

Biodegradation of Textile Dyes by Fungi Isolated from North Indian Field Soil

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Abstract

In this study one azo dye “Congo red”, two triphenylmethane dyes “Crystal violet” and “Methylene blue” have been selected for biodegradation using three soil fungal isolates *A. niger*, *F. oxysporum* and *T. lignorum*. These fungal strains were isolated from field soil. Three methods were selected for biodegradation, viz. agar overlay and liquid media methods; stationary and shaking conditions at 25°C. The experiment was conducted for 10 days and the results were periodically observed. *Aspergillus niger* decolorized maximum Congo red (74.07%) followed by Crystal violet (33.82%) and Methylene blue (22.44%) under liquid medium (stationary) condition. Whereas, under same conditions, *T. lignorum* decolorized maximum crystal violet (92.7%), Methylene blue (48.3%) and Congo red (35.25%). Use of *T. lignorum* as dye bio degrader or decolorizer has been done first time in this study. *Fusarium oxysporum* performed better under shaking conditions compared to stationary and overlay method. It can be concluded that among soil fungus *T. lignorum* could be used as efficient dye decolorizer especially for crystal violet and *A. niger* for Congo red. The excellent performance of *T. lignorum* and *F. oxysporum* in the biodegradation of textile dyes of different chemical structures reinforces the potential of these fungi for environmental decontamination similar to white rot fungi.

Keywords: bioremediation; biosorption; dyes; soil fungi

1. Introduction

Due to rapid industrialization and urbanization, a lot of chemicals including dyes are manufactured and are being used in day-to-day life. About 100,000 commercial dyes are manufactured including several varieties of dyes such as acidic, basic, reactive, azo, diazo, anthraquinone based meta complex dyes with an annual production of over 7×10^5 metric tons are commercially available (Campos *et al.*, 2001). Approximately 50% of the dyes are released in the industrial effluents (Zollinger, 1991). They are used on several substrates in food, cosmetics, paper, plastic and textile industries. Some of them are dangerous to living organisms due to their possible toxicity and carcinogenicity. Dyes in wastewater often lead to instance; the incidence of bladder tumors has been reported to be particularly higher in dye industry workers than in the general population (Suryavathi *et al.*, 2005). Natural pigments used for coloring textiles have been replaced by “fast colors” which do not fade on exposure to light, heat and water. These features unfortunately go with the perils of harmful effluent quality. About 15 % of the dyes used for textile dyeing are released into processing waters

(Eichlerova *et al.*, 2006). Besides being unaesthetic, these effluents are mutagenic, carcinogenic and toxic (Chung *et al.*, 1992).

Following are the names of common dyes other than used in this study, which are carcinogenic in nature; Benzidine, 2-Aminoanthraquinone, o-Aminoazotoluene, 1-Amino-2-methylanthraquinone, 2-Naphthylamine, o-Anisidine Hydrochloride, 4-Chloro-o-phenylenediamine, C.I. Basic Red 9 Monohydrochloride, p-Cresidine, 2,4-Diaminoanisole Sulfate; 2,4Diaminotoluene, 3,3'-Dimethoxybenzidine, Dimethylcarbonyl Chloride, Direct Black 38, Direct Blue 6, 4,4'-Methylenebis (N,N-dimethyl) benzenamine, Michler's Ketone (Anonymous, 1982; CPCB 1990). Dyes with possible mutagenicity are Bemaplex Schwarz C-2B, Bleu Terasil 3R-02, Brun Cibaneone 2RMP, Evercion Navy Blue H-ER, Lanazol Red 6G, Lanazol Rot B, Lumacron Black SEF 300%, Lumacron Red PGA, Olive Cibaneone 2R MD, Orange Minerprint 3RL, Rouge Imperon K-B, Rouge Terasil P3G, Sirius Grau K-CGL, Turquoise Cibacrone P-GR Liq. 50% (Jäger *et al.*, 2004)

Commonly applied treatment methods for color removal from colored effluents consist of integrated

processes involving various combinations of biological, physical and chemical decolorization methods (Galindo and Kalt, 1999; Robinson *et al.*, 2001; Azbar *et al.*, 2004), of these, approximately 10–15 % of unused dyes enter the wastewater after dyeing and after the subsequent washing processes (Rajamohan and Karthikeyan, 2006). Chemical and physical methods for treatment of dye wastewater are not widely applied to textile industries because of exorbitant costs and disposal problems. Green technologies to deal with this problem include adsorption of dyestuffs on bacterial and fungal biomass (Fu and Viraraghavan, 2002; Yang *et al.*, 2009) or low-cost non-conventional adsorbents (Crini, 2006; Ferrero, 2007).

