



# Investigation of the Effect of Redox Mediator on the Decolorization of Anthraquinone and Diazo Dyes Using Peroxidase Enzyme

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## Abstract

Enzymatic methods for dyestuff decolorization have many advantages compared to traditional methods. Laccase and Horseradish peroxidase enzymes are commonly utilized in enzymatic dye decolorization investigations. However, the fact that there are few dye removal experiments using lignin peroxidase enzyme. In this study, the Lignin Peroxidase enzyme was used to decolorization of Naphthol Blue Black (NBB) and Remazol Brilliant Blue R (RBBR) dyestuffs at pH 5.0 and 30 °C. In addition, the effect of 1-hydroxybenzotriazole (HOBt), as a redox mediator, on dye removal at different concentrations was investigated. The decolorization percentage of RBBR increased from 10% to 26% with the addition of a redox mediator to LiP enzyme after 24 hours. The decolorization percentage of NBB dye increased from 5% to 89% after adding HOBt to the LiP enzyme after 24 hours.

**Keywords:** Lignin Peroxidase, 1-hydroxybenzotriazole (HOBt), decolorization, o-dianisidine, dyestuff

## Peroksidaz Enzimi ile Asidik ve Reaktif Boyarmaddelerin Rensizleştirilmesinde Redoks Mediatörün Etkisinin Araştırılması

### Öz

Boyar madde renk gideriminde enzimatik yöntemler geleneksel yöntemlere göre birçok avantajları nedeni ile tercih edilmektedirler. Lakkaz ve Horseradish Peroksidaz enzimleri boyar madde giderme çalışmalarında sıklıkla kullanılmaktadır. Bu çalışmada, Lignin Peroksidaz enzimi, pH:5.0 ve 30 °C'de Naftol Blue Black ((NBB) ve Remazol Brilliant Blue R (RBBR) boyarmaddelerinin renginin giderilmesinde kullanıldı. Ayrıca, bir redoks mediatörü olan 1-hidroksibenzotriazol (HOBt)'ün farklı konsantrasyonlarda boyaların giderilmesine etkisi incelendi. LiP enzimine bir redoks mediatörü eklendiğinde, RBBR boyasının renk giderme yüzdesi 24 saatin sonunda %10'dan %26'ya yükseldi. HOBt'nin LiP enzimine eklenmesinden sonraki ilk günde, NBB boyasının renk giderme yüzdesi %5'ten %89'a yükseldi.

**Anahtar kelimeler:** Lignin Peoksidaz, 1-hidroksibenzotriazol (HOBt), renk giderme, o-dianisidine, boyar madde

### 1. Introduction

The contamination of water caused by the release of colored effluents from textile dyeing factories is one of today's major environmental issues[1]. Dyeing is widely used in plastic, paper, leather, and textile industries. Due to their widespread use in textiles, reactive dyes provide the biggest issue because they are nonbiodegradable and require considerable processing to completely remove color from wastewater[2]. Remazol Brilliant Blue R (RBBR), an anthraquinone derivative with azo, anthrazine, naphthalene, and sulfonated groups in its structure, is an important class of largely hazardous and resistant organic pollutants (see Fig. 1a)[3]. Naphthol Blue Black (NBB) is composed of azo, phenolic, aniline, naphthalene, and sulfonated structures (see Fig. 1b). It is a commercially important acidic diazo dye with strong photo- and thermal endurance[1]. These environmental pollutants could be removed by using physical, chemical, and biological techniques[4].

Polymers are frequently utilized in the chemical industry due to their lightweight, low cost, and ease of molding [5] as automotive parts, building materials, household appliances, electronic

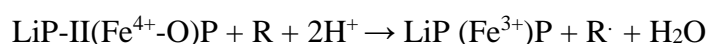
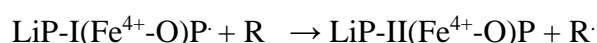
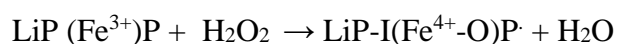
materials [6], and wastewater treatment [7]. Lignin is a complex aromatic polymer that is the second most important sustainable carbon source on the planet after cellulose, and it contains around 30% non-fossil biological carbon. The most significant challenge to utilizing renewable resources is lignin degradation. Enzymes are a specific instance of a biological approach. Enzymes such as lignin peroxidase (LiP) are investigated in dye decolorization. The possible usage of lignin-degrading bacteria and lignin peroxidase has gained interest as they can provide environmentally benign ways for the treatment of dye-containing effluent from different sectors [8], [9].

