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Survey of some Indian soils for laccase producing fungi and their lignin degrading ability

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Abstract. One hundred and fifty soil samples collected from three climatologically different sites were screened for laccase production using lignin-guaiacol agar plates. All the 12 fungi isolated excepting one gave positive test for qualitative lignin degradation. Five fungi were selected further for quantitative study of wood/lignin degradation. The basidiomycetes proved to be better laccase producers and decomposers of wood/lignin. These gave comparable results to a known white-rot fungus, *Polyporus versicolor* (L.) Fr. and degraded wood sawdust causing a total weight loss of 7–8% and lignin loss up to 10% in 30 days.

Keywords. Laccase; lignin; wood; soils.

1. Introduction

Laccase was first detected in the latex of *Rhus vernicifera* Brandis and later in the fruiting bodies of many basidiomycetes (Fahraeus *et al* 1958). The development of Bavendamm test (Bavendamm 1928) was of considerable significance for differentiating white- and brown-rot fungi. The former are characterized by the positive test for phenoloxidase (laccase) whereas the latter lack such activity. Since then, various methods have been evolved to detect laccase production and attempts made to establish correlation with lignin degradation (Davidson *et al* 1938; Fahraeus 1949; Nobles 1958; Kirk and Kelman 1965; Harkin and Obst 1973). Sundman and Nase (1971) found a good conformity between Bavendamm's test and lignin degradation during their studies on 52 wood-rotting and soil fungi. However, the test was not applicable to soil inhabiting ascomycetes and fungi imperfecti. The lignin component has to be removed completely or partially to increase the efficiency of lignocellulosic residues to be used as feed for ruminants, for useful conversion to sugars, alcohols or other organic solvents, or as a raw material for paper industries (Kirk and Harkin 1973; Zadrazil 1977, 1980; Higuchi 1982). The biochemistry of ligninolysis is not completely understood, however, laccase has been considered to be one of the important enzymes. It plays a key role in ligninolysis by causing its demethylation (Kirk and Chang 1975). Its involvement has been fairly substantiated by genetic studies in which laccase-less mutants were unable to degrade lignin (Ander and Eriksson 1976). Microbiology of lignin degradation has revealed fungi (particularly the white-rot basidiomycetes) to be an important group of organisms though certain brown-rot and soft-rot fungi and bacteria can cause ligninolysis to a limited extent (Ander and Eriksson 1978). Till now a number of fungi have been tested for laccase production and lignin degradation. However, no study has been undertaken to directly isolate such fungi from decomposing matter which might yield better lignin degrading species. Keeping this in view a survey of soil samples collected from three different regions was conducted to isolate laccase producing fungi.

To validate their correlation with lignin degradation the fungi were further tested for the latter process both qualitatively and quantitatively.

2. Materials and methods

2.1 *Collection and processing of soil samples*

One hundred and fifty soil samples were collected from three climatologically different sites *viz* subtropical plains of Amritsar, the south-eastern tropical islands of Andaman and hills of Mussoorie (50 samples from each site). The soils of Amritsar are solonized arid brown, saline alkali, light textured sandy loam; of Andaman saline marshy and greyish brown and of Mussoorie forest and hilly. The soil (25 g) was taken in polyethylene bags from sites where some woody matter or leaf litter decomposition occurred. The pH and moisture content of different soil samples varied from 6·5–9·5 and 10–40% respectively. The samples were stored at 4°C till further processing.

Each soil sample (1 g) was suspended in 20 ml of sterilized distilled water in a 100 ml conical flask. The flasks were agitated on a rotary shaker for 30 min and allowed to settle. The supernatant was plated by pour plate method using lignin-guaiacol medium. Four replicates/samples were plated and incubated at 25 °C for a maximum of 10 days.

