Laccase production by *Polyporus sanguineus* under different nutritional and environmental conditions*

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Summary. Laccase production was higher in malt extract medium than in lignin, and *Polyporus sanguineus* appears to be better than *Polyporus versicolor* and *Trametes hirsuta* (syn. *Polyporus hirsutus)* for enzyme production. Phenolic compounds, of which resorcinol was the most active, induced enzyme production; while sugars repressed it. A temperature of 37 °C, pH 3 and indulin AT at a concentration of 0.2% gave the best enzyme yield.
Key words. Polyporus sanguineus; laccase; phenolic compounds; induction; repression.

Extracellular laccase production is a common feature of white-rot basidomycetes[2,3] though it has also been reported in some ascomycetes[4,5]. Different phenolic compounds and protein synthesis inhibitors have been shown to induce laccase in various fungi[5–9]. The enzyme thus induced is considered to cause detoxification of phenolic compounds. Its involvement in lignin degradation has been indicated[10–12], though its exact role is not very clear. It has been reported to cause demethylation of lignin, a key step for its decomposition[11]. Laccase from *Polyporus sanguineus* has been shown to cause demethylation and oxidation of certain lignin-related aromatic compounds to liberate carbon dioxide[13]. Considering the importance of laccase, the best suited nutritional and environmental conditions for its production have been studied.

Materials and methods. The culture of *Polyporus sanguineus* Klotzsch (FRI 970) was obtained from the Forest Research Institute, Dehradun, India, and was maintained on yeast glucose agar medium at 4 °C with periodic subculturing. The pattern of enzyme production was studied in lignin (indulin AT) and malt extract liquid media for 42 days at 7 day intervals. To find out the best substrate for laccase production different lignin preparations obtained from Westvaco Chemical Division, USA (indulin AT, polyfon, reax), phenolic compounds (gallic, tannic, and salicylic acids; resorcinol, orcinol) and sugars were added to mineral solution at a concentration of 0.1%, except for tannic and salicylic acids (0.05%) whereas in the case of sugars the concentration was 2%.

Flasks containing 25 ml of the medium were each inoculated with 2 mycelial discs (4 mm diameter) and incubated at 25 ± 1 °C as stationary cultures for 10 days for phenolic compounds and 25 days for sugars, since there was no enzyme production for up to 7 days in the latter. Triplicate flasks were analyzed for each substrate. Fungal biomass produced was determined in terms of dry weight, and visually in the case of indulin AT because of its insolubility and the poor growth obtained on it. The filtrates were centrifuged at 8500 × g for 30 min at 4 °C and the supernatant was used to estimate the laccase activity as described previously[9]. For laccase assay 5 ml of reaction mixture containing 3.9 ml acetate buffer (0.01 M, pH 5.0), 1 ml guaiacol (0.00176 M) and 0.1 ml of the enzyme extract was incubated at 25 °C for 2 h and absorbance was read at 450 nm. In the blank, guaiacol was replaced by buffer. The enzyme activity has been expressed in relative terms as colorimetric units (cu/ml) whereas the specific laccase activity denotes the enzyme activity per mg of culture-filtrate protein. The proteins were estimated by Lowry's method[14]. The effect of temperature and pH was recorded by growing the fungus on malt extract medium for 7 days at different incubation temperatures ranging from 15 to 42 °C and pH from 3 to 9. Lignin (indulin AT) was added to malt extract medium at a concentration varying between 0.1 and 0.8% to find out its influence on laccase production.

Different media used were (w/v): a) yeast glucose agar (pH 5.5) containing 5 g yeast extract; 10 g glucose; 20 g agar in 1000 ml

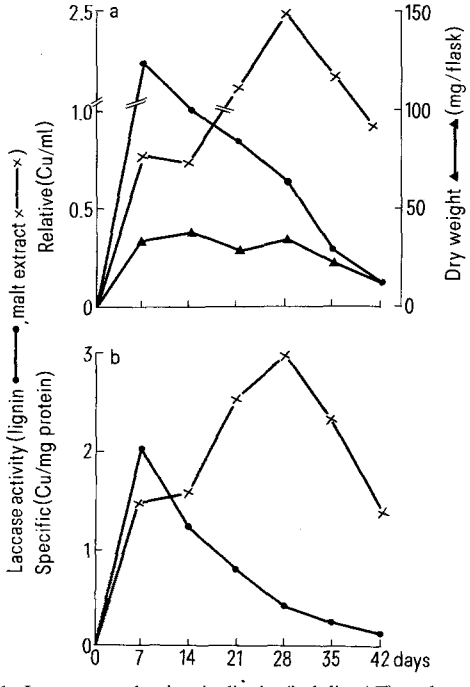

Figure 1. Laccase production in lignin (indulin AT) and malt extract media (a) relative activity (b) specific activity.

