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# Decolorization of textile dye by laccase from newly isolated endophytic fungus *Daldinia* sp.

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## ABSTRACT

**In view of application of laccase in bioremediation of textile dyes, a total of six endophytic fungi were isolated from needle of healthy Gymnosperm plant,*Cupressus torulosa* D. Don of Garhwal, Himalayan region. The colonization rate of these fungi on PDA was higher for various sampling sites in S1 (90) >S2(60) > S3 (40) but colonization rate of fungi in KMM was S3(73) > S1(60)> S2(40). All endophytic fungi were screened for laccase assay as it plays a crucial role in detoxification of toxic dye for human health. Out of six fungi only one endophytic fungus known as KCT34 showed significant laccase activity in solid and liquid media. Initial screening on the malt extract agar plates supplemented with 200 mg $L^{-1}$ of each Congo red, Rosebangal, Orange G and Rhodamine B dye showed that KCT34 was able to decolorize all of the four dyes. Quantitative analysis of the dye decolorization showed that KCT34 was able to decolorize textile dyes efficiently and dye decolorization percentage was maximum on 16$^{th}$ day with percentage ranging from 90.45% for Congo red, 87.91% for Rose bangal, 85.81% for Orange G and 54.39% for Rhodamine B. Molecular identification of the fungal isolate KCT34 using primer ITS1 and ITS4 showed 99% sequence similarity with *Daldinia* sp, an Ascomycetous fungus. The ability to decolorize different types of textile dyes by the isolated *Daldinia* sp. suggested a possible application of fungal endophytes in the decolorization of dyestuff effluents from textile, food and aquaculture industries.**

**Keywords:** Decolorization, Laccase, bioremediation, textile dye, endophytic fungi, *Daldinia* sp.

## INTRODUCTION

Water is basic need of life and used for number of household as well as industrial activities. Being one of the essential natural resource, it is unfortunately exploited the most. Because of its unrestricted and excessive exploitation, presently the world is facing crisis of drinking water. Increased urbanization of natural resources like, increase in textile industries, possess a threat to the aquatic bodies as these industrial effluents with various toxic components, basically dye compounds deteriorate the quality of water and makes it harmful for further usage. Approximately 10,000 dyes are commercially available, with an annual production of over 7 x $10^5$ metric tons (Campos *et al.,* 2001). Over 50% of these dyes are released in the industrial effluent (Zollinger, 2003). These dyes contain high amount of heavy metals and organic compounds and they contribute in textile industries water pollution as 93% of the intake water comes out as colored wastewater (Gupta *et al.*, 2014).

Wastewater released from various textile industries are highly variable and complex mixture of many polluting substances, especially dyes, inducing color coupled with organic load which leads to disruption of the total ecological balance thus disturb the whole aquatic ecosystem (Hassani *et al*., 2008). The non-biodegradable nature of the dyes in the spent dye baths of textile industries constitutes a serious environmental hazard. Most of the dyes induce toxological problems in cardiovascular, dermatologic, gastrointestinal, genito-urinary, hematologic and central nervous system (Harvey and Keitt, 1983).

In general, the direct discharge of the chemicals especially dyes in the effluents from various textile industries in open environment is the principle sources of water pollution (Ramesh Babu *et al*, 2007). Hence, the removal of dyes and other chemical substances from industrial effluents is very important before its discharge into open water (Balaji *et al.*, 2012).

Physical or chemical processes could not be effectively used for treatment of dyes from wastewater as those processes are not economically viable (Grag *et al.*, 2003). Due to low biodegradability of dyes, conventional biological treatment processes are also not much successful in treating dye containing wastewater. Therefore, innovative treatment technologies such as low cost biological conversion based technologies of ecofriendly nature are needed to be investigated for adsorbing or decolorizing dyes from waste water released from textile industries. Many microorganisms including fungi and its enzymes have the ability to decolorize textile dyes (Forgacs *et al.*, 2004). Recently, *in situ* degradation of dyes in wastewater using fungal metabolic activities is the subject of many research studies.

Fungal laccases are known to be effective to degrade azo dyes (Blanquez *et al.*, 2004). Laccases are used in commercial textile applications to improve the whiteness in conventional bleaching of cotton process. Laccases of fungi are of particular interest with respect to potential industrial applications as a result of their capability to oxidize a good range of industrially relevant substrates. Oxidization reactions are comprehensively utilized in industrial processes, for instance, within the textile, food, wood process and pharmaceutical and chemical industries (Onuki *et al.,* 2000). Laccases from fungi provide many advantages and thus are of great interest in biotechnological applications of industrial effluent treatment. Laccases exhibit broad substrate specificity. They can bleach Kraft paper pulp or detoxify agricultural byproducts as well as olive mill wastes or even coffee pulp (Watanabe *et al.,* 2007).

In this context, the present study is aimed to isolate the laccase producing endophytic fungi from leaves of *Cupressus torulosa* D. Don from different sites of Pauri, Garhwal region, Uttarakhand, India and to check its decolorizing ability for different dyes used in textile industries.

## MATERIAL AND METHODS

**Isolation and sterilization of endophytic fungi:** Mature leaves and twigs of *C. torulosa* tree were collected from

different sites of GBPEC, College campus, Ghurdauri, Pauri, located at latitude 3018'35"N and longitude 7869'30"E. The plant was taxonomically identified and authenticated by Botanical survey of India, Dehradun. The voucher specimen was deposited there with accession number 115744. The plant materials were transported to the lab in sterile polythene bags and stored at 4ºC until processed.

Isolation of endophytic fungi were carried out using the protocol given by Atalla *et al*.,( 2010) with slight modifications as described by Sharma *et al*. (2016) . In short, the healthy *C. torulosa* plant tissues were washed under running tap water and samples (leaves and twigs) were cut into size of an inch. Samples were then surface sterilized sequentially with 70 % alcohol for 3 min, 0.5% sodium hypochlorite for 1 min, rinsed twice with sterile water followed by drying on sterile filter paper (Arnold *et al*., 2000) and excess water was drained from the surface of the sample by touching to the sides of the beaker before placing them on the Potato dextrose Agar (PDA) media supplemented with antibiotic streptomycin (200 mg/L).

