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RESEARCH ARTICLE

ANALYSIS ON VARIOUS ENZYMES INVOLOVED IN BIODEGRADATION OF LIGNOCELLULOSE BY FUNGAL ISOLATES FROM WOOD AND SOIL

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ABSTRACT

Three fungal strains that produced ligninolytic enzymes were isolated from palm oil plantation soils. Isolate MIRT 1 was identified as *Aspergillus niger* isolate MIRT 2 was identified as *Aspergillus flavus*, and isolate MIRT 3 was identified as *Trichoderma viridae*. These strains utilized lignin as the sole source of carbon and produced the main ligninolytic enzymes; lignin peroxidase, manganese peroxidase, and laccase. Based on the results obtained in this study, the isolate *Aspergillus niger* MIRT 1 was chosen as the best of the ligninolytic fungal strains, as it achieved maximum manganese peroxidase production of 2221.4 U/L on the third day under the optimum conditions of pH 8 and 30 ºC. Strain *Aspergillus niger* MIRT1 also produced the highest lignin peroxidase, at 204.45 U/L, on the fifth day of fermentation under the optimum conditions of pH 8 and 30 ºC. Strain *Aspergillus flavus* MIRT2 showed the highest laccase enzyme production of the three selected fungal strains in the present study. Although the production of manganese peroxidase and lignin peroxidase by strain *Aspergillus flavus* MIRT2 was lower than *Aspergillus niger* MIRT1, *Aspergillus flavus* MIRT2, *Trichoderma viridae* MIRT3 showed the highest laccase enzyme at 11.12 U/L on the fifth day under the optimum conditions of pH 7.5 and 35 ºC. The *Trichoderma viridae* strain has been considered a novel ligninolytic fungal isolate from palm oil plantation soil. *Phanerochaete chrysosporium* Standard culture showed bet results compared to the isolates though the isolates compete with that. For any environment if there is a mixture of these fungi then there will be great degradation of lignocellulose, which could be used commercially and/or industrially.

INTRODUCTION

Lignocelluloses biodegradation

Lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradative capacity of the microbial population **(Waldrop *et al*., 2000)**. The composition of the microbial community charged with lignocellulose biodegradation determines the rate and extent thereof. The efficient hydrolysis of cellulose requires the concerted action of at least three enzymes: (1) endo-glucanases to randomly cleave intermonomer bonds; (2) exoglucanases to remove mono- and dimers from the end of the glucose chain; and (3) β -glucosidase to hydrolyze glucose dimmers **(Deobald & Crawford, 1997;Tomme *et al*., 1995)**. The concerted actions of these enzymes are required for complete hydrolysis and utilization of cellulose. The rate-limiting step is the ability of endo-glucanases to reach amorphous regions within the crystalline matrix and create new chain ends, which exo-cellobiohydrolases can attack.

Although similar types of enzymes are required for hemicellulose hydrolysis, more enzymes are required for its complete degradation because of its greater complexity compared to cellulose. Of these, xylanase is the best studied (**Kuhad *et al*., 1997)**.Although the model described in Figure 1 was developed from data obtained from *Trichoderma koningii* and *Phanerochaete chrysosporium*, it does well to describe the general aspects of enzymatic hydrolysis of cellulose (**Tomme *et al*,. 1995)**. However, a fundamental difference exists in the mechanism of cellulose hydrolysis between aerobic and anaerobic fungi and bacteria (**Leschine, 1995; Tomme *et al*., 1995)**.

Lignin degradation by white-rot fungi is an oxidative process and phenol oxidases are the key enzymes **(Kuhad *et al*,. 1997; Leonowicz *et al*., 1999)**. Bacterial and fungal feruloyl and *p*-coumaroyl esterases are relatively novel enzymes capable of releasing feruloyl and *p*-coumaroyl and play an important role in biodegradation of recalcitrant cell walls in grasses **(Kuhad *et al*., 1997)**. These enzymes act synergistically with xylanases to disrupt the hemicellulose-lignin association, without mineralization of the lignin **(Borneman *et al*., 1990)**.

MATERIALS AND METHODS

Site And Sample Collection

Soil sample were collected from three different sampling sites in around Arcot, from the area where an old dead wood identifies. The samples were collected from decayed wood

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surroundings. The soil samples were collected from the surface to a depth of about 0 to 10 cm using sterile spatulas and transported to the laboratory and stored at -20˚C.

