Ganoderma Lucidum Degrades Synthetic Dye: A Review

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ABSTRACT: ***In a variety of sectors, synthetic dyes are frequently employes. Such dyes are extremely poisonous, mutation-causing, cancer-causing and also causes various ill effects to the environment. Current study demonstrates the potential of Ganoderma lucidum to decolorize synthetic textile dyes that are recalcitrant. In the course of fermentation of natural lignocellulosic substrate i.e., wheat bran, G. lucidumi predominantly secretes laccase as major lignolytic enzyme. Pure enzyme had a very high decolorizing efficiency against Remazol Brilliant Blue R (RBBR), an anthraquinone dye, without the involvement of a redox mediator, but it needed a redox mediator to decolorize Remazol Black-5 (RB-5), a di-azo dye. The role of laccase enzyme as the main enzyme behind the degradation of both the dyes were confirmed by Polyacrylamide Gel Electrophoresis (PAGE). 1mM concentration of N-Hydroxybenzotriazole (HBT) was established as the prime redox mediator. Time taken by crude enzyme (25U ml^{-1}) to decolorize RBBR (50mg/L) by 90% was 2h when no redox mediator was present whereas it took 2h to decolorize RBBR by 92% when a redox mediator was present. In case of RB-50 dye, the crude enzyme took 1h and 2h to decolorize RBBR by 62% and 77.4%, respectively without the use of any redox mediator.***

KEYWORDS: Ganoderma lucidum, Laccase, Dye degradation, Remazol Brilliant Blue R (RBBR), Remazol Black-5 (RB-5)

1. INTRODUCTION

Chemically synthesized dyes are now widely employed in a variety of industries, including paper, food, pharma, textile, cosmetic, photography and leather dying. These dyes are categorized on the basis of structure as anthraquinone, triphenylmethane, heterocyclic, phthalocyanine and azo dyes. Reactive dyes, dispersive dyes, acidic dyes, and direct dyes are all types of dyes that are categorized based on the dying process. Such chemically synthesized colors are extremely highly poisonous, mutation-causing, carcinogenic, may also cause various ill effects to the environment. Furthermore, these compounds are usually resistant to degradation. Such compounds can be treated by various physical and chemical treatment methods like adsorption, precipitation or chemical degradation but such methods are costly, time-taking, labor intensive and moreover, such methods have low efficacy.

Extracellular oxidative enzymes such as laccase, manganese peroxidase, lignin peroxidase produced by various species of fungus such as *Ganoderma lucidum, Trametes versicolor, Rhizopus arrhizus,* etc., can degrade these dyes. Laccase is the major enzyme produced in the process of dye degradation[1].

1.1Laccase Enzyme:

Laccases are the enzymes classified as oxidoreductases corresponding to International Union of Biochemistry (IUB). These enzymes are majorly secreted by bacteria, plant, fungi and some insects. Although most laccases investigated so far are extracellular proteins, intracellular laccases can also be found in insects and fungus. Function of laccase differ from species to species. In plants, laccase is main function in the synthetic processes such as formation of lignin whereas, in fungi, laccase is involved in the process of toxic phenol removal [2]. Mechanisms of action of laccase include cleavage of aryl-alkyl bond, oxidation of Cα and cleavage of Cα-Cβ bond.

Laccases are involved in the breakdown of pharmaceutical products, biodegradation and bioremediation, healthcare products, and the removal of phenolic compounds from wine, to name a few. Because of their wide spectrum of substrates, they have a great use. Fungal laccases are the most investigated and largely dispersed in fungus causing degradation of wood fungus particularly in white rot fungi like *Ganoderma lucidum*. Enzyme production may vary from one species to another. Moreover, the extent of enzyme production may depend upon the selection of natural producer and optimization of conditions of the culture medium. Presence of metal ions, carbon and nitrogen sources or lignin derivatives are various external factors that may play a role in increasing the gene expression of laccase encoding gene [3]. Furthermore, presence of copper ions, lignocellulosic substrates and phenolic compounds do act as inducers and promote laccase production. Higher yield of laccase enzyme can also be obtained by using low-cost substrates such like agro-industrial and forest residues[4].