The petri dishes were sealed with parafilm and incubated at 28ºC ± 2ºC for 2-4 weeks in incubator. Fungal growth was observed from the tissue segments inoculated on the water media plates as colored cottony outgrowth. Hyphal tips, from germinating fungi, were isolated, sub cultured onto PDA and brought into pure culture by incubating at 28ºC for 5-7 days. The purified fungal isolates were maintained on Potato dextrose agar slant at 4ºC in refrigerator for further studies.

**Morphotypic identification of endophytic fungi:** Fungal growth and sporulation was facilitated by placing the isolates onto PDA growth medium. The plates were continuously visually monitored for spore formation. Fungal isolates were identified on the basis of cultural characteristics, color and morphology of fruiting bodies and spores. Fungal isolates were stained with Lacto Phenol Cotton blue and examined under light microscope (Olympus, USA) (Bhardwaj *et al*., 2015).

**Molecular Identification of the endophytic fungi:** The isolated endophytic fungus was initially identified through 18S rRNA sequence. For molecular identification, total genomic DNA of the endophytic fungus was isolated directly from actively growing mycelium growing in potato dextrose broth (PDB), using DNA extraction kit, Genei. Fungal DNA was extracted in the laboratory using the protocol of Kariyawasam *et al.* (2012). The extracted DNA was subjected to the polymerase chain reaction (PCR) using primers ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTGATATGC (White *et al*., 1990; Sharma *et al*., 2016). Amplified DNA was subjected to DNA sequencing and this DNA sequence was compared with already existing DNA sequences in NCBI GenBank database (http://www.ncbi.nlm.nih.gov.blast) to identify the fungi. PCR and DNA sequencing was done by the Gujarat State Biotechnology Mission, India. The obtained sequence was submitted in National Centre for Biotechnology Information with accession number KT355731.

**Endophytic fungal diversity analysis:** The Colonization rates of fungi were calculated as the percentage of segments colonized by one or more isolate(s) from the total number of segments of each tissue plated following the method of Petrini and Fisher (1988). Samples were incubated and growth was analyzed regularly for 6 weeks and colonizing rate was calculated.

$$\text{Colonization Rate (\%)} = \frac{\text{Total no. of segments infected by fungi}}{\text{Total no. of segments analyzed}} \times 100$$

**Evaluation of laccase activity in solid media:** The fungal isolates were screened for laccase production by growing them on plates of GYP medium containing 4mM guaiacol, 2mM ABTS, 0.5mM syringaldehyde, 0.5µM Tannic acid (Kumar *et al*. 2011). The petri dishes were inoculated with the isolated fungus and were incubated at 28˚C in dark for 2 weeks. The production of intense brown color, reddish brown colour, purples colour under and around the fungal colony was considered as a laccase positive reaction resulting from respectively guaiacol and tannic acid, syringaldehyde and ABTS oxidation.

**Evaluation of laccase activity in liquid media:** Laccase activity was assayed at 28˚C using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)1 or guaiacol or syringaldehyde as substrates as follows. (*a*) The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (e =36,000 $M^{-1}$ $cm^{-1}$). Enzyme activity was expressed in international units (IU). (*b*) The assay mixture contained 4mM guaiacol, and the McIlvaine's citrate-phosphate buffer adjusted to pH 6.0. Oxidation was followed by the absorbance increase at 460 nm. (e=6740 $M^{-1}$ $cm^{-1}$) One unit of activity is the amount of enzyme producing a 1.0 *A* increase/min. (*c*) The assay mixture contained 0.5 mM syringaldazine (dissolved in ethanol) and 50 mM phosphate buffer, pH 6. Oxidation of syringaldazine was followed by an absorbance increase at 525 nm (e=65,000 $M^{-1}$ $cm^{-1}$). Enzyme activity was expressed in IU.

**Enzyme Assay:** For this purpose following relation was employed

Enzyme activity (U/ml) = $\Delta A_{470}$ nm/min×4×Vt×dilution factor/€×Vs

Where, Vt=final volume of reaction mixture (ml) =5

Vs=sample volume (ml) =1

€=extinction coefficient of guaiacol=6,740/M/cm

4=derived from unit definition and principle

**Evaluation of dye decolorization activity in solid media:** Fungal endophytes were grown onto Glucose Minimal (GM) agar plates and initially screened for their ability to decolorize azo dyes eg. Orange G, Congo red, and rhodamine and rose bangal (Ngieng *et al*., 2013). The GM agar media contained (g/L): $K_2HPO_4$, 1; $ZnSO_4.7H_2O$, 0.01; $CuSO_4.5H_2O$, 0.05; $MgSO_4.7H2O$, 0.5; $FeSO_4.7H_2O$, 0.01; KCl, 0.5; glucose, 10; $NaNO_3$, 3; and agar, 20. The pH of the agar medium was adjusted to 5.5 before being autoclaved at 121ºC for 15 min. Dyes were added into the agar media from a sterile stock solution to a final concentration of 200 mg $L^{-1}$. The fungi were

cultivated during 20 days at 28˚C in the dark. Uninoculated plates with the respective dyes were used as negative control. Observation for the rate of decolorization was made twice every week (Narkhede *et al*., 2013). The decolorizing activity was described as positive when the solid media exhibited partial or total loss of color imparted by the respective dyes. The fungal strains with decolorizing activity were further selected for the liquid media evaluation.

**Evaluation of dye decolorizing activity in liquid media:** Dyes were added to the 50 mL GM liquid medium in 250 mL Erlenmeyer flask to a final concentration of 50 mg $L^{-1}$. Each flask was inoculated with 2 pieces of $5mm^2$ agar plugs from a 7day old fungal culture and incubated in the dark at room temperature under static condition. Flask with the respective dye and no fungal inoculum was used as control. Each culture condition was prepared in triplicate, incubated for a period of 16 days, and sampled at 4-day interval. During the sampling, each culture was harvested and centrifuged at 6000 rpm for 10 minutes to separate the fungal mycelium from the culture medium. Fungal biomass was determined by drying the fungal mycelium to a constant weight at 70ºC. Dye decolorization by the isolated fungus was measured by monitoring the absorbance of each dye in the culture medium at its respective maximum absorption wavelength (475 nm for Orange G, 497 nm for Congo red, 590 nm Rhodamine, and 549 nm for rose bangal) using a UV-Vis spectrophotometer (Lab India). Percentage of decolorization was calculated according to the following formula:

$$\% \text{ Dye Decolorization} = \frac{\text{Initial Absorbance (Ac) - Final Absorbance (As)}}{\text{Initial Absorbance (Ac)}} \times 100$$

Where $A_c$ is the absorbance at the maximum absorption wavelength of dye in the control flask at time, $t$ and $A_s$ is the absorbance at the maximum absorption wavelength of dye in the sample flask at time, $t$ (Ozsoy *et al*., 2005).

