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LIGNINOLYTIC ENZYME PRODUCTION AND REMAZOL BRILLIANT BLUE R (RBBR) DECOLORIZATION BY A NEWLY ISOLATED WHITE ROT FUNGUS: *BASIDIOMYCOTA* SPP. L-168.

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ABSTRACT

Recently, extensive research on basidiomycetous fungi has been conducted to isolate new organisms with enormous secretion of ligninolytic enzymes with potential industrial applications. The present study reports the screening of 13 phenol tolerant isolates from 51 rotted wood samples collected from Jalgaon district, Maharashtra (India). The isolates were tested for ligninolytic enzyme production by a two step screening based on phenol tolerance and gallic acid oxidation. Also ability of isolates for Remazol brilliant blue R decolorization was studied. Interestingly, *Basidiomycete sp*. L-168 showed 98.53% decolorization in 5 days of incubation. The isolate was characterized with respect to its set of extracellular phenol oxidases. Laccase was the only ligninolytic enzyme detected. Neither lignin peroxidase nor manganese peroxidase was detected. Maximum of 507.92 U l^{-1} of laccase was produced on 7^{th} day at 30^{o}C. This organism appears to be an ideal model for studies of group of fungi that lack LiP and MnP and operate with laccase as the major phenol oxidase. This is first report on isolation of ligninolytic fungi from Jalgaon district.

KEYWORDS: Ligninolytic enzymes, guaiacol, Remazol brilliant blue R, Laccase.

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INTRODUCTION

The most abundant constituent of plant tissue and crop residue is lignocellulosic material. Lignin is an aromatic and heterogeneous constituent of plant cell wall that ensures strength and resistance towards microbial attack. In nature there is a continuous degradation of dead plant materials by saprophytic microorganisms[1]. Lignin degrading basidiomycetes are collectively referred to as white rot fungi. They are common inhabitants of forest litter and fallen tree. They are the only microbes that have been shown to efficiently depolymarize, degrade and mineralize all components of plant cell wall including cellulose, hemicelluloses and the most recalcitrant lignin [2,3,4] The three major classes of extracellular enzymes designated manganese peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (LiP, EC 1.11.1.14) and laccase (EC 1.10.3.2, benzene diol: oxygen: oxidoreductase) are believed to be important in the fungal degradation of lignin[5,6,7]. A new group of ligninolytic heme containing peroxidase, combining structural and functional properties of the LiP and MnPs, are the versatile peroxidase (VPs). In addition, enzyme involved in hydrogen peroxidase production such as glycoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) are considered to belong to the ligninolytic system[6]. Some white rot fungi produce all three types of ligninolytic enzymes; some of them secrete only one or two types[8]. The enzymes involved in ligninolytic system are effective against a broad spectrum of aromatic compounds. This ability has attracted increasing scientific attention on the use of white rot fungi and their enzymes[6]. The enzymes have high biotechnological interest as demonstrated by several studies reporting their role for green processes such as wood pulp delignification, dye decolorization in textile industry, ethanol production, wine processing, treatment of polycyclic aromatic hydrocarbon, bioremediation and for the realization of biosensors[6]. The increasing demand for these enzymes has intensified the search for microorganisms having high level of enzyme activities and for improved fermentation process for their production. The present study aims to isolate efficient white rot fungi producing ligninolytic enzymes from forest cover of Jalgaon district (North Maharashtra, India). During this study a two step screening was used in order to facilitate isolation of potential organism. The locally isolated strains of WRF were then screened for their ability to decolorize the anthraquinone dye, Remazol Brilliant Blue R (RBBR). The local isolate exhibiting the greatest decolorization ability was then selected to investigate the ligninolytic enzyme activities.

MATERIALS AND METHODS

2.1. Chemicals and reagents

Remazol Brilliant Blue R (RBBR) was purchased from Sigma Aldrich (USA). 2,2'-azino-bis(3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS) was purchased from Fluka Analyticals (USA). O-phenyl phenol was purchased from Lancaster. All the other reagents and media were purchased from HiMedia, Mumbai, India. All other chemicals used in this work were of analytical grade.

