J.Mycopathol.Res. 62(3) : 615-623, 2024, ISSN : 0971-3719 (Print), 2583-6315 (Online) 10.57023/JMycR.62.3.2024.615

Exploitation of *Neolamarckia cadamba*, an excellent source of an endophytic fungus, *Aspergillus ochraceus in bioremediation*

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Received : 31.03.2024 Accepted : 11.06.2024 Published : 30.09.2024

Water pollution is a big problem in India, which is due to several factors responsible for polluting water. Industrial effluent is one of them. Dyestuff containing industrial effluent plays a pivotal role in water pollution. Various methods are available for the treatment of dye containing effluent. Bioremediation has been recognised as the most suitable method for this. Fungi can be used to clean up dyestuff effluent by releasing certain extracellular enzymes, responsible for dye degradation or discoloration. Plants provide a residence of endophytic fungi. A medicinal plant, kadam or cadamba (*Neolamarckia cadamba*) was chosen for isolation of endophytic fungi. *Aspergillus ochraceus* was identified as an endophytic fungus. It showed azo dye (congo red, metanil yellow) discoloration activity tested by using potato dextrose broth and potato dextrose agar. Congo red was more effectively discolored by thisfungus than metanil yellow. The activity of an extracellular enzyme, laccase,was screened by using the dye bromophenol blue. Natural source like medicinal plants can be explored to have endophytic fungi for mitigating the environmental problems, such as water pollution.

Keywords : Azo dye, congo red, endophytic fungi, laccase, metanil yellow, water pollution

INTRODUCTION

Man-made environmental pollution has become a serious problem these days. Population growth, deforestation, urbanization, and industrial development have been considered to be the main factors contributing to this pollution (Appannagari, 2017). Pollution, or human action, has touched every part of the environment. Water pollution occurs when the quality of water deteriorates due to the presence of pollutants like dyes, heavy metals, etc. Azo dye-related waste plays a pivotal role in water pollution (Alzain *et al.* 2023). Azo dyes are commonly used in the textile industry, paper manufacturing, and printing as synthetic colorants. Azo dyes account for approximately 70% of total dyes used in industries (Benkhaya *et al*. 2020). They are mainly characterised by azo groups (-N=N-) linking benzene, naphthalene, or aromatic heterocyclic rings. Metanil yellow, or monoazo dye, is used in industries like colouring paper, textile dyeing, polishes, and lacquers.

Congo red, another azo dye, can be used in the textile industry for the coloration of paper (Horobin). As dyes have complex structures, they are difficult to degrade (Rudakiya *et al.* 2019). Several methods, including physical, chemical, biological, or combined methods, have been developed to remove dye from the effluent of industries to decrease their impact on the environment (Adane *et al.* 2021; Rudakiya *et al*. 2019). Generation of sludge, uses of chemicals, disposal problems, and operating costs make less use of physico-chemical treatment. Hence, bioremediation is the best option due to its cost effectiveness and ability to maintain environmental safety (Rudakiya *et al*. 2019). Bioremediation is an eco-friendly, cost-effective process by which living systems like bacteria, fungi, and algae are used to eliminate

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pollutants and hazardous waste from the environment through degradation, detoxification, and immobilization. The breakdown of organic substances by microorganisms provides nutrients and energy to the microbes for their growth (Bala *et al.* 2022). Specific enzymes catalyse the degradative pathway. Enzymes may be intracellular or extracellular. Extracellular enzymes play a pivotal role in the degradation of macromolecules. Several factors, like the contaminant itself and environmental factors, contribute to microbial activity as well as the rate of degradation. Structure, bioavailability, and physiochemical properties of the contaminants are important factors in the action of microbes on them. Similarly, environmental factors are responsible for the survival and activity of microbes in that particular environment. Redox condition, organic matter, nitrogen source, pH, temperature, salinity, and water activity.

Fungi, microscopic, and macroscopic eukaryotic organisms are ubiquitous in the environment. They can grow on different substrates. They may be tolerant of high temperatures, acidic or alkaline pHs, or higher concentrations of metal. In mycoremediation, fungi can degrade or deteriorate substrates. Several fungi have been reported to degrade azo dyes. Lignocellulolytic enzymes like laccase, Mn peroxidase, and lignin peroxidase are involved in the degradation of lignin as well as dyes, because there is a structural similarity between the dye molecule and the phenolic ring of lignin (Rudakiya *et al*. 2019).