**Statistical analysis:** All experiments were carried out in triplicate and the results obtained were statistically analysed.

## RESULT AND DISCUSSION

**Collection and isolation of endophytic fungi and identification:** A systematic study about the endophytic fungal biodiversity in a forest plant, *C. torulosa* which were growing in Govind Ballabh Pant Engineering College Campus, Pauri, Garhwal, and Uttarakhand was carried out to evaluate its bioremediation potential for removal of various textile dyes. A total of six endophytic fungi were isolated from the leaves of *C. torulosa* by using different culture media and were morphotypically characterized (**Fig. 1 and Table 1**) (Williamson, 1994).

Table 1. Morphotypic characterization of endophytic fungi

| S. No. | Code of isolate | Colony charecterstics on PDA media | Probable endophytic fungus |
|---|---|---|---|
| 1. | PCTS13 | Appears olivaceous brown in colour | *Penicillium* sp |
| 2. | PCTS21 | Appears grayish green in colour | *Alternaria alternata* |
| 3. | PCTS25 | Appears olive green in colour initially but colony becomes white after 10 days | *Penicillium* sp |
| 4. | KCTS14 | Appears whitish gray in colour | *Pestalotiopsis* sp |
| 5. | KCTS15 | Appears gray in colour | *Alternaria alternata* |
| 6. | KCTS34 | Appears cottony white in colour | Unidentified |

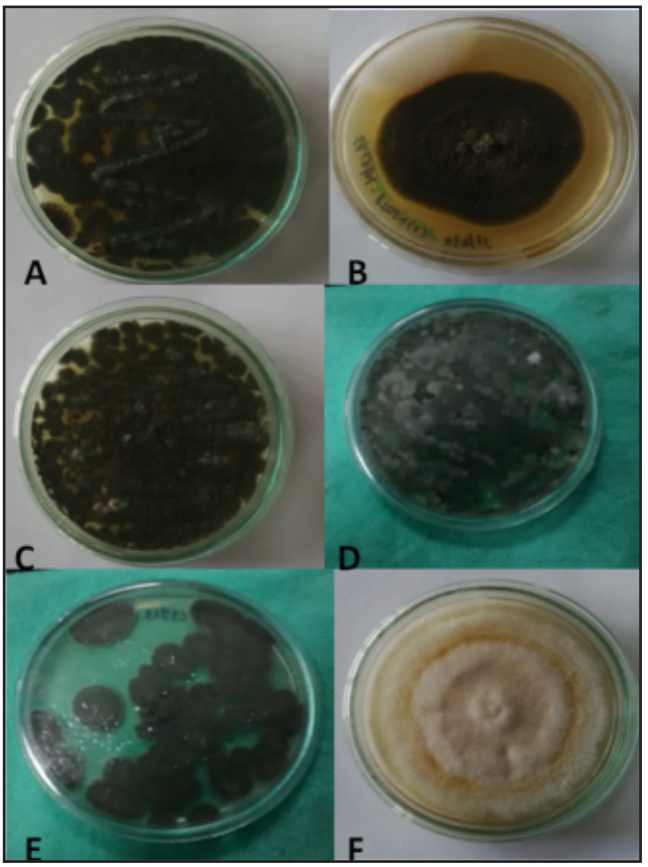


**Fig.1.** Colony morphology of fungal endophytes such as A: PCTS13, B: PCTS21, C: PCTS25, D: KCTS14, E: KCTS15, F: KCTS34 on PDA

The Majority of the recovered endophtytes belong to the *Ascomycota*. Fungal endophytes are especially common among the *Ascomycota*, representing at least five classes, dozens of families, and large numbers of previously unknown species (Gehlot *et al*., 2008). Only one species from the collected isolates in this study belong to the *Dothideomycetes* (Petrini, 1986).

**Diversity analysis of endophytic fungi:** A total of 59 isolates of endophytic fungi were recovered from 90 tissue segments (15 segments from each site) of *C. torulosa* in two media (**Table. 2**). The CR of fungi on PDA was higher in S1(90) > S2(60) > S3 (40) but CR of fungi in KMM was S3(73) > S1(60)> S2(40). So, overall CR of fungi was higher in PDA (**Fig. 2**). All isolated fungi were assembled in six morphogroups; five of known and one of unknown genera.

In Korea, total 59 isolates and 19 species of endophytic fungi were isolated from the leaves of *Juniperus rigida, Larix kaempferi* and *Pinus densiflora* and identified using morphological and molecular characteristics (Kim *et al.,*

**Table 2:** Colonization rate of endophytic fungi on different culture media

| S. No. | Collection of leaves from various sites | No of fungi colonized on segment in KMM | Colonization Rate on KMM | No of fungi colonized on segment in PDA | Colonization Rate on PDA |
|---|---|---|---|---|---|
| 1. | S1 | 6 | [illegible] | 9 | 90±0.3 |
| 2. | S2 | 4 | 40±0.4 | 6 | 60±0.2 |
| 3. | S3 | 11 | 73.33±0.15 | 4 | 40±0.1 |

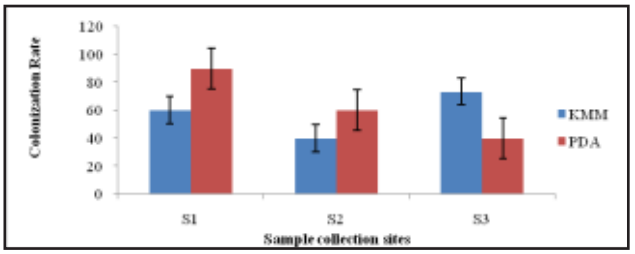


**Fig. 2:** Colonization rate of endophytic fungi

2013). Endophytic fungi isolated from healthy plant parts such as stem, root, rhizome of seven plant species and one thousand five hundred and twenty-nine fungal isolates were obtained from 5200 fragments. Stem fragments harbored more endophytes (80.37%) than roots (19.22%). 31 fungal taxa comprised of coelomycetes (65%), hyphomycetes (32%), and ascomycetes (3*%).* Fungal isolates such as *Fusarium, Acremonium, Colletotrichum, Chaetomium, Myrothecium, Phomopsis,* and *Pestalotiopsis* were commonly isolated (Nalini *et al*., 2014) .

