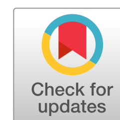
Technical Note



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Evaluation of the Ability of Endophytic Fungi from *Cupressus torulosa* to Decolorize Synthetic Textile Dyes

Pavan Kumar Agrawal[1]; Pooja Upadhyay[2]; Rahul Shrivastava[3]; Swati Sharma[4]; and Vijay Kumar Garlapati[5]

Abstract: Ecofriendly dye decolorization is a prerequisite for textile industries to tackle hazardous textile effluents. In the present study, we have reported a laccase producing (19.12 ± 3.45 U/mL) fungal endophyte, namely, *Penicillium megasporum Orpurt* et *Fennell NFCCI* 3977. The endophytic laccase was evaluated for synthetic textile dyes (congo red, orange G, and rhodamine B) decolorization by considering the carbon, nitrogen, and pH effects. The endophytic-based laccase decolorization showed promising results with the decolorization of congo red ($89.4 \pm 3.2\%$), orange G ($76.2 \pm 2.5\%$), and rhodamine B ($54 \pm 2.8\%$) using glucose and beef extract as carbon and nitrogen sources at pH 6.0. Thus, the present study demonstrated the efficiency of new endophytic fungi for decolorization ability of synthetic textile dyes in an ecofriendly manner. The research findings showcase the handling of the proper disposal of hazardous synthetic textile dyes into the environment through the biological approach by replacing the existing toxic chemical-based disposal approaches. **DOI: 10.1061/(ASCE)HZ.2153-5515.0000569**. *© 2020 American Society of Civil Engineers.*

Author keywords: *Cupressus torulosa D. Don*; Dye decolorization; Endophytic fungi; *P. megasporum Orpurt* et *Fennell*; Synthetic dyes.

Introduction

Textile industry effluents mainly comprise toxic dye components along with heavy metals and organic compounds that eventually deteriorate the quality of aquatic bodies. Approximately 10,000 commercial dyes are produced worldwide with an annual production rate of over 7×10^5 metric tons (Khandare and Govindwar 2015). The nonbiodegradability of spent dyes from textile industries presents a severe environmental concern. Hence, the decolorization of industrial effluents from textile industries in an ecofriendly manner is a prerequisite before discharging them into aquatic bodies. The conventional biological treatment processes (e.g., activated sludge process, carbon absorption, chemical coagulation, chemical oxidation, electrochemical treatment) suffer from little biodegradation ability of the complicated molecular structures of dyes. Hence, innovative ecofriendly biological decolorization processes of dyes must be developed for efficient processing of the textile industry effluents (Khandare and Govindwar 2015).

Fungal enzyme machinery plays a significant role in decolorization of dyes through altering the structure and degradation. Extracellular white-rot fungal laccases (EC 1.10.3.2) have been well documented for their ability to decolorize the azo dyes (Garg et al. 2012). For better results, the fungal laccases have to withstand the harsh environmental conditions of the textile effluents (Piscitelli et al. 2011). In the recent past, endophytic fungi, the inhabitants of asymptomatic plant tissues, have attracted considerable attention from environmental researchers due to their role in natural wood decay processes and plant tissue degradation after plant death (Noman et al. 2019). As an inhabitant of complex lignocellulosic material, the endophytic fungi have a complex set of lignocellulolytic enzymes, which have found application in the synthetic textile dye decolorization process (Neetha et al. 2019). The high penetrating ability of lignocellulosic enzymes into the complex dye structures along with the sustainable process conditions makes their usage preferable over chemical-based treatments (Shang et al. 2019) Moreover, usage of laccase mediated system (LMS) for decolorization of dyes is ecofriendly due to the utilization of mild temperatures in the process (Martín et al. 2013). Research attempts have been started to explore the role of these endophytic fungi in different environmental sectors, such as the biodegradation of wood, lignin, and environmental burden recalcitrant compounds. Chen et al. (2013) reported the N-heterocyclic indole degradation ability of laccase from a novel endophytic fungus, *Phomopsis liquidambari.* In another study, laccase from endophytic fungus *P. liquidambari* was reported for its ability to degrade ferulic acid and sinapic acid (Xie and Dai 2015; Xie et al. 2016). Laccase from endophytic fungi has also been used in the pretreatment step to enhance the enzymatic saccharification process of Eucalyptus globules (Martin-Sampedro et al. 2015). Given the importance of ecofriendly decolorization processes for textile industrial effluent treatment, the present research aimed to evaluate the efficiency of new endophytic fungi in decolorization of synthetic textile dyes, namely, congo red, orange G, and rhodamine B.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in the present study were of analytical and reagent grade. Congo red, orange G, and rhodamine B

