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Decolorization Efficiency of Crude Laccase Extract of *Cladosporium magnoliigena* LACF6 on different Textile Dyes

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ABSTRACT: Many industries use synthetic dyes, but their use pollutes the environment and endangers the lives of people., Despite its high color concentrations, only a tiny percentage of textile effluent is treated. They pollute waterways and harm terrestrial and aquatic ecosystems. Therefore, textile dyes must be removed from wastewater before their final discharge into the receiving water bodies. However, their removal from wastewater remains a significant challenge due to the stable nature of dyes. These effluents are typically treated using physical and chemical processes, but these methods are costly, complex, and may produce harmful intermediates. Dye decolorization using microorganisms and their enzymatic systems is a method that is both cost-effective and helpful to the environment. It can reduce the pollution caused by the dyeing industries. In this work, crude laccase enzyme from *C. magnoliigena* LACF6 was extracted. It is found to have good dye removal efficacy for the various dyes used in this investigation. The color removal efficiency of crude laccase extract was around 75.73 ± 3.00 when applied to Victoria Blue B dye at a concentration of 1 mg/ml. However, at the same conditions, the decolorization percentages for Congo Red, Alizarin Red S, and Methylene Blue were 31.69 ± 3.11, 28.30 ± 1.54, and 11.09 ± 1.44, respectively. The significance of crude laccase (*C. magnoliigena* LACF6) in treating industrial effluents containing dyes is seen in the results of this experiment, which indicate that this crude laccase extract has a high potential for decolorizing various structurally different textile dyes.

Keywords: ABTS, crude enzyme, Dye decolorization, Fungus, Synthetic textile dyes, Tannic acid.

INTRODUCTION

Synthetic dyes are widely employed in the textile dyeing, printing, and leather industries. Their contamination in the water effluents from these industries is frequently a significant source of water pollution, reducing the water quality, transparency, and oxygen content. The annual disposal of 4,500,000 tonnes of dyes and degraded products is an environmental and socioeconomic concern (Rawat *et al.*, 2016). The bioaccumulation of dangerous dyes increases their teratogenicity, carcinogenicity, and mutagenicity in humans (Wang *et al.*, 2017; Lellis *et al.*, 2019; Mishra *et al.*, 2022). Removing the dyes from wastewater is a significant challenge as these dyes have a complex structure; they are highly stable and toxic too (Theerachat *et al.*, 2012).

The chromophore group is the primary structural component that determines the dye's color. This group differentiates the dyes into classes such as anthraquinone, azo, indigo, nitro, nitrous, phthalein, and triphenylmethane (Tavares *et al.*, 2019). More than 70% of global industrial demand (9 million tonnes) is for Azo dyes. They have been used in the food, paper, paint, cosmetic, leather, textile, and pharmaceutical sectors. Anthraquinone dyes are the second most significant textile dyes (Legerska *et al.*, 2016). Due to their reinforced structure, anthraquinone dyes present a significant environmental hazard as they are difficult to degrade naturally (Routoula and Patwardhan 2020). Triarylmethane dyes have a high tinctorial value; typically, less than one ppm of dye in water causes noticeable coloration. The widespread usage of these dyes has resulted in brightly colored effluents that may influence gas solubility in water bodies and significantly reduce the amount of photosynthetic activity in aquatic organisms because of lower light penetration. Additionally, triarylmethane dyes are commonly considered hazardous carcinogenic or derived from other known carcinogens (Ogugbue and Sawidis 2011). Therefore, creating efficient treatment strategies for removing dyes from textile wastewater is the need of the hour.