**Laccase production by fungal endophytes by solid state fermentation:** Six different endophytic fungal isolates were sub-cultured and screened for laccase activity on Glucose Yeast Extract Peptone Agar with Guaiacol, ABTS, Tannic acid and Syringaldehyde. Laccase enzyme react with Guaiacol to give intense brown color product (**Fig. 3**), ABTS give Purple color product (**Fig. 4**), Tannic acid give dark brown product (**Fig 5**), Syringaldehyde to give intense brown color product (**Fig. 6**). Out of the above six fungal isolates, only three such as KCTS34, KCTS14 and PCTS21 exhibited significant laccase activity (**Table 3**). The obtained results are in conformity with the report of Kiiskinen *et al.* (2004) for the isolation of laccase producing fungus. Muthezhilan *et al*. (2014) reported various morphotypes of endophytic fungal strains from different segments (leaf, stem, and root) of sand dune plants and 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. Laccase activity was shown by *Pleurotus* sp.

**Table 3:** Laccase enzyme activity of fungal isolates from *Cupressus torulosa*

| Sample | guaiacol | ABTS | Tannic acid | Syringeldehyd |
|---|---|---|---|---|
| KCTS34 | ++++ | ++++ | ++++ | ++++ |
| PCTS21 | ++ | ++ | ++ | ++ |
| PCTS25 | – | – | + | + |
| PCTS13 | – | – | + | ++ |
| KCTS14 | +++ | +++ | +++ | +++ |
| KCTS15 | – | – | + | + |

(++++)-High activity, (+++)-Good activity, (++)-Medium activity, (+) - Low activity, ()-No activity

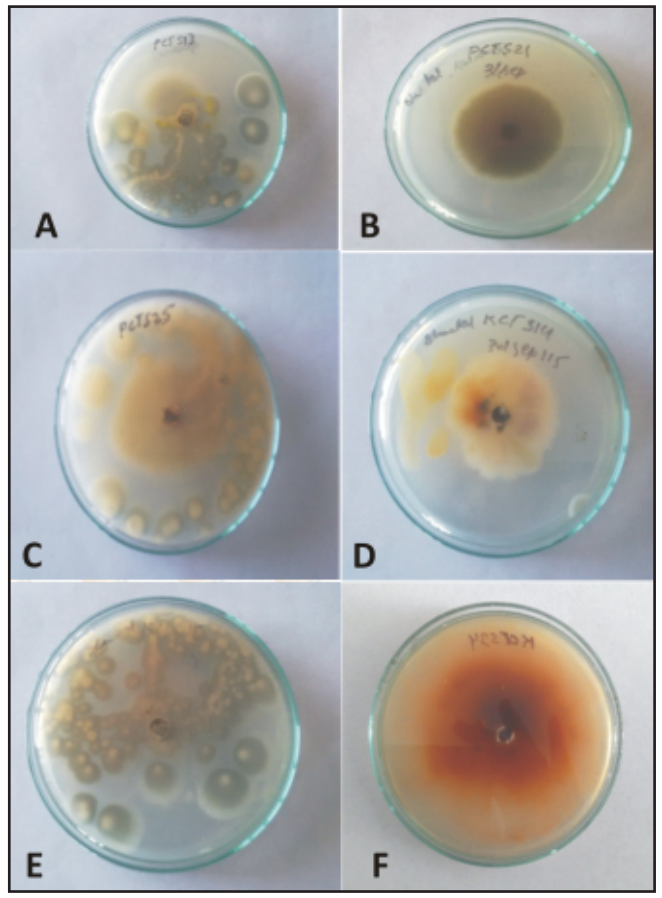


**Fig 3:** Laccase producing fungal endophytes A: PCTS13, B: PCTS21, C: PCTS25, D: KCTS14, E: KCTS15, F: KCTS34 on using Guacol

containing 0.02% guaiacol since laccase catalyzes the

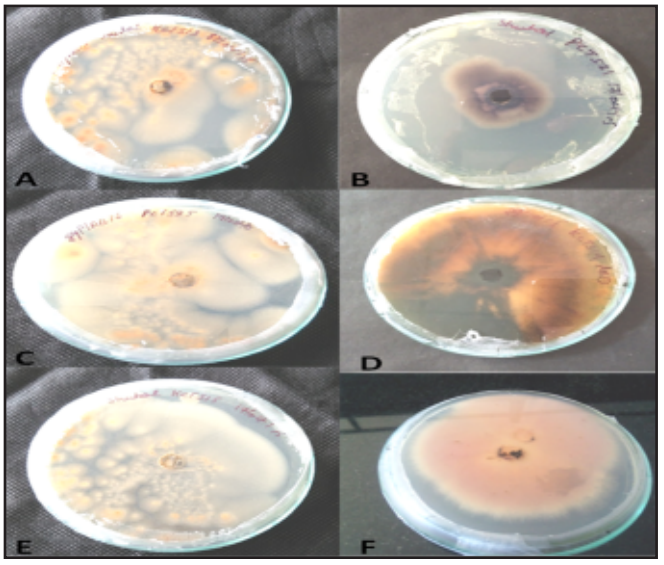


**Fig 4:** Laccase producing fungal endophytes A: PCTS13, B: PCTS21, C: PCTS25, D: KCTS14, E: KCTS15, F: KCTS34 using ABTS