[1]Associate Professor, Dept. of Biotechnology, G. B. Pant Engineering College, Ghurdauri, Pauri, Garhwal, Uttarakhand 246194, India. Email: p_k_agarwal@rediffmail.com

[2]Doctoral Student, Dept. of Biology, Univ. of Louisiana at Lafayette, Lafayette, LA 70503. Email: poojaupadhyay100@gmail.com

[3]Associate Professor, Dept. of Biotechnology and Bioinformatics, Jaypee Univ. of Information Technology, Waknaghat, Himachal Pradesh 173234, India. ORCID: https://orcid.org/0000-0003-2031-8369. Email: rahulmicro@gmail.com

[4]Doctoral Student, Dept. of Biotechnology and Bioinformatics, Jaypee Univ. of Information Technology, Waknaghat, Himachal Pradesh 173234, India. Email: swati790sharma@gmail.com

[5]Assistant Professor (Senior Grade), Dept. of Biotechnology and Bioinformatics, Jaypee Univ. of Information Technology, Waknaghat, Himachal Pradesh 173234, India (corresponding author). ORCID: https://orcid.org/0000-0002-8914-3678. Email: shanepati@gmail.com

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were obtained from Sigma and Fluka, USA. Guaiacol and standards were procured from Sigma, USA.

Collection of Leaves

Mature leaves and twigs of *Cupressus torulosa* D. Don. plant (Accession number 115744, Botanical Survey of India, Dehradun) were collected from different locations of Ghurdauri, Pauri, Uttarakhand, India (latitude 30°18′35″N and longitude 78°69′30″E). The collected material was transported to the workplace aseptically in sterile polyethylene bags and stored at 4°C until further processing.

Isolation, Subculturing, and Identification of Endophytic Fungi

The collected healthy plant tissues were surface sterilized with ethanol (70%, 3 min), sodium hypochlorite (0.5%, 1 min), and finally with sterile water and were dried on sterile filter paper. The dried samples were plated on Water Agar (WA) media amended with Streptomycin (200 mg/L) and incubated at 27°C ± 2°C for 2–4 weeks. Fungal growth was observed in the form of a cottony outgrowth from the incubated WA media plates inoculated with plant tissues. Hyphal tips were isolated from the germinating fungi and subcultured on Potato Dextrose Agar (PDA) medium by incubating at 28°C for 5–7 days (Atalla et al. 2010). Pure culture of the fungal isolate was maintained at 4°C. Pure isolates of fungal culture were submitted to the National Fungal Culture Collection of India (NFCCI), Pune, India, and identified as *Penicillium megasporum Orpurt* et *Fennell* NFCCI 3977 belonging to the family of Trichocomaceae.

Screening of Endophytic Fungi for Laccase Activity

Laccase activity was qualitatively tested initially by growing the fungi on Glucose Yeast Peptone (GYP) agar medium (Peptic digest animal tissue 10 g/L, Yeast extract 5 g/L, Dextrose 20 g/Ll, Agar 15 gLl, pH 6.0) amended with 2 mM ABTS (2,2′-azinobis 3-ethyl-benzothiazoline-6-sulfonate) (pH 4.5), Guaiacol (4 mM, pH 5.4), Tannic acid (0.50 μM), *Syringaldehyde* (50 mM, pH 4.5), and incubated at 25°C ± 2°C for two weeks by wrapping the Petri plates in black polythene bags. The fungi grow on guaiacol, ABTS, tannic acid, and *syringaldehyde* free media were served as controls in their experiments (Sathish et al. 2012). The ligninolytic activity was observed for two weeks by recording the brown oxidation zone (below and around the cultures) and measuring the colored zone and colony diameters. The laccase activity index was calculated as

$$\text{Laccase activity index} = \frac{\text{Diameter of colored zone}}{\text{Diameter of colony}} \tag{1}$$

Quantitative Screening for Laccase Production

Quantitative screening of laccase was carried out using Potato Dextrose broth (100 mL) taken in an Erlenmeyer flask (250 mL). Two mycelial agar plugs (4 mm) from PDA plates were used as an inoculation media to assay laccase production. Flasks were incubated at 28°C with shaking at 120 rpm. Sampling from broth cultures was done at regular intervals for laccase activity (Abd El Monssef et al. 2016).