A variety of physicochemical treatments have been devised previously for the dyes and textile wastewater. However, these suffered from some serious drawbacks in terms of their limited applications or their high cost. Besides, chemical treatments created an additional chemical load in water bodies that eventually resulted in sludge disposal problems. Several factors determine the technical and economic feasibility of each single dye removal technique. These include; dye type and its concentration, wastewater composition, operation costs (energy and material), environmental fate and handling costs of generated waste products. A very small amount of dye in water (10-50 mg/L) is highly visible and reduces light penetration in water systems, thus causing a negative effect on photosynthesis (Cooper, 1993; Vandevivere *et al.*, 1998).

Recently, dye removal became a research area of increasing interest, as government legislation concerning the release of contaminated effluent becomes more stringent. Various treatment methods for removal of dyes from industrial effluents like chemical coagulation using alum, lime, ferric chloride, ferric sulphate and electro coagulation are very time consuming and costly with low efficiency. Among the numerous water treatment technologies, research interest in the fungal bioremediation due to their biomass compared to the bacteria, has increased significantly for decolorization and degradation of synthetic dyes. Common bacteria used for bioremediation were mainly species of *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Corynebacterium*, *Mycobacterium*,

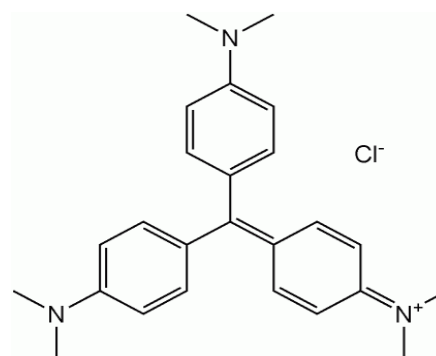
Escherichia, while the common fungal strains are *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Piptoporus betulinus*, *Funalia trogii*, *Coriolus versicolor*, *Cyathus stariatus* and sp. of *Fusarium*, *Aspergillus* (Azmi *et al.*, 1998).

Keeping the above points in view, the main objectives of the problem was to screen and employ selected potential soil fungal sp. capable to decolorize and biodegrade the textile dyes using solid and liquid media under shaking and stationary conditions.

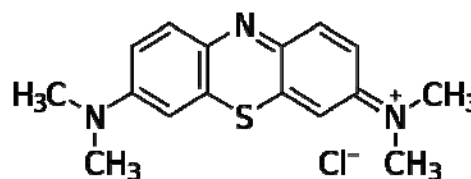
2. Methods and Materials

2.1. Dyes

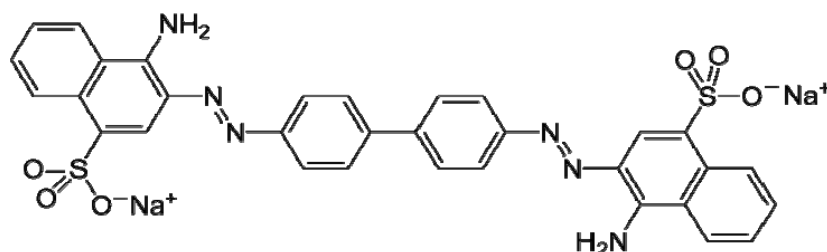
Analytical grade dyes (i) Crystal violet $C_{25}N_3H_{30}Cl$, IUPAC name Tris (4- (dimethylamino) phenyl), methylium chloride



(ii) Methylene blue $C_{16}H_{18}N_3S_2Cl$, IUPAC name 3,7-bis (Dimethylamino)- phenothiazin-5-ium chloride



and (iii) Congo red $C_{32}H_{22}N_6Na_2O_6S_2$, IUPAC name disodium 4-amino-3-[4-[4-(1-amino-4-sulfonato-naphthalen-2-yl) diazenylphenyl] phenyl] diazenyl-naphthalene-1-sulfonate were procured from HiMedia, Mumbai, India.



