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Dye Degrading Potential of Immobilized Laccase from Endophytic Fungi of Coastal Sand Dune Plants

Muthezhilan R[1]*, Vinoth S[1], Gopi K[1] and Jaffar Hussain A[2]

[1]Department of Marine Biotechnology, AMET University (U/S 3 of UGC Act 1956), Kanathur, Chennai, India
[2]Centre for Marine Bioprospecting, AMET University (U/S 3 of UGC Act 1956), Kanathur, Chennai, India

***Corres.author: mycomuthu@gmail.com**

Abstract: The dyes discharged from different industries are the source of organic pollutants which are introduced into the natural environment and spoil the biotic as well as the abiotic systems. Although, decolourization of these dyes from water is a challenging process to the industries. Recently, the use of fungi or its enzymes for dye decolourization is an alternative method. In this present study, three different Coastal sand dune plants such as, *Canavalia rosea*, *Ipomea pescaprae* and *Spinifex* sp. were collected from Kanathur Coastal area, East Coast of Tamil Nadu, India. Morphologically 29 different endophytic fungal strains were isolated from different segments (Leaf, Stem, and Root) of sand dunes plants, and they were named as AEF01 to AEF29. These strains were qualitatively screened for laccase production by agar well diffusion method. Among that, four strains namely, AEF17, AEF19, AEF22 and AEF25 have showed maximum laccase production, and they were cultured for laccase production in solid state and submerged fermentation. Aafter the incubation period the laccase was qualitatively assayed. The endophytic fungal strain AEF17 produced maximum laccase in solid state fermentation and it was potentially chosen for optimization studies in one parameter at a time method under different parameters such as pH, temperature, salinity, incubation period and different cheaper substrates. The laccase production was found to be superior at when using a wheat bran as substrate and the optimum pH, temperature, salinity and incubation time were found to be 5, 40^0C,5% and 168 hours. So, the potential fungal strain AEF17 was mass cultured in optimized medium and the laccase was partially purified by ammonium sulphate precipitation and dialysis. The partially purified laccase was immobilized in sodium alginate beads and tested for their ability to decolorize nine different textile dyes. From the results the maximum decolourization activity was found in Blue M2R (BM2R), Black-B (BB) and Orange M2R(OM2R) followed by minimum decolourization activity in Yellow MR(YMR), Red BSID (RBSID), Manenta MP (MMP), Blue MR (BMR), Orange 3R (O3R) and Brown GR (BGR) decolourization. Based on the microscopic identification, the endophytic fungal strain AEF17 was belonging to *Fusarium* sp.
Keywords: Coastal sand dune plants, Endophytic fungi, Immobilized laccase, Dye decolourization.

Introduction

Dyes are intensely coloured complex organic compounds and indispensable part of major industries to colour the textiles, leather, paper, food substances and other materials. Most of the dyes are potent to induce toxological problems in cardiovascular dermatologic, gastrointestinal, genito-urinary, hematologic and central

nervous system[1]. In normal, the direct discharge of these dyes and chemicals containing industrial effluents in open environment is the principal sources of water pollution[2]. So, the removal of dyes and other chemicals pollutants from industrial effluents is a very essential aspect before discharge[3].

The technologies used for dye treatment can be divided into three categories such as physical, chemical and biological methods[4]. Currently, some conventional treatment methods such as activated sludge process, chemical coagulation, carbon absorption, chemical oxidation, photo decomposition, electrochemical treatment, reverse osmosis, hydrogen peroxide catalysis are used for removal of dyes from wastewater[5, 6]. But, these conventional biological treatment processes is not very effective in treating dyes from wastewater, due to low biodegradation of dyes, and the physical or chemical processes are also very expensive and could not be effectively used to treat the wide range of dyes from wastewater[7]. So, there is urgent need of biological products at low cost with ecofriendly for adsorption or decolorization of dyes from industrial waste water.

Many microorganisms especially fungi and its enzymes have the ability to absorb or degrade textile dyes [8]. Recently, fungal laccases have been studied for their degradation of azo dyes[9, 10]. However, due to the dye structures complexity and to the unawareness of enzymatic transformation mechanism, the capacity to describe laccase decolourization pathways still remains incomplete[11] and still now the laccase from endophytic fungi particularly from coastal sand dunes are unexplored. In fact, only very few publications are available on the floral diversity of Indian coastal sand dunes[12]. In this context, the present study was aimed to isolate the laccase producing endophytic fungi from different coastal sand dune plants (such as *Canavalia rosea, Ipomea pes-caprae* and *Spinifex* sp) from Kanathur Coastal area, East Coast of Tamil Nadu, India and to check the decolourization ability of immobilized laccase in nine different dye degradation.

