Determination of the color removal efficiency of laccase enzyme depending on dye class and chromophore
Deniz İzlen Çifçi, Rıza Atav, Yalçın Günes and Elçin Günes

ABSTRACT
The aim of this article was to clarify which type of dye chromophores could be decolorized efficiently with the use of laccase enzyme. For this purpose, enzymatic degradation of different type of dye classes (4 reactive, 2 acid and 1 basic dye) having various chromophore groups was investigated by using commercial laccase from Cerrena unicolor. It was observed that the chromophore structure of dye is very important on enzymatic color removal efficiency. According to the experimental results, it was found that color removal efficiencies (20 mg/L initial dye) were 98.7% for RB220 (0.1 g/L enzyme after 6 h), 95.1% for RB19 (0.1 g/L enzyme after 48 h), 90.8% for AR42 (0.1 g/L enzyme after 48 h) while they were 60.9% for AR114 (0.25 g/L enzyme), 58.6% for RB21 (0.5 g/L enzyme), 39.7% for RR239 (0.25 g/L enzyme) even after seven days. As a result, it can be said that the highest decolorization rate was achieved for the reactive dye having formazan copper complex (RB220) chromophore. On the other hand, the enzymatic degradation of basic dye (BB9) was found to be rather difficult compared to the acid and reactive dyes used in this study and the maximum color removal was 42.8% after seven days.

Key words | chromophore, decolorization, dye, enzymatic degradation, laccase enzyme

INTRODUCTION
Textile industry uses an extensive amount of synthetic dyes, which commonly have toxic and carcinogenic effect on the environment (Nyanhongo et al. 2002; D’Souza et al. 2006). Among the various chromophores, azo dyes have the most common use in the textile industry and they are recalcitrant to remove with biological process (Stolz 2001).

Laccases are multi copper-containing enzymes and could catalyze the oxidation of most aromatic compounds (Cristóvão et al. 2008; Casas et al. 2013; Afreen et al. 2017). Although the main usage of laccase enzymes in textile industry is the removal of backstaining of denim garments, in recent years, many research articles appeared in the literature on the use of laccase enzymes for color removal of textile dye effluents (Casas et al. 2007; Montazer & Maryan 2010; Koyani et al. 2013; Iracheta-Cardenas et al. 2016). Laccase produced from white rot fungus could easily degrade most of synthetic dyes (Senthilkumar et al. 2014; Collivignarelli et al. 2019). Furthermore, it is cheaper and non-toxic for the environment compared to the other color removal processes (Ma et al. 2014; Singh et al. 2015). However, researches have generally focused on the laccase species of Phanerochaete chrysosporium, Trametes versicolor for the degradation of dyes and there are few studies on the color removal with Cerrena unicolor (Fu & Viraraghavan 2001; Asgher et al. 2008; Michniewicz et al. 2008; Singh et al. 2015). Cerrena unicolor belonging to Polyporaceae family is known to produce highly efficient extracellular laccases (Wang et al. 2017). When the literature is examined in detail, it is seen that up to now there is not any article in which the color removal efficiency of laccase enzymes from Cerrena unicolor depending on dye class and chemical (mainly chromophore) structure was investigated. For this reason, the aim of this article was to clarify which type of dye chromophores could be decolorized efficiently with the use of laccase enzyme. For this purpose, four reactive dyes, two acid dyes and one basic dye were chosen and color removal efficiencies of each dye solutions at different concentrations were determined in the presence of laccase enzyme at various concentrations and durations.
MATERIALS AND METHODS

Dyestuff and enzyme

In this study three different dye classes, namely reactive, acid and basic dyes were used. All synthetic dyes used in experiments were purchased from DyStar Textilfarben GmbH&Co. Commercial name, CI No., molecular weight, chromophore structure and maximum absorption wavelength of dyes used in experiments are given in Table 1.

Commercial laccase (Prima Green EcoFade LT100; activity > 5,700 GLacU g⁻¹), which was provided by GENENCOR International Inc. (Palo Alto, CA, USA), is principally composed of a laccase from modified Cerrena unicolor origin which also contains the mediator (3,5-dimethoxy-4-hydroxybenzonitrile) together with the other auxiliary components (Plácido et al. 2013).

