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Advances in Bioresearch

## ORIGINAL ARTICLE

# Screening and Identification of Laccase Producing Fungi from Various Environment Samples of East Gujarat

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**ABSTRACT**
*Laccase is a copper-containing polyphenol oxidase that works on a number of different substrates. This enzyme is found in many plant species and fungi, including wood-rotting fungi, where it is commonly linked with lignin peroxidase, manganese dependent peroxidase, or either. Laccase-catalyzed dye molecule synthesis is an eco-friendly alternative to traditional synthetic processes. The textile industry will benefit from this green technology because the synthesised dyes can be used to colour various fabrics but also it is important in bioremediation, and removal of environmental effluents. In this research work, primary and secondary screening of fungal cultures were done using the plate test method and the indicator compound guaiacol and ABTS. 12 of the 25 cultures tested were laccase-positive, and confirmed using spot assay techniques. Fungal cultures with potent oxidation zone were processed for further assessment.*
***Key words:*** *Laccase, guaiacol, isolation, dye-degradation.*

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## INTRODUCTION

Fungi represent the second largest group of eukaryotic organisms on earth, with estimates ranging from 1.5 to 5.1 million species. ( 1,2) . Members of the fungal kingdom play significant roles in human life and have the ability to occupy a wide variety of natural and artificial niches. (3) Identification of fungi to species level is paramount in both basic (ecology, taxonomy) and applied (genomics, bioprospecting) applications in scientific research. This is especially true for natural products researchers working with fungi as a source of bioactive secondary metabolites. Scientific names are crucial in communicating information about fungi, enabling researchers to identify other closely related species to better predict evolution of chemical gene clusters, (4) or to prioritize taxonomically related strains, when a productive strain may attenuate production of key bioactive compounds. (5) More importantly, taxonomic identification of fungi is essential if industrial, agrochemical, or pharmaceutical products are to be derived from a fungal strain. Synthetic dyes are widely used for coloring the products of several industries such as textiles, leather, cosmetics, paper, printing materials, and plastics. It is estimated that 1–2% of dye production is lost, and 5–10% is discharged to the environment when the dyes are used.( 6,7). Several dyes and the chemicals used to produce them are often toxic, carcinogenic, or even explosive (8,9,). Effluents from industries that use various dyes are considered as pollutants that can cause severe environmental problems as well as medical and esthetic problems (8,9). The decolorization of this industrial waste is a challenging task because certain dyes are resistant to degradation. (7,8). Physical and chemical decolorizing methods have been developed to remove dyes from wastewater; however, several of them have disadvantages such as high costs and/or limited applicability.( 7,10). Studies on the capability of microorganisms to perform dye decolorization has received increasing attention because the use of microorganisms is considered a cost effective and environmentally friendly method for removing organic dyes from wastewater before they are discharged.( 11 , 12) Likewise, the capabilities and

mechanisms of decolorization by bacteria have been studied.( 12,13). However, the application of bacteria was limited due to the narrow substrate range of various degrading bacteria (13). Moreover, to complete the degradation of dyes, different groups of bacteria are required in an alternation process from anaerobic to aerobic conditions.( 14)

Research on the fungal decolorization of dye wastewater has been performed in recent years. (15,16). Several fungi with the capability to decolorize a wide range of dyes have been reported. For example, the white-rot fungi and brown-rot fungi are well-studied fungi groups with decolorization abilities.( 21,22) Other fungi, such as *Aspergillus niger*, *Rhizopus arrhizus*, and *R. oryzae*, can also decolorize and/or absorb diverse dyes and possess excellent color removal capabilities. (18)

The mechanism of fungal decolorization mainly involves two aspects, biodegradation and biosorption.( 21) The biodegradation capability of fungi is due to their extracellular, non-specific and non-selective enzyme system. Fungal enzyme production depends on nutrient limitations, and their subsequent dye decolorization ability is achieved depending on the growth conditions.(23) Considering the complex environmental factors involved in the dye wastewater conditions, the screening of more fungi is necessary for use in dye decolorization. Aquatic fungi are the main decomposers of aquatic ecosystems and play crucial roles in the cycling of nutrients. (S. Duarte et al 2006) In addition, a unique characteristic of fungi is their ability to produce several non-specific enzymes.(1,2) These non-specific extracellular and/or exoenzymes enable the aquatic fungi to attack structurally diverse organic compounds that correspond to different pollutant classes. (15)Hence, these fungi may serve as a new resource to treat wastewater. However, little research has been performed on the process of decolorizing wastewater by freshwater fungi,(15,16) and more fungi with potential for the biodegradation of synthetic dyes need to be explored. In the present study, several freshwater fungi were isolated from streams in the Zhejiang Province, PR China, and their ability of decolorizing multiple synthetic dyes was evaluated.

