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Screening and Isolation of Laccase Producing Fungi from Saw Mill Wastes

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Fungal enzymes play a vital role in the biodegradation of various organic and inorganic compounds. Lignin is one such compound which causes considerable problems in the environment. The lignin degradation process is performed with the help of enzymes, mainly of microbial origin. The enzymes mainly involved in this process are oxidases and peroxidases especially laccases. The screening and isolation of laccase producing fungi from various saw mill wastes were undertaken in this study.

Key words: Lignin, Biodegradation, Enzymes, Laccase, ABTS, Syringaldazine.

Forests represent approximately 27% of the world's land area and wood is the predominant commercial product from forests. Global wood consumption is around 3500 million mt/year and has increased over 65% since 1960 (Angel T. Martinez *et al.* 2005). Wood and other lignocellulosics are composed of cellulose (insoluble fibers of glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans and glucans) and lignin (a complex polyphenolic structure). Wood in angiosperm trees generally contains 42–50% cellulose, 25–30% hemicelluloses, 20–25% lignin and 5–8% extractives (Raj Kumar *et al.* 2008). Lignin is the second most abundant natural polymer on the earth, which constitutes up to 15-30% cell walls of gymnosperm (softwood) and angiosperm (hardwood) (Gold *et al.* 1993).

Ascomycetes, Basidiomycetes and Deuteromycetes are the impotant fungal groups involved in the biodegradation of wood . The extensive degraders of wood are the White rot and brown rot basidiomyceteous fungi (Tuor *et al*. 1995). While the Ascomycetes and Deuteromycetes colonizes the wood and causes its mechanical destabilization, called soft rot. Soft-rot fungi can degrade wood under extreme environmental conditions (high or low water potential) that prohibit the activity of other fungi. Moreover, some basidiomycetes also cause a soft rot- type decay pattern. Finally, a limited number of ascomycetous fungi, called stain fungi, can colonize wood through parenchymatic rays and resin channels causing discoloration of softwood tissues but a very limited degradation, which mainly affects extractives and water-soluble materials (Ángel T. Martínez1 *et al*.2005).

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The lignin degradation process is performed with the help of enzymes. The enzymes mainly involved in this process are oxidases and peroxidases: mainly lignin peroxidase (ligninases), Mn-dependent peroxidases, Mn-independent peroxidases and laccases (Leonowicz *et al*. 1999).

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) has a rather wide distribution, being found in higher plants, many fungi, certain bacteria and insects. Since fungi are ubiquitous in nature and can occupy any habitat that offers adequate moisture, temperature and organic substrates (Tuor *et al*. 1995), they are the very common sources of laccase enzyme.

The laccase producing fungi can be isolated from various habitats like decaying woods, composting yards, forest soil, paper mill effluents etc.,. Many wood decaying fungi produces laccase enzyme and this also suggests their role in lignin biodegradation (Youn *et al*.,1995). This study involves the isolation of laccase producing fungi from the saw mill wastes.

MATERIALS AND METHODS

Sample collection

The samples were collected from the saw mill wastes which included the wood dust, wood pieces of various sizes, bark and other plant portions which can not be used in the saw industry. The samples were collected aseptically in polythene bags and were transported to the laboratory for further study.

Culture media

Malt Extract Agar (MEA) was used for the initial fungal isolation and the pure cultures were maintained on Potato Dextrose Agar (PDA) slants.

Indicator compounds

The indicator compound which were used in the laccase producing fungal identification are: ABTS (2,2%-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) (0.05% w/v), Syringaldazine, guaiacol (0.01% w/v), gallic acid (0.5% w/v) and Tannic acid (0.5% w/v). All these indicator solution were sterilized by filtration.

Sample processing

The samples were homogenized and sieved (2-mm mesh) and 5 g of samples were added into 250-ml flasks containing 20 ml sterilized physiological sodium chloride solution and glass beads and kept on a rotary shaker for 30 min for full distribution. The prepared suspensions were used for inoculating onto plates (Zhiyu Liu *et al*., 2009).

Isolation of fungi

The samples were placed on the MEA plates having the indicator compounds. The plates were incubated at 30°C for a week. The colonies were identified by the positive reactions (Table 1).