Plants not only act as a source of bioactive compounds against diseases but also provide endophytes, a group of microbes (fungi, bacteria) that do not cause any harm to the plants and colonise the internal parts of the plants. They have a symbiotic relationship with their host plants through all or part of their life cycle. Endophytes can produce hormones or bioactive compounds or help to tolerate biotic or abiotic stress on their host plant, by which plants get more nutrients, leading to enhanced plant growth. They may be present in every part of the plant, like the seed, buds, fruits, petiole, leaves, stem, roots, etc. They are highly variable in number and depend on environmental conditions, host species, developmental stage of the host plant, density of the inoculum, etc. Every single plant is occupied by one or more species of endophytes (Gouda *et al.* 2016). Similarly, endophytic fungi are beneficial and harmless and present within plant tissues. Endophytes have been evolving with their host plants, host community, and surrounding ecosystem. Endophytic fungi can be isolated by the culturing method. A small piece of plant tissue is surface-sterilised and put on agar plates. After a few days, fungal growth is observed. Endophytic fungi mainly belong to Ascomycota (Moore *et al*. 2011). They may produce secondary metabolites like flavonoids, alkaloids, taxol, phenol, terpenoids, and enzymes like amylase, protease, lipase, cellulase, pectinase, etc. They may be sources of an antidiabetic agent, an anticancer agent, an antioxidant agent, or an or an antimicrobial agent (Jaiswal *et al.,* 2023). In addition, endophytic fungi are involved in the decolorization of dyes. Hence, endophytic fungi can be applied to decolorize the dye effluents from the food and textile industries (Chanyal and Agarwal, 2017). The aim of this paper was to identify endophytic fungi from medicinal plants nearby our university campus and show their dye degradation or discoloration ability.

MATERIALS AND METHODS

Chemicals

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Sisco Research Laboratory Pvt. Ltd. (SRL). Agar was obtained from Himedia. Lactophenol cotton blue was purchased from Nice chemicals. Congo red and metanil yellow were purchased from Nice chemicals.

Preparation of fruit peel filtrate agar (FPFA) medium using mosambi peel

Fruit peel filtrate agar (FPFA) medium was prepared according to the protocol obtained from Manna *et al*. (2023). Mosambi peel was taken from a local fruit juice shop. After cleaning with water, it was sun-dried. Next, powder was formed after grinding in the mixer grinder. Filtrate medium was prepared by filtration of 4 gm of mosambi peel powder dissolved into 200 ml distilled water. 3% agar was poured into the filtrate, followed by

autoclaving at 15 psi for 15 min. Solidified petri plates were used to isolate endophytic fungi.

Collection of plant sample

A medicinal plant, kadam or cadamba, was chosen for the isolation of endophytic fungi. It was found in surrounding areas of West Bengal State University campus, North 24-Parganas, West Bengal. *Neolamarckia cadamba* was identified by its external morphological features. A plant sample was collected in a sterile polythene bag, and brought to the laboratory, and was processed for isolation of endophytic fungi.

Isolation of endophytic fungus

For the removal of dirt, the collected plant sample was carefully cleaned with water. The whole process of isolating endophytic fungi was performed in an aseptic condition. Then, the surface sterilization process was performed. Leaves were cut to around 1 cm in length. Leaves were immersed in 70% ethanol for 5 seconds, then dipped into 4% sodium hypochlorite solution for 90 seconds, followed by being rinsed with sterile distilled water for 10 seconds (Amirita *et al*. 2012). Finally, surface sterilized leaves were placed over FPFA supplemented with gentamicin solution (10 mg/ml) and incubated at 25^{0}.C -28^{0}.C for 5-7 days. The plates were observed every day for the fungal growth.

Identification of fungus

Colony characterization and morphological identification were used to identify the fungi. Lactophenol-cotton blue wet mount-tease mount preparation was performed for microscopic identification. A small segment of fungal mycelium was placed on the drop of lactophenol cotton blue stain in the clean slide. Mycelium was teased with a mounted needle. A coverslip was placed over it examined the under the microscope (45X) (Aneja, 2018).

Maintenance of pure culture

Frequent subculture of isolated fungus was carried out in FPFA for the maintenance of pure culture.