Materials and Methods

Isolation of Endophytic Fungi

Three different Coastal sand dune plants such as *Canavalia rosea, Ipomea pes-caprae* and *Spinifex* sp were collected from Kanathur Coastal area, East Coast of Tamil Nadu, India. The plant materials were washed under running tap water for 10 minutes to remove the dirt. Before surface sterilization, the materials such as, leaf, stem and root were cut into small pieces (0.5 cm) and they were sterilized with 70% ethanol and 1.0 % sodium hypochlorite (NaOCl) (v/v) for 1 minute and further cleaned by passing through sterile distilled water[13]. The surface sterilized samples were placed on petri plate containing potato dextrose agar (PDA) with 200 mg/L concentration of streptomycin to suppress the bacterial contamination. The parafilm wrapped petri plates were incubated for 7 days at room temperature. The pure cultures of fungi were sub cultured in potato dextrose agar (PDA) slants for further study[14].

Screening of endophytic fungi by laccase assay

All the morphologically different strains were inoculated in Potato Dextrose Broth (PDB), the presence of laccase in the obtained mycelium free culture filtrate was qualitatively assayed by water agar medium containing 0.5% Guaiacol substrate. The laccase producing endophytic fungal strains were selected by based on the colour formation around the wells[15].

Mass culture of laccase producing endophytic fungal strains

Inoculum preparation

The laccase producing endophytic fungal strains were transferred into a 250ml Erlenmeyer flask containing 50ml of potato dextrose broth (PDB) and incubated at 30± 0.2^{o}C in rotary shaker at 150 rpm for 7 days. After the incubation period, after which 5.0 ml of this culture was transferred into a 500 ml of Erlenmeyer flask containing 100ml potato dextrose broth (PDB). Similarly, the desired inoculums volume was developed by subsequent transfers [16].

Solid State Fermentation (SSF)

Twenty gram wheat bran moistened with 100 ml mineral salt solution (pH: 5 to 5.2) was sterilized and 1ml of each potential endophytic fungal spore suspension was inoculated in the medium and kept for 7 days incubation in rotary shaker at 200 rpm at 30^{0}C. After, the incubation period the laccase was extracted from fermented bran by 50 ml of citrate buffer (pH 4.8). The pooled extract was filtered with muslin cloth and

centrifuged at 3000-3500 rpm and the laccase production was measured by protein estimation, qualitative and quantitative assay[17].

Liquid State Fermentation (LSF)

Two gram wheat bran moistened with 100 ml mineral salt solution (pH: 5 to 5.2) was sterilized and 1ml of each endophytic fungal spore suspension was inoculated in the medium and kept for 7 days incubation in rotary shaker at 200 rpm at 30^0C. After, the incubation period the laccase production was measured by protein estimation, qualitative and quantitative assay[17].

Optimization of potential Endophytic fungal strain for laccase production

For optimization one parameter at one time method was followed in solid state fermentation. The potential endophytic fungal strain was inoculated in mineral salt medium containing different cheaper sources such as, Wheat bran, Rice bran, Groundnut cake and Cotton seed cake. After incubation period, based on the laccase production the cheaper source was selected. Then, the potential endophytic fungal strain was inoculated in medium containing potential cheaper source and parameters such as, different pH (3.0, 5.0, 7.0 and 9.0), temperature (25^0C, 30^0C, 35^0C and 40^0C), salinity (3%, 6%, 9% and 11%) and incubation period (72, 96, 120, 144, 168 and 192 hrs) were optimized[16].

Purification of laccase

After mass scale culture of optimized medium, 100ml of sterile distilled water was added in mass cultured medium and it was filtered through muslin cloth. The filtrate was centrifuged at 10000 rpm for 10 minutes and the obtained supernatant was partially purified by ammonium sulphate precipitation and dialysis. The laccase assay and total protein was estimated by above mentioned procedures.

Immobilization of laccase

The partially purified laccase were suspended in 3% sodium alginate and this mixture was dripped into cross linking solution made of 0.2 M CaCl2 to form calcium alginate beads. The diameter of beads was found to be in the range of 3 mm to 4 mm. The beads were left in the calcium chloride solution for 3 hours to attain desirable hardness.