Proteins are a type of biodegradable polymer derived from biomass [10]. Enzymes are protein-structured biological catalysts [11]. Wastewater treatment with enzymes and nanoparticles has been discovered to be very successful and less harmful since enzyme has a wide range of uses such as catalytic conversion, biological remediation, and biosensors [12]. Although the use of enzymes in wastewater treatment was initially advocated in the 1930s, the concept of environmental biocatalysis, which is the use of enzymes to eliminate and degradation of target contaminants, became apparent in the 1970s [13]. Enzymes can degrade pollutants by increasing their water dissolution, allowing for additional degradation by microorganisms, or by promoting insolubility and later removal from the waste from the industry system [13], [14]. In recent years, researchers have used a variety of enzymes to remove colors from wastewater, including Horseradish Peroxidase [15]–[18], Lignin Peroxidase [9], [19]–[21], Manganese Peroxidase [22], [23], Soybean Peroxidase [24], and laccase [25].

Peroxidases (EC 1.11.1.x) are chemically composed proteins (metalloproteins) that catalyze the oxidation of a variety of substrates by using hydrogen peroxide or other organic peroxides as electron acceptors [26]. Most peroxidases are glycoproteins with N-linked oligosaccharides. These enzymes can be found in practically every field in nature and perform a variety of physiological activities. The most well-known peroxidase enzymes include chloro peroxidase (CPO) from *Caldariomyces fumago*, lignin peroxidase (LiP) from *Phanerochaete chrysosporium*, manganese peroxidase (MnP), and horseradish peroxidase (HRP) from *Coriopsis gallica*. To remove dyes, lignin peroxidase, manganese peroxidase, soybean peroxidase, horseradish peroxidase (HRP), and laccase enzymes are utilized [15], [27], [28].

Lignin peroxidases (EC 1.11.1.14) are glycolysis enzymes with a molecular weight range from 38 to 43 kDa, acidic isoelectric points, and pH values as low as 3.3–4.7. In their active areas, proteins and protoporphyrin IX coexist. LiP acts as a conventional peroxidase, being oxidized by H<sub>2</sub>O<sub>2</sub> to the two electron-deficient Compound I. Compound II was the portion that lacked an electron. While most peroxidases exclusively oxidize phenols and aromatic amines, LiPs also oxidize non-phenolic molecules [29], [30]. In lignin structures, LiP-catalyzed oxidation removes one electron from the aromatic ring to generate an aryl cation radical. Different products are generated in the presence of molecular oxygen [31].

LiP catalyzes H<sub>2</sub>O<sub>2</sub>-dependent oxidation in various lignin model compounds via the chemical sequence outlined below.



P: is pofrin, R: is an aromatic substrate such as veratryl alcohol. LiP Compound I (LiP-I) has two equivalent structures, one of which is the oxyferyl point, which is oxidized to H<sub>2</sub>O<sub>2</sub>, and the other is the deporphyrin (pi) cation radical. In contrast, LiP compound II can only have one oxidized equivalent structure. Compound I is formed by oxidizing Substrate R. LiP is catalytically transformed into the inactive intermediate Compound III in the presence of high H<sub>2</sub>O<sub>2</sub>. Native LiP can be

regenerated by spontaneous dissociation from Compound III, which results in the release of superoxide[32]. In general, LiP catalyzes the breakage of C<sub>alpha</sub>-C<sub>beta</sub> bonds in the model compound's propyl chain [31].

