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SCREENING OF NOVEL ASCOMYCETES FOR THE PRODUCTION OF LACCASE ENZYME USING DIFFERENT LIGNIN MODEL COMPOUNDS

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ABSTRACT

The objective of this present study was to isolate a potential laccase producing fungi from the different environmental samples from Gujarat. Eighteen morphologically different fungi were isolated on mineral salts medium having lignin (sole carbon source) and screened qualitatively for their ability to produce laccase by plate screening method using the indicators ABTS, Brilliant Blue R (RBBR), Syringaldazine and Guaicol. Six selected fungal isolates were further screened quantitatively in liquid medium and assayed using ABTS as a substrate. The highest laccase productivity in liquid medium was 166.2 U/ml (3rd day) with BL-21/4. The genotypic characterization of selected fungal isolates was performed with partial 18S rRNA sequencing using ITS primer set ITS1/ITS4. The fungal isolates were identified as *Aspergillus versicolor*, *Curvularia aeria*, *Curvularia lunata and Curvularia akaii.* The conclusions of this study confirm that ABTS and Syringaldazine is very efficient phenolic compound for qualitative screening of novel ascomycetes that produces laccase.

KEY WORDS: Laccase, Ascomycetes, ABTS, Syringaldazine, Guaicol.

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INTRODUCTION

Laccase enzyme (EC 1.10.3.2) is a benzene diol, a multi-copper enzyme, and one of the three main ligninases. Laccase catalyze the oxidation of wide range of phenolic substrates including diphenols, polyphenols, substituted phenols, diamines and aromatic amines, with reduction of molecular oxygen to water. [1] Laccases can act on a wide range of substrates that makes them highly useful biocatalysts for various biotechnological applications. Commercially, laccases have been used to delignify woody tissues, produce ethanol, and to distinguish between morphine and codeine. A very wide variety of bioremediation processes employ laccase in order to protect the environment from damage caused by industrial effluents. Thus for industrial applications discovery of novel laccases with different substrate specificities is important. Research in recent years has been intense, much of it elicited by the wide diversity of laccases, their utility and their very interesting enzymology.Laccases are widely distributed in fungi, higher plants, [2] bacteria and insects. [3] Fungal Laccases also play an important role in physiological processes related to pathogenesis, [4] morphogenesis i.e. fruiting body development, [5-6] pigmentation [7] and cell detoxification. [8] Most of the Laccases described in literature were isolated from higher fungi, especially from Basidiomycetes, such as *Phlebia radiata*, [9] *Pleurotus ostreatus* [10] and *Trametes versicolor*. [11] Laccase production was reported in plant pathogenic species, some soil ascomycetes species from the genera *Aspergillus, Curvularia* and *Penicillium*, [12-14] as well as some freshwater ascomycetes. [15-16] Different genera of Ascomycetes & Deutromycetes also have been reported to produce laccases, but due to lack of systematic search it is not clear that how many ascomycete's species produce laccases. In view of its importance in large scale application, the present endeavor is to search for highly efficient laccase producing novel ascomycetes fungi from different environmental samples from Gujarat. In the present study, laccase-producing ascomycetes were isolated from various environmental samples using four different indicator compounds like ABTS, Guaiacol, Syringaldazine and Remazol Brilliant Blue R (RBBR) by plate assay method. The screening results with different indicators were also compared in order to reduce the number of indicator compounds needed in future screening procedures. Selected fungal isolates were also quantitatively screened for laccase production in submerged cultivation.

MATERIALS & METHODS

Chemicals

2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS), Remazol Brilliant Blue R (RBBR) and Syringaldazine were purchased from Sigma (St. Louis M.O., U.S.A.). Lignin, Bacteriological Agar, Guaiacol, dextrose and peptone were procured from Hi-Media (Mumbai, India). All other chemicals were of analytical grade and procured from S.D.Fine chemicals (Mumbai, India).