Table 1 Soil Characteristics from Selected Sites

S.No.	Soil site	Cellulose (%)	Hemicellulose (%)	Lignin (%)	pH	Total
1	Decayed wood biomass site	29.11	0.17	0.25	6.99	10.88
2	Tree base sample site	61.11	10.38	0.13	5.77	4.64
3	Pruned frond sample site	47.42	0.24	0.21	6.24	5.23

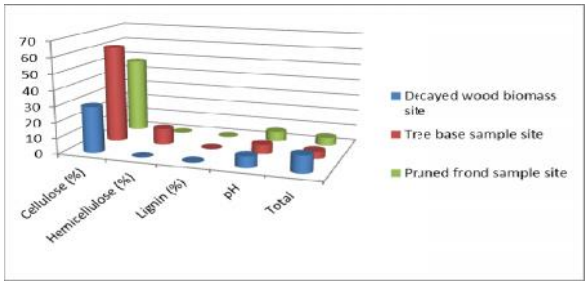

Fig 1 Soil Characteristics from Selected Sites

Soil Characteristics

Soil characteristics were determined using standard methods (APHA, 1985).For each sampling site, three soil samples were combined and mixed together to construct a composite sample. Acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) methods were used to determine the cellulose, hemicellulose, and lignin compositions respectively, of the samples **(Goering and Van Soest, 1970)**.

Table 2 Fungal Isolates Grown on MSM-KL Plate without Glucose and Peptone for measurement of their Tolerances at Different Concentrations of Kraft Lignin

Isolates	Kraft lignin Concentration g/L						
	0.5	0.6	0.7	0.8	0.9	1.0	1.1
MIRT1	+++	+++	+++	++	+	+	-
MIRT2	+++	+++	+++	++	++	+	-
MIRT3	+++	+++	+++	++	+	+	-
MIRT4	++	+	+	-	-	-	-
MIRT5	++	+	-	-	-	-	-
MIRT6	+	-	-	-	-	-	-
***P.chrysosporium* Standard culture**	+++	+++	+++	+++	+++	+	-

+++ (Excellent growth), ++ (Normal growth), + (Poor growth), - (Growth not occurs)

3. Then, 100ml of this solution was plated on Minimal Salt Media containing Kraft lignin (MSM-KL) as a carbone source.
4. MSM-KL media consist of (g/L of deionized water), KL - 0.5, K_2HPO_4 - 4.55, KH_2PO_4 0.53 $CaCI_2$ - 0.5, $MgSO_4$ - 0.5, NH_4NO_3 - 5.0
5. The plates were incubated at 30 °C for 7 days until colonies developed. The isolated fungi were plated onto fresh MSM-KL agar plates repeatedly to obtain pure cultures.
6. The 44 isolates were obtained and grown in Potato dextrose agar(PDA) and potato dextrose broth.
7. Then, 2 mL aliquots of these cultures were frozen as stock cultures in 80% glycerol stock and maintained at -80 ºC.
8. The fungal isolates were further screened using methylene blue dye as an indicator for the oxidation process by ligninolytic enzymes (Bandounas *et al.*, 2011).
9. The isolated fungi were streaked on MSM agar plates containing 0.5 g/L of kraft lignin as lignin substrate and 0.25 g/L Methylene blue dye.
10. The plates were incubated at 30 °C for 7 days. The agar plates were observed daily for fungal growth and decolorization of the methylene blue.
11. Six potential isolates were selected from the large clear (decolorized) zones of methylene blue dyes.
12. Then, these isolates were grown on MSM-KL agar media with different concentrations of kraft lignin (0.5 g/L, 0.6 g/L, 0.7 g/L, 0.8 g/L, 0.9 g/L, 1.0 g/L, and 1.1 g/L) at 30 ºC for 7 days.
13. Fungal strains showing rapid growth on MSM-KL agar with 900 mg/L of kraft lignin and were selected for further study.
14. The isolates were purified by at least five successive passages onto fresh MSM-KL.
15. The purity of each strain was ensured by microscopic observation of colony shape, texture and pigmentation.

Table 3 Highest Lignin Peroxidase, Manganese Peroxidase, and Laccase Enzyme Activities Produced by Selected fungal Strains

Isolates	Lignin Peroxidase		Manganese Peroxidase		Laccase	
	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Aspergillus niger MIRT1	163.90	6	2018.80	4	2.20	5
Aspergillus flavus MIRT2	111.04	5	960.40	5	5.76	6
Trichoderma viridae MIRT3	156.40	6	1512.30	4	1.80	4
P.chrysosporium Standard culture	214.1	5	2305.50	4	2.68	4

***Phanerochaete Chrysosporium* Standard Culture**

Spore inoculum was prepared from 7-8 day old petri plates of PDA-grown ATCC **24725** obtained from MTCC, Chandigarh(grown at 39°C) harvested with 0.85% sterile saline with the aid of a glass hockey stick. Spore counts were determined by hemocytometer.

Fungal Isolation And Screening

1. For isolation of the soil fungi, 1g of soil sample was added to 100 ml of sterile 0.9% NaCl. The solution was stirred vigorously and then allowed to settle.
2. One milliliter of the liquid was mixture was serially diluted until a dilution of 10^6.

