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DECOLOURIZATION OF THE SYNTHETIC DYES USING LACCASE ENZYME DERIVED FROM A FUNGUS

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ABSTRACT

This study describes the isolation of fungal strains from mangrove sediment samples, screening for their ability to produce laccase and to decolourize the synthetic dyes and molecular characterization of the potential laccase producer strain. The study indicated promising decolourizing ability by the isolated *Aspergillus* spp. This fungal strain can therefore be used in developing an eco-friendly technology for bioremediation of the dye containing effluents from dyestuff industries.

KEYWORDS: *Aspergillus*, Fungus, Laccase, Enzyme, Dye Decolourization, Phylogeny.

INTRODUCTION

In the recent years, enzymes have gained great importance in Industries owing to their variety of applications. Laccases form a group of enzymes present widely in the nature. These multicopper enzymes catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. These enzymes contain 15–30% carbohydrate and have a molecule mass of 60–90 kDa.[1]

Laccases are well known for their crucial role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation, biodegradation of environmental phenolic pollutant, pesticide or insecticide degradation, waste detoxification

and removal of endocrine disruptors.[2,3,4] Recent studies have reported efficient application of laccases as biosensors in analytical techniques and in the field of nano-biotechnology.

Laccases are widely distributed in higher plants, bacteria, fungi, and insects.[5] Among these, fungal laccase have accounted for the most important group of multicopper oxidase and have been extensively explored.

The present study was undertaken to isolate laccase producing fungi from natural habitats, to screen these laccase positive isolates for synthetic dye decolourization and to identify potential laccase producing fungal strain that are able to decolourize dyes using molecular and phylogentic analysis.

MATERIALS AND METHODS

Sample Collection

Samples for isolation of laccase producing fungi were obtained from Ratnagiri, Maharashtra (West Coast of India). Mud samples from the mangrove areas were collected in sterile zip lock bags and brought to the laboratory.

Isolation of Fungi

Sediment samples were crushed in sterile seawater and 10^{-2} dilutions were spread plated on PDA plates. The plates were incubated at 30°C for 72 hours.

Screening for Laccase production

Total 8 fungal isolates were obtained from sediment samples. The ability of the fungal strains to secrete extracellular laccase was visualized according to the method of Kiiskinen et al.[6] The assay plate containing 15 ml of 4% potato dextrose agar amended with 0.01% of guaiacol was used for screening. Plates were incubated for 72 hours at 30°C. The presence of brick red colour around the mycelium was considered as laccase producing strain. The strain that showed promising production ability was selected for further studies.

Extraction and Purification of laccase enzyme

The fungi culture was fermented by using shake-flask fermentation in PDB. The laccase enzyme was extracted from culture media. The enzyme was partially purified by using Tangential flow filtration (TFF) technique.

Dye Degradation Study using the extracted laccase

The partially purified laccase enzyme was used for the decolourization of Congo red and Crystal violet dyes. Reaction composition employed for the study was as shown in the table1.

Table 1: Reaction composition for the dye degradation study by laccase enzyme.

Reagent	Test	Blank	ABTS Blank
Buffer	20mM	20mM	20mM
Dye	5µg	5µg	0
ABTS	10mM	10mM	10mM
Enzyme	10µl	0	10µl
Final volume by Distilled water	1ml	1ml	1ml

Identification of the fungal isolate by sequence of the amplified ITS region

Molecular phylogeny of the fungal isolate was determined by amplifying genomic partial ITS region. Two primers specific to ITS region used in this study were ITS1F and ITS4R in order to amplify approx. 600bp sequence of fungal ITS gene. DNA Extraction was carried out using Uniflex DNA Isolation Kit (GeNei, 612117000051730). The DNA isolated from the fungal sample was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). For sequencing of PCR product, ITS1F - 5’ CTTGGTCATTTAGAGGAAGTAA 3’ sequencing primer was used. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of the fungus. The tree was constructed by using MEGA 5 software.[7, 8, 9] The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Tamura-Nei method. Evolutionary analyses were conducted in MEGA5.

RESULTS

Isolation of fungus and Screening for laccase

A total of eight fungal isolates were obtained from the mangrove sediment samples after culturing them on PDA (Fig. 1). The colony characteristics were studied and they were screened for the ability to produce laccase. The fungal strain KRDF2 which showed the best ability was selected for further studies (Fig. 2).

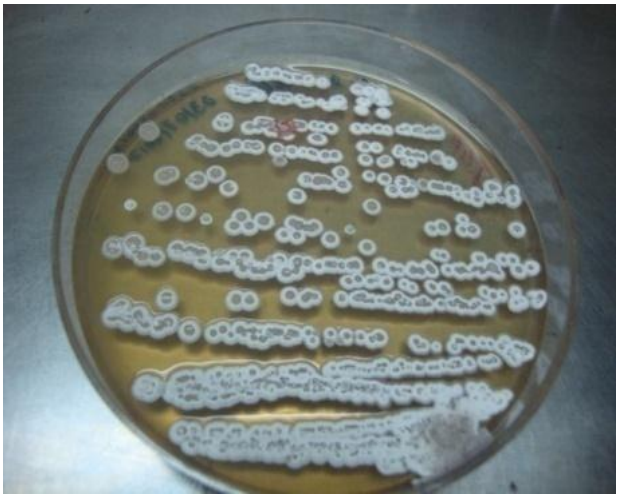

Fig.1. Isolation of mangrove sediment fungi on PDA.

