Role of Lignocellulolytic *Bipolaris* sp. in the Decolourisation of Textile Dyes

Revathi K., Jameson T Joseph, Sreethu E G., Jasmina T., Panneerselvam K., Manikandan P. and Shobana C S.

1 Department of Microbiology, Dr. G.R. Damodaran College of Science, Coimbatore - 641 014, India
2 Department of Microbiology, M R Government Arts College, Mannargudi – 614 001, India
3 Department of Microbiology, Aravind Eye Hospital and Post Graduate Institute of Ophthalmology, Coimbatore - 641 014, India
4 Department of Medical Laboratory Technology, College of Applied Medical Sciences, Majmaah University, Al-Majmaah, Kingdom of Saudi Arabia.
5 Department of Microbiology, PSG College of Arts and Science, Coimbatore - 641 014, India
* Corresponding Author: shobanasenthilkumar@gmail.com

ABSTRACT

The ability of the fungi to degrade lignocelluloses is due to their possession of extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). These enzymes have been shown to degrade not only lignocelluloses but also recalcitrant environmental pollutants such as textile dye effluents. In the present study, a total of 28 fungal isolates from the tree barks of tropical evergreen Pinavu forest, Idukki district, Kerala, were screened for their potential extracellular enzyme activity. Of these, one isolate exhibited α – amylase, cellulase, xylanase, lipase, ligninase, lignin modifying enzyme and laccase activities. The detailed morphological analysis revealed that the isolate belonged to *Bipolaris* sp. This potential isolate was subjected for biodecolourisation assay of aniline blue, trypan blue, Congo red and orange G. The results suggested that the potent isolate of *Bipolaris* sp., efficiently decolourised aniline blue and showed weakly positive result for trypan blue in the plate method. But it did not decolourise both Congo red and orange G. When employed for the colorimetric assay method of azo dye decolourisation, the potent isolate decolourised aniline blue to 91.3%, whereas the decolourisation percentage of trypan blue was not satisfactory.

Key Words: Lignin degrading enzymes, *Bipolaris* sp., Azo dyes and decolourisation.

INTRODUCTION

Wood and other lignocellulosic materials are formed by three main polymeric constituents, cellulose, lignin, and hemicelluloses. Lignin is an amorphous hetero-polymer. It is non-water soluble and optically inactive; it consists of phenyl propane units joined together by different types of linkages[1]. It is indigestible by animal enzymes, but some fungi and bacteria are able to secrete ligninases (also named lignases) that can biodegrade the polymer. Degradation of lignin is mainly by the action of the enzyme ligninase. Well understood lignolytic enzymes are manganese peroxidase, lignin peroxidase, laccase[2]. Laccase is identified as one of the most important enzymes that play an important role in degradation of lignin and xenobiotics.

Biotechnological methods adopting fungi and their enzymes in the dye degradation has been well appreciated globally, because of their potential use in detoxification and degradation of dyes. A great number of dyes and other chemicals are used in textile industry. Among all the dyestuff, the azo dyes are considered as the most important and a major group of dyes mostly used in industry[3]. Some of them are dangerous to living organisms due to their possible toxicity and carcinogenicity[4].

MATERIALS AND METHODS

Collection of samples for the isolation of potential fungi

The bark samples were collected from Pinavu forest, Idukki district of Kerala. The collected
samples were processed by inoculating in 100 ml Sabouraud’s dextrose broth and incubated for 7 days at 25°C. Single spore culture method was followed for the fungal strain purification using dilution plating method.

Screening for the extracellular enzymes produced by fungal isolates

To determine the extracellular enzyme activity, the fungal isolates were point inoculated at the center on various agar plates supplemented with the substrate specific to the enzymes and checked for the production of enzymes viz., cellulose, xylanase, á-glucosidase, phenol oxidase, lipase, alkaline protease, ligninase, tyrosinase, laccase & peroxidase, manganese peroxidase, and lignin peroxidase.