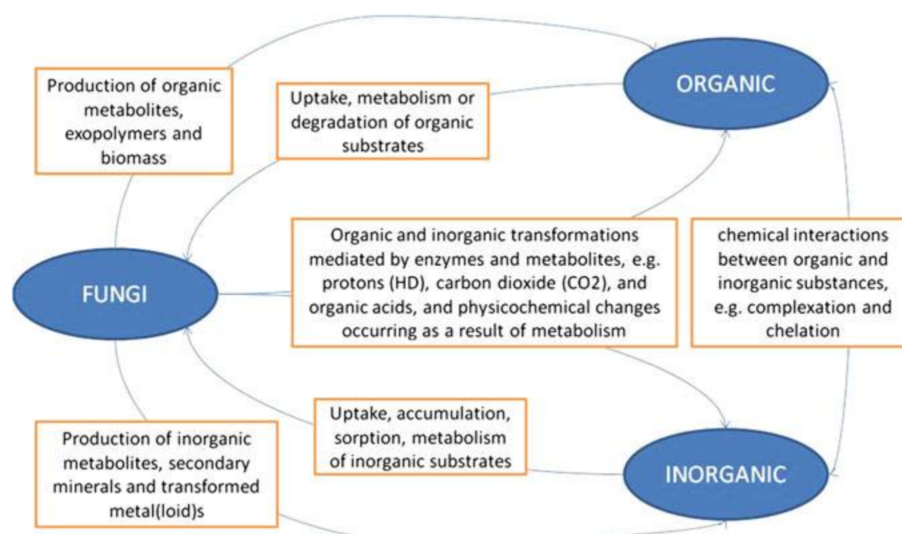


**Fig. 1- Simple model of fungal action on naturally-occurring and/or anthropogenically—derived organic and inorganic substrates (17)**

## MATERIAL AND METHODS

### Screening of Laccase Producing Fungi

The samples were homogenized and suspensions were made with dilution up to 10-5 in sterile distilled water. The prepared suspensions were used for inoculating onto the plates. Plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 4 mM tannic acid as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of dark brown coloured halo around the fungal colonies. The isolates showing positive results were used for further studies. Another plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 3 mM ABTS (2-2’-Azino-bis-[3- ethyl benzthiazoline-6-sulfonic acid] ) as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of green coloured halo around the fungal colonies. (18)

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Bavendam test: Confirmation of laccase production was done by Plate assay technique using 10 mM guaiacol as a substrate. The colour change from colour less to reddish brown colour was to be observed in fungal culture plate for presence of laccase (18).

**Laccase activity*:*** The laccase activity was measured by spectrophotometer using guaiacol as a substrate .Crude enzyme extract from the liquid culture medium was collected under aseptic condition. The reaction mixture consists of 3 ml of 100 mM of guaiacol dissolved in 10% acetone in sodium acetate buffer and 1 ml of culture filtrate (crude laccase). The mixture was incubated at 28°C for 60 min and the absorbance was recorded at 470 nm. One unit of laccase activity was defined as the amount of enzyme catalyzing the substrate (guaiacol) for the production of 1 µl of colored product per min per ml (18). Laccase activity was measured as decrease in absorbance of Guaiacol reagent (substrate) due to laccase enzyme (1ml) in 60 min (18, 19). It was calculated by the following formula;

**Molecular Identification and Phylogenetic Analysis**

Fungal genomic DNA was obtained by culturing monosporic isolates at 28 °C on sterile cellophane membrane overlaid on PDA plates. Fungal cultures were easily detached from the cellophane membrane using sterile spatulas and genomic DNA was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in 50 µL PCR reactions. Reaction conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 34 cycles of DNA denaturation at 94 °C for 45 s, primer annealing at 55 °C for 45 s, and DNA synthesis extension at 72 °C for 45 s, followed by a single step of final extension at 72 °C for 10 min.