Isolation of Fungi

Total 17 soil samples were collected from the Different regions of Gujarat including Municipal Solid Waste dumping Site of Surat & Ahmedabad (Soil from surface & Soil beneath composted waste), Timber mart (soil beneath rotting logs), Dung Dumping Site (Soil Beneath composted dung), textile dye industry (Soil Mixed with Dye) and Gutakha industry. Enrichment of lignin degrading microbial cultures were performed in Mineral Salts lignin Medium containing 5.0 g/L Kraft Lignin, 1.0 g/L KH_2PO_4, 1.0g/L ammonium acetate, 0.01g/L $MgSO_4$ 0.01g/L $CaCl_2$, 0.001g/L $MnSO_4$, 0.001g/l $FeSO_4 \cdot 7H_2O$, 0.0005 g/L $CuSO_4$. Enriched Soil samples were serially diluted and plated on Mineral Salts lignin agar medium and incubated at 30°C. After 5 days of incubation fungal cultures were further purified on Potato Dextrose agar and stored at 4°C. The composition of the mineral salts medium was same [17] except that ammonium tartrate is replaced by 1% ammonium acetate in our experiments. Morphological identification of fungi was done by observing spore morphology under the light microscope.

Qualitative Screening of fungal isolates for laccase production

Laccase production by fungal isolates was screened using composite selective media (pH 6.0) plates containing 5.0 g/L glucose, 5.0 g/L peptone, 1.0 g/L KH_2PO_4, 1.0g/L ammonium acetate, 0.01g/L $MgSO_4$ 0.01g/L $CaCl_2$, 0.001 g/L $MnSO_4$, 0.001g/l $FeSO_4 \cdot 7H_2O$, 0.0005 g/L $CuSO_4$**,** 3% agar supplemented with different Lignin Model Compounds. Four Lignin Model Compounds namely 0.1 % ABTS, 0.1 % Remazol Brilliant Blue R (RBBR) Dye, 0.1% Syringaldazine and 0.06 % Guaicol were used as color indicator. ABTS and Guaicol were incorporated in to agar medium before autoclaving and RBBR after autoclaving as sterile-filtered water solutions. Screening with Syringaldazine was performed by drop test after growth on solid media. The correlation between positive reactions with different indicators was investigated by growing all fungal isolates on all different indicator plates. Single mycelia plug of each fungal isolates were inoculated on the plates and incubated at 30 °C for 5 days. Positive Laccase activity was visualized on the plates as Dark green halo in ABTS medium, Brown halo in Guaicol medium and clear zones around the colonies in RBBR medium. Syringaldazine oxidation was observed by applying few drops of 0.1% Syringaldazine Solution (Prepared in 95% ethanol) on to the edge of fungal colony. [18] Positive reactions were observed as dark pink halo formation due to oxidation of Syringaldazine.

Quantitative Screening of fungal isolates for laccase production

Six fungal isolates were selected for laccase production in submerged fermentation. 2 mycelial plugs from edge of each actively growing cultures were aseptically transferred to Erlenmeyer flasks (250 mL) containing 100 mL of the liquid medium with pH 6.0. The liquid medium contained 5.0 g/L glucose, 5.0 g/L peptone, 1.0 g/L KH_2PO_4, 1.0g/L ammonium acetate, 0.01g/L $MgSO_4$ 0.01g/L $CaCl_2$, 0.001g/L $MnSO_4$, 0.001g/l $FeSO_4 \cdot 7\ H_2O$, and 0.0005 g/L $CuSO_4$**.** Five days old fungal cultures grown on same medium were used as inoculum. Innoculated media were incubated at 30°C and 150 rpm for seven days. The culture broth were removed at 24 h

intervals from each of the culture flasks and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was used for the laccase enzyme assay.

Laccase enzyme assay

Extracellular laccase activity was determined spectrophotometrically with 2.5 mM 2'-azino–bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) as substrate. The reaction mixture contains 1ml of 2.5 mM ABTS, 1.0 ml of 0.1 M sodium acetate buffer (pH 4.5) and 1.0 ml of enzyme. The oxidation of ABTS (ε= 3.6 x 10^4 M^{-1} cm^{-1}) was monitored by measuring the change in absorption at 420 nm for 5 min. One unit of enzyme activity defined as amount of enzyme that oxidizes 1μM of ABTS per min.