For the detection of á-amylase, the fungal isolates were inoculated on starch agar plates and incubated at 28°C for 72 h. After 72 h, the fungal mat was removed from the plates and the plates were flooded with iodine solution. The plates were then observed for zone of hydrolysis. Cellulase activity was screened by filter paper degradation method where the inoculated SDB with 25 × 5 mm strip of sterile filter paper was assessed for degradation. Xylanase activity was screened by inoculating the fungal isolates on Akiba & Horikoshi medium incubated at 28°C for 72h. After 72h, the plates were observed for zone of clearance. á-glucosidase activity was detected on cellulolysis basal medium (CBM) supplemented with 0.5% w/v esculin to which 1 ml of sterile 2% w/v aqueous ferric sulphate solution was added and the plates were inoculated with the test fungus and were incubated at 25°C in dark for 72 h. After incubation, the plates were observed for the development of black colour colonies indicating production of á-glucosidase.

Lipase activity was confirmed by growing the fungal isolates on peptone tributyrin agar and incubated at 28°C for 72 h. After incubation, the plates were observed for the zone formation. For detecting alkaline protease, the fungal isolates were inoculated on skim milk agar plates and were incubated at 28°C for 5–7 d. After incubation, the plates were observed for zone of clearance around the colonies. Ligninase was screened by inoculating the fungal isolates on malt extract tannic acid agar and were incubated at 28°C in dark for 5–7 d. After incubation, the plates were observed for clear zone around the colonies.

For screening phenol oxidase activity, sterile lignin basal medium (LBM) supplemented with 0.25% w/v lignin was prepared. Precisely, 1 ml of sterile 20% aqueous glucose solution was aseptically added to each 100 ml of LBM prepared and the plates were inoculated with the test fungus. The plates were incubated at 25°C in dark for 5 d. After 5 d of growth, the plates were flooded with 1% w/v aqueous solution of ferric chloride and potassium ferricyanide (prepared freshly before use). Phenols in undegraded lignin will stain blue-green, whereas in degraded lignin, clear zone around colonies can be observed.

Laccase and peroxidase were screened by pyrogallol well test as follows: exactly, 1 ml of sterile 20% aqueous glucose solution was aseptically added to 100 ml of LBM and dispensed into Petri dishes. After solidification, the plates were inoculated with the test fungus. The plates were incubated at 25°C in dark for 5 d. After 5 d of growth, a total of three 5 mm diameter wells were cut in the agar medium using sterile syringe. To one well, few drops of 0.1% pyrogallol (in 95% ethanol) was added. To the second well, few drops of 0.1% pyrogallol (in 95% ethanol) and few drops of 0.5% aqueous hydrogen peroxide (H₂O₂) solution were added and to the third well, 95% ethanol was added and used as control. The appearance of yellow-brown colour around each well within 30 min indicates enzyme production. The peroxidase test was regarded as positive, if the laccase test displayed negative or less intense reaction. Tyrosinase activity was screened by adding 1 ml of sterile 20% aqueous glucose solution was aseptically to 100 ml of LBM and dispensed into Petri dishes. After solidification, the plates were inoculated with the test fungus. The plates were incubated at 25°C in dark for 5 d. After 5 d of growth, one 5 mm diameter well was cut in the agar medium using...
sterile syringe and few drops of 0.1% w/v p-cresol in 0.05% w/v aqueous glycine solution was added. The appearance of red-brown colour around the well indicates positive result. The colour reaction may take up to 1 d to develop.

Manganese peroxidase activity was detected by inoculating the fungal isolates on ABTS minus LDM supplemented with 0.1 g/L manganese chloride and were incubated at 28°C for 5–7 d. The formation of black or dark-brown flecks of manganese oxide (MnO$_2$) after incubation indicates the existence of manganese peroxidase (MnP) enzyme. Lignin peroxidase enzyme was screened by inoculating the fungal isolates on lignin peroxidase screening medium supplemented with guaiacol and were incubated at 28°C for 5–7 d. After incubation, the plates were observed for reddish-brown colour change in the medium.

Microscopic observation of the fungal isolates by cellophane tape method
A 1.5 inch (40 mm) strip of clear cellophane tape was taken. Tip of the tape was held securely with forceps and the sticky side was gently pressed against the fungal mycelium. Then the tape was placed on a small drop of lactophenol cotton blue in a glass slide. The preparation was examined microscopically under 10× and 45× objectives.