Redox mediators, initially identified by Bourbonnais and Paice (1990), allow laccases to oxidize nonphenolic substances, thus increasing the substrate range that these enzymes may oxidize[33]. Once substrates are too large to enter the enzyme's active site, mediators, which are tiny molecules, are required. A mediator is a type of molecule that serves as an electron transporter. When the enzyme oxidizes it, a very powerfully oxidizing intermediate chemical is generated. When passed through the enzymatic package, it can quickly oxidize any substrate [34]. As dyes are unstable and complex, they are difficult to remove using traditional water treatment methods. Enzymatic catalysis with redox aids is widely used in the degradation of polycyclic compounds, phenols, aromatic amines, biphenyls, pesticides, insecticides, and other organic molecules. Bromophenol, 2,4-dichlorophenol, guaiacol, 1-hydroxybenzotriazole (HOBt), m-cresol, quinol, syringaldehyde, vanillin, and violuric acid are the most important of these [35], [36].

2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonate (ABTS) and HOBt are the synthetic mediators that are most frequently utilized to improve the oxidation of organic contaminants[33]. Nguyen et. al. investigated the effect of a redox mediator, syringaldehyde, on the degradation of organic pollutants in an enzymatic membrane reactor and concluded that the redox mediator considerably enhanced the degradation of organic pollutants and the toxicity of wastewater[37]. Hirai investigated the role of 1-hydroxybenzotriazole (HOBt) in the oxidation of Guaiacol by laccase from *Trametes versicolor*[38]. Goodwin et. al. investigated the veratryl alcohol (VA)-mediated oxidation of chlorpromazine (CPZ) by using lignin peroxidase (LiP). They concluded that chlorpromazine was a suitable substrate for the LiP enzyme. Also, they demonstrated how the CPZ oxidation kinetics in the presence of VA could operate as a redox mediator for oxidation processes catalyzed by LiP[39]. Jamal et. al. used 9 different redox mediators (HOBt, Syringaldehyde, Guaiacol, Vanillin, Bromophenol, 2-4, Dichlorophenol, Violuric acid, Quinol and m-Cresol) in addition to the peroxidase enzyme in the removal of disperse dyes, and the highest dye removal results were achieved with HOBt-mediated oxidation of the dyes[35]. Matto and Husain removed the direct dyes by turnip peroxidase enzyme using redox mediators such as HOBt ve Violuric acid. They achieved the highest dye removal values at pH: 5.0 and 30 °C[40]. In this study, the enzymatic approach for dye removal was preferred because it is more environmentally friendly than traditional methods. Since the lignin peroxidase enzyme was insufficient to decolorize the NBB and RBBR dyestuffs, a redox mediator was added to the Lip enzyme. According to our knowledge, this study was the first to use HOBt-mediated oxidation of NBB and RBBR dyestuffs by LiP enzyme. According to the above-mentioned research in the literature, the peroxidase enzyme achieved the highest dye removal values at pH: 5 and 30 C, hence it was studied at these optimum conditions.

## 2. Materials and Methods

Remazol Brilliant Blue R (RBBR), Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 30%, and Lignin Peroxidase (specific activity: >0.1 U/mg) were bought from Sigma Aldrich. Dimethyl sulfoxide (DMSO), o-dianisidine, acetic acid, 1-hydroxybenzotriazole hydrate (HOBt), and Naphthol Blue Black were received from Fluka. None of the chemicals were further purified before use. Shimadzu UV-1800 spectrophotometer was used for UV-VIS spectroscopy studies.