Genotyping of laccase producing fungi using ITS primers

Six fungal isolates which showed best laccase activity were subjected to genotypic identification. Genotypic identification was carried out at GSBTM (Gujarat State Biotechnology Mission, Gandhinagar, and Gujarat) by PCR amplification and partial sequencing of the 18S rRNA using ITS 1/ITS 4 primers for the confirmation of morphological identity. A comparative study of other rDNA sequences with rDNA sequence of fungal isolates in this study was done using BLAST algorithm at the website http://www.ncbi.nlm.nih.gov. The nucleotide sequence of selected fungal isolates has been submitted at the NCBI Genbank.

RESULT & DISCUSSION

Isolation and qualitative screening of fungal isolates for laccase production

Eighteen morphologically different fungal cultures were isolated from different environmental soil samples. All fungal isolates were tested for laccase production on different phenolic and nonphenolic lignin model compounds. Laccase productions by microbe have been screened on solid media containing colored indicator compounds that enable the visual detection of laccase production. The traditional use of tannic and gallic acid [19] for qualitative screening have mostly been replaced with synthetic phenolic compounds, such as guaicol and ABTS [20-21] or with the polymeric dyes Remazol Brilliant Blue R. [22-23] Of the eighteen isolates tested, nine isolates showed significant ABTS-oxidation activities with the formation of dark green ABTS cation radical (Fig 1). This dark green or dark purple ABTS cation radical have been reported as an indication of a positive extracellular oxido reductase secretion. [24-25] The diameter of dark green halo and the color intensity were used to evaluate the level of ligninolytic enzyme production of each strain. Fungal isolates BL-21/4 exhibited the highest ABTS oxidation (Table 1).Formation of reddish brown halo around fungal colony indicates oxidation of guaicol. Eight isolates showed Guaicol oxidation activities (Figure 2). Strain BL-21/4, BL-1/4 and BL-18/15 exhibited the highest guaicol oxidation activity on the oxidation scale (Table 1). Since guaicol is a compound that could be degraded by Lignin peroxidase, Manganese peroxidase as well as laccase, the exact nature of the enzyme that effected the utilization of the compound was not obvious at the primary screening stage. Among LiP, MnP and laccase, only laccase can oxidize syringaldazine thus the use of syringaldazine is therefore recommended. Among eighteen isolates tested, nine isolates showed Syringaldazine oxidation activities (Figure 3). Formation of dark pink color around the mycelia growth indicates oxidation of Syringaldazine.Dye-based plate assay is the common method used for the screening of lignin degrading fungi. [26] In present study only three fungal isolates were able to decolorize the RBBR dye after 5 days of incubation (Figure 4). RBBR seemed to be less specific, as it failed to give positive results with 9 fungal isolates that were positive on ABTS. In the present study, ABTS and Syringaldazine are considered unique laccase substrates, therefore it is confirmed that the enzyme is a true laccase and not peroxidase enzyme.Based on microscopic spore morphology all laccase positives genus were identified and they belong to ascomycetes.

Table 1
Qualitative screening of laccase enzyme production with different indicators*.

Sr. No.	Fungal Isolates	Lignin Model Compounds			
		0.1% ABTS	0.01% Syringaldazine	0.05% Guaicol	0.05% RBBR
1	BL-1/4	+	+	+	-
2	BL-2/4	-	-	-	-
3	BL-4/4	+	+	+	+
4	BL-5/4	-	-	-	-
5	BL-6/8	-	-	-	-
6	BL-7/10	+	+	+	-
7	BL-8/10	+	+	-	-
8	BL-9/10	-	-	-	-
9	BL-10/8	+	+	+	-
10	BL-11/10	-	-	-	-
11	BL-12/4	-	-	-	-
12	BL-13/10	+	+	+	+
13	BL-15/8	+	+	+	-
14	BL-16/4	-	-	-	-
15	BL-17/15	-	-	-	-
16	BL-18/15	+	+	+	+
17	BL-19/8	-	-	-	-
18	BL-21/4	+	+	+	-

Fungal isolate that is able to produce color halo with the indicator compound around its colony is shown by (+) symbol and absence of color halo is shown by (-) symbol.