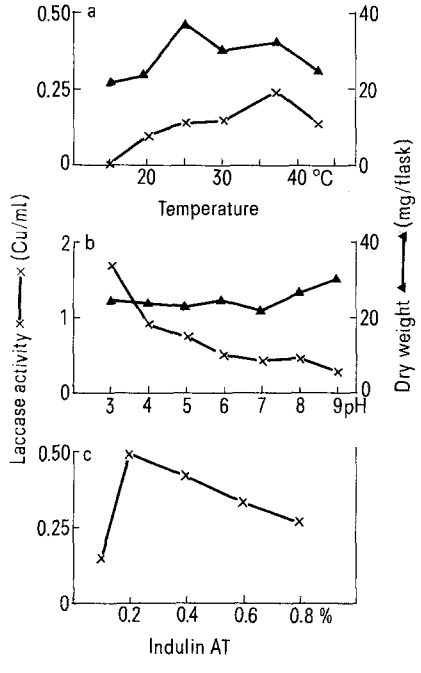

Figure 2. Optimization of laccase production (a) temperature (b) pH (c) indulin AT concentration.

distilled water, b) malt extract medium (pH 5.5) containing 10 g malt extract in 1000 ml mineral solution, c) lignin medium (pH 5.5) containing 10 g indulin AT in 1000 ml mineral solution, d) mineral solution containing 0.5 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7\ H_2O$; 0.1 g $NaNO_3$; 0.1 g KCl; 0.02 g $FeSO_4$; 0.05 g $CaNO_3$ in 1000 ml distilled water. Some media were sterilized by autoclaving at 15 lb pressure for 20 min, whereas the media containing different lignin preparations, phenolic compounds and sugars were sterilized by steaming for 30 min.

Results and discussion. The relative and specific laccase activities of 1.69 cu and 2.0 respectively were obtained in lignin medium on the 7th day, whereas the activity was 2.5 cu and 3.0 respectively in malt extract medium on the 28th day of incubation (fig. 1, a and b), thus indicating the suitability of the latter for a better yield of enzyme. *Polyporus sanguineus* proved to be better than *Polyporus versicolar* and *Trametes hirsuta* in laccase production[8,9]. Comparatively poor growth observed in lignin medium may be because of its poor utilization in the absence of some easily metabolizable carbon source[11,15,16].

All the phenolic compounds except orcinol and salicylic acid were able to induce laccase production (table 1) to variable levels which increased further on supplementing these compounds with malt extract, and enzyme production could be detected in orcinol medium also. The maximum yield obtained on resorcinol was approximately 7 times higher than on malt extract alone. In an earlier study, maximum laccase production in two other *Polyporus* species was observed in the presence of tannic acid and indulin AT for *T. hirsuta*[8] and *P. versicolor*[9] respectively, thus exhibiting species-specificity. Enzyme induction has also been observed in the presence of protein synthesis inhibitors in *Neurospora crassa*[5], and gallic acid in *Botrytis cinerea*[7]. Laccase induced in the presence of phenols has been suggested to cause their detoxification[17], whereas Haars and Huttermann[18] are of the opinion that it is not these phenols which are toxic to fungal growth but the quinones, the oxidized products of the former, catalyzed by laccase. Further, in recent studies the phenols were found not to check growth in *Lactarius* sp. irrespective of the production of phenoloxidase[19]. Comparatively poor laccase yields in the presence of sugars may be attributed to catabolite repression[20,21], as indicated by the present studies also, where no enzyme could be detected on the 7th day, and the exhaustion of sugars by 25 days might have led to the variable levels of enzyme production observed. As observed for phenolic compounds, the addition of malt extract to sugars also increased the enzyme yield and was maximum in lactose (0.85 cu), being 10 times more than in malt extract alone (table 2). The completeness of malt extract, which can provide the necessary amino acids for enzyme synthesis, might be responsible for its effect[22]. No correlation existed between the enzyme and the fungal mass produced, as less growth on phenolic compounds could give more enzyme, while the reverse was the case in sugars (tables 1, 2). Also, during optimization of temperature, pH and indulin AT concentration for laccase production, no correspondence existed between the optimum values for enzyme production and growth (fig. 2). Indulin AT at a concentration of 0.2%, a temperature of 37 °C and pH 3 were best for enzyme production, and for biomass the optimum was 25 °C and pH 9 (fig. 2, a and b) though there was not much difference in growth at different pH values. The low pH value has been considered favorable for the release of extracellular enzyme[23].

Table 1. The effect of different lignins and phenolic compounds on laccase production by *Polyporus sanguineus*

Substrate	Laccase activity (cu/ml)	Fold increase/decrease in laccase activity as compared to malt extract	Growth* (mg/flask)
Malt extract (M)	0.14	–	11
Resorcinol	0.08	0.60	4
M + resorcinol	0.97	6.96	15
Tannic acid	0.23	1.67	4
M + tannic acid	0.54	3.85	14.5
Indulin AT	0.11	0.82	+
M + indulin AT	0.36	2.57	+ +
Gallic acid	0.04	0.28	5
M + gallic acid	0.30	2.14	16.5
Polyfon	0.10	0.71	5
M + polyfon	0.23	1.67	11.25
Reax	0.04	0.28	4
M + reax	0.21	1.50	14.25
Orcinol	0.00	0	3
M + orcinol	0.20	1.42	4

* +, + + relative growth.

Table 2. The effect of different sugars on laccase production by *Polyporus sanguineus*. Period of incubation: 25 days

Substrate	Laccase activity (cu/ml)	Fold increase/decrease in laccase activity as compared to malt extract	Growth* (mg/flask)
Malt extract (M)	0.085	–	12
Lactose	0.04	0.52	23
M + lactose	0.85	10.00	46.5
Glucose	0.04	0.52	26.5
M + glucose	0.44	5.17	39.0
Maltose	0.06	0.76	29.0
M + maltose	0.32	3.76	51.0
Sucrose	0.05	0.64	27.0
M + sucrose	0.27	3.23	46.0
Sorbitol	0.05	0.58	29.0
M + sorbitol	0.25	2.94	36.5

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