Laccase Production

The yeast extract peptone dextrose (YPD) medium, containing glucose 20 g/l, peptone 5 g/l, and yeast extract 2 g/l, supplemented with 100mg copper sulphate was used as the basal medium (YPD–Cu) for laccase production. The pH of above media was adjusted to 6.5 before autoclaving. Flask experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml YPD–Cu medium cultivated at 30°C and 150 rpm on a rotary shaker.

Laccase assay

Culture medium was centrifuged at 8,000 rpm for 10 min. and the supernatant was used for enzyme assay. Laccase activity was assayed spectrophotometrically by measuring the oxidation of ABTS at 420 nm at 30°C. The assay mixture in a total volume of 1 ml contained 0.1 ml cell-free supernatants at various dilutions and 1 mM ABTS in 100 mM citrate buffer (pH 3.4). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute (Bourbonnais and Paice 1990).

RESULTS AND DISCUSSION

The results showed that there are 16 different colonies. Of these 7 colonies showed positive result for all the indicators (SMWF-2, SMWF-5, SMWF-6, SMWF-10, SMWF-11, SMWF-14 & SMWF-16) while the 4 isolates (SMWF-3, SMWF-9, SMWF-12 & SMWF-15) gave negative results for all the indicators. The isolates SMWF-7 SMWF-8 & SMWF-13 didn't give positive result for ABTS but gave positive results for rest of the indicators. Similarly the

isolate SMWF-1 gave positive result for guaiacol alone. The results are shown in the table (Table 2)

Those colonies which gave positive result to all the indicators were placed on YPD-CU media and were tested for laccase production. The flasks were observed for up to 8 weeks. The laccase production was detected with the help of ABTS. The results are given in table 3.

Table 1. Laccase indicators and their reaction

S.No.	Indicator	Positive Reaction
1	ABTS	Green coloured zones around colony
2	Syringaldazine	Dark brown coloured zone around colony
3	Guiacol	Reddish Brown halo around colony
4	Tannic acid	Dark brown coloured zone around colony
5	Gallic acid	Dark brown coloured zone around colony

Table 2. Response of fungal isolates to the indicators

Strain	ABTS	SyringaLdazine	Guaiacol	Tannic acid	Gallic acid
SMWF-1	-	-	+	-	-
SMWF-2	+	+	+	+	+
SMWF-3	-	-	-	-	-
SMWF-4	-	-	+	+	+
SMWF-5	+	+	+	+	+
SMWF-6	+	+	+	+	+
SMWF-7	-	+	+	+	+
SMWF-8	-	+	+	+	+
SMWF-9	-	-	-	-	-
SMWF-10	+	+	+	+	+
SMWF-11	+	+	+	+	+
SMWF-12	-	-	-	-	-
SMWF-13	-	+	+	+	+
SMWF-14	+	+	+	+	+
SMWF-15	-	-	-	-	-
SMWF-16	+	+	+	+	+

- Negative result, + positive result

Table 3. Laccase production

Fungal isolate	Laccase production in			
	1 week	2 weeks	4 weeks	8 weeks
SMWF-2		+		
SMWF-5		+		
SMWF-6			+	
SMWF-10	+			
SMWF-11				+
SMWF-14	+			
SMWF-16		+		

+ laccase production

Table 4. Amount of enzyme produced by fungal isolates

S. No.	Fungal Isolate	enzyme units(U/ml)
1.	SMWF-2	0.028
2.	SMWF-5	0.042
3.	SMWF-6	0.0031
4.	SMWF-10	0.187
5.	SMWF-11	0.0002
6.	SMWF-14	0.11
7.	SMWF-16	0.023

The isolates SMWF-10 & SMWF-14 produced laccase in the first week of inoculation and the isolates SMWF-12 SMWF-15 & SMWF-16 produced laccase in the second week of inoculation, while the isolates SMWF-16 & SMWF-10 produced in the 4 and 8 weeks respectively.

The amount of laccase produced by the 6 isolates were also studied and the results are given in Table 4.

CONCLUSION

All though 16 isolates were there in the saw mill wastes only less than half (43.75%) of them were truly laccase positive. In the 7 isolates which showed the positive result the time interval for laccase production was varying from 1-8 weeks some gave in the very first week itself (SMWF-10 & SMWF-14) while majority of them gave in the 2nd (SMWF-2, SMWF-5 & SMWF-16)and 4th (SMWF-6) weeks. But some (SMWF-11) took exceptionally long times (8 weeks). On the basis of amount of enzyme produced and time period taken for laccase production, it is concluded that the isolate SMWF-10 could be used for further studies.

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