Qualitative screening of dye-decolorizing fungi in solid media

Congo red and metanil yellow were used for the dye decolorizing experiment. PDA medium was used. Dye solution (congo red and metanil yellow) and agar medium (PDA) were separately autoclaved at 15 psi for 15 min. Congo red solution was mixed with PDA. Similarly, metanil yellow was mixed with PDA. PDA with congo red (0.05%) and PDA with metanil yellow (0.05%) were used in this study. Agar plates were inoculated with a $5mm^2$ agar plug from a 7 days old isolated fungal culture. Agar plates were incubated at 25^{0}.C-28^{0}.C for 10-15 days. Control plates for dyes, congo red and metanil yellow, were prepared. These plates contained no inoculation of fungal culture. The experiment was carried out twice in triplicates. The presence of a clear zone around the colony was an indicator of the dye decolorizing ability of the endophytic fungus isolated from the medicinal plant, *N. cadamba*.

Dye decolorization ability of fungus on liquid medium

This experiment was carried out according to the protocol obtained from Ngieng*et al*.,2013 and Rani, *et al*. (2014). PDB supplemented with congo red (0.05%) and PDB supplemented with metanil yellow (0.05%) were used for liquid medium. These two-dye containing PDB media were inoculated with an isolated fungal spore $5mm^2$ agar plug derived from a 7-days old fungal culture and incubated at 25^{0}.C-28^{0}.C for 5-10 days without shaking condition. PDB supplemented with congo red (0.05%) and PDB supplemented with metanil yellow (0.05%) with no fungal inoculum respectively were served as control media. Fungal growth and dye discoloration were observed every day. After incubation time, filtration followed by centrifugation at 5000 rpm for 10 min was carried out for removal of mycelium. Filtrate were undergone spectrometric analysis. The absorbance maxima of congo red and metanil yellow was 498 nm and 440 nm respectively. The percent of dye degradation by isolated endophytic fungi was calculated from the formula:

$$\% \text{ of dye degradation} = (\text{Absorbance}_{control} - \text{Absorbance}_{test} / \text{Absorbance}_{control}) \times 100$$

Absorbance $_{control}$ = Absorbance for control sample
Absorbance $_{test}$ = Absorbance for test sample
Screening of laccase production by fungus by bromophenol blue assay
PDA and PDB were supplemented with bromophenol blue (0.2 g/l). They were then inoculated with fungal mycelium for the degradation of bromophenol blue. PDB without fungal inoculation was as a control. The prescence of a lignolytic enzyme (laccase) was indicated by the discoloration of this dye (Kumar *et al*. 2016).

Data analysis

Using Excel analysis, the mean and standard deviation of the data were calculated. Values were expressed as the mean ± SD for triplicate.

RESULTS AND DISCUSSION

Sustainable environment is one of the biggest challenges in recent times and has received significant attention from researchers, government and non-government organisations, etc. (Arora, 2018). In the management of waste and pollutants, biodegradation of xenobiotics through bioremediation has become a part of it. This present study also focuses on bioremediation with the help of endophytic fungi isolated from medicinal plants.

The medicinal plant, Neolamarckia cadamba, or *Anthocephalus cadamba,* has anti-diabetic activity, anti-inflammatory activity, anti-microbial activity, and antioxidant and wound healing properties (Verma *et al.* 2018). In our study, this plant, located near the university campus area, was chosen for isolation of the endophytic fungus (Fig. 1). In this regard, exploration of plants adjacent to our university's locality was also carried out.

Leaves were collected from the plant. The surface-sterilised leaves of *N. cadamba* were put on the FPFA. Fungus was isolated on the FPFA medium. A pure culture of the fungus was done (Fig. 2). The fungus was identified as *Aspergillus ochraceus* on the basis of morphological and microscopic characterization. The conidiophore of *A. ochraceus* seemed to be a powdery mass. The colour of the colony was yellow-orange or caramel (Arifah *et al.* 2023). The microscopic feature of *A. ochraceus* was small and spherical in shape (Diba *et al.* 2007) (Fig. 3). It is a wide range of pH-tolerant fungus; and can grow well from pH-3 to 10 (https://www. sciencedirect. com.). Attia *et al.* (2020) isolated *Aspergillus ochraceus* MSEF6 as an endophytic fungus from the leaves of *Medicago sativa* (alfalfa) and showed antimicrobial activity, as well as laccase activity. *Aspergillus ochraceus* was isolated from the medicinal plant *Bauhinia forficate* as an endophytic fungus. It also showed antibacterial potential (Bezerra *et al.*2015).

