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Research Article

EFFECT OF PARAMETERS ON REMOVAL OF RB19 DYE BY LACCASE ENZYME AND KINETIC EVALUATION

Deniz İzlen ÇİFÇİ*[1], Rıza ATAV[2], Yalçın GÜNEŞ[3], Elçin GÜNEŞ[4]

[1]*Department of Environmental Engineering, Tekirdağ Namık Kemal University, TEKİRDAĞ;*
ORCID:0000-0001-7527-6130
[2]*Department of Textile Engineering, Tekirdağ Namık Kemal University, TEKİRDAĞ;*
ORCID: 0000-0002-5807-4542
[3]*Department of Environmental Engineering, Tekirdağ Namık Kemal University, TEKİRDAĞ;*
ORCID:0000-0001-8697-3345
[4]*Department of Environmental Engineering, Tekirdağ Namık Kemal University, TEKİRDAĞ;*
ORCID:0000-0002-1457-1504

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ABSTRACT

Dye decolorization of RB19 using commercial laccase (*Cerrena unicolor*) enzyme was evaluated in this study. The effect of pH, laccase concentration, temperature, metal salt, carbon and nitrogen sources and solvents on the removal of RB19 dye were examined in detail. Highest RB19 removal with laccase (*Cerrena unicolor*) was obtained at pH 6, 40°C, 0.3 g/L enzyme concentration and 200 mg/L initial dye concentration. According to the Michaelis–Menten kinetics, the highest V_{max} was 6.61 mg/L.min at these conditions. 0.25 mM of Cu^{2+} or Mn^{2+} enhanced the decolorization of RB19 but further increase in metal concentration decreased the removal of RB19. In the presence of carbon source such as glucose, starch and lactose, removal of RB19 by laccase was not significantly affected. However, in the presence of nitrogen sources such as urea and NH_4Cl, the removal of RB19 by laccase was significantly reduced.
Keywords: *Cerrena unicolor*, enzymatic treatment, kinetic, laccase, Reactive Blue 19.

1. INTRODUCTION

Reactive dyes have a wide range of applications for dyeing cotton, wool and polyamide fibers in the textile industry [1]. They covalently bond with cellulosic fibers and are preferred because of their wide range of color tones, high wet fastness values, ease of application, and bright colors [2]. Among the reactive dyes, anthraquinone reactive dyes are the most commonly used dye after azo dyes, but they are highly resistant to biodegradation than the azo dyes due to the aromatic structures [3]. Reactive Blue 19 (RB19) dye is one of the most important antraquinone reactive dyes for the textile industry and is defined as toxic and persistent organic pollutant [4]. RB19 is also used as starting material to produce the polymeric dyes [5].

Among the color treatment processes, enzymatic degradation is the most favor because of being economics, simple and highly efficient for the most of resistance dyes removal [6]. It is

* Corresponding Author: e-mail: dicifci@nku.edu.tr, tel: (282) 250 23 83

known that laccase enzymes can successfully degrade various dye types such as azo, diazo and anthraquinone structures and can be successfully applied in the treatment of textile industry wastewater [7]. Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) with multi-copper oxidases can catalyse phenols, anilines, and aromatic thiols to the corresponding radicals with the reduction of O_2 to H_2O [8-9]. The advantages of using enzymes to decolorize the dyes may be their rapid dissolution activity and their ability to retain their effectiveness even under adverse conditions [9]. Laccase catalyzes the organic pollutants' oxidation by direct reduction of molecular oxygen to water in the absence of H_2O_2 or other metabolites [10].

In textile applications various dye classes are used depending on fiber type to be dyed. The most commonly used dye classes for cotton, wool (and also polyamide) and polyacrylonitrile are reactive, acid and basic dyes. For this reason in our previous study it was aimed to clarify which type of dye chromophores could be decolorized efficiently with the use of *Cerrena unicolor* laccase enzyme [11]. 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* [11]. It was determined that laccase enzyme can effectively decolorize reactive and acid dyeing effluents. Taking into consideration that acrylic fiber is not very common and it is generally dyed in dyeing mills specialized in this field, laccase enyzmes could be used for decolorization of wastewater of textile dyeing mills in which cellulosic, protein and polyamide fibers. It is important to note that the enzymatic decolorization could be applied after biological treatment of textile wastewater after removal of COD, N, P and some other pollutants. Because, laccase enzymes have only potential to decolorize remaining dye.