1.2 Ganoderma lucidum:

Ganoderma lucidum is one of the laccase producing medicinal white rot fungus used in the current study. It is a member of the family *Ganodermataceae* having double-walled basidiospore [5]. It has been used for almost 200 years due to its high medicinal value. There are various agar mediums that support in vitro growth of *Ganoderma lucidum* [6]. Triterpenes, peptidoglycan and polysaccharides are the dominant active compounds in *G. lucidum*. The growth of fungi can be increased by maintaining growth parameters such as water content, pH, relative humidity and temperature [7]. The amount of these active compounds has vary in natural and commercial products. It also possess various pharmaceutical activities such as hypoglycemic, anti-inflammatory, immune-stimulant, etc[8].

1.3 Degradation of Synthetic Dyes:

1.4 Fungi isolation:

The fungus was isolated from wood trunk of tamarind tree near the IIT established in Chennai, India [9]. The isolated fungus was confirmed as *G. lucidum* by amplification and sequencing of Internal Transcribed Spacer (ITS) DNA employing two primers, ITS1 and ITS2. Following their identification, Potato Dextrose Agar (PDA) slants at 4^{o}C were used to maintain stock culture of the isolated fungi.

1.5Dye Degradation on Agar Plate:

The capability of *G. lucidum* to degrade synthetic dyes were identified on agar plates having synthetic dyes. Mycelium from PDA plate were inoculated in the centers two culture plates containing nitrogen basal medium and synthetic dyes Remazol Brilliant Blue R (RBBR) and Remazol Black-5 (RB-5), respectively, and incubated at 30^{o}C in dark. The plates were checked after interval of every 24h for growth and dye degrading activity.

1.6 Enzyme Production:

KH_2PO_4, $MgSO_4$, Na_2HPO_4, $CaCl_2$, wheat bran (WB), glucose, trace element solution and thiamine hydrochloride were used as the constituents for preparing production media employed in laccase production. Wheat bran is the major substrate for enzyme production. Nitrogen basal media containing glucose (0.2%) was used to moisten wheat bran by 70% prior to preparing production media. A glass bottle containing moistened wheat bran and cotton plugs was autoclaved for 15 minutes at 121^{o}C and 15 psi.

Fungal mycelia were collected from the area of active growth from the slants and were incubated in glass bottle at 30^{o}C in dark and the culture was harvested after every 7 days. 100nM Sodium acetate buffer with 5.0 pH was utilized for soaking the culture for extraction of extracellular enzyme. Clear supernatant containing enzyme was obtained after filtering the culture through a filter made from nylon and centrifuging two times at 10000×g.

1.7 Polyacrylamide Gel Electrophoresis:

To established the role of laccase enzyme in the dye degradation process, SDS-PAGE (12%) and native PAGE (10%) polyacrylamide gel electrophoresis was used on the crude enzyme. For detection of laccase enzyme, the polyacrylamide gel was incubated with ABTS and guiacol in sodium acetate buffer after the separation of proteins.

1.8 Enzyme Assay:

1nM ABTS at 30^{o}C was used as the substrate to measure laccase activity. The constituents of assay mixture are 1nM ABTS stock solution, 100nm Sodium acetate buffer having 5.0 pH and diluted pure enzyme. A spectrophotometer was used to measure the increase in absorbance of the test mixture at 420mn. Enzyme activity is measured in international unit (U). At 30^{o}C, 1U of enzyme is equal to the quantity of enzyme necessary to produce 1µmol of by-product in 1 minute. The dry form of enzyme is expressed in U g^{-1}[10].

1.9 Enzymatic Dye Degradation:

RBBR and RB-5 dyes were used to test the ability of crude laccase enzyme isolated from G.lucidum. 20U/mL crude enzyme, 50 mg/L dye and 50nM sodium acetate buffer having 5.0 pH make up the reaction mixture. The entire volume of reaction mixture was 1 ml which was taken in a sterilized Eppendorf. The Eppendorf was incubated in dark at 30^{o}C and the decrease in absorbance was measured using spectrophotometer at 592nm and 597nm for RBBR and RB-5, respectively. To test the influence of a redox

mediator on the enzyme's ability to degrade RBBR and RB-50, 1nm N-hydroxybenzotriazole (HBT) was added to the reaction mixture[11].

1.10 Impact of Temperature and pH on Degradation of dye:

The enzyme was incubated in a reaction mixture with the dye concentration as 50mg/l at temperatures ranging from 20°C to 70°C to see how temperature affected dye degradation. The impact of pH on the process of dye de-colorization is studied using the same dye concentration at pH levels ranging from 3 to 9. The pH of the reaction mixture was adjusted using citrate-phosphate and Tris-HCI buffers.