### 2.1. Peroxidase Activity

Lignin peroxidase activity is frequently assessed by oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) [41]. In this study, peroxidase activity was measured by monitoring the H<sub>2</sub>O<sub>2</sub>-mediated oxidation of o-dianisidine at 460 nm with extinction coefficients 11.300 M<sup>-1</sup> cm<sup>-1</sup> [42]. The enzyme concentration was prepared to 0.004 mg/mL. The enzymatic activity of the

Peroxidase was determined using the following steps at pH 5.0 and 30 °C. 930 µL sodium acetate buffer solution (0.05 M, pH: 5.0), 20 µL o-dianisidine (10 mM), and 40 µL LiP solution were added to a quartz cuvette and the cuvette was stirred with an orbital shaker. Finally, 10 µL H<sub>2</sub>O<sub>2</sub> from 3% H<sub>2</sub>O<sub>2</sub> was added to initiate the reaction and then, OD<sub>460</sub> of this solution was acquired at each tenth minute. The enzyme activity was calculated using Equation 1. A unit was defined as the amount of enzyme required to oxidize one µmol of substrate per minute [3], [15], [28]. A<sub>460</sub> is the absorbance of the solution at 460 nm, and M<sub>ε</sub> is the molar absorption coefficient of o-dianisidine (11.300). c is Lignin Peroxidase enzyme concentration (milligrams per milliliter) referred to in equation 1 [28].

$$\frac{U}{mg} = \left( \frac{A(460) \times 10^6}{M_{\epsilon} \times C(LiP) \times t} \right) \quad (1)$$

## 2.2. Decolorization

Remazol Brilliant Blue R and Naphthol Blue Black dyestuffs were prepared at a concentration of 40 mg/mL. 100 mM HBT was prepared in 50% Dimethyl sulfoxide (DMSO) and 0.05 M sodium acetate buffer at pH 5.0[43]. Decolorization of RBBR and NBB was determined by measuring dye solution absorbance at 594 nm and 620 nm, respectively for 14 days at 30 °C. The glass tube was filled with 10 µL dyestuff solution, LiP enzyme (30 µL, 50 µL, and 100 µL for different reactions), and/or redox mediator (100 mM) (50 µL, 100 µL, and 150 µL HOBt) respectively. Then, the volume of the glass tube was completed to 2990 µL with sodium acetate buffer (0.05 M, pH:5.0). The decolorization reaction was then initiated with the addition of 10 µL H<sub>2</sub>O<sub>2</sub> (3%). The total reaction volume was 3 mL. The concentration of both dyestuffs (RBBR and NBB) in the glass tube is 133 mg/L. The final concentration of the redox mediator was 1,66 mM, 3.32 mM, and 4.98 mM for 50 µL, 100 µL, and 150 µL pipetted respectively. Dye removal experiments were first carried out in the UV-Vis. cuvette of a temperature-controlled UV-Vis spectrophotometer. However, no decrease was observed in the UV-Vis spectra of the dyes in the measurements made at 5-10-minute intervals during the day. For this reason, experimental studies were continued by making measurements at longer intervals in closed glass tubes.

Percentages of dye decolorization values were calculated using Equation 2, where A<sub>b</sub> is the absorbance of the dye solution before the enzyme reaction begins and A<sub>a</sub> is the absorbance of the dye solution after the enzyme reaction begins [15]. Table 1 describes the characteristics of dyestuffs. The chemical structures of dyestuffs are illustrated in Figure 1.

$$(\%)Decolorization\ of\ Dyestuff = \frac{A_b - A_a}{A_a} \times 100 \quad (2)$$

Table 1. General characteristics of Remazol Brilliant Blue R and Naphthol Blue Black

Parameters	Remazol Brilliant Blue R	Naphthol Blue Black
Color Index (C.I)	Reactive Blue 19	Acid Black 1
Molecular Weight (M <sub>w</sub> )	624.54 g/mol	616.49 g/mol
Maximum Absorption Wavelength (λ <sub>max</sub> )	594 nm	620 nm
Chemical structure	Anthraquinone	Diazo

