE PRODUCTION IN THE FUNGUS *THELEPHORA*

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ABSTRACT

White rot fungus, *Thelephora,* isolated by its high production levels of ligninolytic enzymes from stumps of a burnt tree, was examined for its optimum growth conditions such as pH and temperature as well as carbon sources (glucose, cellulose and lignin) and nitrogen sources (diammonium tartrate, yeast extract and urea). For the maximum growth of *Thelephora* the 6 day incubation period was required at pH 5.0 and 35°C with 3.0 g/L lignin as a carbon source and 0.88 g/L yeast extract as a nitrogen source. The lignin peroxidase (LiP) production was maximal on the 7th day at pH 4.5 and 40°C with 3.0 g/L lignin and 0.44 g/L diammonium tartrate. The manganese-dependent peroxidase (MnP) production was achieved on 7th day incubation at pH 4.5 and 40°C, with 2.5 g/L glucose and 0.22 g/L diammonium tartrate as carbon and nitrogen sources respectively. The highest amount of laccase was obtained on the fourth day of incubation at pH 7.0 and 35°C using 3.0 g/L cellulose and 0.44 g/L diammonium tartrate as carbon and nitrogen sources respectively.

INTRODUCTION

White rot fungi produce extracellular lignin-degrading enzymes that consist of lignin peroxidases (LiP), manganese-dependent peroxidases (MnP), laccases and hydrogen-producing oxidase (Barr & Aust 1994). The fungi belong to heterogeneous groups but have in common the capacity to degrade lignin as well as other wood components. Ligninolysis by white rot fungi is initiated only after the primary growth of fungi has been ceased due to the carbon, nitrogen or sulphur limitation (Jeffries et al. 1981). The ligninolytic enzymes seem to be simply formed as a part of the secondary metabolism. The culture conditions such as agitation speed, incubation period, pH, temperature, and types and concentrations of the carbon source as well as a nitrogen source greatly influence the growth and the enzyme production in white rot fungi. Furthermore, oxygen concentration also strongly influences the rate and the extent of the lignin degradation, since experimental evidences indicate that the lignin degradation is oxidative and nonspecific (Kirk & Farell 1987). When the fungi are grown in thin layer cultures under elevated O_2 concentration, lignin can be degraded rapidly. The physiological importance of the lignin biodegradation is the destruction of the lignin matrix so that microorganisms can gain better access to the real substrates such as hemicellulose and cellulose. In white rot fungi, lignins do not seem to be sole carbon source or primary growth substrates, since it has been postulated that fungi may obtain little net energy from lignin (Leatham 1986). However, *Phanerochaete chrysosporium* and *Lentinula edodes* can metabolize various lignin preparations, when an alternate carbon/energy source is present. Laccase is one of important ligninolytic enzymes (Ander & Erikksson 1976), although the well-known lignin decomposer, *P. chrysosporium*, has been reported as lacking it (Kirk & Farell 1987). However, in the studies carried out by Srinivasan et al. (1995), laccase is apparently produced by *P. chrysosporium* in a defined culture medium containing cellulose. In the present work, the optimization of the ligninolytic enzyme production by

Thelephora was analysed, which was isolated by its high ligninolytic enzyme production. The effect of carbon and nitrogen sources on the enzyme production was also investigated. The enzyme activities under the optimised conditions were higher than those reported previously.

MATERIALS AND METHODS

The fungus, *Thelephora* was isolated by its higher production levels of ligninolytic enzymes from the stumps of a burnt tree in the Western Ghats region of Tamilnadu, India. The fungus was identified based on the keys provided by Bakshi (1971), Gilbertson & Rywarden (1986) and Herbarium Cryptogamae Indiae Orientalis (HCIO), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi. Fungal growth was cut out, sterilized with 1% mercuric chloride solution, repeatedly washed with sterile distilled water as described previously (Roy Watling 1971) and inoculated on 2% malt agar medium. The fungal growth on the plate was sub-cultured for 6 days at 37°C and maintained on malt agar slants. Then, the spores were harvested without disturbing the mycelial growth using camel hair brush and filter-sterilized. The spore concentration was adjusted to 10^5 spores/mL and used as an inoculum for further studies. Growth kinetics and the ligninolytic enzyme production were examined in the C-limited medium (M14) of Janshekar & Fiechter (1988), to which spores in the one-tenth volume of the medium were inoculated.