Domestic waste, agricultural waste, industrial waste, and run-off from urban waste have been considered to be major water pollutants. Inorganic pollutants, toxic metals, organic pollutants, including detergents, pesticides, and dyes, and suspended matter have been identified (Sharma, 2001). Around 17–20% of total water pollution in the world is caused by effluent released from the textile industries, according to the World Health Organisation (WHO) (Sarkar *et al*. 2021). Azo dyes and synthetic compounds are widely used in the textile industry. Azo dyes have received popularity because they are cheap and stable, may be easily generated in laboratories, and have easy availability of chemicals for synthesis. Direct coupling between a diazonium salt and a phenol or amine leads to the development of azo dye. They may be grouped into monoazo, disazo, trisazo, etc, based on the number of azo groups in the molecule. Again, it is categorised as acidic (anionic) or basic (cationic). Dyestuff of azo dye as toxic effluent is released into water bodies like rivers and causes water pollution. Aquatic organisms and water resources are damaged by this effluent. Humans may get these toxic chemicals through the food chain and may experience several health problems (Dayal, 2015). Azo bonds and sulfonic groups make them more recalcitrant in nature. The quality of water is deteriorating after the dispersion of azo dye into the bodies of water (Mani *et al.*2019). Diazo dye and congo red may be present in the effluent of textile industries (Sharma *et al.*2008). Congo red is a direct or substantive azo dye because it does not need mordant for dyeing cellulosic fibre. It is

Fig. 1 : Cadambda tree

Fig. 2: Pure culture of *Aspergillus ochraceus*

Fig. 3 : Microscopic observation of *Aspergillus ochraceus*

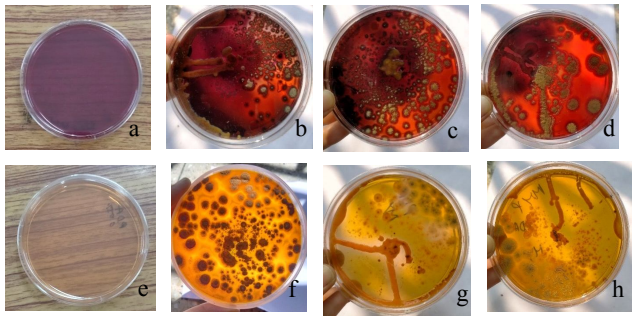

Fig .4: a and e - control plate with PDA in congo red (0.05%) and metanil yellow (0.05%.) respectively. **b,c,d** and **f,g,h** showed PDA containing congo red (0.05%) and metanil yellow (0.05%.) inoculated with *Aspergillus ochraceus respectively. Discoloration of congo red and metanil yellow by Aspergillus ochraceus was observed.*

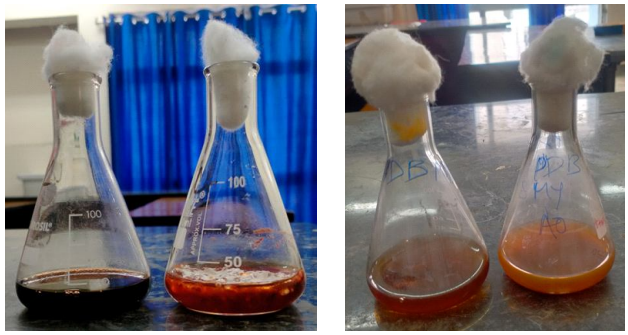

Fig. 5 : a and c -control for congo red and metanil yellow in PDB medium. **b and d** were the *Aspergillus ochraceus*-treated congo red and metanil yellow in PDB medium. Discoloration of congo red and metanil yellow in PDB medium was observed after treatment with *Aspergillus ochraceus*

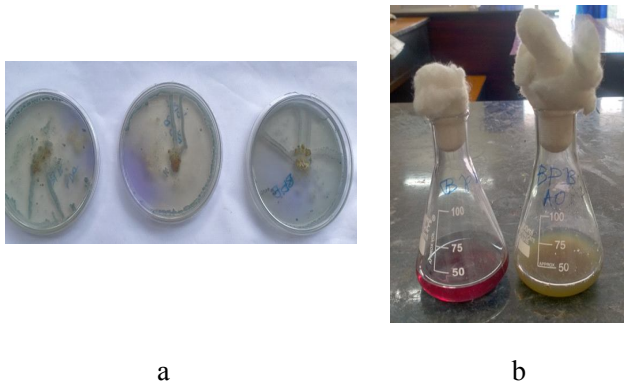

Fig. 6: The discoloration of bromophenol blue in PDA (a) and PDB (b) indicated the production of laccase by *Aspergillus ochraceus* in our study