2.2. Source of fungal strains

The soil-derived fungal strains were obtained from the culture collection of College of Applied Education & Health Sciences, Meerut, India. All three strains *Aspergillus niger*, *Fusarium oxysporum* and *Trichoderma lignorum* were isolated from fields of Meerut region, North India and maintained on Potato Dextrose Agar (Himedia, Mumbai, India) and sub cultured periodically to maintain their viability. Identification of these soil fungal strains was done previously based on their morphological characters (Yao et al., 2009). These soil fungi have selected in present study because *A. niger* and *F. oxysporum* has been widely studied for dye decolorization, while the potential of *T. lignorum* has been exploited first time.

2.3. Screening of soil-derived fungi for dye decolorization activities

Fifty six field soil fungal strains were screened for their ability to degrade dyes using the tube overlay method. Initially, the fungal strains were grown on culture plates pre-filled with Potato Dextrose Agar (PDA) and incubated at room temperature for 14 days. Following incubation, mycelial agar plugs (~5 mm²) were cut approximately 5 mm from the colony margin and inoculated on test tubes (in triplicates) containing 5 ml of PDA overlaid with 1 ml of PDA with 0.01% (w/v) respective textile dye. All culture tubes were incubated at room temperature (~25°C) and observed weekly for up to four weeks. Clearing of the overlaid dye indicates full decolorization (+++). Partial dye decolorization (++) was indicated by less dye intensity in comparison with the control (uninoculated PDA overlaid with PDA + 0.01% dye). All the three fungal strains were selected on the basis of full or maximum (+++) decolorization.

2.4. Decolorization of dyes in solid medium (Tube overlay method)

The three selected soil isolated fungal strains were further tested for their ability to decolorize on PDA and Sabouraud Dextrose Agar (SDA) medium, Himedia, Mumbai, India. This was done to select which medium support better growth and dye decolorization activities of selected fungal isolates. Initially, all the three fungal strains were grown as previously described. Following incubation, fungal mycelial agar plugs (~5 mm²) were cut approximately 5 mm from the colony margin and inoculated on test tubes (in triplicates) each pre-filled with 2 ml of the Potato Dextrose Agar (PDA) medium, supplemented separately with either with following dye

0.01 % (w/v) crystal violet, methylene blue and Congo red, respectively (Lopez et al., 2006). The culture tubes were then incubated at room temperature (~25°C). The growth of the fungi and its ability to decolorize the dye were observed weekly up to four weeks. The depth of dye decolorization (in mm) indicated by clearing of the dye was then measured.

2.5. Assay for the dye decolorization activities of fungi in liquid media

The spores and mycelia were then dislodged using a flame-sterilized inoculating loop. Then, 10 µl of the inoculum were added on culture vials (in triplicates) pre-filled with 25 ml Potato Dextrose Broth (PDB) supplemented with 0.01% of either one of the following dyes: Congo red, methylene blue, and crystal violet. Three sets were prepared and were incubated either under constant agitation/shaking (100 rpm, Yoriko Scientific Orbital Shaker) or under stationary/without shaking condition (Park et al., 2007). All culture vials were incubated at room temperature (~25°C) for 10 days and all assays were performed in triplicate. Growth and dye decolorization were noted every day. Following culture for 10 days, the culture filtrates were decanted and subjected to spectrophotometric analysis. For the crystal violet, the absorbance reading was done at 590 nm wavelength. Congo red and methylene blue were read at 497 nm. The extent of dye decolorization by the soil fungal strains on liquid media was calculated using the formula below:

$$\text{Percent dye decolorization (\%)} = \frac{\text{Absorbance control} - \text{Absorbance inoculated}}{\text{Absorbance control}} \times 100$$

Finally, the mycelial biomass were harvested on clean Petri plates and observed directly. Fungal hyphae were also mounted on clean glass slides and observed under a compound light microscope for the biosorption of dyes.