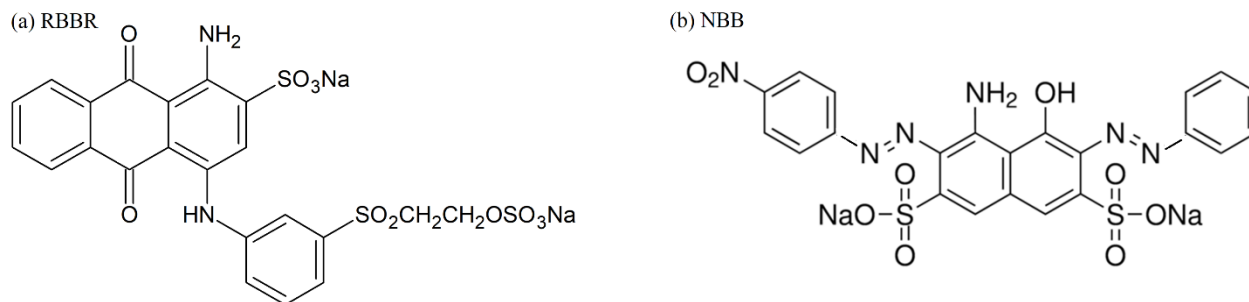


Figure 1. Molecular structure of Remazol Brilliant Blue R (RBBR) (a) and Naphthol Blue Black (NBB) (b) dyestuffs.

### 3. Results and Discussions

The peroxidase activity of the lignin peroxidase enzyme was calculated as 27.5 IU/mg in this study at pH 5.0 by using the o-dianisidine substrate. The Lignin Peroxidase (LiP) enzyme activity was assessed by Giap et al. using veratraldehyde substrate at pH 3.0 (0.25 M sodium tartrate) and 10 mm  $\text{H}_2\text{O}_2$ . LiP from an isolated strain of *Pleurotus pulmonarius* CPG6 was purified with a specific activity of 6.59 U/mg by Giap et al. in their research [20].

The decolorization percentage of RBBR increased from 11% to 37% with the addition of the redox mediator (100 $\mu\text{L}$ ) to the LiP enzyme (30 $\mu\text{L}$ ) on the 14th day, as seen in Figure 2. The decolorization percentage of RBBR On the first day, increasing the amount of redox mediator to the LiP enzyme (50 $\mu\text{L}$ ) from 100 $\mu\text{L}$  to 150  $\mu\text{L}$  enhanced from 38% to 73%.