2.2. Screening and isolation of ligninolytic fungi

A total of 51 rotted wood samples were collected from forest area in Jalgaon District, North Maharashtra, India. Prior to sampling, information related to climate, geology and vegetation was gathered. The fungi were screened at primary stage for phenol tolerance ability on O-phenyl phenol agar (0.3%). Wood chips measuring 5 mm × 5 mm × 1 mm were removed from the rotten wood and aseptically transferred to Petri plates containing two different cultural media: 2% malt extract agar amended with 250 µg ml^{-1} streptomycin sulphate and 2% malt extract agar amended with 0.3% O– phenyl phenol (OPP). The plates were incubated at 30 °C for 7–10 days. Once

fungal colonies formed in the agar plates, each colony was transferred to a new agar plate to grow as a pure culture to be used for further analysis. The secondary screening was performed as per Hedger (1982)[9] and Lopez (2006)[10] on Lignin agar to check the dephenolization ability of isolates. To test phenol oxidase production ability, the isolates were grown on Bavendamm's medium[11] containing 0.5% gallic acid, 2% malt extract and 2% agar, pH 4.5 adjusted with 0.1 N sodium hydroxide. Gallic acid was added to the media before autoclaving. The medium was inoculated with a single agar plug cut from edge of a growing culture and incubated at 30°C for 7-10 days.

2.3. Identification of the isolate

The morphological identification of the isolates was attempted by microscopic observations using illustrated Atlas of Common Plant Pathogenic Fungi. The molecular analysis was conducted by small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. The evolutionary history was inferred using the Neighbor-Joining method. The phylogenetic analysis was conducted in MEGA4 software.

2.4. RBBR decolorization study using solid media

The ability of fungi to decolorize of the anthraquinone dye Remazol Brilliant Blue R (RBBR) was evaluated using malt extract agar plates supplemented with 500 mgl^{-1} of the dye. A disc 1 cm in diameter, that contained mycelia from the edge of a growing colony maintained on PDA, was placed in the center of the plate. In addition, un-inoculated plates containing dye was used as control. The plates were then incubated at 30°C for 7 to10 days. The plates were examined daily for the visual disappearance of color, when compared to control.

2.5. RBBR decolorization study in liquid medium

Decolorization experiments were carried out in 100 ml Erlenmeyer flasks with 20 ml of medium containing glucose-1%, peptone-0.1% and malt extract 1%. The medium was supplemented with RBBR at a concentration of 500 mg l^{-1} and the pH was adjusted to pH 5.0. The medium was inoculated with two plugs of one cm diameter cut from the growing edge of PDA cultures and incubated stationary at 30^0C for 7 days. Cultivations were done in triplicate with biotic (without dye) and abiotic (without fungus) controls. The mycelium was separated by centrifugation (10 min, 8000 x g at 40 ^{0}C) and color reduction of the supernatant was recorded at 595 nm using Shimadzu UV-1800 spectrophotometer. The decolorization efficiency of the different isolates was expressed as percentage RBBR decolorization[10] as per the following equation: Decolorization (%) = (A_λ initial - A_λ final) / A_λ initial X 100 Where A_λ initial = initial absorbance and A_λ final = absorbance after 7 day culture.

2.6. Extracellular Ligninolytic enzyme production using liquid media

Lignolytic enzymes were produced using submerged culture method by employing isolated fungal strains. A general medium, 2% malt extract broth (glucose-1%, peptone-0.1%, malt extract- 1%, pH-5.0 adjusted with 0.1N NaOH), was used to study the constitutive production of ligninases. In addition, to study the production of LiP and MnP, the basal medium described by Tien and Krik (1988)[12] were used. To study MnP production, 100 mg l^{-1} $MnSO4. H_2O$ was added to basal medium. The medium described by Eggert et al. (1996)[13] was used for laccase production. Cultivation was carried out in 100-ml Erlenmeyer flasks with 20 ml of medium. The medium was inoculated with two plugs of one cm diameter cut from edge of a growing culture and incubated stationary at 30^0C for 7-10 days.

2.7. Extracellular Ligninolytic enzyme production using solid media

The production of peroxidases (LiP and MnP) and laccase was assessed by qualitative approach using solid media[14]. The medium contained (g l^{-1}) KH_2P0_4-1, $MgS0_4$•$7H_20$- 0.5, $CaCl_2$•$2H_20$ -0.01, Yeast Extract- 0.01, $CuS0_4.5H_20$- 0.001, $FeS0_4$ - 0.001, $MnS0_4.H_20$ - 0.001, and Agar-20.0. The medium was supplemented with 0.02 % w/v Azure B (to determine peroxidases) and 0.01% ABTS and 0.005% α-napthol (to determine laccase). Each medium was inoculated with a single agar plug cut from edge of a growing culture and incubated at 30^{o}C for 7-10 days.