Treatment of these effluents often involves physical and chemical techniques such as adsorption, coagulation-flocculation, and filtration. However, these techniques are costly, occasionally ineffective, and may result in harmful by-products. In addition, they may increase sludge formation, resulting in secondary pollution and making their implementation difficult (Mohanty and Kumar 2021). These constraints prompted the development of biological and environmentally friendly approaches. Biological approaches for removing dyes

from textile effluent offer an alternative favorable to the environment. It induces the degradation of synthetic dyes to a less harmful inorganic chemical by breaking the bond (i.e., the chromophoric group) and ultimately helps in color removal (Bhatia *et al.*, 2017). In order to degrade dyes, fungi, algae, and bacteria naturally found in contaminated environments are used (Kamal *et al.*, (2022). Furthermore, prior studies have demonstrated that microbial enzyme preparations may be helpful in the breakdown of synthetic colors (Iark *et al.*, 2019). Breaking down textile dyes by microbes requires providing nutrients and regulated or optimal growth conditions. In contrast, the breakdown of dyes by enzyme treatment has been shown to be efficient and more versatile in its application. (Ifriadi *et al.*, 2021). Enzymes are very effective in catalyzing reactions and may be controlled to have either high or low substrate specificity (Senthivelan *et al.*, 2016; Ardila-Leal *et al.*, 2021). These enzymes are found in plants, insects, bacteria, and fungi. Chemical reactions have low energy requirements, are simple to regulate, and do not produce toxicity. It is reported that fungi can degrade complex organic molecules by secreting extracellular ligninolytic enzymes such as laccase, manganese peroxidase, and lignin peroxidase (Isanapong and Mataraj 2018).

Among lignolytic enzymes, laccase has the most potential for dye degradation since it does not require a mediator or an expensive co-substrate other than molecular oxygen. (Singh *et al.*, 2020). Laccase is a member of the multi-copper oxidase enzyme family and is classified as benzenediol oxygen reductase (EC 1.10.3.2) and is also referred to as urushiol oxidase and p-diphenol oxidase. Due to its low substrate specificity, it is regarded as a flexible enzyme capable of oxidizing a wide variety of chemicals, including diphenols, polyphenols, diamines, aromatic amines, benzenethiols, substituted phenols, and many types of colored pollutants, while producing water as a by-product (Forootanfar *et al.*, 2012; Arregui *et al.*, 2019). The primary processes catalyzed by lignolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation, and aromatic ring opening (Kochher and Kumar 2011).

In the current study, a crude extract of laccase enzyme was prepared from the fungus *C. magnoliigena* LACF6. The laccase activity of the crude extract was determined and studied for its ability to decolorize dyes of different classes.

MATERIALS AND METHODS

Chemicals. Synthetic dyes: Malachite Green, Congo Red, Methylene Blue, Orange G, Alizarin Red S, Trypan Blue, and Victoria Blue B were procured from Himedia and Thermoscientific and were of laboratory grade. Potato Dextrose Agar (PDA)medium, ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)],and Tannic acid were purchased from Sisco Research Laboratory.

Fungal isolate. *C. magnoliigena* LACF6 was isolated and purified in the Environment Molecular Microbiology Laboratoryfrom soil/ sludge samples from Sanganer, Jaipur, where textile dye contaminants were prevalent. This fungal isolate was identified (Accession no. ON246094) and maintained on Potato Dextrose Agar (PDA) medium at 4°C and periodically sub-cultured to preserve its viability.

Screening for laccase enzyme by plate assay method. *C. magnoliigena* LACF6 was screened for laccase enzyme by a plate assay method utilizing ABTS and Tannic acid. A fresh culture of *C. magnoliigena* LACF6 was inoculated on PDA media with 0.1% ABTS (Patel and Bhaskaran 2016) and incubated at 25 °C for five days for the ABTS plate assay. The laccase enzyme from the isolate produced a green and reddish -purple oxidation zone surrounding and under the fungal growth. The tannic acid plate assay employed 0.5% tannic acid in the media (Yadav *et al.*, 2019). A brown oxidation zone around and under the fungal growth showed a positive result.