Qualitative screening of fungal isolates for laccase production on ABTS containing medium

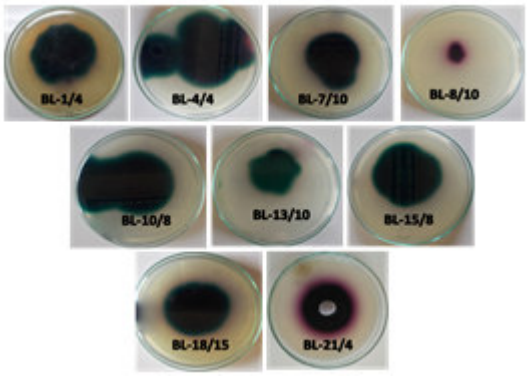


Figure 1
Green or purple halo around colony indicates oxidation of ABTS by laccase producing fungi

Qualitative screening of fungal isolates for laccase production on Guaicol containing medium

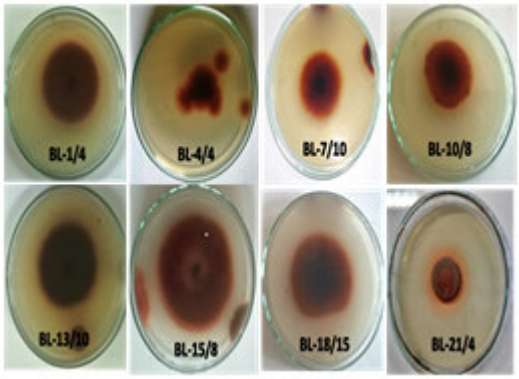


Figure 2
Reddish brown halo around colony indicates oxidation of Guaicol by laccase producing fungi.

Qualitative screening of fungal isolates for laccase production using Syringaldazine drop test

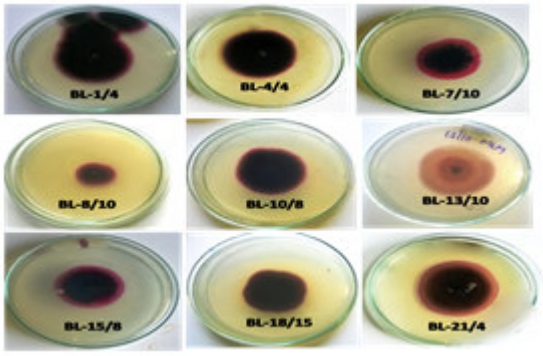


Figure 3
Dark pink halo around colony indicates oxidation of Syringaldazine by laccase producing fungi.

Qualitative screening of fungal isolates for laccase production on RBBR containing medium

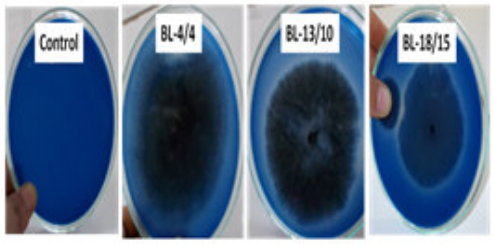
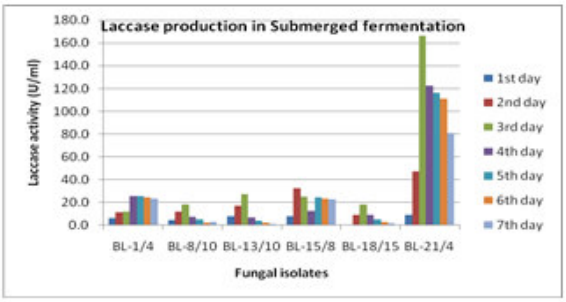


Figure 4
Zone of clearance around colony indicates dye degradation by laccase producing fungi