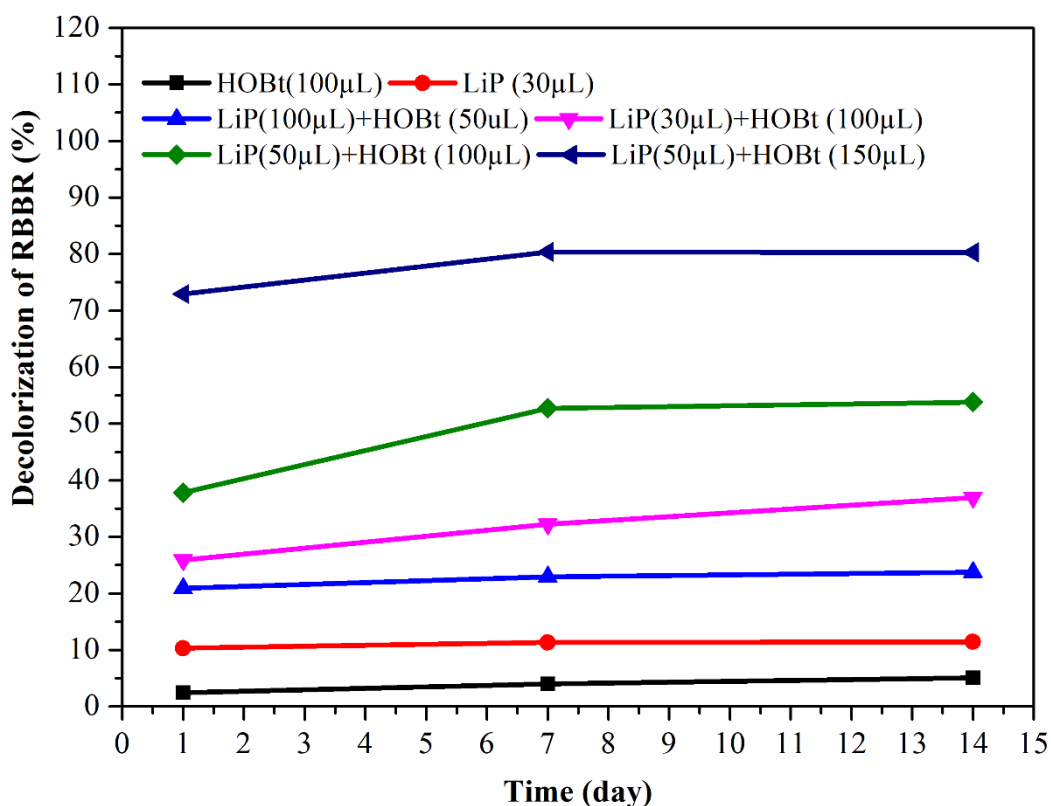


Figure 2. Decolorization of RBBR dyestuff with LiP, HOBT and LiP+HOBT at pH: 5.0 and 30 °C.

RBBR dyestuff was eliminated by 32% using LiP enzyme after 12 hours, according to Giap et. al. (2009) [20].

Figure 3 shows that when the HOBt (100  $\mu$ L) was added to the LiP enzyme (30 $\mu$ L), the percentage of NBB dye removed increased from 5% to 89% after 24 hours and from 6% to 97% after 14 days. The percentages of decolorization values were 43%, 89%, 87%, and 87% after 24 hours when the HOBt volume was kept constant at 100  $\mu$ L and the LiP enzyme was increased to 10  $\mu$ L, 30  $\mu$ L, 50  $\mu$ L, and 75  $\mu$ L, respectively. Using more than 30  $\mu$ L of LiP enzyme together with HOBt to decolorize the NBB had no significant effect on dyestuff decolorization; on the contrary, it promoted the production of additional by-products.