2.6. Enzyme assay

Laccase activity was measured by using syringaldazine as a substrate as per the method of (Valmaseda et al., 1991). The activity was assayed using using 1.0 ml of 0.2 M sodium phosphate buffer (pH 5.7) and 0.2 ml syringaldazine (1.6 mg/ml) in absolute ethanol, (4.47 Mm). Reactions were initiated by the addition of syringaldazine and after mixing; incubations were conducted at 30°C for 1h, because after 1h highest enzyme laccase activity was observed. The absorbance was measured in a spectrophotometer (ELICO SL 150)

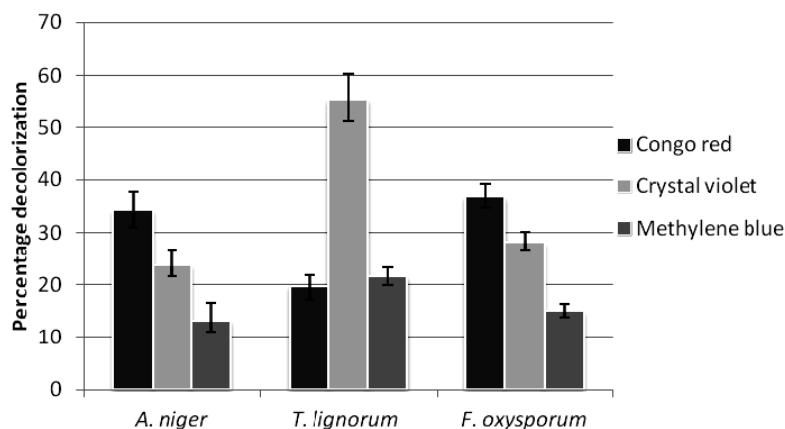


Figure 1. Dye decolorization by soil fungi in tube overlay method

before (0 time) and after incubation (60 min) at 526 nm and the increase in absorbance was calculated. One unit activity was defined as the enzyme producing one absorption unit/min at 526 nm.

3. Results

Out of 56 fungal isolates, three soil fungal isolates selected after comprehensive screening of the textile dyes biodegradation for further studies. In tube overlay method, *F. oxysporum* decolorized maximum (36.7%) Congo red followed by *A. niger* (34.21%) and *T. lignorum* (19.73%), while the highest percentage of crystal violet (55.26%) was degraded *T. lignorum*, 28.1% by *F. oxysporum* and 23.68% by *A. niger*, respectively. Similar trend was also observed in methylene blue biodegradation, (21.5%) by *T. lignorum*, (14.9%) by *F. oxysporum* and (13.15%) by *A. niger* (Fig. 1). The depth of decolorization of textile dyes by tested soil fungal isolated using tube overlay method exhibited the trend of Fig. 1. The range of depth of decolorization ranged from 7.5 to 13 mm in Congo red, 9 to 21 mm in crystal violet and 5 to 8 mm in case of methylene blue (Fig.2).

Under stationary conditions, biodegradation of Congo red was maximum (78.9%) by *F. oxysporum*, followed by *A. niger* (74.07%) and least by *T. lignorum* (35.25%). In case of crystal violet *T. lignorum* biodegraded (92.7%) followed by *F. oxysporum* (53.4%) and *A. niger* (33.82%), respectively. The trend was reversed in case of methylene blue, where *T. lignorum* and *F. oxysporum* biodegraded equally i.e. 48.3% and 48.5% respectively, while *A. niger* showed least biodegradation activity i.e. 22.44% (Fig. 3). Under constant shaking conditions the over all dye biodegradation was better compared to stationary state. Congo red was biodegraded almost equally by the three test fungal isolates, ranged from 84.7-91.4% decolorization. Highest percentage of crystal violet was biodegraded by *T. lignorum* (96.6%), followed by *A. niger* (78.8%) and *F. oxysporum* (56.1%), similar trend was followed in case of mythylene blue, where highest biodegradation was by *T. lignorum* (85.2%) and least was by *F. oxysporum* (53.4%) (Fig. 4). The laccase activity was also measured of all the test fungi and the three exhibited positive test, which showed that this enzyme also plays a role in biodegradation of textile dyes.