Laccase Assay

The identified fungal strain was further grown under submerged conditions using GYP broth medium supplementing guaiacol, ABTS and syringaldehyde as substrates. The culture filtrate was quantified for laccase activity with 6,740 M^{-1} cm^{-1} ε at 470 nm (Bhattacharya et al. 2011). The reaction mixture consisted of guaiacol (3 mL, 100 mM) dissolved in acetone (10% v/v), sodium acetate buffer (100 mM, pH 5.0), and culture filtrate (1 mL). The reaction components were incubated for 15 min and then absorbance taken at 470 nm. The culture filtrate was also assayed using 2 mM ABTS as substrate for laccase activity with the assay components of sodium citrate buffer (pH 3.0, 0.1 M). Oxidation of ABTS was followed by absorbance increase at 420 nm ($e = 536{,}000$ M^{-1} cm^{-1}). Laccase activity was also determined using 0.5 mM syringaldehyde (dissolved in ethanol) as substrate dissolved in 50 mM phosphate buffer, pH 6. Oxidation of syringaldehyde was followed by an absorbance increase at 525 nm ($e = 65{,}000$ M^{-1} cm^{-1}). Laccase activity was expressed in International Units (IU), where one IU defined as the amount of laccase required to oxidize 1 μmol of guaiacol, ABTS, and syringaldehyde per min (Sharma et al. 2016), which can be shown as

$$\text{Laccase activity (U/mL)} = A \times \frac{V}{t} \times e \times v \tag{2}$$

where A = absorbance; V = total mixture volume; v = enzyme volume; t = incubation time; and e = extinction coefficient of Guaiacol = 6,740 M^{-1} cm^{-1}.

Decolorization Studies of Textile Dyes

The decolorization ability of endophytic fungus was qualitatively tested through the agar plate method. Seven-days-old mycelial discs (6 mm) were placed on malt extract agar medium [MEA, 3% (w/v) malt extract, 0.5% (w/v) peptone, and 1.5% agar] along with the 50 mg/L of individual dyes addition and incubated at 25°C ± 2°C for 15 days. Agar plates without fungal inoculum served as a control. The decolorization rate was checked twice a week (Garg et al. 2012).

Further, decolorization of textile dyes was quantified in the liquid broth method. Nine-millimeter endophytic fungal culture plugs were transferred to sterilized malt extract broth (50 mL) amended with 50 mg/L of individual dyes (congo red, orange G, and rhodamine B) in the 250 mL conical flasks and incubated at room temperature in a static condition with periodic shaking for 14 days. After 14 days of incubation, an aliquot of culture filtrate (0.5 mL) was drawn aseptically from each tube periodically and diluted with an equal volume of acetate buffer (pH 5.0). The diluted samples were centrifuged and optical density (OD) of the supernatants were measured spectrophotometrically at 514, 478, and 540 nm for congo red, orange G, and rhodamine B, respectively. The difference in the OD before and after incubation gives the rate of dye decolorization. A sample without fungal inoculation was considered as a control.

Decolorization ability was calculated as

$$\%\ \text{Dye decolourization} = \frac{\text{Initial absorbance} - \text{Final absorbanc}}{\text{Initial absorbance}} \times 100 \tag{3}$$

Effect of Carbon and Nitrogen Sources, pH and Laccase Activity on dye Decolorization

Decolorization of dyes in malt extract broth using endophytic fungi could influence experimental conditions and were studied for the effect of carbon sources (glucose, sucrose, and maltose, 1%), nitrogen sources (peptone, yeast extract, and beef extract, 0.25%), pH (4, 6, and 8), and laccase activity (0–3 U/mL). All the experiments