The LiP activity was assayed with a veratryl alcohol as a substrate by the method of Linko (1988). One unit of the enzyme activity was defined as the amount of the enzyme capable of oxidizing 1 mole of veratryl alcohol in a min. The MnP activity was assayed by the method of Kuwahara et al. (1984). One unit of the enzyme activity was expressed as the amount of the enzyme capable of increasing one optical density at 610 nm (OD_{610}) in a min. The laccase activity was measured using guaiacol as a substrate by the method of Evans (1985). One unit of the enzyme activity was expressed as the amount of the enzyme oxidizing 1 mole of the substrate in a min. At regular time intervals, the dry weight of mycelial biomass obtained by filtration was determined. The cell-free culture filtrate was used immediately to measure the enzyme activities using the boiled cell-free filtrate as a blank. The optimum pH for the growth and the enzyme production were determined from 3.0 to 8.0 at room temperature. The effect of temperature was examined from 30 to 80°C. Glucose, cellulose or lignin at different concentrations (2 to 4.0 g/L), and diammonium tartrate, urea or yeast extract at different concentrations (0.22 to 1.1g/L) were examined as carbon sources and nitrogen sources respectively.

RESULTS AND DISCUSSION

Optimum Incubation Period, pH and Temperature for the Enzyme Production

Immobilised mycelium of *P. chrysosporium* produces the highest amount of lignin peroxidase on the 6th and 7th day (Asther et al. 1988). *Pogonomyces hydnoides* produces the highest laccase activity in the four-day culture filtrate (Kreitsberg et al. 1981). In *Phlebia radiata* the maximum laccase production has been observed on the 3rd to 4th day and the activity rapidly disappears before the MnP and LiP activities are increased (Niku-paavola et al. 1990). *Pleurotus florida* produces the highest amount of laccase in malt extract broth after 12 days under stationary conditions (Dhaliwal et al. 1992). In the present study, the optimum growth conditions for isolated fungus was determined. In shaking culture the maximal growth of *Thelephora* sp. occurred on the 6th day (Fig. 1). However, in the fungus, LiP and MnP were in the highest amounts on the 7th day and laccase on the 4th day.

The medium pH has been known as being critical to lignin decomposition. In *P. chrysosporium,* the optimum culture pH for the lignin decomposition has been analysed as 4.0 to 4.5 with the marked reduction above 5.5 and below 3.5 (Kirk 1981). Comparably, the optimum pH of 3.5 or 4.5 has been observed for the ligninase production in *P. chrysosporium* (Janshekar & Fiechter 1988, Asther et al. 1988, Kirk et al. 1986). Therefore, it should be identified what pH is optimal for the ligninolytic enzyme synthesis in *Thelephora* sp. The optimum pH for the growth and the peroxidase (Lip and MnP) production is 4.5. The laccase production in *Thelephora* sp. was favoured at higher pH at 7.0 (Fig. 2).

Temperature can also influence the enzyme production. Several reports have shown that the optimal temperature for the ligninolytic enzyme production in *P. chrysosporium* and *P. radiata* ranges from 37 to 40°C with some exceptions. On the other hand, it has been also reported that the optimum lignin peroxidase production in *P. chrysosporium* is at 30°C or 45°C (Asther et al. 1988, Niku-Paavola et al. 1988). Therefore, the optimal temperature for the mycelial growth and for the enzyme production should be analysed. The optimum temperature for *Thelephora* sp. mycelial growth was 35°C, whereas the ligninases production required slightly higher temperature; for the LiP and MnP production the optimum temperature was 40°C, and for the laccase production 35°C (Fig. 3). All the three enzymes were produced up to the culture temperature of 70°C.

