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Original Research Paper

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DECOLORIZATION OF TEXTILE DYE BY ENDOPHYTIC FUNGAL ISOLATE OF NIGROSPORA HAINANENSIS PRODUCING LACCASE ENZYME

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ABSTRACT

Advanced industrialization has led to an increase in the continuous discharge of hazardous waste into the environment. In this case, it is necessary to solve the polluted environment without causing any harm. In order to find a solution, the endophytic fungus of Nigrospora hainanensis was found in seaweed and its decolorization ability was studied on textile dye effluent. Molecular identification of the fungal isolate using primer ITS showed 99% sequence similarity with Nigrospora hainanensis, an endophytic fungus. The potential isolate has shown promising result (91.6%) on the dye decolorization. Further, laccase synthesized by potential strain to decolorize the Reactive dye than isolate. This efficacy result was obtained with 48hrs of incubation with 3U of laccase enzyme. Therefore, the present study suggests that the potential use of fungal endophytes and its producing laccase can ensure decolorization of dye effluents and replace existing toxic chemical-based disposal approaches.

KEYWORDS : Endophytic fungus, Nigrospora haenanensis, Laccase, Decolorization, Textile dye

INTRODUCTION

Water contamination is a serious issue for the ecosystem. According to reports, a number of industries discharge trash into ponds, lakes, rivers, and streams in an effort to elude hide them from Environmental Protection organizations. Among the industrial wastes, textile wastes mainly contain toxic dye components and heavy metal s and organic compounds that eventually deteriorate the quality of water bodies. About 10,000 commercial dyes are produced worldwide; with an annual production rate of 7×10^5 metric tons (Kandare and Govindwar 2015). An important environmental hazard is the spent dyes from the textile industries' inability to biodegrade. Therefore, decolorizing industrial effluents from textile companies in an environmentally responsible way is necessary before releasing them into waterways.

The conventional methods of decolorization of dye wastes are not effective, so it is necessary to deal with new strategies, in that way, innovative eco-friendly biological decolorization processes should be developed, especially the method of decolorization by enzymes produced by fungi is currently developing (Khandare and Govindwar 2015). Endophytes are endosymbiotic fungi with high redox potential and low-specificity enzymes that aid in dye degradation. They have excellent dye-degradation capabilities with extraordinarily high efficiency (Mishra and Maiti, 2019). Besides various marine organisms, algae are a ubiquitous source of marine endophytic fungi for biochemical research. The endophytic fungi, which colonize the internal tissues of their hosts without any deleterious effect, have proven to be important sources of bioactive natural products with microstructure and potent beneficial activities especially removing xenobiotic dyes from wastewater effluents (Ahamed and Murugan, 2019).

It is well known that fungi laccases can destroy azo dyes (Blanquez *et al*., 2004). In commercial textile applications, laccases are used to enhance the whiteness of cotton during the traditional bleaching process. Despite advances in biodegradability, the widespread use of algae endophytes in dye degradation has yet to be realized. Previous study of Chen *et al*. (2014) reported the N-heterocyclic indole degradation ability of laccase from a novel endophytic fungus. Xie and Dai 2015also separate the laccase from endophytic fungus of *P liquidambari* and utilized for the degradation of ferulic acid and sinapic acid. Realizing the importance of eco-friendly decolorization processes for textile industrial waste treatment; this study was conducted to decolorize textile dyes by extracting laccase enzyme from new endophytic fungi of *Nigrospora hainanensis*, which was from seaweed algae of *Gracilaria debilis*.

MATERIALS AND METHODS

Collection Of Seaweed

The seaweed (*Gracilaria debilis)* sample was collected from the southeast coastal region (Gulf of Mannar) and confirmed with the references available from CSMCRI, Mandapam Camp, and Ramanathapuram (District). The latitude and longitude of the sampling site is 8°31'-9°25'N latitude and 78°08'-29°30'E longitude at an altitude of 9 feet MSL. The sample was carried in poly bags by covering it with wet cotton tissue, and brought to the laboratory and subsequently stored at a deep freezer with -20°C (Neethu *et al.,* 2017).

Isolation And Identification Of Endophytic Fungi From Seaweed

The seaweed was surface sterilized and inoculated on Sabouraud dextrose agar (SDA) media with streptomycin (150 mg/L) was inoculated with seaweed, sealed with parafilm, and incubated at 30 °C under light for one week. (Noorjahan *et al.,* 2019). The slide culture technique and lactophenol cotton blue mount were used for fungal identification (Riddell 1950). Morphological (color, shape, and growth cultured type) and microscopic (structure of hyphae, conidia, and conidiophores) patterns of fungi were observed and compared with standard mycological manuals (Singh *et al.,* 2017).