2.2 *Screening of laccase producing fungi*

Phenolic compounds get oxidized to coloured quinonic products by phenoloxidase (laccase) produced particularly by the white-rot fungi. In the present study production of reddish brown colour under or around the colony was recorded as a positive test for laccase. Each fungus was isolated and subcultured on yeast glucose medium. These were further tested for colour production on lignin and guaiacol media separately and for Bavendamm reaction (Bavendamm 1928) using tannic acid as the substrate. To find any correlation between laccase production and lignin degradation the fungi isolated were tested qualitatively for the latter phenomenon according to Sundman and Nase (1971).

2.3 *Laccase production in lignin-malt extract broth*

Hundred ml conical flasks, each containing 25 ml of lignin-malt extract broth were sterilized and after inoculation were incubated at 25 ± 1°C as stationary cultures for 10 days. Triplicate flasks were processed for each fungus. The contents of each flask were filtered through Whatman filter paper No. 1 and the filtrate was centrifuged at 8500 g for 30 min at 4°C. The supernatant thus obtained was used for estimating laccase using guaiacol as the substrate (Sandhu and Arora 1984). Five ml of the reaction mixture containing 3·9 ml acetate buffer (10 mM, pH 5), 1 ml guaiacol (1·76 mM) and 0·1 ml enzyme extract were incubated at 25°C for 2 hr and the absorbance was read at 450 nm. In the blank, guaiacol was substituted by buffer. The formation of coloured products was taken as indicative of laccase activity which was expressed in relative terms as colorimetric units per ml of the enzyme.

2.4 *Degradation of wood sawdust*

Five fungal isolates including *Allescheriella crocea* (Montagne) Hughes, *Moniliella* sp., two basidiomycetes and one unidentified fungus (24 And) were selected to study their

wood/lignin degrading ability since these gave comparatively better laccase yield in broth. Two gram (dry weight) of angiospermic wood sawdust moistened with 15 ml of 0·5 % malt extract taken in 100 ml flasks were sterilized and inoculated. Uninoculated substrate served as the control. After 30 days of incubation at 25 ± 1°C triplicate set of flasks for each fungus were processed. Ten ml of acetate buffer (10 mM, pH 5) was added to each flask and after shaking on rotary shaker for 20 min the contents were filtered through Whatman filter paper No. 1. These were dried to a constant weight and weight loss caused by the organism was worked out with reference to uninoculated substrate. The residual matter was homogenized thoroughly and used for lignin estimation (Effland 1977) and the filtrate was used for laccase estimation.

2.5 *Media*

Different media used for the above studies include lignin-guaiacol medium: 1 g lignin (indulin AT) dissolved in 10 ml dioxan suspended in 1 litre of mineral salt solution was supplemented with 0·2 % malt extract and 0·4 ml guaiacol. The medium was solidified with 15 g agar. Lignin and guaiacol media were prepared separately in a similar way without adding guaiacol and lignin respectively. However, the concentration of lignin was doubled and no dioxan was used in case of lignin medium employed for Sundman's test. Lignin-malt extract broth medium contained 0·4 % lignin and 0·5 % malt extract in mineral salt solution. The composition of the latter was KH_2PO_4, 0·5 g; $MgSO_4 \cdot 7H_2O$, 0·2 g; $NaNO_3$, 0·1 g; KCl, 0·1 g; $FeSO_4$, 0·02 g; $CaNO_3$, 0·05 g; distilled water, 1 litre.

All the media and wood sawdust were sterilized by autoclaving at 15 lb pressure for 20 min. The pH value of different media before autoclaving was in the range of 5–5·5. The inoculum used for different experiments consisted of 4 mycelial discs (4 mm diam)/flask obtained from the fungal cultures grown on yeast glucose agar plates for 8–10 days. For control studies known brown and white-rot fungi were used for different tests.

2.6 *Identification of the fungi isolated*

Various fungi isolated were identified from their colonial morphology and microscopic study. *Allescheriella crocea*, *Gilmaniella humicola* Barron and *Moniliella* sp. were identified with the help of literature available (Barron 1972; Ellis 1971, 1976). Basidiomycetes were identified on the basis of clamp connections. The identification was confirmed from Commonwealth Mycological Institute (CMI), England. However, a few cultures could not be identified in our laboratory or at CMI.