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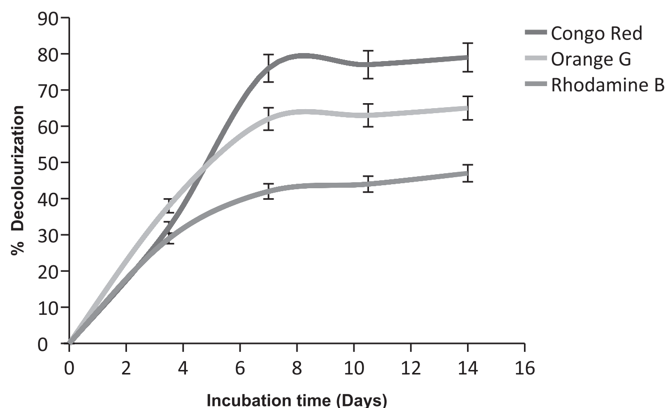

Fig. 1. Effect of incubation time on dye decolorization by *P. megasporum Orpurt* et *Fennell*. All values are represented as mean ± s.d. of three replications.

were carried out with 1% (v/v) inoculum (10 spores/mL); whereas the malt extract broth without fungal culture was treated as control. All the cultures were incubated at 27°C under shaking conditions for 14 days.

Results

Screening of Endophytic Fungi for Laccase Activity

Lignin-degrading laccase enzymes present in the endophytic fungus are responsible for the oxidation of ABTS (expand) (2 mM, pH 4.5), guaiacol (4 mM, pH5.4), tannic acid (0.50 μM), *Syringaldehyde*, which showed a change in the color at the center of the Petri plates. Laccase reacts with guaiacol and gives an intense brown color product, a purple color product with ABTS and a dark brown and brown color product with tannic acid and syringaldehyde.

Quantitative estimation by liquid culture method reveals the higher laccase activity of 57 ± 2.5 U/mL using guaiacol as a substrate, followed by 47 ± 2.2 U/mL by ABTS and the least 32.12 ± 1.8% U/mL with syringaldehyde have been observed after the 10th day of incubation at 27°C and 120 rpm (results not shown).

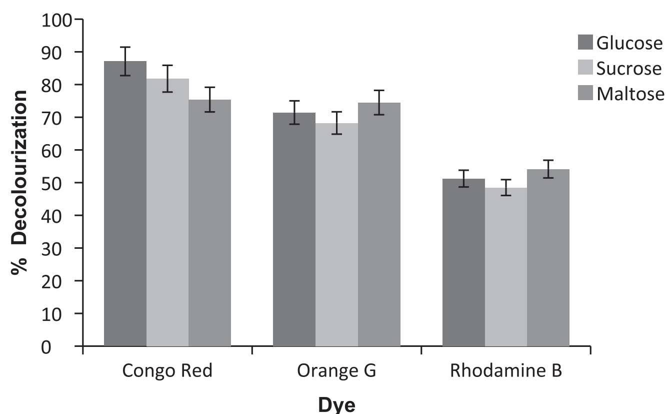

Fig. 2. Effect of carbon sources on dye decolorization by *P. megasporum Orpurt* et *Fennell.* All values are represented as mean ± s.d. of three replications.

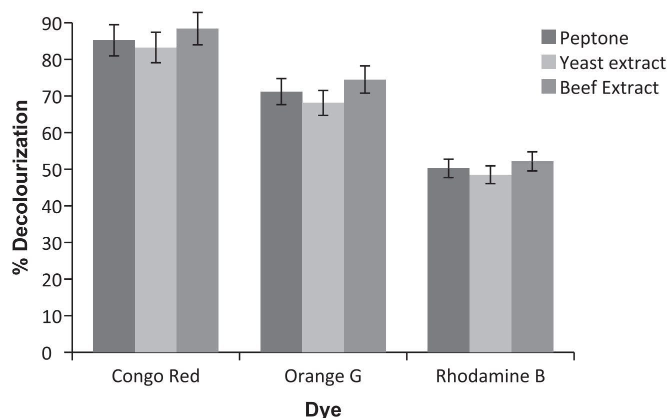

Fig. 3. Effect of nitrogen sources on dye decolorization by *P. megasporum Orpurt* et *Fennell.* All values are represented as mean ± s.d. of three replications.

Dye Decolorization Studies by Endophytic Fungal Isolate

Congo red, orange G, and rhodamine B have been utilized to evaluate the decolorization efficiency of endophytic fungi using solid agar plate medium. After seven days of incubation, congo red and orange G were decolorized, whereas rhodamine B was partially decolorized (results not showed).