Screening And Submerged Fermentation For Laccase Enzyme Producer

Laccase production was carryout in GYP media (glucose- 20 g/L, yeast extract- 5 g/L, peptone- 10g/L, agar-15g/L, and pH-5.4) supplemented with 4mM guaiacol by incubating for 2 weeks at 25°C in dark condition (Yasser *et al.,* 2019). The production of intense brown color, reddish brown colour around the fungal colony was considered as a laccase positive reaction.

Laccase Enzyme Activity

The fungal isolate was incoculated in to GYP broth media supplemented with 4mM guaiacol and incubated at room temperature for 2 weeks. Laccase activity was determined by

the oxidation of ABTS method. The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and incubated for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1µmol of ABTS substrate per min. Protein concentration was determined by the dye-binding method of Bradford using BSA as standard (More *et al*., 2011).

Sequencing And Phylogeny Analysis

Fungal genomic DNA was extracted using the mini-prep method (Lee *et al*., 1988). The 1µl of thawed DNA was subjected to agarose gel electrophoresis and was visualized using a gel documentation system (Bellemare *et al*., 2018).The fungal identity was confirmed by using universal ITS primer targeting 5.8 S ITS-rDNA by direct sequencing (White *et al*., 1990).Fungal genomic DNA was isolated and amplified in thermal cycler using universal primer set ITS1-(5'-TACTACCACCAAGATCT-3'), and ITS4- (5'-ACCCGCTGAACTTAAGC-3'). Sequencing reactions were done using ABI analyzer. Nucleotide sequence matching was performed using NCBI BLAST against the closely related fungal species sequence, followed by multiple sequence alignment using the program tool MUSCLE 3.7. The phylogeny tree was constructed using PhyML software (Pandey *et al*., 2018).

Decolorization Assay With Isolate

The GM agar medium contained (g/L): K_2HPO4, 1; $ZnSO_4 \cdot 7H_2O$,0.01; $CuSO_4 \cdot 5H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$,0.01; KCl, 0.5; glucose, 10; $NaNO_3$, 3; and agar, 20. The pH of the agar medium was adjusted to 5.5 before being autoclaved at 121^0C for 15 minutes. Reactive blue 4 was added into the agar from a stock solution to a final concentration of 1gm L^{-1}. The agar plates were inoculated with a 5 mm 2 agar plug from a 7-day old fungal culture and incubated in the dark at room temperature. Uninoculated plates with the respective dyes were used as the control. Plates were regularly monitored and observed for visual disappearance of colour for a period of 3, 7 and 15 days (Narkhede *et al*., 2013).

Decolorization Assay With Laccase

The potential of the crude laccase for dye removal was evaluated on Reactive Blue 4 (RB4). About 50 mg/L of each dye was dissolved in sterile tap water. Purified laccase and each dye solution were allowed to react at a 1:1 ratio. The 3mL reaction volume contained 1mL acetate buffer (pH 5.5), 1mL dye solution, and 1mL of laccase; decolorization was detected by measuring the decrease of color absorbance with time (2, 4, 24, and 48 h). After, absorbance was read at 595 nm using a spectrophotometer (Edoamodu *et al*., 2022). The percentage decolourization was calculated using the following equation:

$$\%\text{decolourization} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is the initial absorbance of the mixture immediately after the addition of the enzyme and A_t is the absorbance at a given time.

RESULTS AND DISCUSSION

A total number of three endophytic fungi were successfully isolated from the host seaweed of *Gracilaria debilis* (*Aspergillus sp, Mucor* and unknown species). The fungal isolates of morphological characterization were observed on SDA plate media. While isolates were subjected to screening of laccase producers, unknown fungal isolate was showed positive laccase producers (Fig.1). Therefore, such isolate only carryout for fungal identification process. It has smooth hyphae, branched & septate and range in between 6 - 17.5 µm diameters. The slide culture has appeared clustered pattern of spherical condiogenous cells (Wang *et al*., 2017).

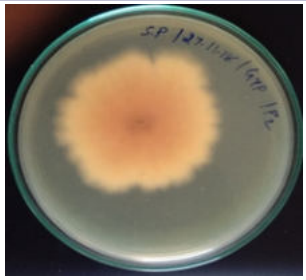
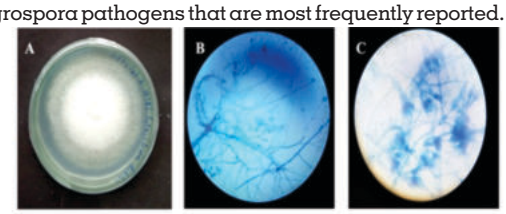


Fig.1 Screening Of Laccase Producing Isolate

More likely the filamentous fungus has diverse hyphae with spherical conidia in size 6 - 12 µm diam. Small, flattened; thick pale colored conidiospore has formed. A conidia cell has monoblastic, discrete, solitary, hyaline & smooth globose. Condiogenous cells had diverse spherical globoses. Most commonly, the Ascomycota family of endophytic fungi emerged from seaweed. Lacto phenol cotton blue was to examine fungal elements macro & microscopic structures were observed through specimen (Fig.2). Basidiomycetes have smaller conidia, and diverse spherical pale-structured patterns have been confirmed through morphological identification (Basava *et al,* 2016).