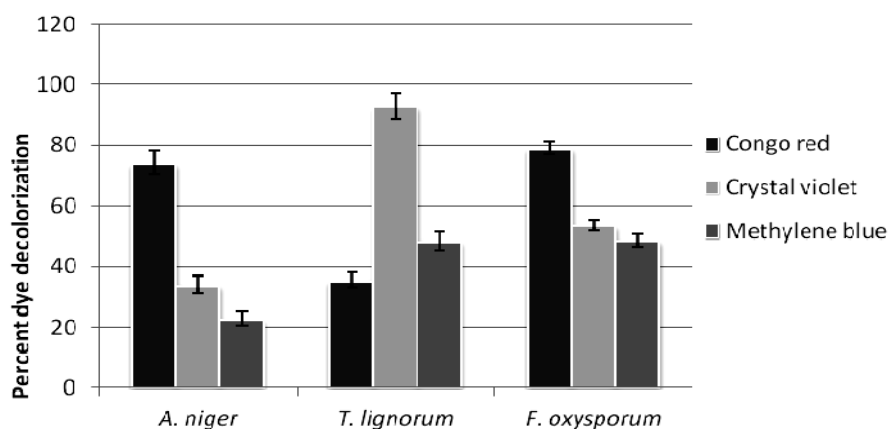


Figure 2. Depth of dye decolorization by soil fungi in solid medium

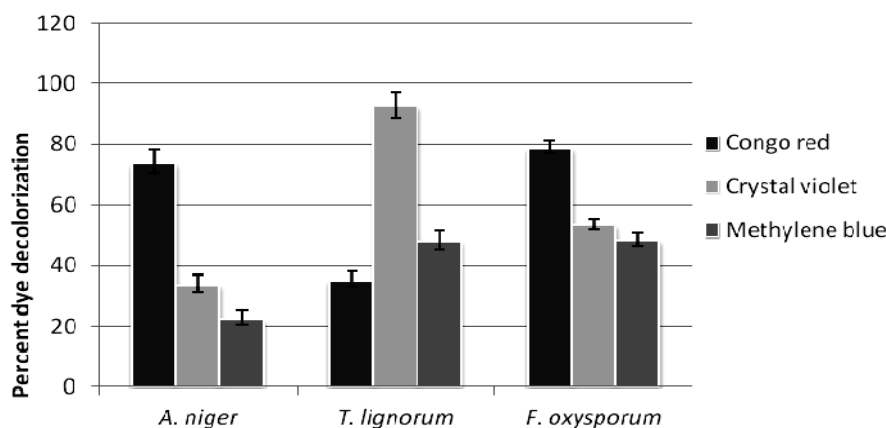


Figure 3. Dye decolorization by soil fungi in liquid media under stationary conditions

4. Discussion

The use of these soil fungi, thus could offer a much cheaper and efficient alternative treatment of wastewaters contaminated heavily with textile dyes. However, even though qualitative assays using the tube overlay method are powerful tools in screening fungi for extracellular enzyme production, they are not conclusive in that a negative reaction is not an absolute confirmation of a species' inability to produce a particular enzyme (Abdel-Raheem and Shearer, 2002). Hence, the tube agar overlay method (Lopez *et al.*, 2006) only provides an easier and quicker method to screen a large number of fungal isolates for further studying dye decolorization activity.

The removal of the dye color is vital in the potential application of soil fungal organisms as bioremediation agents in wastewater treatment plants and in runoff waters. Thus, it is essential to test soil fungal strains for dye decolorization in liquid medium. In the present study, soil fungus *Aspergillus niger* decolorized maximum Congo red (74.07%) followed by Crystal violet (33.82%) and Methylene blue (22.44%) under liquid

medium, stationary condition. Whereas, under the same conditions, *T. lignorum* decolorized maximum Crystal violet (92.7%), Methylene blue (48.3%) and Congo red (35.25%). *Fusarium oxysporum* also exhibited encouraging results under shaking conditions with decolorization of Congo red was 78.9%, Crystal violet 53.4%, while the Methylene blue was 48.5%. Ollikka *et al.* (1993) reported that Congo red was a substrate for the ligninolytic enzyme lignin peroxidase.

Use of *T. lignorum* as dye bio degrader or decolorizer has been studied first time in this report and the efficient decolorization may be attributed to either through the action of extracellular enzymes such as laccase and/or biosorption by the fungal biomass. Laccase production by the soil fungal species have been studied, this test was performed to know, whether laccase enzyme plays any role in biodegradation of dyes. Tatarko and Bumpus (1997) confirmed that Congo red was readily degraded in liquid cultures as also shown in this study. Furthermore, the soil fungal strains also showed promising decolorization activities against crystal violet (Figs. 3-4). Results of the dye biodegradation by soil fungi in this study using