Decolourization of textile dyes by using immobilized laccase

In this study the textile dyes such as, Yellow MR (YMR), Blue M2R(BM2R), Black-B (BB), Red BSID (RBSID), Manenta MP (MMP), Blue MR (BMR), Orange M2R(OM2R), Orange 3R (O3R) and Brown GR (BGR) were taken and prepared in the ratio of 100 mg/L. The immobilized laccase beads were inoculated into 25 ml of respective dye containing distilled water. This was incubated for 9 days in static condition at room temperature. The respective dye (100 mg/L) solution incubated without beads was taken as control. After the incubation period, the supernatant was obtained by centrifugation at 10,000 rpm for 10 minutes and the OD values were noted under UV- Vis Spectrophotometer from 594 nm. The decolourization of dyes by immobilized fungal Laccase was calculated by following formula [18].

$$\% \text{ Decolourization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Identification of potential Endophytic fungal strain

The potential endophytic fungal strain was identified by referring standard mycological books and manuals[19-23].

Results and Discussions

In general, fungal laccases have several applications in paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation of dye and their precursors, enzymatic conversion of chemical intermediates and production of chemicals from lignin. In this present study, three different Coastal sand dune plants such as, *Canavalia rosea*, *Ipomea pescaprae* and *Spinifex* sp. were collected from Kanathur Coastal area, East Coast of Tamil Nadu, India. Morphologically 29 different endophytic fungal

strains were isolated from different segments (Leaf, Stem, and Root) of sand dunes plants, and they were named as AEF01 to AEF29. When comparing with plant segments, most of the fungal strains were isolated from root, stem and leaf respectively (Table- 1). Regarding plants twelve different endophytic fungal strains were isolated from *Ipomoea pescaprae,* eleven strains from *Canavalia rosea* and six from *Spinifex species* respectively. Similarly 220 endophytic fungal strains were isolated from 180 root segments of three different sand dune plants such as, *Ipomoea pes-caprae*, *Launaea sarmentosa* and *Polycarpaea corymbosa* and they reported among that 31 species are filamentous[24].

Table 1: Isolation of Morphologically different endophytic fungi from Coastal sand dune plants

Plant Segments	Name of the Coastal sand dune plants		
	Canavalia rosea	**Ipomoea pescaprae**	**Spinifex**
Leaf	2	3	2
Stem	5	4	1
Root	4	5	3
Total No. of strains	**11**	**12**	**6**

While screening all the isolated 29 endophytic fungal strains for laccase by well diffusion assay, four strains namely AEF17, AEF19, AEF22 and AEF25 have showed positive results for laccase production. So, all the four strains were tested for laccase production in solid state and submerged fermentation. Among the four strains the strain AEF17 showed maximum Laccase production in both submerged and solid state fermentation compared with other strains. Within two types of fermentation minimum production (10.00 U/ml) was observed in liquid state fermentation (Figure- 1) and maximum (11.03 U/ml) was observed in solid state fermentation (Figure- 2). So, the potential strain AEF17 was potentially chosen for laccase production in solid state fermentation. In general, very few endophytic strains were able to produce laccase[25]. Endophytic fungi such as *Phomopsis longicolla* (Bo13) of *Bixa orellana*, was significantly highest producer of laccase enzyme followed by *Discosia* sp. (Ci5) from *Calophyllum inophyllum* and *Fusicoccum* sp. (Ac26) followed by *Chaetomium* sp. (Ac 4) from *Alpinia calcarata*[26].

Figure 1: Production of laccase by potential Endophytic fungal strains In Liquid State Fermentation (LSF)