Dye decolorization experiments

Dye decolorization experiments were performed by using a commercial laccase enzyme. Laccase enzyme was inoculated in a 1 L Erlenmeyer flask containing acetic acid/sodium acetate buffer solution (pH 5.0) with stirring 2 h at room temperature (25 °C). Dye stock solutions at 1 g/L concentration were also prepared in the presence of acetic acid/sodium acetate buffer (pH 5.0). To determine the effect of enzyme concentration on the decolorization efficiency, the enzymatic degradation of each dye was carried out at 40 mg/L initial dye concentration by using different concentrations of enzyme (0.05–1.0 g/L) followed by incubation in complete darkness at pH 5. Then the color removal values (%) were determined at different time intervals.

To determine the effect of initial dye concentration, solutions including optimum concentration of enzyme prepared with varying concentration of dyes (10–100 mg/L) incubated in complete darkness at pH 5. Then the color removal values (%) were determined at different time intervals.

Table 1 | Commercial name, CI no., molecular weight, chromophore structure and maximum absorption wavelength of dyes used in experiments

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>CI no.</th>
<th>Molecular weight (g/mol)</th>
<th>Chromophore</th>
<th>Wavelength λmax (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remazol Turquoise Blue G</td>
<td>Reactive Blue 21 (RB21)</td>
<td>1,079.54</td>
<td>Phthalocyanine</td>
<td>624</td>
</tr>
<tr>
<td>Remazol Blue R Special</td>
<td>Reactive Blue 19 (RB19)</td>
<td>626.53</td>
<td>Anthraquinone</td>
<td>592</td>
</tr>
<tr>
<td>Remazol Brilliant Red 3BS</td>
<td>Reactive Red 239 (RR239)</td>
<td>1,120.28</td>
<td>Monoazo</td>
<td>540</td>
</tr>
<tr>
<td>Remazol Blue BB</td>
<td>Reactive Blue 220 (RB220)</td>
<td>733.10</td>
<td>Formazan copper complex</td>
<td>610</td>
</tr>
<tr>
<td>Telon Red BN</td>
<td>Acid Red 42 (AR42)</td>
<td>505.50</td>
<td>Monoazo</td>
<td>512</td>
</tr>
<tr>
<td>Telon Red M-R</td>
<td>Acid Red 114 (AR114)</td>
<td>830.81</td>
<td>Diazo</td>
<td>521</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>Basic Blue 9 (BB9)</td>
<td>319.85</td>
<td>Thiazine</td>
<td>620</td>
</tr>
</tbody>
</table>

Determination of kinetic parameters

The enzyme kinetic is identified according to the Michaelis-Menten kinetic model. The kinetic parameters (Km and Vmax) of the commercial laccase enzyme were determined at varying initial dye concentrations (20–100 mg/L) for each dye. The enzyme concentration was 0.5 g/L for RB21 dye and 0.1 g/L for RB220 dye, whereas it was 0.25 g/L for RB19, RR239, AR42 and AR114 dyes. The experimental data were analyzed according to the Michaelis-Menten model by Lineweaver-Burk plots as follows (Salazar-López et al. 2017):

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \times \frac{1}{S}
\]

where V is the apparent reaction rate (mg/(L.min)), Vmax is the maximum apparent reaction rate (mg/(L.min)), S is the dye concentration (mg/L) and Km is the Michaelis-Menten kinetic constant (mg/L).

Analysis

The pH was measured using a pH meter (WTW pH 315i). Absorbance values of dye solutions were measured using a UV spectrophotometer (Shimadzu UV-2401 PC instrument). The dye decolorization efficiency by laccase enzyme was determined at the maximum absorbance wavelength of each dye (see Table 1). Dye concentrations were calculated from calibration curves of each dye and the dye decolorization efficiency (%) was calculated as follows:

Dye decolorization (%) = \( \frac{C_0 - C_t}{C_0} \times 100 \) (2)
where $C_0$ is the initial concentration of dye (mg/L) and $C_t$ is the final concentration of dye for a certain period of time (mg/L).

## RESULTS AND DISCUSSION

**Effect of enzyme concentration on dye decolorization**

To determine the optimum enzyme concentration for decolorization of each dye, the initial dye concentration and pH value were fixed at 40 mg/L and 5, respectively. Effect of laccase enzyme concentration on decolorization of various reactive dyes having different chromophore groups and molecular weights is given in Figure 1.