Screening of fungal isolates for decolourizing azo dyes
Aniline blue and trypan blue were the dyes used to test the decolourisation. Sabouraud’s Dextrose agar was prepared, 0.005mg of dyes were added to each 100 ml of medium and autoclaved and poured into Petri dishes. Fungal isolates were inoculated in the centre of the plate and incubated at room temperature for 5 - 7 days. After incubation, the plates were observed for the zone of clearance.

Bio-decolourization assay of azo dyes
Sabouraud’s dextrose broth supplemented with 0.005 mg of azo dye per 100 ml of the medium was prepared and autoclaved. After sterilization, the flasks were inoculated with the test fungal isolates and incubated at 28°C for 5–7 d. After incubation period, decolourisation of the dyes by the selected isolates was determined using colorimeter. Precisely, 2 ml of the culture broth was centrifuged at 8,000 rpm for 10 min and the optical density (OD) of the culture supernatant was determined using colorimeter at the respective $l_{max}$ of the azo dyes. From the absorbance values measured during the assay procedure, dye decolourisation percentage of each isolate was calculated using the formula as follows:

\[
\text{Percentage} \ (%) \ \text{of dye decolourisation} = \left( \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \right) \times 100
\]

RESULTS AND DISCUSSION
Lignin is a complex aromatic polymer, highly recalcitrant towards both chemical and biological degradation. Like lignin, recalcitrant materials, azo dyes are also resistant to biological degradation. Hence the present study was undertaken to isolate various fungi from bark sample and analyse various enzyme activity to degrade lignin, azo dyes and other recalcitrant materials.

A total of 18 different bark samples from the tree barks as well as decayed wood were obtained from Pinavu forest, Idukki district, Kerala. By microbiologically processing the bark samples, a total of 28 isolates of mold fungi were obtained. All these fungal isolates were screened for the production of various extracellular enzymes, *viz.*, α-amylase, cellulase, xylanase, α-glucosidase, lipase, alkaline protease, ligninase, lignin modifying enzyme, phenol oxidase, peroxidase, tyrosinase, laccase, lignin peroxidase and manganese peroxidase. This investigation on enzyme production was done in order to detect the potency of the organism to produce various extracellular enzymes so that these molds could be used in the bioremediation of recalcitrant environmental pollutants. Among the 28 fungal isolates, 21 isolates produced α-amylase (*n* = 21), 11 isolates produced cellulase (*n* = 11), 5 isolates produced α-glucosidase (*n* = 5) and 11 isolates...
showed the production of xylanase \( n = 11 \). Similarly, we also investigated the production of lipase, among the 28 fungal isolates, 20 isolates produced lipase \( n = 20 \). In recent years, lignin-degrading enzymes have been extensively studied because of their potential biotechnological applications in the degradation of various environmentally hazardous compounds. In the same aspect, we too narrowed down our work towards screening of lignin degrading enzymes.

Production of lignolytic enzymes such as laccase, lignin peroxidase and manganese peroxidase by the fungal isolates was investigated. Among the 28 fungal isolates screened for ligninase production, 13 isolates were ligninase positive \( n = 13 \) and 9 isolates were able to produce lignin modifying enzyme \( n = 9 \). A total of 13 isolates showed positive result for the production of laccase \( n = 13 \) and 6 isolates showed positive result for the production of peroxidase \( n = 6 \) determined by pyrogallol well test.

Since ABTS is a good substrate for the production of the enzyme laccase, it was also used to determine the laccase productivity. ABTS plus LDM was used for the screening of laccase producing fungi. Among 28 fungal isolates, 5 isolates produced the positive result for laccase production \( n = 5 \). Similarly, MnP producing fungi was determined using ABTS minus LDM supplemented with 0.1 g/L manganese chloride. A total of 2 isolates produced positive result for MnP production \( n = 2 \). In the same way, LiP activity was screened for all the 28 isolates in LiP screening medium with guaiacol as a substrate. As a result, three isolates produced positive result for LiP production \( n = 3 \).

Among these 28 isolates, 5 isolates, viz., GRDBF2, GRDBF5, GRDBF17, GRDBF21 and GRDBF23 showed the presence of most of the enzyme activities. These were selected for further characterization. The fungal isolates were subjected to analysis like colony morphology on SDA and LCB wet mount for the identification of fungal genera. Based on the SDA and LCB wet mount characteristics the potent isolates were identified as *Fusarium* sp. (GRDBF2), *Bipolaris* sp. (GRDBF23), *Aspergillus* sp. (GRDBF17) and other two unidentified fungi (Table 1).