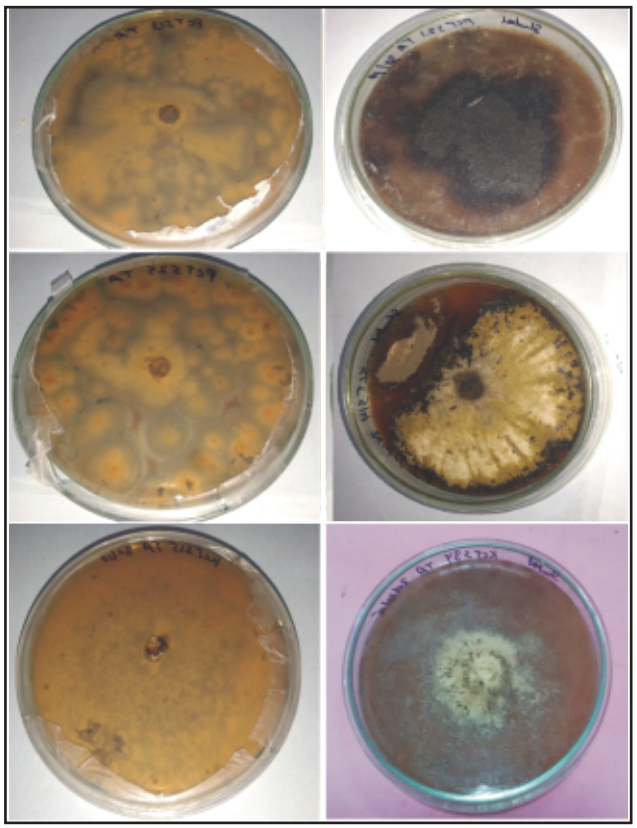

**Fig 5:** Laccase producing fungal endophytes A: PCTS13, B: PCTS21, C: PCTS25, D: KCTS14, E: KCTS15, F: KCTS34 using Tannic acid

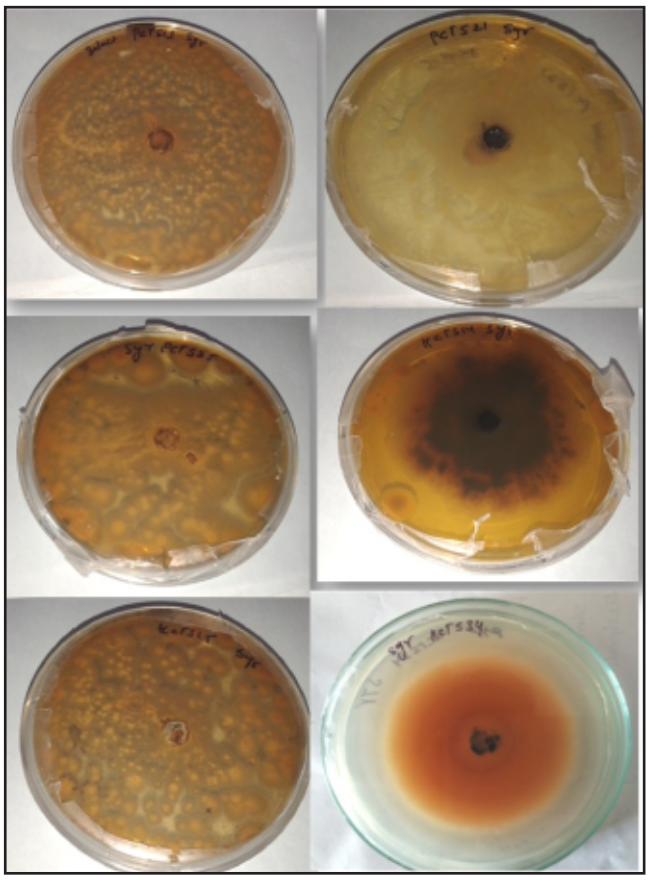

**Fig 6:** Laccase producing fungal endophytes using A: PCTS13, B: PCTS21, C: PCTS25, D: KCTS14, E: KCTS15, F: KCTS34 using syringaldehyde

oxidative polymerization of guaiacol to form reddish brown zones in the medium (Kumar, 2012). Syringaldehyde substrate showed a very strong ability to facilitate the growth and the isolation of the White rot fungus *P. ostreatus* with the laccase activity (Palmieri *et al.,* 1997).

**Quantitative estimation of laccase production:** Fungal endophytes that showed positive reaction in the plate test for the secretion of laccase, were further analyzed in liquid culture for the quantitative estimation. Laccase activity was calculated with respect to the incubation time. Maximum secretion of laccase was seen in 10$^{th}$ day of the culture age. Among them fungal isolates, KCTS34 produced the maximum laccase activity (57 U/mL) by using Guaicol as substrate, 47U /mL by using ABTS and least 32.12 U/ml by syringaldehyde (**Fig. 7**). Initial screening of crude enzyme showed complete oxidation of ABTS and Guaiacol after 7days of incubation. *Alternaria arborescence* showed maximum production of laccase enzyme (800U/l) at 30˚C in 4.5 pH followed by *Fusarium oxysporium* (JQ 950134) with 600 U/l at 45°C in 5 pH after 15 days of incubation (Christie and Shanmugam, 2012)

Desai *et al*., (2011) reported that when *Tricoderma genusi* was cultivated on potato dextrose agar (PDA) plates containing guaicol and tannic acid as indicator compounds, higher level of laccase activity was observed under solid state condition production (15.7 U/mL) in comparison to submerged condition (10.68 U/mL). It was also found out that the laccase was active over broad range of temperature and pH, with optimum temperature of 45ºC and pH 5. The isolated strain can be further evaluated for other characteristics and it could prove to be a potent source of enzyme for lignin degradation.

**Dye decolourization studies by endophytic fungal isolate:** Textile dyes Congo red, Rose bangal, Orange G and Rhodamine B were used to study the ability of laccase

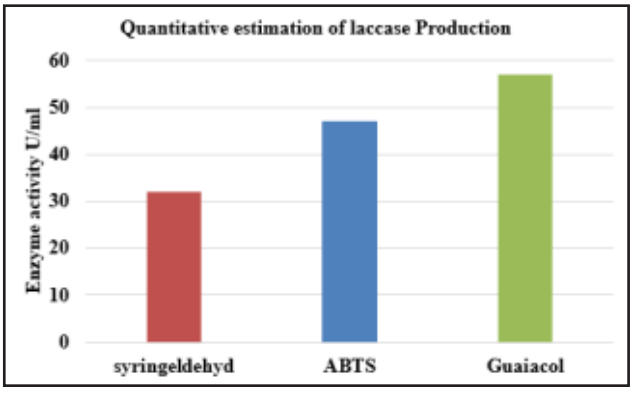


**Fig 7:** Quantitative estimation of laccase production

producing endophytic fungi KCT 34 to decolorize textile dye. As per the result of decolorization obtained in the malt extract media supplemented with textile dye, KCT 34 was able to decolorize all the four different dyes tested (**Fig. 8**). Further, KCT34 was used for detailed study using spectrophotometry method. Poojary *et al*., (2012) conducted a similar study and isolated two fungal strains capable of dye decolorization. Another study by Levin *et al*., (2004) also showed the dye decolorizing ability of fungi isolated in Argentina. Decolourization of textile dyes by a *Clitocybula dusenii*, white-rot fungus producing laccase was reported by Wesenberg *et al*., (2002). Liu *et al*., (2004) also reported that laccase is solely responsible for the decolorization and degradation of dyes.