Growth conditions and preparation of crude laccase extract. After seven days of fresh culture on PDA, two pieces ($1cm^2$) of *C. magnoliigena* LACF6 from the colony margin were used to inoculate 200 ml of laccase production medium, i.e., glucose peptone broth containing (gL^{-1}) Glucose 10; Peptone 3; KH_2PO_4 0.6; $ZnSO_4$ 0.001; K_2HPO_4 0.4; $FeS0_4$ 0.0005; $MnSO_4$ 0.05; $MgSO_4$ 0.5; $CuSO_4$ 0.01 (Vantamuri and Kaliwal 2015) in 500 ml Erlenmeyer flask. The fungus was then cultivated for 15 days at 25°C with constant agitation at 120 rpm**.** The fungal culture broth was filtered using Whatman filter paper No. 1 and centrifuged at 4 °C at 10000 rpm for 10 minutes. The clear supernatant was used as a crude laccase enzyme extract for enzyme activity assays and dye decolorization studies**.**

Laccase activity assay. A spectrophotometric measurement of laccase activity was done using ABTS as a substrate. 50 µl of 2 mM ABTS was dissolved in 50 µl sodium acetate buffer (0.1 M pH 5) and 50 µl of the enzyme (crude extract). A heat-denatured enzyme extract with ABTS and buffer was used for the blank. After ten minutes, absorbance was measured at A_{436} (Sivakumar *et al.*, 2010). The enzyme required to oxidize 1 mole of ABTS per minute is defined as one unit of enzyme activity. The laccase activity was estimated in U/ml using the following formula:

$$\text{Enzyme activity} = \frac{A * V}{t * e * v}$$

A = Absorbance,
V = Volume of the total reaction mixture,
t = Incubation time,
e = Extinction coefficient for ABTS (i.e., 2.9×10^{-3} cm^{-1} μM^{-1})
v = Volume of enzyme

Dye decolorization experiment. Seven different dyes, namely Malachite Green, Congo Red, Methylene Blue, Orange G, Alizarin Red S, Trypan Blue, and Victoria Blue B (Table 1), were studied for decolorization using a crude laccase extract from the fungus *C. magnoliigena* LACF6. The dyes were prepared at 1mg/ml concentration using sodium acetate buffer (0.1 M; pH 4.5), filtered (0.45 µm pore membrane), and used in each decolorizing experiment. The decolorization reaction mixture comprised an equal proportion of enzyme extract and dye solution. The reaction mixture was incubated in a shaker incubator at

30 ± 1°C for 24 hours. After 24 hours, the decolorization was assessed using a spectrophotometer to determine the maximum absorbance of Malachite Green, Congo Red, Methylene Blue, Orange G, Alizarin Red S, Trypan Blue, and Victoria Blue B at 620nm, 496nm, 665nm, 488nm, 428nm, 590nm, and 593nm respectively. Distilled water was used as the analytical control rather than the enzymatic extract. The following formula was used to calculate the decolorization percentage:

$$\text{Decolorization (\%)} = \left[\frac{Ai - Af}{Ai}\right] \times 100$$

Ai = initial absorbance
Af = final absorbance of dye after incubation time

RESULTS AND DISCUSSION

Screening for laccase enzyme. In the ABTS plate assay, *C. magnoliigena* LACF6 exhibited a reddish-purple color oxidation zone around and under the fungal colony. However, in the tannic acid plate assay, the fungal isolates showed a brown color oxidation zone (Fig. 1a and b). The screening results explain that the medium for each substrate turned a particular color exhibiting that the isolated fungus can produce laccase enzyme (Senthivelan *et al.*, 2016).

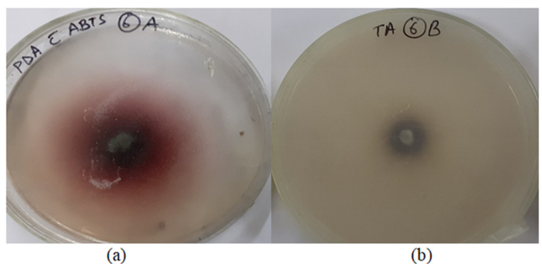

Fig. 1. Screening of laccase enzyme (a) ABTS plate showed violet oxidation zone around the fungal colony, and (b) tannic acid plate showed brown oxidation zone around the fungal colony

Laccase enzyme activity assay of *C. magnoliigena* LACF6. The first reading was taken on the third day of the incubation for the assay, and subsequent readings were taken at three-day intervals after that. According to Fig. 2, *C. magnoliigena* LACF6 started producing laccase on the sixth day. By the ninth day, it had reached its maximum level of 12.75 ± 1.47 U/ml, after which it began to decrease gradually. Probably, a lack of macro- and micronutrients in the medium in which the enzymes were produced was the reason for the drop in enzyme synthesis. These findings are consistent with those of Kalra *et al.* (2013); Monssef *et al.* (2015).