Quantitative screening of laccase production

Laccase production can be monitored by measuring enzyme activity during submerged cultivations. Six fungal isolates which gave positive results with ABTS were quantitatively screened by submerged fermentation. Highest laccase activity were observed with fungal isolate BL-21/4 (166.2 U/ml) of incubation followed by BL-13/10 (27.5 U/ml), BL-1/4(25.4 U/ml), BL-15/8 (25.1 U/ml), BL-8/10 (18.2 U/ml) and BL-18/15 (18.1 U/ml) (Graph 1). The highest activity was observed on 3rd day for BL-21/4, ,BL-13/10,BL-18/15 and BL-8/10 and in other isolate BL15/8 the activity was maximum on second but the activity was less when compared to other isolates. Highest laccase production was reported when glucose concentration was decreased to 1%, and the medium was supplemented with 0. 5% peptone. [26] This is in good agreement with the result obtained in present study as glucose and peptone concentration were 0.5% in the medium.

Graph 1
Quantitative screening of Laccase production by different fungal isolates belongs to class Ascomycota



Genotyping of laccase producing fungi using ITS primers

The morphological characters of the isolated strain spores were uninform and confused with other related genera. Hence the molecular method was used for the identification of isolated strain. ITS regions of fungal rDNA have been successfully used for species identification. The sequence of the isolate studied was compared with that of NCBI databases using BLAST network. The isolate was analyzed based on the similarity with the best-aligned sequence of the BLAST search. Based on BLAST search of partial 18s rRNA gene sequence and complete sequence of ITS region, the fungus was identified and submitted to GenBank, NCBI (Table 3). The 18srRNA sequence of the isolated fungus BL-1/, BL-13/10, BL-15/8 are *Curvularia aeria* ,but the spore color and the morphological characteristics of BL-1/, BL-13/10, BL-15/8 are different, indicating they are belonging to different strains of *Curvularia aeria*. So further characterization have to be carried out to differentiate between these three isolates.

Table 3
Identification of the laccase producing potential fungal strains isolated in the screening.

Sr. No.	Fungal isolates Code	Identified Ascomycetes	GeneBank Accession no.
1	BL-1/4	*Curvularia aeria*	KT355733
2	BL-8/10	*Aspergillus versicolor*	KT454795
3	BL-13/10	*Curvularia aeria*	KX184725
4	BL-15/8	*Curvularia aeria*	Not submitted to NCBI
5	BL-18/15	*Curvularia akaii*	KX184726
6	BL-21/4	*Curvularia lunata*	KX184727

CONCLUSIONS

Present research work aimed at the screening of Ascomycetes that produces laccase from environmental samples. For screening of laccase production different indicators like ABTS, Guaicol and Syringaldazine gave were used. Guaiacol showed a very strong ability to facilitate the growth and the isolation of the interested fungi with the laccase activity. Syringaldazine have not been reported as indicator compound for qualitative screening of laccase producing Ascomycetes. The color reactions with ABTS and Syringaldazine are only specific for laccase thus these compounds reliably be used for laccase activity screening. 18 isolates were obtained after enriching with lignin in the medium. Of the 18 isolates six were selected for further studies. Fungal isolate BL-21/4 showed highest laccase activities (166.2 U/ml) on 3rd day of incubation) in submerged medium and it was identified as *Curvularia lunata.* Fungal isolates BL-1/4, BL-13/10 and BL-15/8 were identified as *Curvularia aeria* but colony morphology of all three isolates as well as amount of laccase enzyme they produced were different. Fungal isolate BL-18/15 was identified as *Curvularia akaii* that produced 18.1 U/ml laccase on 3rd day of incubation. *Curvularia* have been reported to produce Laccase but species level identification has not been reported. [12] BL-8/10 was identified as *Aspergillus versicolor* that produces laccase in good amount (18.2 U/ml). In present study *Curvularia akaii* and *Aspergillus versicolor* showed laccase activity and have not been reported till now. The present study reports, novel laccase producing ascomycetes like *Curvularia lunata* , *Curvularia aeria* , *Curvularia akaii* and *Aspergillus versicolor*.

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CONFLICT OF INTEREST

Conflict of interest declared None

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