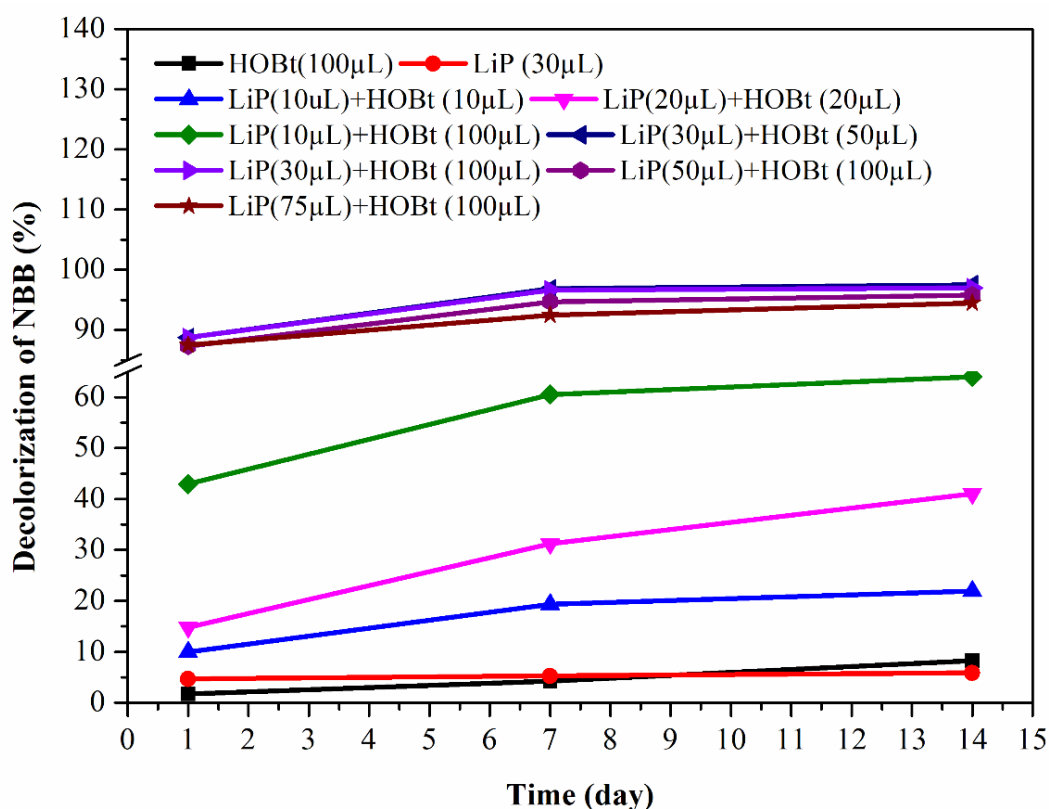


Figure 3. Decolorization of NBB dyestuff with LiP, HOBt and LiP+HOBt at pH: 5.0 and 30  $^{\circ}$ C.

Ferreira-Leitao et. al. investigated the decolorization of Methylene Blue (MB) dye with the Lignin Peroxidase enzyme from *Phanerochaete chrysosporium* at various MB: H<sub>2</sub>O<sub>2</sub> molar ratios. The dye decolorization percentage of MB dye (50 mg/L) was measured to be approximately 90%, 10%, and 10% at 1:5, 1:10, and 1:20 MB: H<sub>2</sub>O<sub>2</sub> molar ratios, respectively[13]. Immobilization of Ca-alginate in a packed bed reactor system, LiP was also used to decolorize the Remazol Brilliant Blue R dye by Bilal and Iqbal (2019). Bilal and Iqbal (2019) found that the ability of immobilized LiP to decolorize the RBBR in multiple batch cycles indicated its potential for bioremediation [44]. Tartarazine, an azo dye, was eliminated by 20% after 8 days with *Pleurotus sajorcaju* by Chagas et al. Their findings revealed that *P. sajorcaju* had the maximum Glucose-1-oxidase (GOD) activity on the second day of growth. The authors found that this fungus does not produce lignin peroxidase and that the initiation of degradation could be caused by free radicals produced by GOD or laccase enzymes [45].

LiP is a glycosylated enzyme that can biodegrade and biotransform highly hazardous phenolic chemicals found in bleach plant effluents. The peroxidase enzymes have been used in a variety of commercial applications, including dye decolorization, bio-delignification for biofuel production, organic pollutant bioremediation, biosensor creation, and sewage treatment [44]. Enzymatic

treatments are highly useful because enzymes operate on wastewater even when they are present in highly diluted solutions and are resistant to the action of certain bacteria involved in dye degradation. However, some resistant dyes are not decolorized in the presence of such enzymes (HRP, LiP, MnP, and laccase). Redox mediators increase the availability of substrates and improve the efficiency of difficult chemical degradation [46].

While the percentage of LiP enzyme-related dye decolorization was relatively low in this investigation, the addition of a redox mediator (HOBt) resulted in a significant rise in dye decolorization values, as shown in Figures 2 and 3. While the HRP enzyme removed over 100% of these dyes in a relatively short time at pH: 5 and 30°C [3], [47], the dyestuffs decolorization values were very low with the LiP enzyme, and the dye removal period was weeks in this study.

#### 4. Conclusions

Dye wastewater from the textile and dyestuff industries must be treated since it causes visual pollution and poses a risk to aquatic and terrestrial life. Traditional wastewater treatment technologies are ineffective due to the properties of this wastewater. Therefore, redox-mediated enzymatic oxidation of organic compounds has been used as an environmental method. Redox-mediated decolorization of RBBR and NBB was performed by the Lignin Peroxidase enzyme at optimum conditions in this study. The decolorization percentage of RBBR was enhanced with increasing enzyme and redox mediator concentration. However, the redox mediator (HOBt) did not affect the decolorization of NBB when the LiP enzyme concentration was more than 30 µL. HOBt showed a particularly effective influence on NBB decolorization at low enzyme concentrations. It was concluded that the LiP enzyme with redox mediator decolorized the NBB dye more effectively than the RBBR dyestuff. These encouraging findings recommend the use of a redox-mediated decolorization of dyestuffs by the Lignin Peroxidase enzyme to degrade various resistant substances.

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