For laccase enzymes to be used in industrial applications, it should also be clarified how various factors such as salts, heavy metals solvents and etc., which may present in textile dyeing effluents, effect this enzyme's performance.

Researches have shown that enzymatic degradation of RB19 is possible with laccase such as *Trametes trogii*, *Ganoderma lucidum*, *Pleurotus florida*, *Pleurotus ostreatus*, *Coriolus versicolor*, *Funalia trogii* [4, 12-16]. However, *Cerrena unicolor* laccase enzyme has little researches on the color removal [17-19]. In general, researches have been focused on the effect of parameters such as pH, temperature, enzyme concentration, and obtained the enzyme kinetics parameters on the decolorization by *Cerrena unicolor*. However, textile wastewater commonly contains high salt concentration and various heavy metals and it is known that salts and most heavy metals or cations have toxic effects on the enzyme activity [12,20].

The aim of this study was to investigate the effect of temperature, enzyme concentration and pH value on the color removal of RB19 dye by the laccase enzyme originated from *Cerrena unicolor*. In addition, the impact of various salts, heavy metals, cations, solvents, carbon and nitrogen sources found in the textile industry wastewater were investigated. Also, the kinetic parameters were determined by the Michaelis-Menten kinetics.

2. MATERIALS AND METHODS

2.1. Laccase enzyme and dye

Commercial laccase (Prima Green EcoFade LT100; activity > 5700 GLacU/g), which was provided by GENENCOR International Inc. (Palo Alto, CA, USA), is composed principally by a laccase from modified of *Cerrena unicolor* [11].

Remazol Brilliant Blue R (Reactive Blue 19, RB19) dye used in the experiments was purchased from DyStar Textilfarben GmbH&Co. RB19 is one of the anthraquinone dye and chemical structure of RB19 is given in Figure 1. Constitution number of RB19 is 61200 and the molecular formula is $C_{22}H_{16}N_2Na_2O_{11}S_3$ with a molecular weight of 626.54 g/mol.

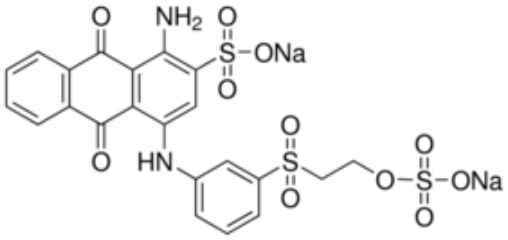

Figure 1. Chemical structure of RB19 dye

2.2. Experimental Study

RB19 dye decolorization experiments were performed by using a commercial laccase (*Cerrena unicolor*) enzyme. Laccase enzyme inoculated in acetic acid/ sodium acetate buffer solution at the desired pH by stirring 1 h at room temperature (20-25°C). Dye stock solutions at 1 g/L concentration were prepared in the presence of sodium acetate buffer solution. Experimental studies were performed in an active volume of 50 mL and it was kept in the incubator at the desired temperature and reaction time to provide dye removal. Then the RB19 dye concentration and dye removal were determined from the absorbance values obtained by using spectrophotometer.

2.2.1. Effect of pH, laccase concentration, initial dye concentration and temperature on dye decolorization

The effect of pH on the decolorization of RB19 dye was studied at 200 mg/L of RB19, 0.25 g/L laccase and 40°C in the pH range 2-8 using the sodium acetate and acetic acid buffer solution. The decolorization of the RB19 of monitored each 15 or 30 min. during 3 hours of reaction.

The effect of laccase concentration ranging from 0.1 to 0.6 g/L on the dye decolorization was monitored with 200 mg/L dye concentration, pH 6 and 40°C. The decolorization of the RB19 was monitored again each 15 or 30 min during 3 hours of reaction.

In order to determine the effect of the initial dye concentration and temperature on dye decolorization, experimental studies were carried out at the initial dye concentration of 50-500 mg/L and at temperatures between 30-60°C with the constants of pH 6 and 0.3 g/L enzyme concentration. The decolorization of the RB19 was monitored each 15 or 30 min during 3 hours of reaction.

2.2.2. Effect of metal salts on dye decolorization

The effect of metals on RB19 decolorization was investigated under 200 mg/L initial RB19 concentration, at pH 6, 0.3 g/L enzyme concentration and 40°C. NaCl, KCl, $HgCl_2$, $SnCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, $CoCl_2$, $AlCl_3$, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, $AgSO_4$ were examined between 0.25-4.0 mM concentration. The dye decolorization was calculated after 3 hours of enzymatic degradation.