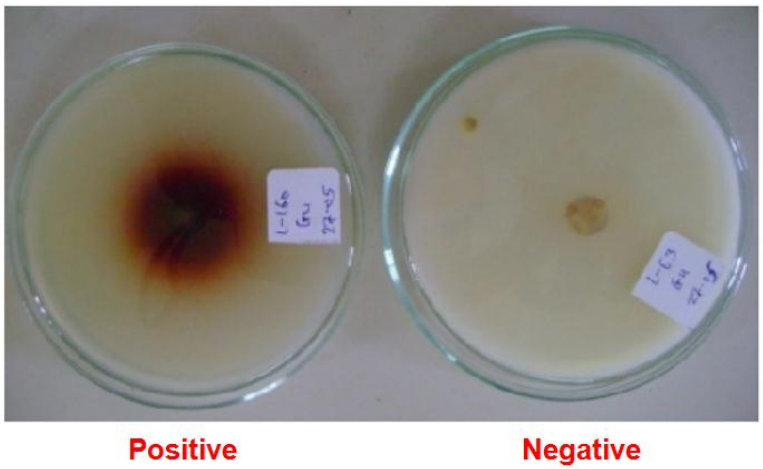

Fig. 2. Laccase enzyme production by fungal isolate on Guaiacol agar plate.

Table 2: Dye Degradation Study using the extracted laccase.

Dye	ABTS	pH	Percentage degradation
Crystal violet	0	2	18.2%
	10µM	2	48.7%
Crystal violet	0	5	5%
	10µM	5	32.9%
Congo red	0	5	1.6%
	10µM	5	76.6%

Dye Degradation Study using the extracted laccase

Results for the dye degradation studies can be seen in Table 2 and Fig. 3. The extracted laccase enzyme from the fungal isolate KRDF2 showed a very promising activity in degrading both the synthetic dyes, crystal violet and congo red employed in the present study.

The decolourization of Congo-red was observed to be 76.6% whereas the decolourization of crystal violet was found to be 48.7% at pH 5.

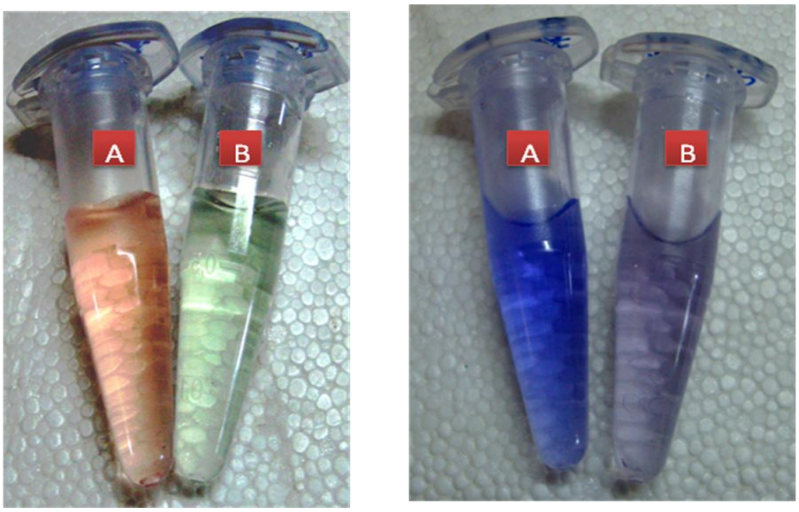

Fig. 3. Decolourization of Congo red (left) and Crystal violet (right) by partially purified laccase. A= Before dye decolourization and B= After dye decolourization.

Identification of the laccase producer fungal strain by sequence of the amplified ITS region

The amplified ITS region produced a sequence of 608 base pairs. The sequence is shown in the Fig. 4. The BLAST results for sequence alignment indicated that the fungal strain was *Aspergillus* spp. as the amplified internal transcribed spacer (ITS) region showed 99% sequence similarity with many *Aspergillu*s spp. existing in the nucleotide database. Phylogenetic tree constructed by using neighbor joining method also confirmed that the laccase producer fungal isolate KRDF2 belonged to genus *Aspergillus* (Fig. 5).

Partial ITS gene Sequence (length 608bp)
>KRDF2
GGGTTTTAGATTCGTAGTGACCTGCGGAGGATCATTACCGAGTGAGGGCCCTC
TGGGTCCAACCTCCCACCCGTGTCTATCGTACCTTGTTGCTTCGGCGGGCCCG
CCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCGCC
GAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCG
TAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGT
CTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGC
GTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCC
CGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCG
TATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCCGGCGCCAGCCGACACCC
AACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
AAGCATATCAATAAGGCCGGGGGGAA

Fig. 4. Sequence of the amplified ITS region of the laccase producer fungal isolate KRDF2.