A decolorization test using Basic fuchsin, Congo red, Crystal violet, Malachite green and Orange G was carried out using partially purified immobilized laccase enzyme of *Trametes hirsuta*, in which maximum decolorization was observed in Malachite green and Crystal violet in 15 days (Rasheeda *et al*., 2014). Congo red showed 86% decolorization, followed by Orange G (71%) and Rhodamine B (52%). Another decolorization test using the same dyes along with fast green was carried out which showed decolorization rate of 93% with Congo red, followed by 87 % with fast green, 81% with Orange G and 71 % with Rhodamine B (Kumar, 2012). This study results were supported by Eggert *et al*., (1996), who showed the decolorization ability of laccase in *Phlebia tremmellosa* (Robinson *et al*., 2001) and *Pleurotus sajor-caju* (Chagas *et al*., 2001) .

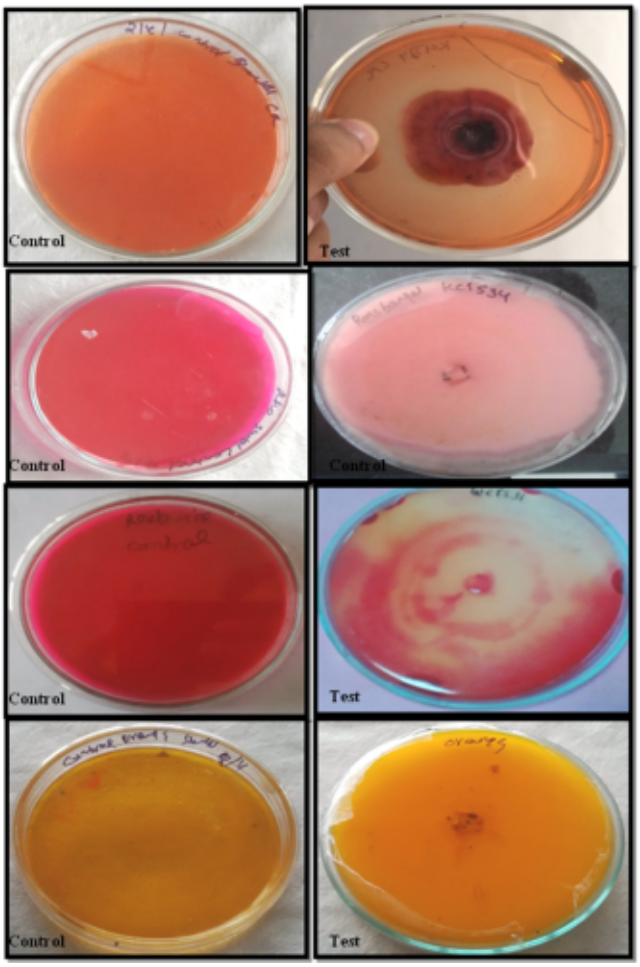


**Fig 8:** Dye decolorization by *Daldinia* sp. resulting in the formation of halo on agar plate medium containing (A) Congo red (B) Rose bangal (C) Rhodamine B (D) Orange G

**Quantitative assay for synthetic textile dye decolorization:** All of the 4 textile dyes were tested for decolorization by KCT34 in Malt extract broth. Fungal isolate showed variable potential to decolorize Congo red, Rosebangal, Orange G and Rhodamine B in liquid medium. All the selected dyes were degraded by *Daldinia* sp. fungal strains but per cent decolorization was different for all dyes. Per cent of dye decolorization by KCT34. showed minimum dye decolorization percentage on 4$^{th}$ day 56.20% at 497 nm, 7.38% at 475 nm, 4.42% nm at 590, 4.11% at 549 nm and maximum on 16$^{th}$ day 90.45%, 85.81%, 54.39%, 87.91%. Congo red, Orange G, Rhodamine B, Rose bangal, respectively (**Fig. 9**).

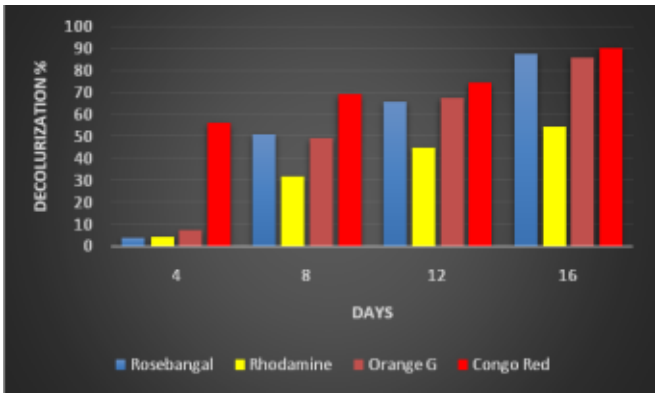


**Fig. 9:** Per cent dye decolorization of synthetic textile dye by the KCTS 34

Dye decolorization activity of endophytic fungi were screened using an agar plate based and liquid culture based method. Maximum decolorization exhibited by the liquid method in comparison of agar media method. Only one endophyte, KCT34 was able to decolorize all the four different dyes tested (Congo red, Rosebangal, Rhodamine and Orange G), after 16 days of fungal incubation. *Alternaria alternata* CMERI F6 decolorized 99.99% of 600 mg/L Congo red within 48 h in yeast extract-glucose medium at 25°C, pH 5 and 150 rpm (Chakraborty *et al*., 2013). Decolorization of textile dye industrial effluents by white-rot fungus producing laccase as the major lignin-degrading enzyme as seen in this study was reported in white-rot fungus, *Clitocybula dusenii* by Wesenberg *et al*., (2002). The results of this study lead to a conclusion that endophytic fungi having potential ligninolytic enzymes can be used in industrial effluent treatments.