Effect of Carbon and Nitrogen Sources on the Enzyme Production

For the production of ligninolytic enzymes, various carbon sources have been used, such as glucose and cellulose. In some cases, a mixture of carbon sources has been also used, for example, glucose and tannin (Gurusamy 1997). All of the above results led to analyse the effect of a type and a concentration of carbon source on the ligninolytic enzyme production in *Thelephora* sp. Three kinds of carbon sources up to the concentration of 4.0 g/L were analysed (Table 1). In glucose amendment, maximum growth was observed at 3.5 g/L (34.0 mg dry mycelium) concentration. LiP production was maximum at 3.5 g/L (202 U/mL) concentration, and MnP and laccase production at

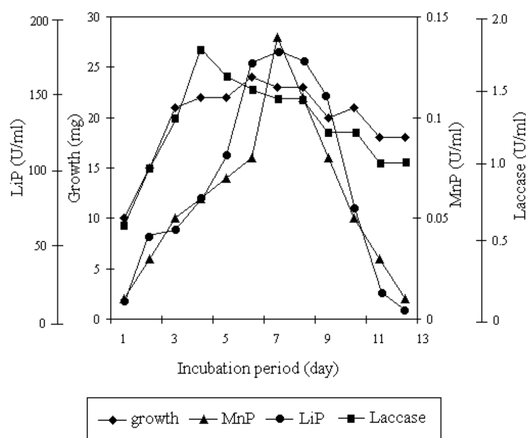

Fig. 1: Kinetics of the mycelial growth and the production of two peroxidases and laccase.
LiP = lignin peroxidase; MnP = manganese-dependent peroxidase.

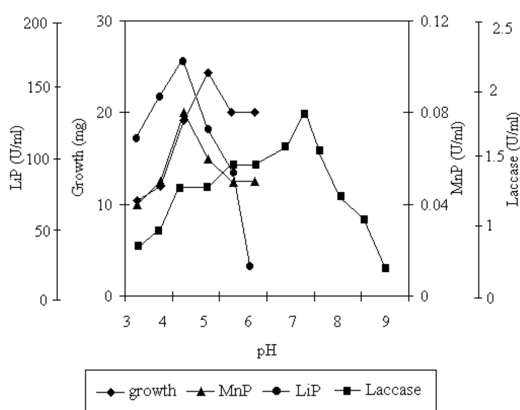

Fig 2: Effects of pH on the mycelial growth and the production of two peroxidases and laccase.
LiP = lignin peroxidase; MnP = manganese-dependent peroxidase. LiP and MnP activity was measured on 7th day. Laccase activity was measured on 4th day.

Table 1: Effect of various carbon sources on growth and ligninase production by *Thelephorce* sp.

Substrate Concentration (g/L)	Growth* (Mycelial dry weight in mg)	Enzyme production(U/mL)			Growth** (Mycelial dry weight in mg)
		LiP*	MnP*	Laccase*	
Control	18.0	12	0.02	0.60	18.0
Glucose					
2.0	27.0	22	0.12	1.80	26.0
2.5	29.0	68	0.19	2.60	27.0
3.0	29.0	112	0.13	2.20	27.0
3.5	34.0	202	0.10	2.20	27.0
4.0	28.0	140	0.10	0.80	25.0
Cellulose					
2.0	24.0	66	0.04	1.80	21.0
2.5	29.0	100	0.04	2.40	27.0
3.0	33.0	172	0.07	2.60	29.0
3.5	38.0	116	0.05	3.20	31.0
4.0	37.0	80	0.03	2.60	30.0
Lignin					
2.0	31.0	108	0.03	2.80	27.0
2.5	39.0	172	0.04	3.00	30.0
3.0	48.0	216	0.06	3.20	38.0
3.5	47.0	148	0.05	2.00	42.0
4.0	43.0	116	0.03	1.40	35.0

Values are mean of three replicates ; LiP = Lignin peroxidase; MnP = Manganese dependent peroxidase
*Growth and enzyme production was estimated on 7th day; **Growth and enzyme production was estimated on 4th day

Table 2: Effect of various nitrogen sources on growth and ligninase production by *Thelephorce* sp.

Substrate Concentration (g/L)	Growth* (Mycelial dry weight in mg)	Enzyme production(U/mL)			Growth** (Mycelial dry weight in mg)
		LiP*	MnP*	Laccase**	
Control	19.0	20	0.01	0.80	14.0
Diammonium tartrate					
0.22	21.0	94	0.06	3.80	17.0
0.44	24.0	216	0.04	5.80	20.0
0.66	30.0	184	0.03	5.40	27.0
0.88	32.0	154	0.02	4.00	24.0
1.10	24.0	116	0.01	3.80	23.0
Urea					
0.22	25.0	80	0.02	1.20	16.0
0.44	29.0	112	0.03	1.60	16.0
0.66	32.0	144	0.04	2.00	19.0
0.88	26.0	212	0.01	1.80	25.0
1.10	25.0	130	0.01	1.20	15.0
Yeast extract					
0.22	36.0	112	0.01	0.80	23.0
0.44	37.0	164	0.02	1.20	27.0
0.66	45.0	204	0.05	1.00	40.0
0.88	60.0	180	0.03	0.80	50.0
1.10	55.0	150	0.01	0.60	46.0