For RB19 and RB220 dyes, experimental studies were carried out at 0.25, 0.5 and 1 g/L, but as 95% decolorization was observed within first 24 h of period when using the enzyme concentration of 0.25 g/L, results of 0.5 and 1 g/L enzyme concentrations were not shown in Figure 1 and further experiments were set at 0.05 and 0.1 g/L enzyme concentrations. Color removal values for the RB19 dye after 48 h were 30.6%, 74.6% and 95.1% at 0.05, 0.10 and 0.25 g/L enzyme concentrations, respectively. Moilanen et al. (2012) obtained 45% and 80% decolorization (100 mg/L initial RB19 concentration, 500 U/L enzyme concentration, pH 4.5) of RB19 after 19.5 h enzymatic degradation using *Trametes hirsuta* and *Cerrena unicolor*, respectively. Osma et al. (2010) obtained 44% decolorization of RB19 (133 mg/L initial concentration) after 42 h enzymatic degradation with 500 U/L laccase from *Trametes pubescens*.

When Figure 1 is examined, it can be seen that decolorization efficiency of laccase treatment at 0.25 g/L enzyme concentration in descending order was as follows: RB220 (Formazan copper complex) > RB19 (Anthraquinone) > RB21 (Phthalocyanine) > RR239 (Monoazo). While color removal efficiency higher than 95% could be obtained for RB220 and RB19 dyes after 4 h and 48 h, respectively, color removals were 60.9% and 32.9% for RB21 and RR239 even after seven days at an enzyme concentration of 1 g/L for 40 mg/L initial dye concentration. When chemical structures of dye molecules given in Figure 2 are investigated, it is seen that RB220 and RB21 contain Cu$^{2+}$ ion in their chemical structure. In the study carried out by Ratanapongleka & Phetsom (2014), maximum laccase activity was determined in the presence of Cu$^{2+}$; also Zhuo et al. (2017) found the synergetic effects of Cu$^{2+}$ ions on the extracellular laccase activity. It is thought that this could be the reason of obtaining high color removal results with RB220 dye.

As can be seen from Figure 1, the decolorization of RB21 and RR239 increased up to four days and then no
Figure 2 | Chemical structures of the dyes used in experiments.
further improvement in dye decolorization was observed. On the other hand, in case of RB21 dye, decolorization efficiency increased with increasing enzyme concentration and 32.5%, 51.7% and 60.9% color removal was obtained for 0.25, 0.5 and 1.0 g/L enzyme concentrations after 4 days, respectively. For RR239, 22.4%, 31.4% and 32.9% color removal values were achieved at 0.25, 0.5 and 1.0 g/L enzyme concentrations respectively after 4 days.

Effect of laccase enzyme concentration on decolorization of various acid dyes having different chromophore groups and molecular weights are given in Figure 3. For AR42, experimental studies were initially carried out at 0.25, 0.5 and 1.0 g/L enzyme concentrations and over 93% of dye decolorization was achieved above 0.25 g/L enzyme concentration within 24 hours. For this reason results of 0.5 and 1 g/L enzyme concentrations were not shown in Figure 3 and further experiments were set at 0.05 and 0.1 g/L enzyme concentrations. 23.4, 68.8 and 93.4% color removal were obtained for AR42 dye after 48 h of reaction at 0.05, 0.1 and 0.25 g/L enzyme concentrations, respectively.

When Figure 3 is examined, the first attracting point is that the AR42 dye was much more easily decolorized by laccase enzyme compared to AR114 dye. When their chemical structures given in Figure 2 are examined, it is seen that AR42 dye has a monoazo chromophore which has smaller size and hence lower molecular weight. Therefore, it could be decolorized more easily compared to diazo dye (AR114). On the other hand, it is known that laccase enzymes act over phenolic hydroxyl groups. For each dye only one hydroxyl group is bound to the dye structure. But by taking molecular weights of dyes into consideration, it can be said that the relative hydroxyl group content of the smaller dye molecule (AR42) is higher. Which again explains why higher color removal efficiency was obtained for this dye. Furthermore, it is known that electron-donating substituents (-OH, -CH₃, -NH₂, -N(CH₃)₂) contribute to increased biodegradability while electron-withdrawing substituents (-COOH, -SO₃H, -NO₂, -Cl, -Br) make a ring a challenge to biological oxidation (Suzuki et al. 2001; Moilanen et al. 2010). This could be the other reason for obtaining lower color removal values for AR114 dye which contains more -SO₃H groups. AR42 dye decolorization increased with the rise of enzyme concentration. The AR42 dye decolorization after 24 h was 8.6%, 38.3% and 92.8% at 0.05, 0.10 and 0.25 g/L enzyme concentrations respectively.