Obtained PCR products were visualized on 1.5% agarose gel with 1× tris TAE buffer and E-Gel Sizing DNA Ladder (Invitrogen) was used as a reference to estimate amplicon size. PCR products were prepared for sequencing by purification with a GenElute PCR purification kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. After the PCR products were sequenced using Sanger sequencing protocol, the obtained sequences were blasted against the NCBI database to identify the isolates. Published sequences representative for fungal ITS regions were retrieved and aligned with obtained sequences using MEGA11 software . A phylogenetic tree was constructed with the maximum likelihood algorithm and validated with 1000 bootstrap replicates.(20)

## RESULTS AND DISCUSSION

**Primary screening of laccase producing fungi:**

Fungi were screened using plate assay techniques for laccase enzyme using 3mM ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]), 10mM guaiacol(Bavendem test), and 4mM tannic acid as a substrates on PDA plates.

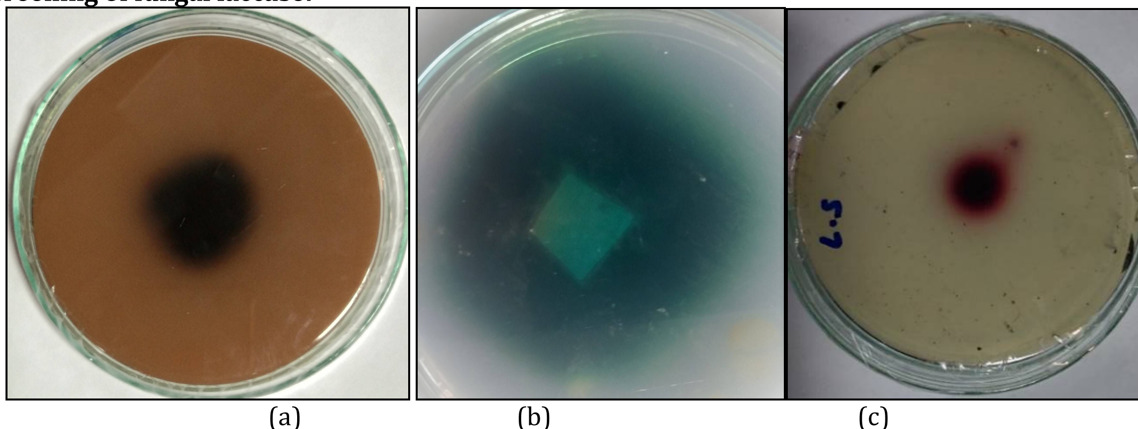
Where different potential reduction zone was observed in laccase producing fungi using various substrate.

**Secondary screening of laccase producing fungi:**

For confirmation of potent laccase producing fungi, bavendem test is done using 4mM guaiacol where , reddish brown reduction zone is been observed.

13 fungal isolates which were showing potential reduction zone on PDA plate which were considered for further process.

**Screening of fungal laccase:**




(a) (b) (c)

a. tannic acid screening-dark brown oxidation zone
b. ABTS screening-dark green zone
c. guaiacol screening-reddish brown oxidation zone

## Specificity of substrates

Reactions of three different substrates such as tannic acid, ABTS, and guaiacol with fungal isolates on Potato dextrose agar plate. From given isolates few isolates failed to give reactions with all three isolates . where few showed potential reduction on all three substrates.

**Table -1 Comparison of reactions of the fungal isolates on potato dextrose agar**

| Fungal Isolate | ABTS | Guaiacol | Tannic acid |
|---|---|---|---|
| Sk1 | + | + | + |
| Sk2 | + | + | + |
| Sk3 | + | + | + |
| Sk4 | + | + | + |
| Sk5 | + | + | - |
| Sk6 | - | + | - |
| Sk7 | + | + | + |
| Sk8 | + | + | - |
| Sk9 | + | + | + |
| Sk10 | - | + | + |
| Sk11 | + | - | - |
| Sk12 | + | - | - |
| Sk13 | + | + | - |

From 13 isolates, fungus showing potential zone in secondary screening were proceeded for further activity. From the results of primary and secondary screening laccase producing fungi , three fungi that were showing prominent result with highest zone of reduction in all the substrates were further processed for identification using 18S rRNA gene sequencing technique.