Endophytic fungi intervention in synthetic textile dye decolorization has been further quantified spectrophotometrically by studying the decolorization ability for seven days. The results showed the decolorization efficiencies of 76 + 2.4%, 62 + 2.8%, and 42 + 3.2% had been observed with the congo red, orange G, and rhodamine B, respectively (Fig. 1). Endophytic fungal isolate decolorizes congo red more rapidly than the orange G and rhodamine B.

Selection of Dye Decolorization Process Parameters

The medium used for decolorization studies is usually devoid of sufficient carbon and nitrogen sources. Moreover, the working of fungal isolates in decolorization processes needs to have compatible pH conditions to exert their maximum effect (Khelifi et al. 2009). Selection of carbon and nitrogen sources and the pH facilitates a better working environment for endophytic fungi, which helps in achieving enhanced dye decolorization efficiencies.

The addition of sugars (glucose, sucrose, and maltose) facilitates better decolorization efficiencies. A maximum decolorization efficiency of 87.15 + 2.5% has been observed with congo red using glucose as a carbon source. The presence of maltose as a carbon source results in higher decolorization efficiencies with the orange G (74.5 + 3.2%) and rhodamine B (54.15 + 2.8%) (Fig. 2).

After observing the enhanced decolorization results with the addition of carbon sources, the addition of nitrogen sources (Peptone, Yeast extract, and Beef extract) to the media has been tested for its decolorization of congo red, orange G, and rhodamine B by the endophytic fungal isolate. Enhanced decolorization results have been observed for all tested synthetic dyes using beef extract as a nitrogen source. The decolorization results seem to be equal to the 88.4 ± 2.8% for congo red, 74.5 ± 4.2% for orange G, and 52.15 ± 3.3% in the case of rhodamine B (Fig. 3).

The pH compatibility is another important parameter for efficient decolorization results by any endophytic fungal isolate. Various pH conditions (pH 4.0, pH 6.0, and pH 8.0) have been studied for decolorizing congo red, orange G, and rhodamine B by the endophytic fungal isolate. Enhanced decolorization results have been observed using pH 6.0 rather than pH 4.0 and pH 8.0 for all tested synthetic dyes. The enhanced decolorization results

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were found to be equal to 89.4 ± 3.2% for congo red, but 76.2 ± 2.5% and 54 ± 2.8% for orange G and rhodamine B, respectively (Fig. 4). The effect of laccase activity (0–3 U/mL) on synthetic textile dyes revealed that the usage of 2, 2.3, and 0.5 U/mL laccase activities result in higher decolorizing efficiencies with congo red, orange G, and rhodamine B, respectively (results not shown).

Discussion

Oxidation of 1-naphthol showed the qualitative laccase activity with the fungal growth characteristics of 25 mm zone diameter and fungal colony diameter of 15 mm and laccase activity index of 0.66. The obtained results have good agreement with the previous report of Muthezhilan et al. (2014), where laccase activity was reported from endophytic fungi of coastal sand dune plants. Higher titer values of laccase (19.12 + 3.45 U/mL) have been observed in seven days, utilizing 120 rpm at 27°C and 120 rpm. After seven days, less titer values of laccase have been seen, which may result from subsequent laccase degradation (Mazumder et al. 2009). Endophytic fungi of *N. austral* (Fillat et al. 2016) and *Phomopsis liquidambari* (Xie et al. 2016) have exhibited similar laccase activities.

The seven-day solid agar plate medium decolorization studies have shown the efficient decolorizing efficiency of endophytic fungi with congo red and orange G and partial decolorization efficiency with rhodamine B. The decolorization abilities of an endophytic fungal isolate from *P. megasporum* was found to be 76 ± 2.4%, 62 ± 2.8%, and 42 ± 3.2% for congo red, orange G, and rhodamine B, respectively. Endophytic fungal isolate decolorizes congo red faster than the orange G and rhodamine B. This study was in corroboration with the decolorization report of laccase from *Ceriporiopsis subvermispora* CZ-3 (Yavuz et al. 2014). The results showed the endophytic fungal laccase ability in decolorization of synthetic textile dyes.