3. Results

Out of 150 soil samples screened only 12 gave positive results to yield the same number of laccase producing fungi (table 1). The fungi isolated include two genera of basidiomycetes and three of fungi imperfecti *viz*, *Allescheriella crocea*, *Gilmaniella humicola* and *Moniliella* sp. while the remaining could not be identified. In general, all the cultures were able to cause colouration in lignin and guaiacol media separately. However, the colour intensity was maximum in lignin-guaiacol followed by guaiacol and lignin medium. *Allescheriella crocea* and an unidentified strain failed to cause

Table 1. Laccase producing fungi isolated from different soils.

Place of collection	Culture number	Fungi
Amritsar	13 Asr	Basidiomycete
	20 Asr	NI*
	42 Asr	*Gilmaniella humicola*
Andeman	1 And	NI
	3 And	*Moniliella* sp.
	6 And	NI
	8 And	NI
	23 And	NI
	24 And	NI
	37 And	*Allescheriella crocea*
Mussooree	61 Mus	NI
	78 Mus	Basidiomycete
Total number of samples tested		150
Number positive		12

* NI = Not identified

colouration in guaiacol whereas 24 And had no effect in lignin medium (table 2). All the white-rot fungi included as control gave positive results while the brown-rot fungi gave negative test on the three media.

3.1 *Bavendamm and Sundman's test*

All the twelve fungi isolated from soil and the white-rot fungi were positive for both the tests except for a single isolate (24 And) which gave negative Sundman test for lignin degradation but showed positive Bavendamm reaction. Both the brown-rot fungi gave negative results (table 2).

3.2 *Laccase production in lignin-malt extract broth*

All the fungi were able to produce the enzyme in broth culture to variable levels except 20 Asr, 61 Mus and a brown-rot fungus, *Poria monticola* Murrill (table 3). The two basidiomycetes gave good enzyme production, comparable to *Polyporus versicolor* (1·1 CU).

3.3 *Degradation of sawdust*

Of the five fungi selected to study wood/lignin degradation only the basidiomycetes 13 Asr and 78 Mus caused sufficient weight loss of 8·2% and 7·5% with a respective lignin loss of 10·4% and 9·4% over a period of 30 days. Though the loss was less as compared to that caused by *P. versicolor* it was higher than the rest of the fungi (table 4) which could cause a lignin loss of only 2–5%. Both the basidiomycetes ranked close to *P. versicolor* in laccase production.

Table 2. Laccase production on lignin-guaiacol, lignin and guaiacol media by fungi isolated from soil and their performance for Bavendamm and Sundmans test (lignin degradation).

Culture No.	Type of fungus	Colour production on			Bavendamm test	Sundman test
		Lignin guaiacol	Lignin	Guaiacol		
Fungi isolated from soil						
37 And	*Allescheriella crocea* (Montagne) Hugh	+	+	−	+	+
42 Asr	*Gilmaniella humicola* Barron	+	+	+	+	+
3 And	*Moniliella* sp.	+	+	+	+	+
13 Asr	Basidiomycete	+	+	+	+	+
78 Mus	Basidiomycete	+	+	+	+	+
20 Asr	NI*	+	+	+	+	+
1 And	NI	+	+	−	+	+
6 And	NI	+	+	+	+	+
8 And	NI	+	+	+	+	+
23 And	NI	+	+	+	+	+
24 And	NI	+	−	+	+	−
61 Mus	NI	+	+	+	+	+
White-rot fungi						
7 FRI	*Daedalea flavida* Lev.	+	+	+	+	+
824 FRI	*Fomes annosus* (Fr.) Cooke	+	+	+	+	+
PAU	*Pleurotus ostreatus* (Jacq.) Fr.	+	+	+	+	+
983 FRI	*P. sajor-caju* (Fr.) Singer	+	+	+	+	+
534 FRI	*Polyporus hirsutus* Fr.	+	+	+	+	+
970 FRI	*P. sanguineus* Klotzsch	+	+	+	+	+
165 FRI	*P. versicolor* (L.) Fr.	+	+	+	+	+
Brown-rot fungi						
528 FRI	*Polyporus palustris* Berk. Curt.	−	−	−	−	−
180c FRI	*Poria monticola* Murrill	−	−	−	−	−