Submerged Fermentation

a) The isolated fungal strains are named as TRY1, TRY2 and TRY3.
b) One loopful of each selected fungal strain was inoculated into 10 mL of PDA broth medium at an initial concentration of 10^7 cells /mL.
c) Samples were incubated at 30 ºC with shaking at 120 rpm for 10 h to obtain a final OD value of 1.0 at 600nm.
d) Then, the inoculums were transferred to 250 mL Erlenmeyer flask containing 90 mL of PDA medium and 0.9 g/L of lignocellulose loaded soil.

e) The cultures were incubated at 120 rpm and at 30 ºC for 7 days.
f) The initial pH was set at 7.6. Sampling was performed at every 24 h to observe daily fungal growth (OD). *Escherichia coli* strain was used as a negative control.

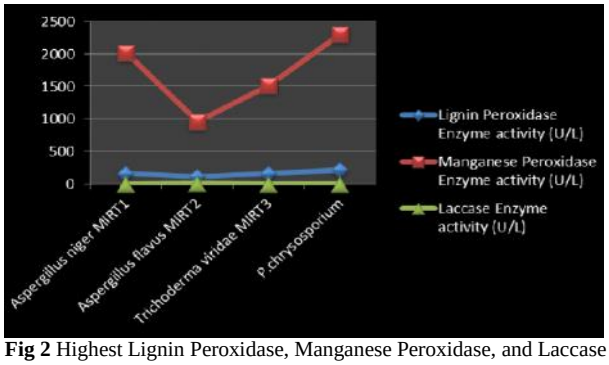

Fig 2 Highest Lignin Peroxidase, Manganese Peroxidase, and Laccase Enzyme

Preparation Of Crude Enzymes

Samples were collected at every 24 h and their turbidity was measured by reading the OD at 600nm. The supernatants and pellets were separated by centrifugation at 8,000 rpm for 10 minutes. The supernatants were collected and were used to measure extracellular ligninolytic enzyme activity.

Lignin Peroxidase Enzyme Assay

The lignin peroxidase (LiP) activity was assayed via the oxidation of veratryl alcohol to veratryaldehyde at 310 nm (TienandKirk,1988).

Manganese Peroxidase Enzyme Assay

Manganese peroxidase (MnP) activity was measured via the oxidation of guaiacol to a colored product using the UV-Vis spectrophotometer at 465 nm (Li *et al*., 2008).

Laccase Enzyme Assay

Laccase activity was determined by oxidation of 2,2'-azinobis-(3-ethylbenzethia- zoline-6-sulphonate) (ABTS) using the method of Wolfenden and Willson (1982).

Effect of pH concentration

The effect of initial pH was evaluated via submerged fermentation of the selected isolates in PDA broth medium with the addition of 0.5 g/L lignocellulose loaded soil at 5 different initial pHs.

The optimum initial pH for enzyme production was determined by incubating triplicate samples of the selected isolates at pH 7.0, 7.5, 8.0, 8.5, and 9.0, using 1 M HCl or 1 M NaOH to adjust the pH. The experiments were all conducted in an incubator shaker set at 120 rpm and 30ºC for 7 days. Samples were collected at every 24 hours.

Effect of temperature variation

The optimum temperature for ligninolytic enzyme production was investigated by incubating triplicate samples of the selected isolates in PDA broth medium with the addition of 0.5 g/L lignocellulose loaded soil at temperatures of 30, 35, 40, 45, and 50 °C. The flasks were incubated at 120 rpm on a rotary shaker for 7 days, and samples were collected at every 24 hours.

RESULTS & DISCUSSION

Three newly isolated fungal strains that produced ligninolytic enzymes were isolated from palm oil plantation soils. Isolate MIRT 1 was identified as *Aspergillus niger* isolate MIRT 2 was identified as *Aspergillus flavus*, and isolate MIRT 3 was identified as *Trichoderma viridae*. These strains utilized lignin as the sole source of carbon and produced the main ligninolytic enzymes; lignin peroxidase, manganese peroxidase, and laccase. Based on the results obtained in this study, the isolate *Aspergillus niger* MIRT 1 was chosen as the best of the ligninolytic fungal strains, as it achieved maximum manganese peroxidase production of 2221.4 U/L on the third day under the optimum conditions of pH 8 and 30 ºC. Strain *Aspergillus niger* MIRT1 also produced the highest lignin peroxidase, at 204.45 U/L, on the fifth day of fermentation under the optimum conditions of pH 8 and 30 ºC.