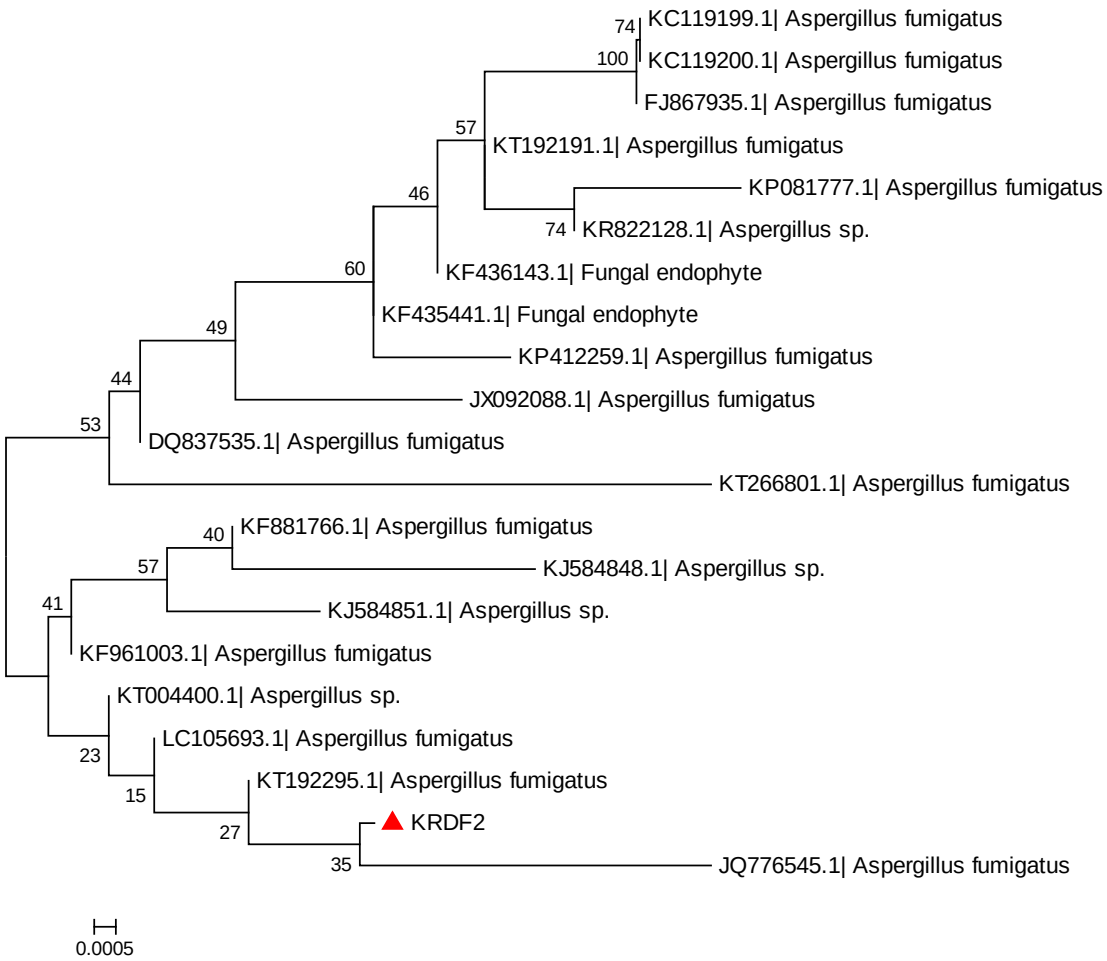

Fig. 5. Phylogenetic tree for the fungus KRDF2 on basis of partial ITS region analysis.

DISCUSSION

The present study supports the previous reports which indicate that fungi prove to be the best source to extract the enzyme laccase with good quality and quantity. Laccases are found to be in large quantities in various higher fungi as they are involved in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis.[10, 11] However, majority of studies report the extraction of laccases from white rot fungi and there are very few reports available on extraction of laccases from *Aspergillus* spp. Therefore, the present study holds a significant importance.

The extracted laccase enzyme in the present study showed promising decolourization of the synthetic dyes. This observation is in agreement with those who have clearly indicated that certain fungal laccase are efficient in dye decolourization.[12-16]

It is already known that the laccase has a great potential application in several areas of food, Biotechnology and Environmental industry. However, one of the limitations for the large-

scale application of laccase is the lack of capacity to produce large volumes of highly active enzyme at an affordable cost. The use of inexpensive sources for laccase production is being explored in recent times. In this regard, as highlighted in this paper, mangrove areas could prove as a promising site to explore such laccase producing strains.

CONCLUSION

To conclude, *Aspergillus* sp. isolated from mangrove sediments was found to have potential in producing laccase enzyme. This laccase enzyme obtained from the fungus was found to be effective in degrading both the dyes. The decolourization of Congo-red was observed to be 76%. The decolourization of crystal violet was 48%. This investigation highlights the importance of fungi derived laccase enzyme for the decolourization of synthetic dyes.

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