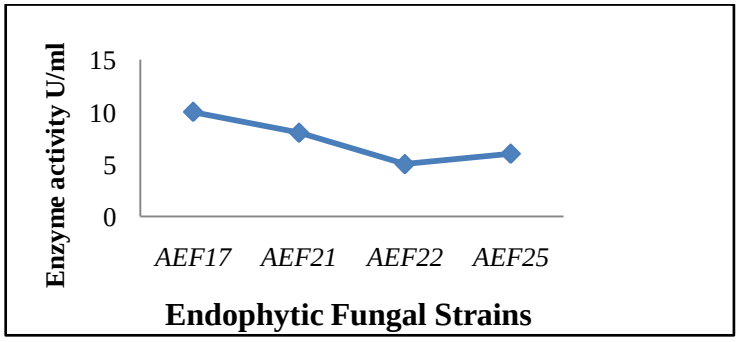
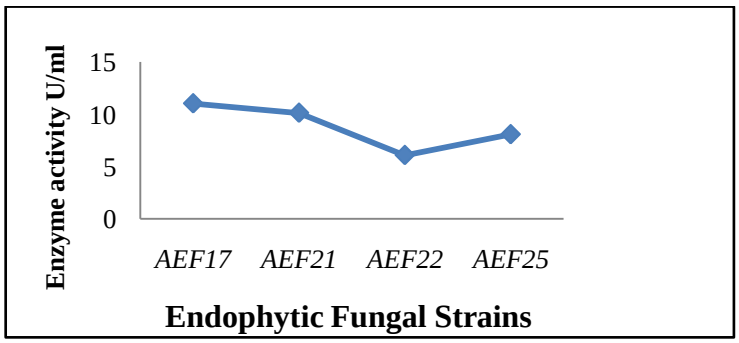


Figure 2: Production of laccase by potential Endophytic fungal strains in Solid State Fermentation (SSF)



The potential endophytic fungal strain AEF17 was optimized by using five different cheaper substrates, the maximum laccase production (15.04 U/mI) was found in wheat bran amended medium. So, the wheat bran was selected for further optimization studies. Similarly the maximum production of endoglucanase enzyme when optimizing using wheat bran in solid state fermentation also observed[27]. While optimizing the laccase production at different physicochemical parameters, the maximum laccase production was obtained at pH 5 (15.07 U/ml), 5% Salinity (16.02 U/ml), 40^{0}C temperature (16.04 U/ml) at 168 hours incubation (17.01 U/ml) (Figure- 3-7). The mass culture laccase was partially purified by ammonium sulphate precipitation and dialysis.