2.8. Enzyme assays

After the mycelium was removed by centrifugation (10 min, 8,000 x g at 40 ^{0}C), laccase activity in the culture supernatant was determined by measuring the oxidation of 1 mmol l^{-1} ABTS buffered with 50 mM phosphate buffer (pH 4.0). Formation of the cation radical was monitored at 420 nm (ε_{max} = 3.6 x 10^4 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme able to oxidize one µmol of the substrate per minute[15]. Peroxidase activity was determined by adding to the laccase assay solution H_2O_2 to 100 mmol l^{-1} final concentration and subtracting the increase in absorbance caused by laccase activity[13]. LiP and MnP activities were separately measured by azure B assay[16] and phenol red assay[5] respectively. All spectrophotometric measurements were carried out at 25^{0}C using a Shimadzu UV-1800 spectrophotometer with uv probe software.

RESULT AND DISCUSSION

Screening and isolation of ligninolytic fungi

The importance of phenol tolerance in screening of white rot fungi is emphasized since long period. A selective medium was reported for the isolation of white-rot fungi (WRF) containing 0.006 percent o-phenyl phenol (OPP)[17]. Hedger, 1982[9] also used OPP medium containing 0.003 percent OPP for selective isolation of ligninolytic fungi. However in the present study we used 0.3 percent OPP to isolate high phenol tolerant fungi. When 51 rotted wood samples were plated onto malt extract agar, fungal growth was observed on all the plates. However, as phenol tolerance ability is prime necessity for lignin degradation, the fungi were screened at primary stage for this ability on O-phenyl phenol agar (0.3%). From 51 rotted wood samples, altogether 13 phenol tolerant fungal isolates were obtained. The fungal isolates were maintained on potato dextrose agar (PDA) and stored at 4^{o}C (sub-cultured monthly). The phenol tolerant isolates were then subjected to secondary screening using lignin agar to check the dephenolization ability of isolates. Among 13 isolates, all isolates showed lignin dephenolization ability indicating the presence of ligninolytic enzymes.

Similarly the oxidase enzyme production ability of phenol tolerant isolates was evaluated by Bavendamm reaction. Among 13 isolates, 10 isolates showed dark-brown colored zone around the fungal colonies indicating the presence of phenol oxidase enzymes. It was observed that some isolates (L-56, L-109, L-143, L-149 and L-168) showed very strong reaction with gallic acid and some (L-86 and L-113) showed very poor reaction. Interestingly, the isolates L-24, L-81 and L-170, which showed lignin dephenolization ability, did not oxdise gallic acid. The Bavendamm reaction, involving the oxidation of aromatic acids such as gallic acid to various brown quinines, has been used as indicator for ligninolytic enzymes production by several researchers[10,11,14,18]. However, this test gives a positive or negative indication of enzyme production and is particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data is not required[19]. Thus, by primary and secondary screening, 10 phenol tolerant fungal isolates with oxidase production ability were selected for further study. The screening profile of samples is summarized in Table 1.

Sr.no	Sample code	Growth on Malt agar	Growth on Malt agar with O-phenyl phenol	Dephenolization on lignin agar	Oxidase production on Gallic acid agar [a]	% RBBR decolorization
1	L24	√	√	+	-	5.02
2	L56	√	√	+	52	1.78
3	L62	√	√	+	24	0.30
4	L81	√	√	+	-	44.2
5	L86	√	√	+	20	28.18
6	L96	√	√	+	46	62.00
7	L109	√	√	+	50	0.80
8	L113	√	√	+	28	59.3
9	L139	√	√	+	41	33.5
10	L 143	√	√	+	63	64.81
11	L149	√	√	+	54	18.75
12	L168	√	√	+	67	98.53
13	L170	√	√	+	-	53.33

√= Growth is present
+= Dephenolization of lignin
- = No oxidase production
[a] Zone diameter (mm) of colored reaction measured after 7 day incubation at 30^{0}C on gallic acid agar:

RBBR decolorization study using solid and liquid media

Since the capacity of WRF in decolorizing dyes results in part from the activity of enzymes that take part in lignin depolymerization process, the polymeric dyes are frequently used as model compounds in studies of ligninolytic fungi[15,20]. The correlation between decolorizing and ligninolytic abilities of white-rot fungi has been commented upon by several authors[8,21,22,23,24]. RBBR (also known as Reactive Blue 19) has been widely used, since as an anthracene derivative this compound represents an important group of organo-pollutants. The structure of RBBR is shown in figure 1.