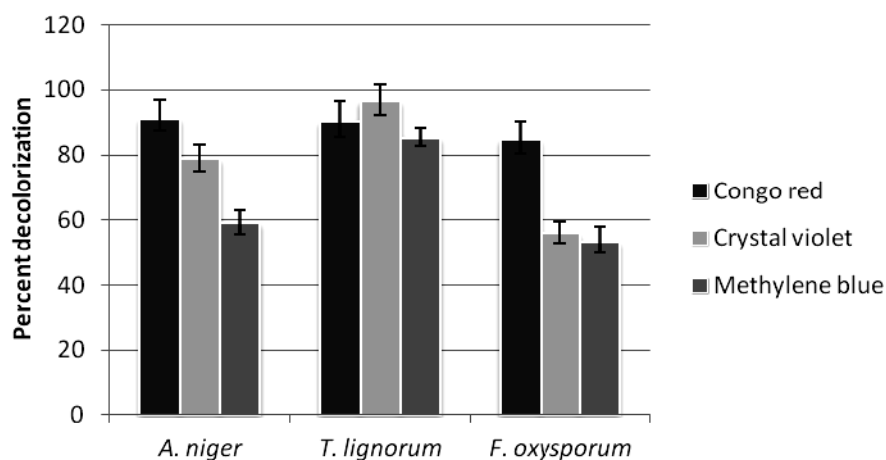


Figure 4. Dye decolorization by soil fungi in liquid media under shaking conditions

spectrophotometric analysis were even comparable with the percent dye decolorization exhibited by the white-rot fungus *Trametes versicolor* and *Pleurotus ostreatus* (Yao et al., 2009), and even *Phanerochaete chrysosporium* (Bumpus and Brock, 1988).

Soil fungi possess ligninolytic enzymes and play an important role in the degradation of lignocellulose in soil ecosystems (Okino et al., 2000). These lignin-degrading enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds, including dyes. Moreover, ligninolytic enzymes have been reported to oxidize many recalcitrant substances such as chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorus compounds, and phenols (Wesenberg et al., 2003).

Biosorption of dyes occur essentially either through complexation, adsorption by physical forces, precipitation, entrapment in inner spaces of fungal mycelium, ion exchange due to surface ionization, and by formation of hydrogen bonds (Yeddou-Mezenner, 2010). Due to an increased cell-to-surface ratio, fungi have a greater physical contact with the environment. Thus, some fungi have demonstrated better dye adsorption potential exceeding that of activated charcoal (Fu and Viraraghavan, 2002). Additionally, it is not unusual for some species to demonstrate both enzyme-mediated degradation and biosorption in the decolorization of textile dyes (Park et al., 2007). It is thus feasible that in addition to the production of extracellular enzymes, the ability of the soil fungi to decolorize synthetic dyes is coupled also with their biosorption abilities (Kaushik and Malik, 2009). We have also observed dye absorption by the test fungal mycelium under light microscope (1500 ×). This may account for the more efficient Congo red and crystal violet biodegradation by the soil fungal strains (Kirby et al., 2000) (Figs.1-4). It is therefore, possible that the ability of soil-derived fungi to degrade crystal violet, methylene blue and Congo red as revealed in this study can be largely attributed to the lignin-degrading enzyme system of the organism. In addition to extracellular enzymes, it is also likely that dye decolorization activity of these fungi could also be attributed to the ability of their mycelia to adsorb/absorb the dye.

5. Conclusions

We have studied the decolorization of dyes under stationary and shaking conditions; we have got encouraging results after 5 days, but maximum decolorization of all the dyes were obtained after 10 days. In this study we have observed higher decolorization under shaking conditions, which could be due to better oxygenation of the fungus and regular contact of secreted enzymes with

dye molecules to decolorize it, moreover agitation also helps the fungus to grow better. Disappearance of dye color may be due to biodegradation of chromophore in dye molecule because of extracellular enzyme production by fungi. Due to the environmental friendly techniques it utilizes, bioremediation has been characterized as a soft technology. Its cost-effectiveness and the little disturbance in the environment render this technology a very attractive and alternative. The identification and research of new fungal strains with the aid of molecular techniques will further improve practical application of fungi and it is anticipated that fungal remediation will be soon a reliable and competitive dye remediation technology.

Acknowledgments

The authors are grateful to the Management, College of Applied Education & Health sciences, Meerut, India for providing research facilities to carry out this work.

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Received 26 February 2013

Accepted 19 March 2013

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