The decolorization of the basic dye BB9 by laccase enzyme is rather more slowly compared to the other dye classes. 42.8% decolorization efficiency was achieved for BB9 after seven days at the maximum enzyme concentration (1 g/L) and 10 mg/L of initial dye concentration.

**Effect of dye concentration on dye decolorization**

Varying dye concentrations were used to determine the effect of initial dye concentration on the decolorization efficiency of reactive dyes using laccase enzyme (Figure 4). For RB220, increasing the initial dye concentration did not reduce the color removal efficiency. Above 90% of the color removal efficiency was obtained up to 100 mg/L RB220 concentration within 1.5 h and it reached over 98% after 48 h. For RB19, maximum dye decolorization was 95.0% at 20 mg/L dye concentration and it reduced gradually to about 74% when the initial dye concentration increased to 40 mg/L. Moilanen et al. (2010) achieved 80% of RB19 color removal with Cerrena unicolor laccase enzyme after 19.5 h enzymatic degradation and Murugesan et al. (2007) achieved 87–100% decolorization of RB19 between 25–300 mg/L concentration with laccase from Ganoderma lucidum after 12 h incubation. Maximum dye decolorization was observed at 20 mg/L for RR239 as 39.7%, while it was 58.6% at 20 mg/L for RB21 and further increasing the dye concentration caused to decrease in decolorization efficiency. In the study by Tavares et al. (2008),
58% of RR239 decolorization was obtained using laccase enzyme from *Aspergillus* species.

Like RB19, similar effects of the initial dye concentration was observed for AR42. Dye decolorization decreased from 90.8% to 68.8% when the initial dye concentration increased from 20 to 40 mg/L (Figure 5). The color removal rate of laccase enzyme for AR42 is much higher than that of AR114. As explained before, the reason of this result is related to the molecular weight of dyes.

Increasing the basic dye (methylene blue) concentration leads to decrease in the dye decolorization efficiency and maximum dye decolorization was obtained as 42.8% for 10 mg/L dye concentration and it reduced gradually with increasing the initial BB9 concentration (Figure 6). When the initial BB9 concentration was increased to 30 mg/L, the BB9 decolorization decreased to 12.1% after seven days at 1 g/L of enzyme concentration. In literature, decolorization efficiency below 20% was obtained with laccase from *Aspergillus oryzae* and *Trametes versicolor*, but it was stated that it could be increased when the laccase mediator is added (Forootanfar et al. 2012).
Kinetic evaluation of enzymatic dye decolorization

Laccases can degrade a wide range of organic pollutants having different chemical structures by oxidation, but this depends on both the enzyme and the presence of different functional groups in the dye structure (Legerská et al. 2016). Lineweaver-Burk plot of the enzymatic decolorization kinetics of dyes is given in Figure 7. The kinetic study results are summarized in Table 2. The $V_{\text{max}}$ value which shows the degradation rate, decreases in the following order: RB220 > RB19 > AR42 > RB21 > AR114 > RR239. When the $V_{\text{max}}$ values are examined, RR239 (monoazo) dye has the lowest $V_{\text{max}}$ value. This, results from the fact that the triazine ring in its structure is more recalcitrant compared to the benzene or naphthalene rings (Franciscon et al. 2013). The $V_{\text{max}}$ value was lower even than the AR42 and AR114 dyes, because the competition between the nitrogen atoms in the triazine ring and the nitrogen from the azo bond decreases the degradation rate (Jamal et al. 2011). Formazan copper complex dye has the highest removal rate due to the copper ion. Although phthalocyanine dye also has a copper ion in the structure, it has a lower $V_{\text{max}}$ than formazan copper complex dye. This is thought to be due to the higher molecular weight of phthalocyanine dye (Khan et al. 2015). $K_m$ expresses the affinity between the substrate and the enzyme and the lower $K_m$ means the higher affinity (Shi et al. 2016). Comparing the $K_m$ values of each dye, the affinity of the laccase enzyme for dye increases in following order: RB21 < AR114 < RB220 < RB19 < RR239 < AR42.

In the case of acid dyes, the color of monoazo dyes can be removed faster than diazo dyes. Previous studies also had shown that dye removal efficiency and the removal rate decreased with the increase of azo group number of dyes (Ashrafi et al. 2016; Forootanfar et al. 2019). Besides, AR42 dye has a lower $K_m$ than AR114 dye, which means laccase enzyme used in experiments has higher affinity for AR42 dye.