Table 4 Optimum pH for Selected Fungal Strains and Maximum Ligninolytic Enzyme Activities

Isolates	Optimum pH	Lignin Peroxidase		Manganese Peroxidase		Laccase	
		Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Aspergillus niger MIRT1	5.5	197.25	5	2121.4	2	2.79	4
Aspergillus flavus MIRT2	6.0	144.56	5	1080.19	6	6.17	5
Trichoderma viridae MIRT3	6.5	187.33	7	1560.94	4	2.16	4
***P.chrysosporium* Standard culture**	6.5	220.10	6	2330.10	5	2.83	5

Table 5 Optimum Temperature with Maximum Ligninolytic Enzyme Activities from isolated Fungal Strains

Isolates	Optimum T°	Lignin Peroxidase		Manganese Peroxidase		Laccase	
		Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Aspergillus niger MIRT1	25	204.45	5	2221.4	3	2.98	3
Aspergillus flavus MIRT2	30	164.50	5	1180.19	6	11.12	5
Trichoderma virida MIRT3	35	203.13	6	1860.94	4	4.10	4
***P.chrysosporium* Standard culture**	35	265.30	5	2420.30	4	3.1	4

Strain *Aspergillus flavus* MIRT2 showed the highest laccase enzyme production of the three selected fungal strains in the present study. Although the production of manganese peroxidase and lignin peroxidase by strain *Aspergillus flavus* MIRT2 was lower than *Aspergillus niger* MIRT1, *Aspergillus flavus* MIRT2, *Trichoderma viridae*MIRT3 showed the highest laccase enzyme at 11.12 U/L on the fifth day under the optimum conditions of pH 7.5 and 35 ºC. The *Trichoderma viridae* strain has been considered a novel ligninolytic fungal isolate from palm oil plantation soil. *Phanerochaete chrysosporium* Standard culture showed bet results compared to the isolates though the isolates compete with that. For any environment if there is a mixture of these fungi then there will be great degradation of lignocellulose, which could be used commercially and/or industrially.

For determination of optimum temperature condition for production of ligninolytic enzymes in isolated fungal strains, a temperature range from 25 °C to 35 °C was selected due to

previous reports by Kuwahara*et al.,* (1984) and Zhu et *al.,* (2013). It was stated in a previous report that some of the ligninolytic fungi were also isolated from soil are thermophilic strains (Oliveira *et al.,* 2009). Based on the results, at 35 °C, 204.45% of the LiP activity was retained, while further increases in the temperature have resulted in drastic decreases in activity. This indicated that higher temperatures resulted in gradual inactivation of the LiP (Bibi and Bhatti, 2012). For manganese peroxidase, *Aspergillus niger* MIRT 1 produced the highest enzyme activity at a temperature of 30 °C. For laccase, the fungal *Aspergillus flavus* MIRT 2 produced a maximum activity of 11.12 U/L on day 5, even though at 35 °C, the LiP and MnP enzyme activities were highest for *Trichoderma viridae* MIRT 3. The laccase enzyme activity was lower at a temperature of 30 °C. In previous studies, most of the isolated ligninolytic fungal could only produce two main ligninolytic enzymes, especially a combination of MnP and Lac enzyme production (Shi *et al.,* 2013; Chen *et al.,* 2012 ; Oliveira *et al.,* 2009). Meanwhile, in this study, all three isolated strains produced all three main ligninolytic enzymes: LiP, MnP, and Lac. Regardless of the other findings, only MnP and LiP showed comparable enzyme activity with previous studies, while the Lac enzyme activity results showed the lowest activity. The maximum Lac obtained in this work was from *Aspergillus flavus* MIRT 2 which was 11.12 U/L, while *Phanerochaete chrysosporium* sp. B-9 is at 1250 U/L based on Chen *et al.,* (2012). Shi *et al.,* (2013) reported that *Cupriavidus basilensis* B-8 can produce Lac up to 815.6 U/L. Based on the present work, *Aspergillus niger* MIRT 1 can produce a maximum MnP at 2221.4 U/L, which is higher than for *Cupriavidus basilensis* B-8 at 1685.3 U/L (Shi *et al.,* 2013). However, the MnP activity from *Phanerochaete chrysosporium* is still the highest at 2903.2U/L, as reported in Chen *et al.,* (2012). Based on these results, it can be concluded that the present isolated fungi can produce ligninolytic enzymes at comparable activity when compared with previous studies.

The results of the present study confirmed that three newly isolated fungal strains from palm oil plantation soils can grow on kraft lignin as a sole carbon source and can produce three main types of ligninolytic enzymes in vitro and in vivo. Further studies are required to measure lignin degradation of lignocellulosic biomass by these fungal strains. Moreover, future studies may determine other optimum conditions in addition to pH and temperature, such as nutrients, dissolved oxygen level, and fungal concentrations to maximize the production of ligninolytic enzymes by pure or mixed cultures of these newly isolated strains.

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