2. LITERATURE REVIEW

Prabin Shrestha et al. published a paper describing the procedure of isolating and characterizing laccase enzyme from Ganoderma lucidum. Three fungal strains, from Nepal, were initially isolated and screened for the ability to produce extracellular laccase. The fungi that was found to produce highest level of laccase was G. lucidum-CDBT. The dominant substrate that was used to isolate laccase from G. lucidum-CDBT was lignin that was extracted from rice straw. They stated that the production of laccase enzyme was maximized by addition of copper sulfate. Ammonium sulphate was used to fractionate the enzyme, and DEAE Sepharose anion exchange chromatography was used to purify it. The authors used SDS-PAGE to evaluate the molecular mass of isolated enzyme that came out to be 43kDa. Optimum temperature of 30°C and pH of 5 were determined by measuring absorbance with the help of UV-Vis spectrophotometer. This study doesn't discusses the application of laccase enzyme and doesn't include the method for enhancing enzyme production whereas in the given review paper the method for increasing enzyme production and the application of laccase enzyme for degradation of industrial dye is given[12].

Kumarasamy Murugesan et al. in a study emphasized on the function of laccase enzyme in the process of dye degradation against synthetic dyes particularly, RBBR and RB-5. G. lucidum is medicinally important fungi and thus, grown on a large scale due to which it can act as a source of production. The role of laccase enzyme in dye degradation is confirmed by native and SDS-PAGE using ABTS and guiacol and sodium acetate buffer. Dye degrading potential of laccase enzyme against RBBR and RB-5 was checked in the presence and absence of inducer by measuring absorbance using UV-Vis spectrophotometer. Maximum de-colorization was observed at pH 4 and 60°C was the temperature at which the enzyme showed high thermostability. Moreover, it was found that sodium azide completely inhibits the dye degradation. Inhibition of enzyme helps to prevent any undesirable color formation. This paper doesn't include the materials and methods for production of laccase enzyme. The present review paper mentions the procedure for isolation of crude laccase enzyme from *Ganoderma lucidum*[13].

Ana PM Tavares et al. mentioned the process to optimize degradation of dye by using system to enhance laccase secretion. Degradation of six different dyes were performed by utilizing various mediators namely, (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate)) (ABTS), polioxometalates, (2,2,6,6-tetramethylpiperidin-1-yloxy) (TEMPO), violuric acid (VA), N-hydroxyacetanilide (NHA) and 1-hydroxybenzotriazole (HBT). They observed that the highest de-colorization was observed when ABTS was used as the enzyme mediator. The various parameters that govern the efficiency of ABTS are pH, dye, dye concentration and temperature. Reactive yellow 15 and Reactive blue 114 showed maximum de-colorization at pH 5 and temperature 300C. However, 4.5 pH and 400C were the optimum conditions for Reactive red 239 dye. Reactive blue 114 is the only dye where ABTS showed effect even at low concentration and 93% of maximum de-colorization was observed. They also calculated the percentage drop in absorbance at each dye's maximum wavelength of absorption to compare different reactive dyes. Authors doesn't mention the procedure to isolate crude laccase enzyme and doesn't include the degradation of laccase enzyme of commonly used industrial dyes that are Remazol Brilliant Blue R and Remazol Black-5. The current study is focused on degradation of these industrially important dyes via laccase produced by *Ganoderma lucidum*[14].

Emrah A. Erkurt et al. examined three white rot fungi namely, *Coriolus versicolor, Pleurotus ostreatus and Funalia trogii* which degrades two textile dyes, Drimaren Blue Cl-BR (DB) and Remazol Brilliant Blue Royal (RBBR). The enzyme that was responsible for degrading both the dyes were identified by analyzing the degree of protein content, pH, dye concentration, dry mycelium weight and laccase activity. *F. trogii* showed maximum de-colorization whereas, *P. ostreatus* showed minimum degradation. Both dyes were likewise discovered to be toxic to P. ostreatus. Laccase was established to be the only enzyme that carried out degradation of both dyes using SDS-PAGE. This research report doesn't include the process for isolation of laccase enzyme from *Ganoderma lucidum* and the method for increasing enzyme production however,

the given review paper is focused on dye degradation by laccase produced by *Ganoderma lucidum* and the method for increasing laccase production[15].