**Identification of endophytic fungi:** On PDA medium, the isolated fungal culture KCT34 appeared cottony white in colour (**Fig. 10**). The slide cultures prepared from this fungus showed the presence of stolons and pigmented rhizoids, multi-spores, generally globose sporangia. In addition to the morphological characterization, molecular methods were carried out to confirm the identification of most promising endophytic fungal strain KCTS34 isolated from the *C. torulosa* D. Don.

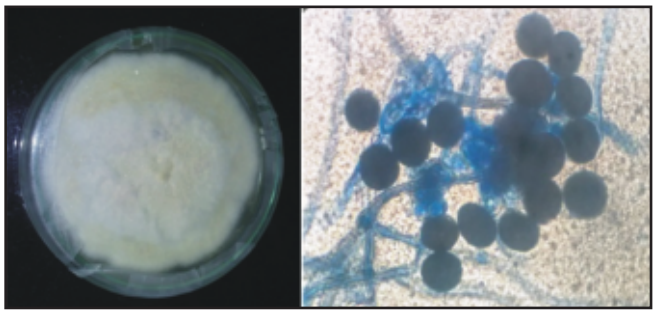

**Fig. 10:** (A) Colony Morphology on PDA of KCTS34; (B): Shape of Conidia by staining techniques

Molecular identification techniques were used to determine the identity of the fungus to generic level. On the basis of its 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence, it can be concluded that the fungus KCTS34 belongs to the genus *Daldinia sp.* The obtained sequence was submitted in National Centre for Biotechnology Information with accession number KT355731. The percentage of similarity between the fungus and database suggests it to be a novel strain of species of genus *Daldinia,* an Ascomycetous fungus.

## CONCLUSION

The direct discharge of the chemicals, basically dye containing industrial effluents in open environment is the principle source of water pollution. So, the removal of dyes and other chemical substances from industrial effluents is a major concern before its discharge into open water bodies. There are different chemio-physical and biological methods used for the treatment of dye. Dye decolorization with microorganisms is cost effective and environment friendly method for controlling pollution generated by textile industries. In this work, six strains of endophytic fungi from *C. torulosa* were recovered and screened out for laccase activity. Out of them, 3 fungal strains, namely *Alternaria alternata, Pestalotiosis neglecta* and *Daldinia* sp. exhibitted their ability to secrete laccase comparable to other lignin-degrading enzymes by 10$^{th}$ day of incubation. Out of these *Daldinia* was selected for textile dye decolorization test because of better showing in comparison in this regard. This is the first preliminary report on endophytic *Daldinia* sp. from Gymnospermous tree *C. torulosa* growing in Garhwal region.

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## REFERENCE

Arnold, A.E., Maynard, Z., Gilbert, G.S., Coley, P.D. and Kursar, T.A. 2000. Are tropical fungal endophytes hyperdiverse. *Ecology Lett.* **3:**267-274.

Atalla, M.M., Zeinab, H.K., Eman, R.H., Amani, A.Y. and Abeer, A.A.E.A. 2010. Screening of some marine-derived fungal isolates for lignin degrading enzymes (LDEs) production. *Agriculture and Biology Journal of North America* **1**(4): 591-9.

Balaji, V., Vinayagamoorthi, D., Palanisamy, A. and Anbalagan, S. 2012. Degradation of Reactive Red HE7B and Yellow FN2R dyes by fungal isolates. *J. Acad. Indus. Res.***1** (3): 132-136.

Bhardwaj, A., Sharma, D., Jadon, N., and Agrawal, P.K. 2015. Antimicrobial and Phytochemical screening of endophytic fungi isolated from spikes of *Pinus rouxburghii. Archievs of Clinic Microbiol.* **6** (3): 1-9.

Blanquez, P., Casas, N., Font, X., Gabarrell, X., Sarra, M., Caminal, G., and Vicent, T. 2004. Mechanism of textile metal dye biotransformation by *Trametes versicolor*. *J. Water Res.* **38:** 2166-2172.

Campos, R., Kandelbauer, A., Robra, K.H., Cavaco-Paulo, A., and Gubitz, G.M. 2001. Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii. J. Biotechnol* **8:**131-139.

Chagas, E.P. and Durrant, L.R. 2001 Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sojar caju*. *Enzy. Microb. Technol.* **29**: 473-477.

Chakraborty, S., Basak, B., Dutta, S., Bhunia, B., and Dey, A. 2013 Decolorization and biodegradation of Congo red dye by a novel white rot fungus *Alternaria alternata* CMERI F6. *Bioresour. Technol.* ***147:*** 662-666.

Christie, S.A.D. And Shanmugam, S. 2012. Analysis of fungal cultures isolated from Anamalai Hills for laccase enzyme production effect on dye decolorization, antimicrobial activity. *Int. J. of Plant Animal and Env. Science* **2** (3):143-148.

Desai, S.S., Tennali, G.B., Channur, N., Anup, A.C., Deshpande, G., and Murtuza, B. A.2011. Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnol. Bioinf. Bioeng.* **1** (4): 543-549.

Eggert, C., Temp, U., Dean, J.F. and Eriksson, K. E. L. 1996. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett*. **391:** 144-148.

Forgacs, E., Cserhati, T. and Oros, G. 2004. Removal of synthetic dyes from wastewaters: a review. *Environ. Int.* **30:** 953-997.

Garg, V.K., Gupta, R., Yadav, A.B. and Kumar, R. 2003. Dye removal from aqueous solution by adsorption on treated saw dust. *Bioresource Technol.* **89:**121-124.

Gehlot, P., Bohra, N.K. and Purohit, D.K. 2008. Endophytic mycoflora of inner bark of *Prosopis cineraria* - a key stone tree species of Indian Desert. *American-Eurasian Journal of Botany* **1** (1): 01-04.

Gupta, V.K., Pathania, D., Singh, P., Kumar, A. and Rathore, B.S. 2014. Development of guar gum based cerium

(IV) tungstate nanocomposite material for remediation of basic dye from water. *Carbohydrate Polymers* **101:** 684-691.

Harvey, J.W. and Keitt, A.S. 1983. Studies of the efficacy and potential hazards of methylene blue therapy in aniline- induced haemoglobinaemia. *Br. J. Haematol.* **54** (1): 29-41.