**Molecular Identification-** The strain which was showing potent ABTS and guaiacol activity was choosen for the molecular identification and the genomic DNA of this strain was extracted using modified CTAB method. Mycelia of the species was grown on MEA culture plates were scrapped off and placed in 1.5 ml tube containing 600 µl CTAB buffer. With a sterilized pestle, the mycelia were ground to release the DNA, and then, incubated at 65oC for 15 minutes in a heat block machine. Afterwards, 600 µl chloroform: isoamyl alcohol (C:IAA 24:1) was added, mixed by inverting the tube slowly for more than 50 times, and then, centrifuged at 13,000 rpm for 15 minutes at 25 oC. The upper aqueous phase was pipetted to a new tube and to this, 300 µl cold isopropanol was added and later kept in the freezer at (-20oC) for 20 minutes. The tubes were again centrifuged at 13,000 rpm for 5 minutes at 4oC. The supernatant was discarded and the DNA pellets was washed with 70% EtOH, and then, air dried until EtOH has totally evaporated. Finally, the DNA was diluted in 50 µl TE buffer. The genomic DNA of the macro fungi were then subjected to PCR to amplify the ITS regions of the nuclear ribosomal DNA using two primers: ITS 1 (5′TCCGTAGGTGAA CCTTGCGG 3′) and ITS 4 (5′TCCTCCGCTTATTGATATGC3′) (White et al., 1990). The PCR reaction included 1 x PCR buffer, 2.5 µM MgCl2, 200 µMdNTP, 0.5 µM of each primer, 1 U Taq DNA polymerase, and 50-100 ng extracted genomic DNA and nanopure water to make a volume of 50 µl. The PCR conditions are as follows: 94 oC for 3 min, followed by 30 cycles at 94 oC for 1 min, 52 oC for 50 sec and 72 oC for 1 min, with a final extension step of 72 oC for 10 min . The PCR products were then purified using QIAGEN purification kit following the manufacturer's instructions and the purified PCR products were sent to Scigenome Cohin, Kerala, India for outdoor DNA sequencing. The sequences of fungus were compared using the GenBank database and basic local alignment search tools (Blast) for nucleotide analysis. The identification was confirmed with >98% similar to the fungal species deposited in GenBank. The sequence of the organisms is identified as shown in table.

Phylogenetic tree for the same was generated using MEGA-10.0 software.

## Characterization of fungal laccases

**3 fungal isolates** which were showing highest yield were proceeded for 18S rRNA gene sequencing MEGA 10.0 software BLAST tool.

**Table 2: Characterization of laccase producing fungi**

| Organism | Cultural Characteristics | Morphological characteristics | Identified as |
|---|---|---|---|
| **SK 3** | Cottony white growth and aerial hyphae | Filamentous, spore forming, Septate mycelia | *Aspergillus fumigatus* strain F029 |
| **SK 9** | White and pale yellow pigment, non-dense | Filamentous, spore forming, Septate vegetative mycelia | *Aspergillus ochraceus strain LW3* |
| **SK 5** | Cottony, White with Grey centred | Filamentous, spore forming, Septate mycelia | *Aspergillus nidulans strain KU20018.62* |