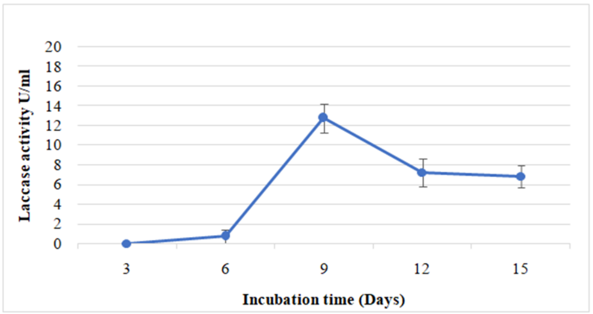

Fig. 2. Laccase activity (U/ml) (Mean ± SD) of *C. magnoliigena* LACF6.

Dye decolorization assay. After 24 hours, the decolorization of seven dyes caused by crude laccase extract from *C. magnoliigena* LACF6 was measured and analyzed (Table 1). This demonstrates that the decolorization activity caused by crude enzymatic extract is not specific to any particular dye class since no distinct pattern of decolorization was observed within the same class. In general, decolorization efficiency is determined by the structure of the dye and the redox potential of the enzyme (Ratanapongleka and Phetsom 2014). These dyes were evaluated at pH 4.5, where the enzyme exhibited a high activity level towards ABTS (acidic condition). The decolorization percentages observed after 24 hours varied in each dye. The decolorization of Congo Red, Methylene Blue, Alizarin Red S, and Victoria Blue B was observed at measurable levels; however, the decolorization of Malachite Green, Orange G, and Trypan Blue was not detected. The degree of decolorization that was seen to be at its highest in Victoria Blue B was 75.73 ± 3.00 (Fig. 3). It is reported that *Phanerochaete chrysosporium* decolorized only 23.8% of Victoria Blue dye in vivo conditions (Gomaa, 2012) and *P. pulmonarius* strain BPSM10decolorized 16.03±0.42 of Victoria Blue B dye (Lallawmsanga *et al*., 2019). These values are lowerthan the results obtained in the current study.The structures of the dyes and the selectivity of the substrates play a considerable role in the amount of color removed by the laccase enzyme. Previous findingshave demonstrated thatlaccase activityis affected by the presence of different substitutions in the benzene rings of the dye molecules. (Easwaramoorthi, 2017; Yuan *et al*., 2016). Even for the same dye, the decolorizing efficacy of laccases differs, and this difference is due to the biological sources of the producing microbe. According to Zhuo *et al.* (2011), after 72 hours of incubation with the laccase of *Ganoderma* sp. En3, 98% of Malachite Green lost its color. On the other hand, 60.5% of Malachite Green (initial concentration of 60 mgL^{-1}) was removed after 15 minutes of incubation of the dye in the presence of laccase from *P. variabile* (Forootanfar *et al*., 2012).

Table 1: Class, chemical structure, and images before and after decolorization of the selected dyes.

Dyes	Class	Chemical Structure	Dye decolorization images Before After
Congo Red (CR)	Azo		
OrangeG (OG)	Azo		
Trypan Blue (TB)	Azo		
Methylene Blue (MB)	Azo		
Malachite Green (MG)	Triarylmethane		
Victoria Blue B (VB)	Triarylmethane		
Alizarin Red S (ARS)	Anthraquinone		

After 24 hours of incubation, Yuan *et al*. (2016) found that the laccase of *Pleurotus nebrodensis* was able to decolorize Congo Red (45%), Methylene Blue (55.56%), and Trypan Blue (86.08%). This was discovered by examining the decolorization capacity of the laccase of *Pleurotus nebrodensis* on twenty-four different synthetic dyes. Asdyes of the same class may not react identically to the same laccase, it becomes necessary to analyze the decolorizing efficacy of specific dyes of interest with individual laccases. This is because dyes of the same class may not react in the same way (Yang *et al*., 2014). Our research used a crude laccase extract from *C. magnoliigena* LACF6, which confirmed this finding clearly and convincingly. It was successful in decolorizing Victoria Blue B with a nearly 75% efficiency rate. However, it could not decolorize Malachite Green, even though both chemicals belonged to the same class (triarylmethane).