Hassani, A.H., Seif, S., Javid, A.H. and Borghei, M. 2008. Comparison of adsorption process by GAC with novel formulation of coagulation flocculation for color removal of textile wastewater. *Int. J. Environ. Res.* **2** (3): 239-248.

Kariyawasam, G.K., Mithrasena, Y.J.P.K., Fernando, T.H.P.S., Wijesundara, R.L.C. and Wijesundara, W.S.S. 2012. A new cost effective method for extracting genomic DNA from fungi. Colombo: Institute of Biochemistry, Molecular Biology and Biotechnology; Abstracts of papers, 5th annual sessions of Institute of Biochemistry, Molecular Biology and Biotechnology, Colombo, vol 5, pp 49

Kiiskinen, L.L., Ratto, M. and Kruus, K. 2004. Screening for novel laccase producing microbes. *J. Appl. Microbiol.* **97:** 640-646.

Kim, C.K., Eo, J.K. and Eom, A.H. 2013. Diversity and seasonal variation of endophytic fungi isolated from three conifers in M. Taehwa, Korea. *Mycobiology* **41**: 82-85

Kumar P.A., 2012. Optimization and purification of laccase from *Pleurotus* sp. and its dye decolorization ability. *J. Acad. Indus. Res.* **1** (1):39-44

Kumar, V.V., Kirupha, S.D., Periyaraman, P. and Sivanesan, S. 2011. Screening and induction of laccase activity in fungal species and its application in dye decolorization. *Afric J. Microbiol. Res.* **5** (11): 1261-1267,

Levin, L., Papinutti, L. and Forchiassin, F. 2004. Evaluation of Argentinean white rot fungi for their ability to produce lignin modifying enzymes and decolorize industrial dyes. *Bioresour. Technol.* **94:** 169-176.

Liu, W., Y. Chao, Y., X. Yang, X., H. Bao, H. and S. Qian, S. 2004. Biodecolorization of azo, anthraquinonic and triphenylmethane dyes by white-rot fungi and a laccase-secreting engineered strain. *J. Ind. Microbiol. Biotechnol*. **31**: 127-132.

Muthezhilan, R., Vinoth, S., Gopi, K. and Jaffar Hussain, A. 2014. A dye degrading potential of immobilized laccase from endophytic fungi of coastal sand dune plants. *Int. J. Chem. Tech. Res.* **6** (9): 4154-4160.

Nalini, M.S., Sunayana, N. and Prakash, H.S. 2014. Endophytic fungal diversity in medicinal plants of Western Ghats, India. *Int. J .Biodive.* Article ID 494213, 9

Narkhede, M., Mahajan, R. and Narkhede, K., 2013. Ligninolytic enzyme production and Remazol Brilliant Blue R (RBBR) decolorization by a newly isolated white rot fungus: *Basidiomycota* sp. L-168. *Int. J .Pharm. Bio. Sci.* **4** (1): 220-228.

Ngieng, N.S, Zulkharnain, A., Roslan, H.A. and Husaini, A. 2013. Decolorisation of synthetic dyes by endophytic fungal flora isolated from Senduduk plant (*Melastoma malabathricum*). *ISRN Biotechnology* Article ID 260730, 7 pages

Onuki, T., Nogucji, M., and Mitamura, J. 2000. Oxidative hair dye composition containing laccase. *Pat. Int. Appl. WO.* **37** (030), 78994m.

Ozsoy, H.D., Unyayar, A. and Mazmancı, M.A. 2005. Decolorization of reactive textile dyes Drimarene Blue X3LR and Remazol Brilliant Blue R by *Funalia trogii* ATCC 200800. *Biodegradation* **16** (3): 195-204.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A. and Sannia, G. 1997. A novel white laccase from *Pleurotus ostreatus*. *J Biol Chem* **272:** 31301-31307.

Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the Phyllosphere* (Eds.: Fokkema, N.J. and van den Heuvel, J.). Cambridge University Press, Cambridge 175-187.

Petrini, O. and Fisher, P. J.1988. Comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *Trans. British Mycol. Soc.* **91:** 233-238.

Poojary, H., Hoskeri, A., Kaur, A. and Mugeraya, G. 2012. Comparative Production of ligninolytic enzymes from Novel isolates of basidiomycetes and their potential to degrade textile dyes. *Nature and Sci.* **10:** 90-96.

Ramesh Babu B., Parande, A.K., Raghu, S. and Kumar, T.P. 2007. Textile technology cotton textile processing: Waste generation and effluent treatment. *J. Cotton Sci.* **11**:141-153

Rasheeda, K., Prasuna, R.G. and Rajasekhar, A.V. 2014. Dye decolorization: laccases by the best fusant strain of *Trametes hirsuta. World. J. Pharma Res.* **3**: 2142-2155.

Robinson, T., McMullan, G., Marchant, R. and Nigam, P.2001. Remediation of dyes in textile effluent a critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.* **77**: 247-255.

Sharma, D., Pramanik, A. and Agrawal P.K. 2016. Evaluation of bioactive secondary metabolites from endophytic fungus *Pestalotiopsis neglecta* BAB-5510 isolated from leaves of *Cupressus torulosa* D. Don. *3 Biotech.* **6** (210): 1-14.

Watanabe, M., Takano, T., Nakamura, K., Watanabe, S., and Seino, K.2007. Water quality and concentration of alkylphenols in rivers used as source of drinking

water and flowing through urban areas. *Environ. Health Prev. Med.* **12** (1):17-24.

Wesenberg, D., Buchon, F. and Agathos, S. N. 2002. Degradation of dye-containing textile effluent by the agaric white-rot fungus *Clitocybula dusenii*. *Biotechnol. Lett.* **4**: 989-993.

White, T.J., Bruns, T., Lee, S., and Aylor, J.W.T. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics in PCR protocols: a guide to methods and applications. In: Innis MA, Gelfand DH , Sninsky JJ, White TJ (eds), Academic Press, New York, pp 315-322.

Williamson, P.R.1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J. Bacteriology* **176** (3): 656-664.

Zollinger, H. 2003. *Color chemistry: Synthesis, properties, and applications of organic dyes and pigments*. Zurich, Switzerland/Weinheim, Germany: VHCA, Verlag Helvetica Chimica Acta/Wiley Third ed., 249.