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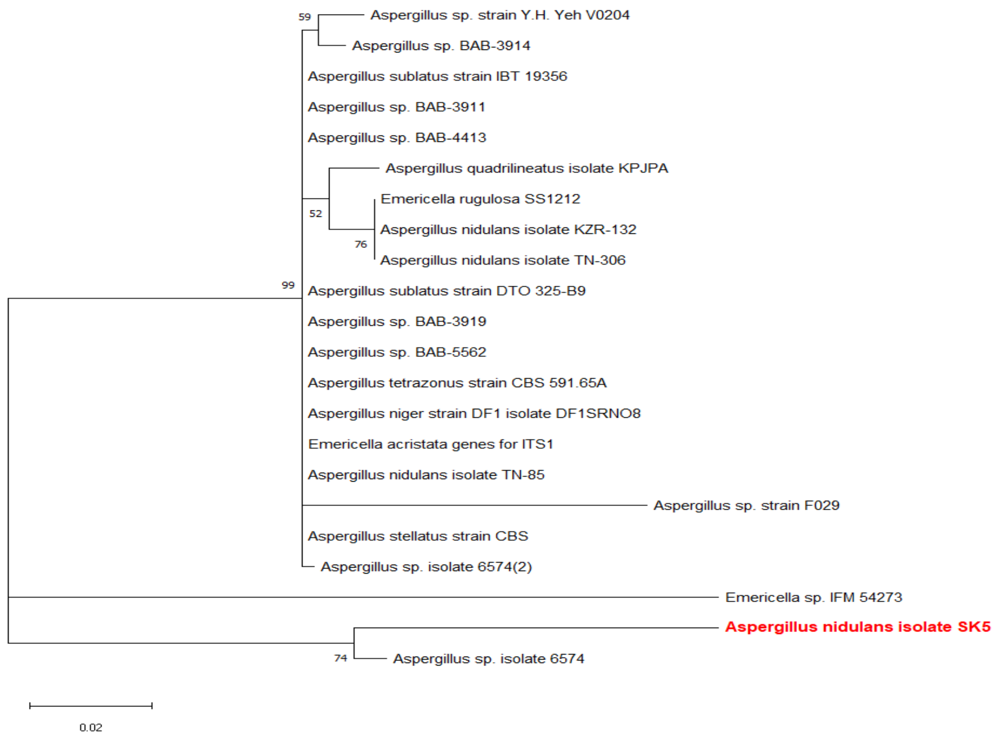