The addition of carbon and nitrogen sources to the medium had a profound effect on the dye decolorization. Moreover, the working of fungal isolates in decolorization processes needs to have compatibility in pH conditions to exert the maximum decolorization efficiency (Khelifi et al. 2009). The addition of sugars such as glucose, sucrose, and maltose enhances the decolorizing ability of entophytic fungal isolate. The role of glucose and other carbon sources for enhanced fungal decolorization abilities have also been reported in the case of cotton bleaching by *Phanaerochaete chysosporium* (Tatarko and Bumpus 1998) and black olive mill wastewater decolorization by *Geotrichum candidum* (Assas et al. 2002). However, maltose addition also gave better decolorization results in the case of solar golden yellow R by *Schizophyllum commune* IBL-06 (Asgher et al. 2008).

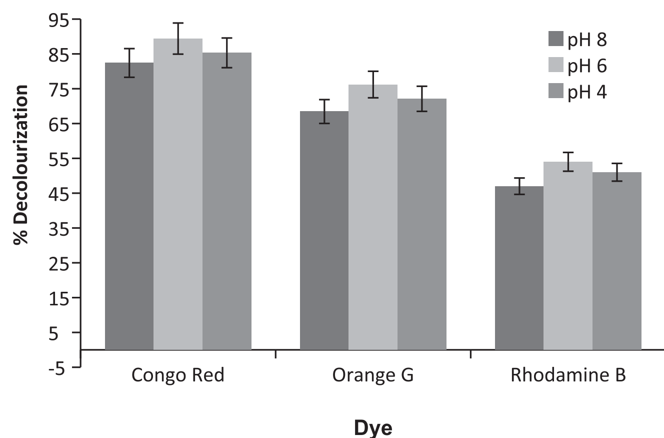

Fig. 4. Effect of pH on dye decolorization by *P. megasporum Orpurt* et *Fennell.* All values are represented as mean ± s.d. of three replications.

Among the tested nitrogen sources, the enhanced decolorization results have been observed using the beef extract as a nitrogen source for all tested synthetic dyes. Nitrogen stimulation of fungal species for decolorization abilities has also been reported in indigo and congo red decolorization by *Aspergillus alliaceus* strain 121C (Khelifi et al. 2009). The study of different pH conditions on dye decolorization showed efficient decolorization with pH 6.0 rather than pH 4.0 and pH 8.0 for all tested synthetic dyes. Suwannawong et al. (2010) reported that optimal congo red and rhodamine B decolorization were achieved with pH 6.0–7.0 using the fungal laccase of *Lentinus polychrous Lév.* The enhanced decolorization with congo red is attributed to the enhanced effect of the endophytic fungus' intrinsic enzyme activities. The decreased decolorization abilities with orange G and rhodamine B is mainly due to the complex structure of the dyes and higher dye concentrations, which results in slower decolorization rates (Abadulla et al. 2000).

Over 80% of dye decolorization took place by laccase-catalyzed oxidation (Abadulla et al. 2000). From various research studies, the mechanism of laccase mediated decolorization is a two-step mechanism via the physical adsorption and enzymatic degradation (Young and Yu 1997). Several studies revealed the importance of Lignin modifying enzyme (LME) in the dye decolorization process through the action of Laccase, Lignin Peroxidase (LiP), and Manganese Peroxidase (MnP) (Vyas and Molitoris 1995). The laccase mediated mechanism for dye decolorization involves two-electron oxidation reactions, which initially generate a phenoxy radical and then a carbonium ion in the phenol ring. The formed carbonium ion triggers a nucleophilic attack of water, which results in a diazine derivative and quinine (Eggert et al. 1996). The higher production yields coupled with lesser fastidious induction conditions makes the fungal laccases the predominant choice rather than the LiP and MnP for different synthetic textile dye decolorization approaches (Thurston 1994; Campos et al. 2001; Poiniting and Vrijmoed 2000).

Conclusion

In the present study, an endophytic fungal strain, *P. megasporum* has shown the efficient decolorization results with seven days of incubation with the synthetic textile dyes, namely congo red, orange G, and rhodamine B. Decolorization efficiency of endophytic fungal isolate has been further studied to check the profound effect of carbon and nitrogen sources and pH conditions. Overall, the present study reveals the efficiency of endophytic fungi in decolorization of synthetic textile dyes, which were the usual components of textile effluents and impinged laccase's profound role toward different bioremediation of industrial effluents in a sustainable way.

Data Availability Statement

No data, models, or code were generated or used during the study.

Acknowledgments

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