The fungal isolates that showed positive result in the screening test were selected for assay. Two different azo dyes viz., aniline blue and trypan blue were used for the assay. All the four potent isolates (GRDBF2, GRDBF17, GRDBF21 and GRDBF23) showed positive result for the assay of aniline blue and one isolate (GRDBF21) showed positive result for trypan blue macroscopically. Decolourisation was further confirmed by measuring the optical density of the culture supernatant (centrifugation at 8,000 rpm for 10 minutes) at 595 nm using colorimeter (Table 2 and 3).

Thus the present study revealed that the most potent isolate, GRDBF23, was able to decolourise only aniline blue to 91.3% but not trypan blue, whereas GRDBF21, was able to decolourise both trypan blue to 91.5% and aniline blue to 82.6%.

### Table 1: Morphological characteristics of the selected fungal isolates on SDA

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Texture</th>
<th>Colour Front</th>
<th>Colour Back</th>
<th>Fungal genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRDBF2</td>
<td>Puffy</td>
<td>Violet with white</td>
<td>Violet</td>
<td><em>Fusarium</em> sp.</td>
</tr>
<tr>
<td>GRDBF5</td>
<td>Puffy</td>
<td>Brownish white</td>
<td>Black</td>
<td>Unidentified fungi</td>
</tr>
<tr>
<td>GRDBF17</td>
<td>Powdery</td>
<td>Green</td>
<td>Light red</td>
<td><em>Aspergillus</em> sp.</td>
</tr>
<tr>
<td>GRDBF21</td>
<td>Flat</td>
<td>Black</td>
<td>Black</td>
<td>Unidentified fungi</td>
</tr>
<tr>
<td>GRDBF23</td>
<td>Puffy</td>
<td>Black</td>
<td>Black</td>
<td><em>Bipolaris</em> sp.</td>
</tr>
</tbody>
</table>
Table 2: Percentage of aniline blue
decolourisation by the potent fungal isolates

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>OD Value Initial</th>
<th>OD Value Final</th>
<th>Decolourisation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRDBF2</td>
<td>0.23</td>
<td>0.05</td>
<td>78.3</td>
</tr>
<tr>
<td>GRDBF17</td>
<td>0.23</td>
<td>0.03</td>
<td>86.9</td>
</tr>
<tr>
<td>GRDBF21</td>
<td>0.23</td>
<td>0.04</td>
<td>82.6</td>
</tr>
<tr>
<td>GRDBF23</td>
<td>0.23</td>
<td>0.02</td>
<td>91.3</td>
</tr>
</tbody>
</table>

Table 3: Percentage of trypan blue
decolourisation by the potent fungal isolates

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>OD Value Initial</th>
<th>OD Value Final</th>
<th>Decolourisation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRDBF2</td>
<td>1.06</td>
<td>0.83</td>
<td>21.6</td>
</tr>
<tr>
<td>GRDBF17</td>
<td>1.06</td>
<td>0.80</td>
<td>24.5</td>
</tr>
<tr>
<td>GRDBF21</td>
<td>1.06</td>
<td>0.09</td>
<td>91.5</td>
</tr>
<tr>
<td>GRDBF23</td>
<td>1.06</td>
<td>0.88</td>
<td>16.9</td>
</tr>
</tbody>
</table>

Kirby et al. have reported that *Phlebia tremellosa* was capable of decolourising eight commercially used textile dyes and an artificial textile effluent, consisting of a mixture of each of these dyes, by greater than 96%. The present study reveals that the fungal isolates such as *Aspergillus* sp., *Bipolaris* sp., *Fusarium* sp. and one unidentified fungi isolated from the bark samples were not only able to degrade the complex heteropolymer structure, lignin but also they possess the capability to decolourise the environmentally hazardous azo dye. Hence these isolates could be further subjected for the degradation analysis of hazardous recalcitrant compounds.

REFERENCES


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Role of Lignocellulolytic *Biopolaris* sp. in the Decolourisation of Textile Dyes