**Figure 1-** Phylogenetic Tree of Sk-3 Fungal Isolate With Accession No - *Oq622074* Using Mega-10 Software

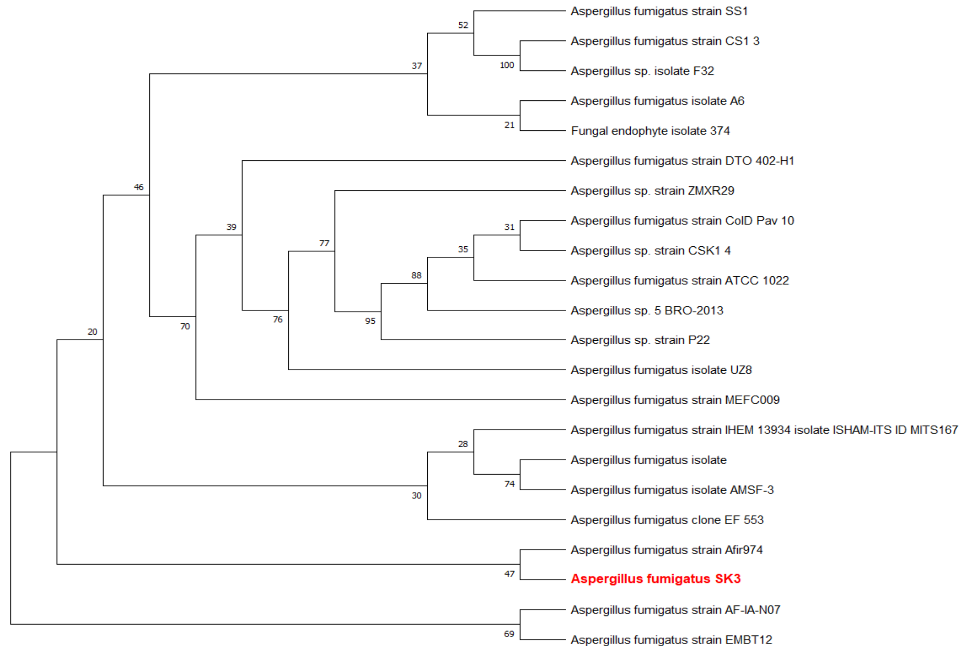


**Figure 2 Phylogenetic Tree of Sk-5 Fungal Isolate With Accession No Oq622228 Using Mega-10 Software**

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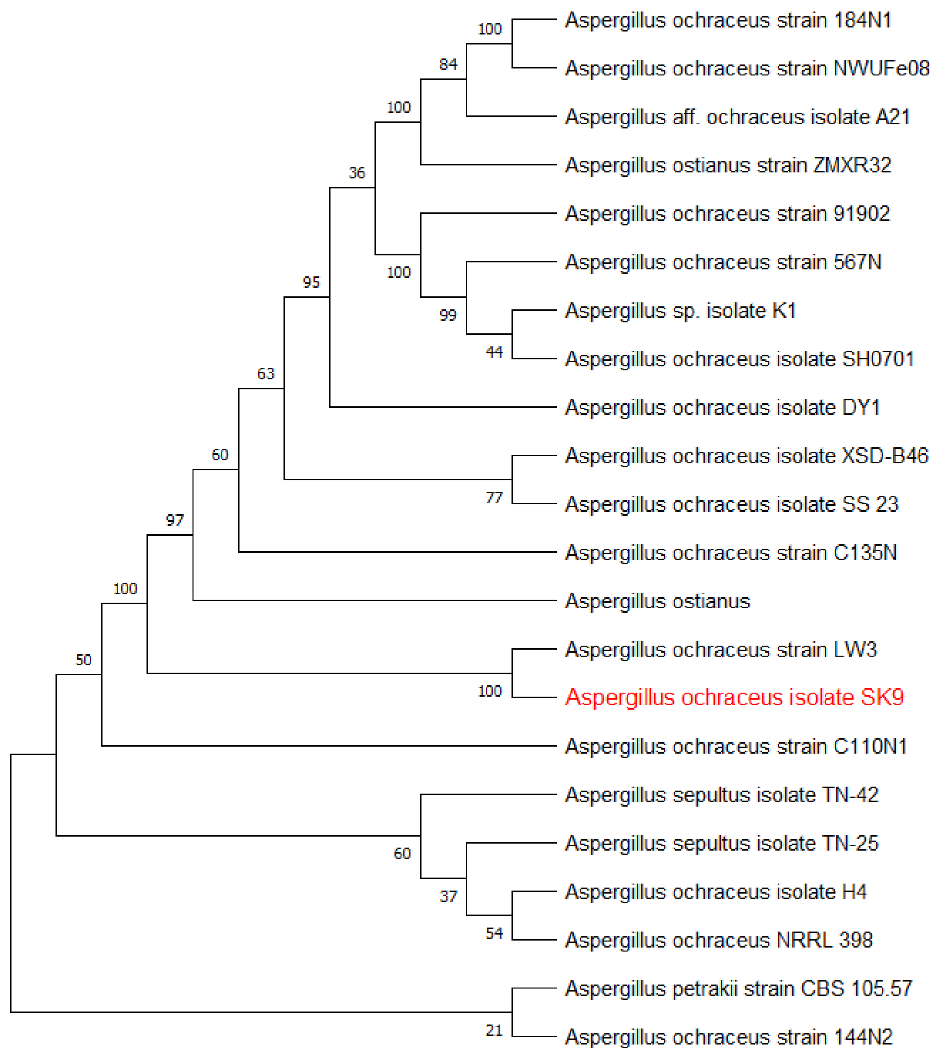


**Figure 3 PHYLOGENETIC TREE OF SK-3 FUNGAL ISOLATE WITH ACCESSION NO - *OQ625837* USING MEGA-10 SOFTWARE**

## CONCLUSION

The laccase producing potential isolate SK3,SK6,SK9 was isolated from bark of tree and used for enzyme production. Based on the screening results using various substrates of ABTS, guaiacol and tannic acid, laccase positive test were confirmed and production was carried out . And after the identification process , the fungal isolates SK3,SK6 and SK9 were identified as *Aspergillus fumigatus sp., Aspergillus ochraceus sp ,Aspergillus nidulans sp. Respectively .* The use of the laccase may conceivably be extended to other anthraquinone-type textile dyes, indeed suggesting a potential application field for the removal of dyes from industrial effluents

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