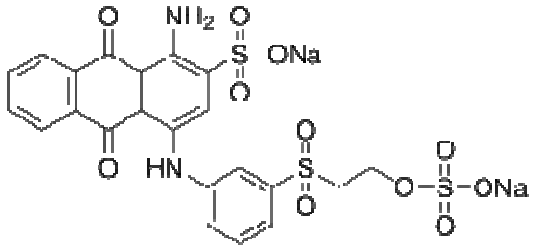

Figure 1
Structure of Remazol Brilliant Blue R.

RBBR decolorization was studied for all phenol tolerant fungal isolates. The ligninolytic efficiency of the test fungi was determined qualitatively based on their RBBR decolorization ability in solid media by visual detection of decolorization as compared to control. In order to get the quantitative data, decolorization studies were also conducted in liquid media. Of the 10 fungal isolates, five isolates (L-96, L-113, L-143, L-168 and L-170) showed more than 50% decolorization. Interestingly, among these five isolates, L-170

showed no reaction with gallic acid, L-113 showed very poor reaction and the remaining isolates (L-113, L-143 and L-168) showed strong reaction. However, the isolate L-168 showed highest decolorization activity (98.53% in 5 days of incubation) than that of remaining isolates. The percentage decolorization of RBBR by the isolates is presented in Figure 2. Even though, the isolate L-170 showed no reaction with gallic acid, it showed 53.33% RBBR decolorization. This might be due to the fact that the range of substrates oxidized by the enzymes involved in ligninolytic system varies significantly from organism to organism.

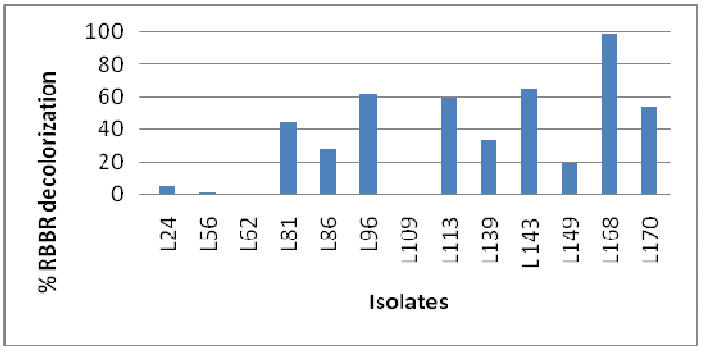

Figure 2
The percentage RBBR decolorization by different isolates

The time taken (5 days) for the isolate L-168 to decolorize the dye RBBR compares favorably with reports on other white-rot fungi which required periods of between 7 and 20 days to achieve 90% decolourisation in a diverse range of synthetic dyes[25,26]. It also compares favourably against *Ganoderma* strains that typically require 14 days to achieve substantial dye decolourisation[27]. These results show the biotechnological potential of ligninolytic enzymes from L-168 in textile industry.

Extracellular Ligninolytic enzyme production using liquid and solid media

In view of the results obtained, the isolate L-168, which gave maximum dye decolorization, was prioritized for a better understanding of ligninolytic enzyme system. The production of main ligninolytic enzymes (LiP, MnP and laccase), was studied in liquid media for 10 days. Each day an aliquot of sample was aseptically removed and assayed for the enzymes LiP, MnP and laccase by the methods described in enzyme assays. The result showed that, laccase was the only ligninolytic enzyme detected in the isolate L-168. No peroxidase activity was detected by ABTS oxidation in the presence of H_2O_2. LiP assay carried out with azure B as per Priscila Brasil de Souza- Cruz, 2004[16] was also negative. Moreover, MnP-type peroxidase was also absent since it could not oxidize phenol red, a standard substrate for MnP's. The study indicates that the isolate produces laccase as the sole enzyme involved in lignin degradation. Our results corroborate with the earlier findings of Saparrat et al, 2008[28] which reports laccase as the only component of the ligninolytic system of white rot fungi.