According to earlier reports, crude extract of laccase from fungal species plays a crucial role in the decolorization of dyes (Monssef *et al.*, 2016). However,as per our knowledge, there is no report on the decolorizing ability of crude laccase isolated from *C. magnoliigena*. So, in the current study, an effort was made to investigate the dye decolorization potential of crude laccase extract of *C. magnoliigena* LACF6. According to our findings, this crude laccase extract has a good potential for decolorizing various dyes, indicating the significance of crude laccase (*C. magnoliigena* LACF6) in treating industrial effluents containing dyes.

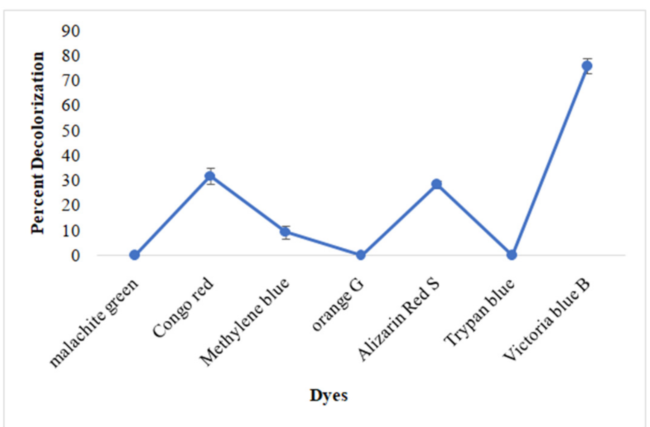

Fig. 3. Percent decolorization (Mean ± SD) of different dyes by crude laccase extract of the fungus *C. magnoliigena* LACF6.

CONCLUSIONS

One of the principal sources of water contamination in the textile dyeing industrial area is the direct dumping of chemicals, primarily dye-containing effluents, into the open environment. The dyes seriously threaten the ecosystem and negatively affect all living organisms. Therefore, removing dyes and other chemical compounds from industrial effluents prior to their discharge into open water bodies is a key concern. There are various physicochemical and biological techniques for dye treatment. Dye decolorization with microorganisms and their enzymatic system is a cost-effective and environmentally beneficial solution for reducing the pollution from the dye industry. In the current preliminary study, crude laccase enzyme was extracted from *C. magnoliigena* LACF6 and has been shown to have 12.75 ± 1.47U/ml laccase activity and good color removal efficiency for the several classes of dyes. For example, at a dye concentration of 1 mg/ml, the color removal efficacy of Victoria Blue B dye by crude laccase extract was 75.73 ± 3.00. However, at the same initial dye concentration, the decolorization percentages for Congo Red, Alizarin Red S, and Methylene Blue were 31.69 ± 3.11, 28.30 ± 1.54, and 11.09 ±1.44 respectively, under the same conditions. On the other hand, it has been seen that Trypan Blue, Malachite Green, and Orange G could not be decolorized effectively with this enzymatic treatment. The findings showed that crude laccase from *C. magnoliigena* LACF6 is a significant biological and eco-friendly approach for treating colored dye wastewater.

FUTURE SCOPE

There is a need for research on the scalability of successful laboratory experiments with the new fungal isolates for future industrial use. Together with decolorization, effluent toxicity should also be tested. Based on positive laboratory results, scale-up and utilization of fungal decolorization methods in industrial effluents can be very promising. Moreover, molecular techniques like proteomics and genomics can be applied to investigate the identification and characterization of effective enzymes involved in textile wastewater treatment. Due to promising research and continuous advances, microbial enzymatic treatment may dominate textile wastewater color and hazard removal effectively.

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Conflict of Interest. None.

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