According to the morphological characterization, such isolate as *Nigrospora sp*. Based on the review of literature, no one reported that *Nigrospora* species were observed from algae. However, Species of this genus are endophytes and saprobes of various hosts. In 2020, Hao *et al*., observed the various *Nigrospora* species from bamboo and Chinese rose. These investigations highlight *N. oryzae* and *N. sphaerica* as the two Nigrospora pathogens that are most frequently reported.



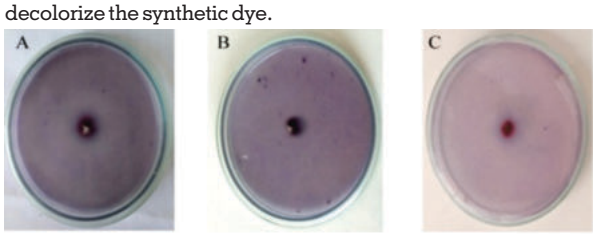
Isolation of *Nigrospora hainanensis*
A.Endo fungi in SDA media, B.Microscopic 10x pattern has shown filamentous fungi morphology (C) 40x visualization has shown clustered condiogenous cells

Fig.2 Isolation of *Nigrospora hainanensis*

However, previous studies have shown that this isolate contains bioactive secondary metabolites that are active against bacteria and cancer (Metwaly *et al*., 2014). According to reviews, no study was done about the isolation of *Nigrospora* species from algae and utilized the dye degradation. However, other fungal and bacterial laccase have been shown to effectively decolorize the chemical dyes Forootanfar *et al*., 2012; Asgher *et al*., 2013). This point of view, the fungal isolate was identified from the reactive dye sample and subjected to decolorization activity. The *Nigrospora hainanensis* was confirmed with ITS gene sequence, which sequence was compared with NCBI data and obtained the accession number was Oq137042.

The confirmed isolate was subjected to dye decolorization ager plant method. The decolorization was started from 7 day of the incubation period. After 15 days of an incubation period, highest zone of clearance was observed; the rate of decolorization percentage was 91.6%. The reducing enzyme has that degrade the azo dye into color less amines by means of reductive cleavage process (Fig.3). The results of this study lead to a conclusion that endophytic fungi having potential enzymes can be used in industrial effluent treatment. Similarly, Agrawal (2017) was decolorized the various textile dye with using endophytic fungus of *Daldinia sp*. Another hand of study was determined the laccase producing isolate

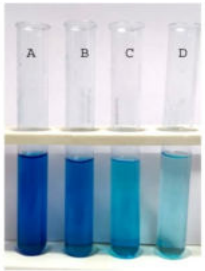
of endophytic fungi of *Cupressus torulosa*, which was highly decolorize the synthetic dye.



Decolorization of Reactive blue 4 with laccase enzyme
A.3 day incubation, B.7 day incubation, 15 day incubation

Fig.3 Decolorization of dye with fungal isolate

Another decolorization test using the same dyes along with *Nigrospora hainanensis* producing laccase enzyme was utilized in the present study, which showed decolorization rate of 95% with laccase enzyme. The *Nigrospora hainanensis* laccase was efficient in the decolorization of dye solution, which became gradually colourless as a function of the incubation time (Fig.4). The decolorization was stated with 24hrs of the incubation period. Compared to isolate, which producing laccase was highly decolorized the reactive blue dye. Decolorization of textile dyes by a *Clitocybula dusenii*, white-rot fungus producing laccase was reported by Wesenberg *et al*., (2002). Liu *et al*., (2004) also reported that laccase is solely responsible for the decolorization and degradation of dyes. Presently, 3U of laccase was able to decolorize the maximum dye, which was correlated with previous study of Agrawal *et al*., 2021, they were higher decolorized the congored and orange G with 2 and 2.3U of laccase respectively.



A.2hrs incubation, B.4hrs incubation, 24hrs incubation, 48hrs incubation

Fig.4. Decolorization of dye with laccase enzyme

CONCLUSION

In the present study, an endophytic fungal strain, *Nigrospora hainanensis* has shown the efficient decolorization results with 15 days of incubation with the synthetic dyes. Furthermore, such isolate producing laccase was capability to decolorize the dye than isolate. This study suggests that laccase is suitable for the enhanced decolorization of synthetic dyes, which was represent a potentially commercially viable, effective and environmentally friendly alternative to traditional physicochemical methods. However, the decolorization ability of endophytic fungal isolates needs to be further investigated to verify the effect of carbon and nitrogen sources and pH levels.

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