The isolate L-168 expressed a high titer (507.92 U l^{-1}) of laccase on 7th day of incubation. The results are in parallel with earlier findings[29]. To compare this value with data from the literature, the laccase activity of the isolated strain exceeds that of *Pleurotus ostreatus* (ranging 1.6–5.9 of U l-1), which has been successfully used in bioremediation applications under various culture conditions[30]. Although the higher laccase titer is reported by some investigators, the laccase titer obtained with L-168 is significant since no specific laccase inducer (e.g. 2,5 xylidine) was used in the medium. Therefore further increase in the titer of laccase by L-168 could be obtained by

induction studies. The presence of laccase and absence of peroxidase was qualitatively confirmed by cultivation of the isolate on Azure-B, ABTS and α-napthol agar. Decolorization of the dye Azure-B by fungi is positively correlated with production of lignin peroxidase and Mn dependent peroxidase, however, this dye is not a substrate for laccase [14]. No decolorization of azure B was found for L-168, however, green coloration on ABTS agar due to the oxidation of ABTS and blue coloration on α-napthol agar confirmed laccase activity.

Identification of the isolate

The microscopic observations of the isolate L-168 revealed that the isolate was non sporulating. The molecular analysis was conducted at National Centre for Cell Science (NCCS), Pune (India). The isolate was identified as *Basidiomycota sp*. L-168 (Genbank Accession no. JF 412304). Phylogenetic relationship of present isolate based on RNA gene sequencing is given in the figure 3.

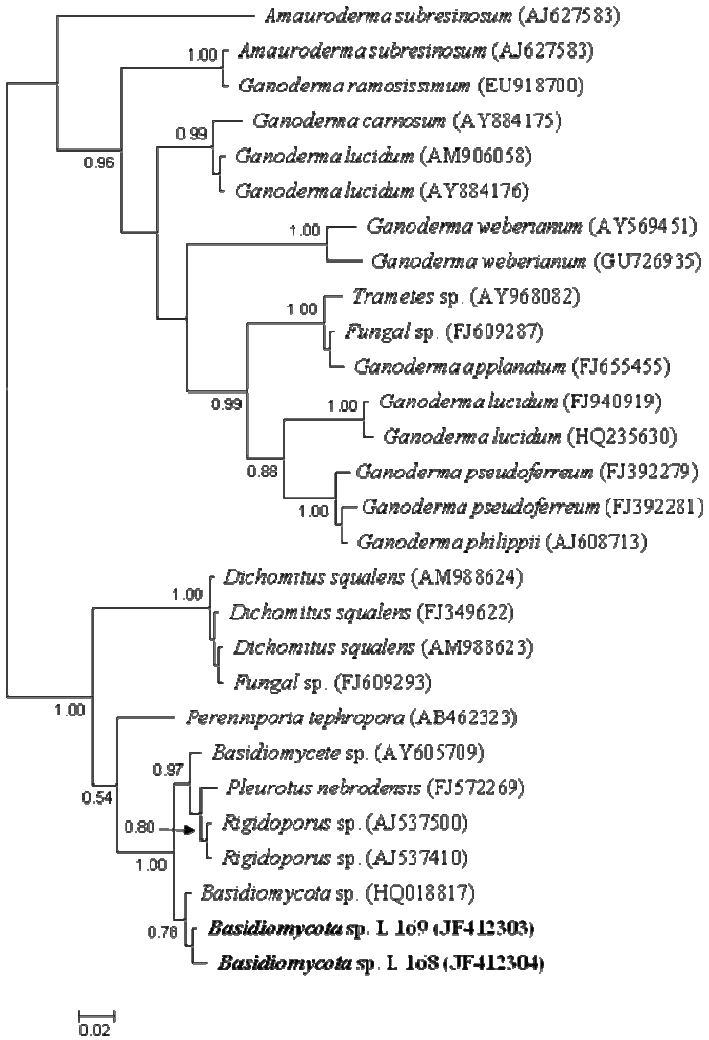

Figure 3

Phylogenetic relationship of present isolate based on RNA gene sequencing. The accession number for the sequence is given in parenthesis.

CONCLUSION

In conclusion, it is clear that during screening of ligninolytic fungi from large number of samples, a combination of plate assay and dye decolorization is found to be more effective. Only laccase of the three major ligninolytic enzymes was detected in culture supernatants of the isolate L-168 obtained from natural habitats in Jalgaon district (India). The isolate was capable of efficiently decolorizing RBBR indicating biotechnological prospects especially in bioremediation.

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