3. DISCUSSION

3.1 Screening for Dye Degrading Capacity:

Simple agar plates having nitrogen basal medium and synthetic dyes RBBR and RB-5 were used for screening of dye degrading ability of extracellular enzyme. 50 % de-colorization and complete de-colorization zones were monitored after 10 and 14 days, respectively. The RBBR decolorization process involves a succession of color changes from blue to pale pinkish blue to colorless. Agar plate containing RB-5 showed mycelium growth but dye degradation was not observed without the addition of redox mediator whereas addition of 1nM HBT lead to complete de-colorization in under 10 days.

Two weeks after the incubation of agar plates, 1nM ABTS in 0.1M sodium acetate buffer having 5.0 pH was added to the plates for screening the presence of laccase enzyme. Laccase enzyme secretion by *G. lucidum* was confirmed by intensive oxidation of ABTS.

3.2 Polyacrylamide Gel Electrophoresis:

To confirm the function of laccase enzyme in the degradation of dye process, SDS-PAGE and native PAGE were used. The crude enzyme was assayed against ABTS and guiacol. In the existence and lack of a mediator of redox reaction, 1nM HBT, the gel was stained with the dyes in sodium acetate buffer at 30^{o}C for 1 hour. RBBR showed a white zone of de-colorization even in the lack of redox mediator whereas, in case of RB-5, no zone of de-colorization was observed without redox mediator. In the presence of HBT, RB-5 showed zone of de-colorization. After the addition of guiacol in sodium acetate buffer, turning of specific band changes appearance from colorless zone to brownish color showed that only laccase enzyme is involved in the degradation of dye[16].

3.3 Production of Enzyme:

The laccase secretion procedure was carried out on moistened wheat bran as substrate. After 24h of incubation, mycelium growth was observed on substrate and complete colonization of mycelium on the substrate was seen within 5 days. The crude enzyme was collected after filtering the culture using a nylon filter and centrifuging.

3.3.1 Dye degradation by Laccase Enzyme:

Two synthetic dyes were used for enzymatic dye degradation namely RBBR and RB-5. In case of RBBR, decolorizing zone was observed in the lack of redox mediator HBT whereas, in case of RB-5, no decolorizing zone was obtained without the addition of HBT.

In case of RBBR, it took 2h for 40% de-colorization without the addition of HBT. The addition of 1nM HBT increased the efficacy of enzyme and de-colorization of 92.4% was observed within 2h. In case RB-5, HBT enhanced the efficiency of the enzyme to decolorize and 62% and 77.4% de-colorization was observed within 1h and 2h, respectively[17].

3.4 Impact of Temperature and pH on Degradation of dye:

3.4.1 Effect of Temperature:

Different temperatures were employed for incubation of reaction mixture to evaluate the impact of temperature on the degradation of synthetic dyes. It was observed that the degradation of dyes increased as the temperature was increased till 60^{o}C. Maximum degradation of 98% and 66.8% was observed for RBBR and RB-5, respectively. Beyond 60^{o}C, a sharp decrease in the process of de-colorization was observed in case of both the dyes[18].

3.4.2 Effect of pH:

Enzymatic degradation of both the dyes were conducted at different pH from 3-9. The results showed that dye degradation by laccase enzymes best occurs at acidic pH ranging from 3-6. There was a sharp decline in the enzyme activity beyond pH 6 and no action was monitored at alkaline pH[19].

4. CONCLUSION

In the present study, dye-degrading ability of laccase enzyme secreted by *Ganoderma lucidum* was tested. The laccase secretion procedure was carried out on moistened wheat bran as substrate. The independent role

of laccase enzyme in the degradation process was identified using native PAGE and SDS-PAGE. The enzyme was then extracted in crude form and assayed against two synthetic dyes specifically RBBR and RB-5. The enzyme showed for efficacy against RBBR as compared to RB-5. The enzyme doesn't require any redox mediator for the degradation of RBBR but a redox mediator i.e., HBT was required for degradation of RB-5.

Although laccase is very efficient against both the test dyes, it also possesses a disadvantage that there is formation of undesired color in case of azo dye. This disadvantage can limit the enzyme's use for bioremediation. Hence, it can be concluded that laccase enzyme can be used in the treatment of industrial effluents that contain various synthetic dyes that pose great threat to animals, plants and environment. Production of laccase enzyme from *G. lucidum* in bulk volumes is easy and cost-effective because this fungus is grown in large quantities due to its high medicinal value and thus, can be a great source of laccase production.

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