The laccases are copper-dependent enzymes produced by a number of mushrooms and plants, and phenols and aniline are oxidized in the presence of oxygen, this makes it possible to oxidize the phenolic rings in the azo dye due to its rich electron (Chivukula & Renganathan 1995). In the enzymatic degradation, the breakdown of azo linkage occurs by the water’s nucleophilic attack and intermediates are formed (Khan et al. 2015). Laccase oxidation of phenols produces a quinone from the phenolic portion of the azo dye and 4-sulfophenylhydroperoxide from the sulfophenyl ring (Chivukula & Renganathan 1995).

Anthraquinone dyes have the delocalized $\pi$-conjugated electron system, both in the nucleus and substituents (Pramodini & Poornesh 2013). Dyes having anthraquinone chromophores can be decomposed to the smaller molecules by hydroxylation, deamination and oxidation reactions by the laccase enzyme (Osma et al. 2010; Legerská et al. 2016).

The main intermediate products of Cu-phthalocyanine dyes are sulfophthalimide and its derivatives occurring the breakdown of the phthalocyanine ring in the sulfonated phthalocyanine dye degradation by white rot fungus or laccase (Heinling-Weidtmann et al. 2001; Kenzom et al. 2014).
On the other hand, there are not any studies in the literature on the degradation of dyes having formazan copper complex chromophore to the best of authors’ knowledge. But in a study carried out on the decolorization of RB220 dye, ligninolytic enzyme (*Myceliophthora vellerea*) was used and it was stated that dye molecule had been degraded to phenyl derivatives, cresol and catechol (*Patel et al. 2013*). Enzymatic degradation of methylene blue (which is a basic dye) with laccase is quite difficult due to the thiazin groups bonded to the aromatic rings and the methyl bonded aromatic structures (*Diwaniyan et al. 2010; Zucca et al. 2015*). Therefore, it can be said that methylene blue is not an ideal substrate for laccases. These observations are not surprising as the dye molecule does not contain any -OH or -NH₂ groups, and for this reason, no hydrogen atom could be abstracted from the molecule. Only a non-bonding electron could be drawn from a -N(CH₃)₂ group or also from the nitrogen and sulfur atoms incorporated in the thiazine ring, therefore adding a supplementary positive charge to the dye cation (*Zucca et al. 2015*). However, laccase from *Aspergillus oryzae* or *Trametes versicolor* could decolorize the methylene blue dye in the presence or absence of hydroxybenzotriazole (*Forootanfar et al. 2012*).

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**CONCLUSIONS**

Enzymatic degradation of different dye classes having various chromophores was investigated in this study. The color removal efficiencies of laccase enzyme from *Cerrena unicolor* for reactive dyes used in this study were found to be high. The most remarkable result found in this study is that the reactive dye having formazan copper complex could be decolorized much more readily using laccase enzyme since the Cu²⁺ ions in this dye structure improved the rate of enzymatic degradation reaction. At the initial dye concentration of 20 mg/L, 98.7% of RB220 (Formazan copper complex) was removed in 6 h while 95% of RB19 (anthraquinione) was removed in 48 h. However, in same initial dye concentration, RB21 (phthalocyanine) and RR239 (monoazo) dye removal reached to 58.6% and 39.7% after seven days, respectively. Furthermore, it was determined that the enzymatic degradation of acid dye containing diazo chromophore was more difficult than the mono azo acid dye that in diazo (AR114) dye, 60.9% dye removal was obtained within seven days, while monoazo (AR42) dye removal was 90.8% after 48 h. On the other hand, it has been found that enzymatic degradation is not suitable for C.I. Basic Blue 9 dye that below 42.8% of decolorization was obtained at 1 g/L enzyme concentration after seven days of treatment. According to the results of kinetic studies, the Vₘₐₓ values decreased in the following order: RB220 (6.3151 μM/min) > RB19 (0.4263 μM/min) > AR42 (0.2477 μM/min) > RB21 (0.2218 μM/min) > AR114 (0.0727 μM/min) > RR239 (0.0205 μM/min). As a result, it can be concluded that the enzymatic degradation could be used as an ecological alternative for the decolorization of textile dye effluents with integration to conventional wastewater treatment systems.


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