* NI = Not identified

4. Discussion

The use of lignin-guaiacol as the test substrate for detecting laccase producing fungi gave an advantage over the earlier studies in which guaiacol alone (Boidin 1951; Kirk and Kelman 1965) or spraying the lignin agar plates with guaiacol after fungal growth (Westermark and Eriksson 1974) has been used to detect the enzyme production. The study helped to detect colonies which might have gone unnoticed in guaiacol (*Allescheriella crocea*, 1 And) or lignin medium alone (24 And). However, *Allescheriella crocea* and 1 And were able to produce colouration in lignin-guaiacol medium which may indicate their requirement for lignin polymer to induce laccase production which then reacts more sharply with guaiacol.

The study revealed a good consistency with Bavendamm test and may provide a good substitute for screening laccase producing fungi. With some exceptions, the fungi giving positive Bavendamm reaction are considered as white-rot fungi capable of lignin degradation (Bavendamm 1928; Davidson *et al* 1938; Nobles 1951; Kirk and Kelman

Table 3. Laccase production by fungi isolated from soil in lignin malt extract broth medium.

Fungi	Laccase activity* (CU/ml)
Basidiomycete (78 Mus)	1·25
Polyporus versicolor	1·10
Basidiomycete (13 Asr)	1·04
Allescheriella crocea	0·225
Moniliella sp.	0·217
NI (6 And)	0·157
NI (1 And)	0·125
NI (23 And)	0·107
NI (8 And)	0·07
NI (24 And)	0·05
Gilmaniella humicola	0·05
NI (61 Mus)	0
NI (20 Asr)	0
Poria monticola	0

* The enzyme activity has been expressed in relative terms as colorimetric units/ml of the enzyme extract.

Table 4. Decay of angiospermic wood sawdust by different fungi after 30 days of incubation.

Fungi	Per cent loss		Laccase activity (CU/ml)
	Total weight	Lignin	
Polyporus versicolor	10·69	13·75	0·06
Basidiomycete (13 Asr)	8·16	10·40	0·06
Basidiomycete (78 Mus)	7·50	9·40	0·05
Allescheriella crocea	*+0·30	5·36	0
NI (23 And)	+0·25	3·69	0
Moniliella sp.	+0·25	2·68	0

* + represents the increase in total weight

1965; Sundman and Nase 1971). Our studies corroborate the above observations as all the fungi isolated gave positive test for qualitative lignin degradation except isolate No. 24 And. Thus it resembles *Aureobasidium pullulans* (de Bary) Arrand in giving good Bavendamm reaction but negative test for lignin degradation (Sundman and Nase 1971).

The quantitative laccase production and lignin degradation studies corroborate the earlier observations where basidiomycetes have been shown to be better enzyme producers as well as lignin decomposers as compared to fungi imperfecti (Eslyn *et al* 1975; Norris 1980; Haider and Trojanowski 1981). The inability of two isolates (20 Asr, 61 Mus) to give quantitative enzyme yield may be due to cell bound nature of the

enzyme which is not excreted into the medium but on coming in contact with the substrate in solid medium causes colouration (Lindeberg 1948; Rosenberg 1980). The slight increase in overall weight of sawdust in some fungi may be due to the fact that the fungal biomass was enough to balance the low weight loss or utilization of various wood components. No enzyme could be detected in the extract of wood decayed by *Allescheriella crocea*, *Moniliella* sp. and 23 And which may be attributed to the low level of the enzyme which gets further diluted during the processing and thus goes undetected.

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