Values are mean of three replicates; LiP = Lignin peroxidase; MnP = Manganese dependent peroxidase
*Growth and enzyme production was estimated on 7th day; **Growth and enzyme production was estimated on 4th day

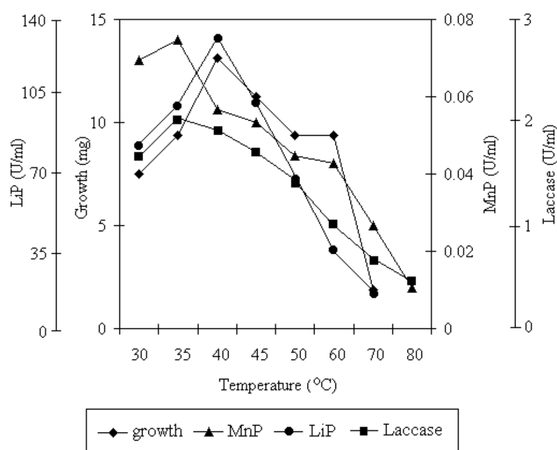

Fig. 3: Effects of culture temperature on the mycelial growth and the production of two peroxidases and laccase.
LiP = lignin peroxidase; MnP = manganese-dependent peroxidase. LiP and MnP activity was measured on 7th day. Laccase activity was measured on 4th day.

2.5 g/L (0.19 U/mL and 2.6 U/mL respectively). Higher concentrations of substrates were found to be inhibitory for growth and enzyme production. In cellulose amendment, the mycelial growth was maximum at 3.5 g/L (38.0 mg dry mycelium), LiP and MnP productions were maximum at 3.0 g/L (172 U/mL and 0.07 U/mL respectively) and laccase production (3.20 U/mL) at 3.5 g/L. In lignin amendment, mycelial growth, and LiP, MnP and laccase productions were maximum at 3.0 g/L concentration (4.8 mg dry mycelium and 216, 0.06 and 3.2 U/mL, respectively).

For the production of ligninolytic enzymes, nitrogen-limited medium stimulates the ligninolytic enzyme production (Kirk 1981, Costa-ferriera et al. 1996). Alternatively, both low and high nitrogen media can enhance the ligninase production (Schmidt et al. 1989). In this report three types and four concentrations of nitrogen sources were examined for the ligninolitic enzyme production (Table 2). In diammonium tartrate amendment, maximum growth (32.0 mg) was observed at 0.88 g/L concentration; LiP and laccase productions were maximum at 0.44 g/L substrate concentration (216 and 5.8 U/mL respectively); MnP production was favoured by 0.22 g/L concentration with a maximum production of 0.06 U/mL. In urea supplementation studies growth was maximum (32.0 mg) at 0.66 g/L concentration; LiP production was maximum (212 U/mL) at 0.88 g/L and MnP (0.04 U/mL) and laccase (2.00 U/mL) at 0.66 g/L concentration. In yeast extract supplementation, growth was maximum (60.0 mg) at 0.88 g/L substrate concentration; LiP and MnP productions were maximum (204 and 0.05 U/mL respectively) at 0.66 g/L concentration; and laccase production was maximum (1.20 U/mL) at 0.44 g/L concentration.

From the present study, it was found that the optimum incubation period for the growth was 6 days, for the LiP and MnP production seven days, and for the laccase production 4 days. The optimal growth and the LiP and MnP productions were found to be in acidic pH 4.5, and the laccase production at nearly neutral pH 7.0. The temperature optima for growth and the enzyme production was in the range of 35-40°C, which is slightly higher than that reported previously. Carbon rich and simultaneously nitrogen-limited medium was favoured for the growth and the enzyme production (Tables 1, 2). It can be concluded from the study that lignin is favoured for the mycelial growth, and the LiP and laccase enzymes production. Diammonium tartrate is better for the Lip, MnP and laccase production.

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