formed by coupling tetrazotized (bis-diazotized) benzidine with two molecules of napthionic acid. Then blue dye can be converted to red disodium salt during its salting out with sodium chloride. This red salt has the ability to dye cotton directly and is very sensitive to acid. The colour changes from red to deep blue in the presence of strong mineral acids and dull purple in the presence of

organic acids (Chatwal, 2022). Hence, congo red solution is red at a neutral or alkaline pH and becomes blue when the pH is lowered. The unprotonated anionic form is present at a pH above 5, and the protonated form is present below 5 (Csillag *et al.*2023). Congo red is one of the recalcitrant azo dyes; hence, it may persist in the environment for a long time (Sarkar *et al.* 2021). In addition, nitrobenzene is a starting compound of the acidic, sulphonated azo dye metanil yellow. It is sulphonated, followed by reduction with iron and sulfuric acid, then diazotized and coupled with diphenylamine (Chatwal, 2022). Metanil yellow is widely used in food- colouring, although it is a non-permitted colourant (Anjaneyaa *et al*. 2011). Metanil yellow was detected in turmeric powder, whereas congo red was detected in red chilli powder (Biswas *et al.*2021).

In our study, the dye degradation ability of endophytic *Aspergillus ochraceus*was screened using the agar plate method. The visible disappearance of dye or the development of a lighter dye colour around the colony was an indicator of dye degradation or decolourization by *Aspergillus ochraceus*. (Fig.4a**)** was a control plate with PDA in congo red, 0.05%. Fig. 4b-4d showed PDA containing congo red (0.05%) inoculated with *A. ochraceus.* In our study, there was a change in red dye colour to a lighter dye colour around the colonies and also the formation of a light purple-violet colour. As congo red is a pH indicator dye, it shows an orange-red colour at pH 5 and a violet colour at pH 3 (Singh *et al*., 2010). Formation of aggregation of protonated congo red and precipitation occurs at highly acidic pH and appear as dark blue (Csillag *et al*. 2023). Hence, it is clearly predicted that the decolorization of congo red by *A. ochraceus* has been associated with lowering the pH of the congo red solution. A reduction in pH also changes the structure of the congo red (Csillag *et al*. 2023). Csillag *et al*. (2023) studied the antifungal effect of congo red. It was found that a reduced growth inhibitory effect of congo red was observed due to lowering pH. In another way, it could be said that a less toxic form of congo red might be formed after treatment with fungi. So, decolorization of congo red by *A. ochraceus* may be associated with the production of less toxic compounds, which meet the use of bioremediation over chemical treatment. The ë $_{max}$ of congo red has been observed at 498 nm (Rafiquea *et al*. 2021). We observed decreased absorption of *A. ochraceus*-treated congo red solution in comparison to the control (without treatment of the fungus) in congo red solution) at 498 nm, indicating that the dye structure was transformed by treatment with *A.ochraceus* (Asses *et al*. 2018). A percentage of congo red decolorization by *Aspergillus ochraceus* was observed in liquid broth medium (Figs.5a and 5b). (Fig.5a) was the control for congo red, and (Fig.5b) was the *A. ochraceus*-treated congo red-PDB medium. After 5 days of incubation, the mean percentage of Congo red decolorization by *A. ochraceus* was 86.19 ± 0.59.

Metanil yellow, another azo dye, is widely used in food as a dye adulterant in India. Fig. 4e was a control plate with PDA in metanil yellow, 0.05%. Fig.4f-4h shows PDA containing metanil yellow inoculated with *A. ochraceus.* A lighter dye colour was observed around colonies of *A. ochraceus.* Fig.5c was the control for metanil yellow, and Fig.5d was *A. ochraceus*-treated metanil yellow-PDB medium. Whereas, the mean percentage of metanil yellow degradation took place at 48.64± 0.50 after 10 days of incubation. This data found that congo red was degraded more effectively than metanil yellow by *A. ochraceus*.