Figure 3: Optimization of laccase production using different cheaper

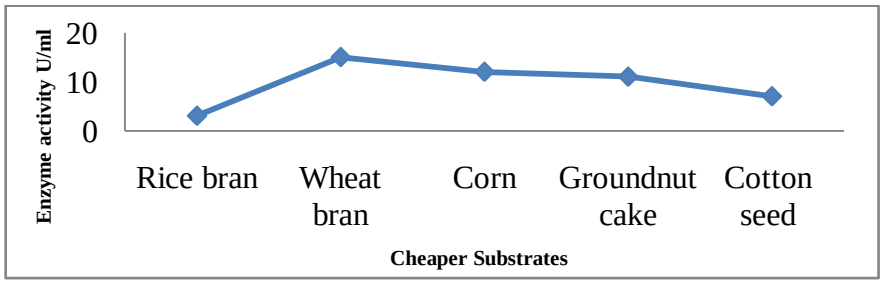


Figure 4: Optimization of laccase production at different incubation time

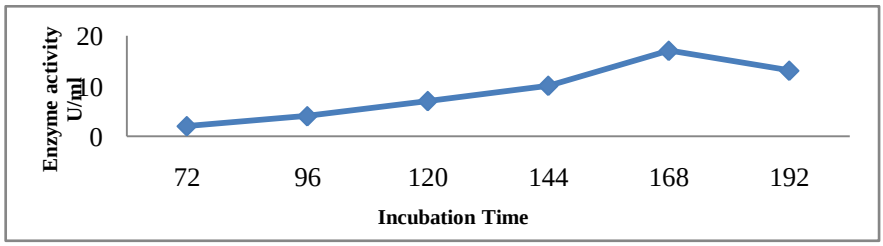


Figure 5: Optimization of laccase production at different pH

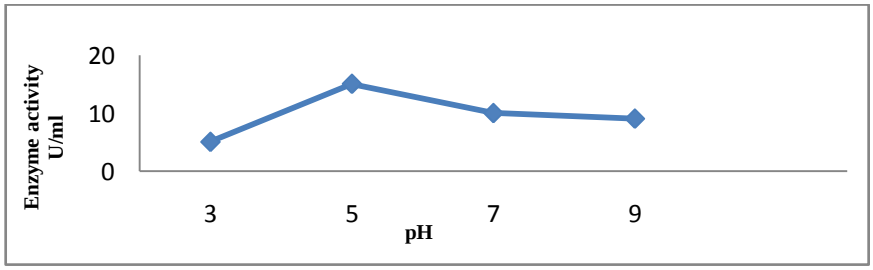


Figure 6: Optimization of laccase production at different salinity

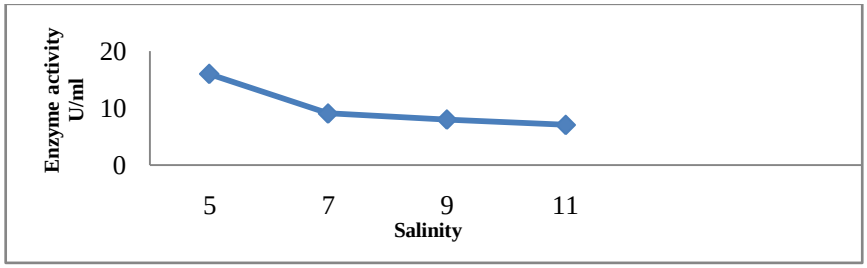
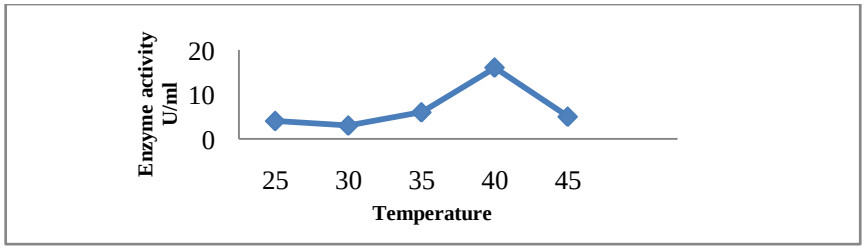
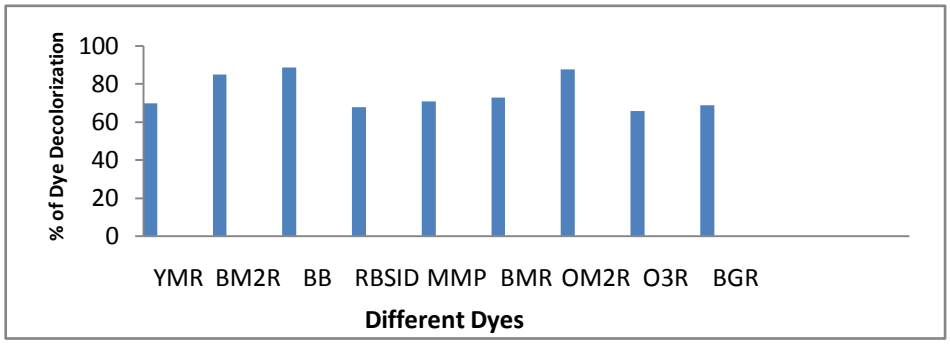


Figure 7: Optimization of laccase production at different temperature



Recently, the laccase from the fungal source have acknowledged the researchers on mycoremediation due to its ability to oxidize both phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants[28]. The obtained laccase was immobilized in sodium alginate beads and tested for their ability to decolorize nine different textile dyes. The white-rot fungi *Phanerochaete chrysosporium, Bjerkandera adusta, Trametes versicolor* or *Phlebia radiata*, are able to degrade dyes[29]. In this study, the maximum decolorization activity was found in Black-B (89%), Orange M2R(88%) and Blue M2R (85%) followed by minimum decolorization activity in Blue MR (73%), Manenta MP (71%), Yellow MR (70%), Brown GR (69%), Red BSID (68%) and Orange 3R (66%) by immobilized laccase of potential endophytic strain AEF17 (Figure- 8). Similarly 34% dye removal prevalence by *Penicillium* sp, *Fusarium* sp and *Aspergillus niger* was found to dominant was reported[30]. And also the decolorizing effect of *Aspergillus niger* in degradation of Red HE7B (82 and 87%) and Yellow FN2R by *Penicillium* sp. (78%) was observed[31]. Similarly after 5 days of incubation decolorizing activity has showed 89% by *Penicillium* sp., and Yellow FN2R was degraded by *Aspergillus niger* (94%) and *Mucor racemosus* (92%) were recorded[32]. The same decolourization activity around 86% by *Aspergillus niger* also observed[33].

Figure 8: Different dyes decolourization activity of immobilized laccase



Based on the microscopic observation it was identified that, the endophytic fungal strain AEF17 was belonging to *Fusarium* spp. From the results, it has been concluded that the immobilized laccase from endophytic fungal *Fusarium* sp. (AEF17) has the dye decolourization ability, and the work also suggested, that using these kind of immobilized fungal laccase for decolourization of dye in industries at large scale level, will definitely prevent the water pollution and keeps the environment clean.

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