A previous report also showed the dye degradation ability of *Aspergillus ochraceus*. Solid-state fermentation was used to cultivate *A.ochraceus* by using waste from the sugar industry. After 7 days of cultivation of *A.ochraceus*, the visible disappearance of methylene blue and congo red began, and after 21 days, almost complete dye decoloration occurred (Tišma *et al*. 2012). Cotton blue and malachite green were decolorized by *Aspergillus ochraceus* (Saratale *et al*. 2006). *Alternaria alternata* CMERI F6 had been observed to decolorize 600 mg/L congo red (Chakraborty *et al*. 2013). Metanil yellow can also undergo bioremediation. A report showed that metanil yellow was degraded by bacteria (Anjaneyaa*et al*., 2011). A halophilic alkalilithermophilic bacterial consortium was able to decolorize metanil yellow (Guo *et al*. 2020.). Another report showed that metanil yellow was decolorized by molybdenum-reducing bacteria (Mansur *et al*. 2017).

Microorganisms can remove or degrade dyes by adsorption or production of enzymes that target dyes or a combination of both. Live cells or dead cells may be involved in adsorption. Adsorption process has limitations; azo dyes cannot be converted into non-toxic products. Hence, microbial enzymes that cause decolorization or degradation have received more attention (Ngo *et al*. 2022). Azoreductase, peroxidase, and phenol oxidases have played a significant role in dye degradation. Azoreductases are intracellular enzymes. Lignin peroxidase and manganese peroxidase are included in the peroxidase group. Laccase, which is an extracellular multicopper oxidase enzyme involved in acting on several phenolic compounds (Legerská *et al*. 2016), Laccase is widely distributed in fungal groups, including Ascomycetes, Basidomycetes, and Deuteromycetes (Devi *et al*. 2012). Fungal laccase has an advantage over bacterial or plant laccase because it has a higher redox potential and is involved in the degradation of various xenobiotics (Iark *et al*. 2019). In addition, fungal laccase shows stability and can be effective without hydrogen peroxidase. They can be used in paper, pulp, textile industries, and biodegradation of environmental pollution due to their broad substrate specificity (Wadhwa *et al*. 2023). A change in the absorption spectrum of a dye is an indicator of the biodegradation ability of laccase (Legerská *et al*. 2016). Microorganisms may be screened for the production of laccase by solid media having coloured indicator substances through visual detection. The colour change of the indicator substance has been associated with fungal laccase activity (Vaidyanathan *et al*. 2019). Bromophenol blue (BPB) is one of the substrates of laccase. Discoloration of bromophenol blue marks the presence of lignolytic enzymes (Kumar *et al.* 2016). In our study, laccase activity was screened by the discoloration of bromophenol blue. The discoloration of bromophenol blue in PDA (Fig. 6a) and PDB (Fig. 6b) indicated the production of laccase by *Aspergillus ochraceus* in our study. At pH 4.6, bromophenol blue shows blue colour and yellow colour at pH 2.8 (Singh *et al*. 2010). The mean percentage of BPB decolorization by *Aspergillus ochraceus*was 40.99± 2.07. Laccase-mediated azo-dye degradation was reported. Phenolic compounds are formed in the first step, followed by the oxidation of phenolic compounds. In addition, non-phenolic compounds can be oxidised by laccase (Mani *et al.* 2019). A report showed that laccase isolated from the fungus *Oudemansiella canarii* functioned on the chromophore of congo red and cleaved covalent bonds. Lowered toxicity was also observed in the presence of laccase (Iark *et al.* 2019). Hence, it can be proposed that laccase secreted by endophytic fungi *Aspergillus ochraceus* may be responsible for congo red and metanil yellow discoloration in this study.

CONCLUSION

The present study shows that the endophytic fungus *Aspergillus ochraceus* has been isolated from a medicinal plant, *Neolamarckia cadamba* or *Anthocephalus cadamba,* located at our university campus. Enzyme laccase-mediated decolorization of two azo dyes, congo red and metanil yellow, has been observed by this fungus. Treatment of industrial effluent by this fungus may be an eco-friendly and cost-effective alternative option for reducing the water pollution mediated by the textile and dye industries. Mycoremediation may be performed on industrial effluent before disposal into water bodies. Endophytic fungi as well as medicinal plants play a pivotal role in maintaining balance in ecosystems by alleviating water pollution. Another interesting point has been disclosed through this study: the local environment should be exploited properly to mitigate global environmental problems. Hence, the local environment is complementary to the global environment.

ACKNOWLEDGEMENT

The authors acknowledge the Department of Biochemistry at West Bengal State University for providing the lab facilities.

DECLARATION

Conflict of interest. Authors declare no conflict of interest.

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