2.2.3. Effect of carbon and nitrogen source on dye decolorization

The effect of carbon and nitrogen source on RB19 decolorization was investigated using glucose, lactose and starch from potato as cosubstrates and peptone, urea, NH_4Cl, KNO_3, $NaNO_3$ as the nitrogen source. The RB19 dye decolorization experiments were performed at the initial RB19 dye concentration (of 200 mg/L), enzyme concentration (of 0.3 g/L), pH (of 6.0) and

temperature (of 40°C). Carbon and nitrogen source concentrations were selected between 2.5-15 g/L and dye decolorization were calculated after the 3 hours reaction.

2.2.4. Effect of organic solvent on dye decolorization

To determine the effect of various organic solvents on RB19 dye decolorization; acetone, isopropanol, ethanol and methanol were selected between 2.5-20% (v/v) concentrations. The RB19 dye decolorization experiments were performed used at the initial RB19 dye concentration (of 200 mg/L), enzyme concentration (of 0.3 g/L), pH (of 6.0) and temperature (of 40°C). The dye decolorization was calculated after 3 hours of enzymatic degradation.

2.3. Determination of kinetic parameters

The kinetic parameters, K_m and V_{max} of the commercial laccase enzyme were calculated according to the Michaelis–Menten model by Lineweaver–Burk plots as follows [21]:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} * \frac{1}{S}$$

where V is the apparent reaction rate (mg/L/min), V_{max} is the maximum apparent reaction rate (mg/L/min), S is the dye concentration (mg/L) and K_m is the Michaelis–Menten kinetic constant (mg/L). Eight different initial dye concentrations between 50-500 mg/L were chosen in kinetic studies at 0.3 g/L enzyme concentration and pH 6 after 3 hours of enzymatic degradation. The apparent reaction rate was calculated by the slope obtained from the time-dependent change of RB19 dye concentration for each dye concentrations [22]. According to a plot against 1/V versus 1/S, V_{max} was calculated from the intercept and K_m was calculated from the slope [21-22].

2.4. Analysis

The pH was measured using a pH meter (WTW pH 315i). RB19 dye concentration was measured using a UV spectrophotometer (Shimadzu UV-2401 PC instrument) at 592 nm. Dye concentrations were calculated using the calibration curve and the percent of dye decolorization was calculated as follows:

$$\text{Dye Decolorization } (\%) = \frac{C_0 - C_t}{C_0} * 100$$

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).

3. RESULTS AND DISCUSSIONS

3.1. Effect of pH, laccase concentration, initial dye concentration and temperature on dye decolorization

The effect of pH was examined in the pH range 2-8 at the temperature of 40°C, 0.25 g/L laccase concentration and 200 mg/L RB19 (Fig. 2a). The maximum dye decolorization was observed at pH 6 and there was no significant change in dye removal between pH 5 and 7. The RB19 dye decolorization was 64.8, 70.1 and 64.6% at pH 5, 6 and 7, respectively. The decolorization was gradually decreased at pH below 5 and the RB19 dye removal was approximately 20% between pH 2 and 4 (Fig. 2b). Similarly, the decolorization began to decrease above pH 7. As it is known enzymes show their maximum activity at a specific pH and temperature. Laccase enzymes have generally the maximum activity at around pH 5-6 [4,23]. It is stated that the highest RB19 dye removal was at pH 5 with *Trametes trogii* enzyme and the removal efficiency is decreased at pH 3 and above pH 7 values [4]. Tavares et al. (2008)

observed similiar results that Reactive Blue 114 decolorization was almost constantly with the pH range of 5-7 using commercial laccase from *Aspergillus* [23].

To determine the effect of enzyme concentration for RB19 dye decolorization, the enzyme concentration was selected in the range of 0.1 to 0.6 g/L at pH 6, 200 mg/L RB19 and 40°C (Fig. 2c). Enhancing the laccase concentration increased the decolorization as well as the decolorization rate. The maximum RB19 dye decolorization was 86% at 0.3 g/L enzyme concentration and the dye removal efficiency did not significantly change when the enzyme concentration was increased above 0.3 g/L (Fig. 2d). These findings are consistent with the literature that excessive amount of enzyme did not increase the decolorization of dye [12,24].

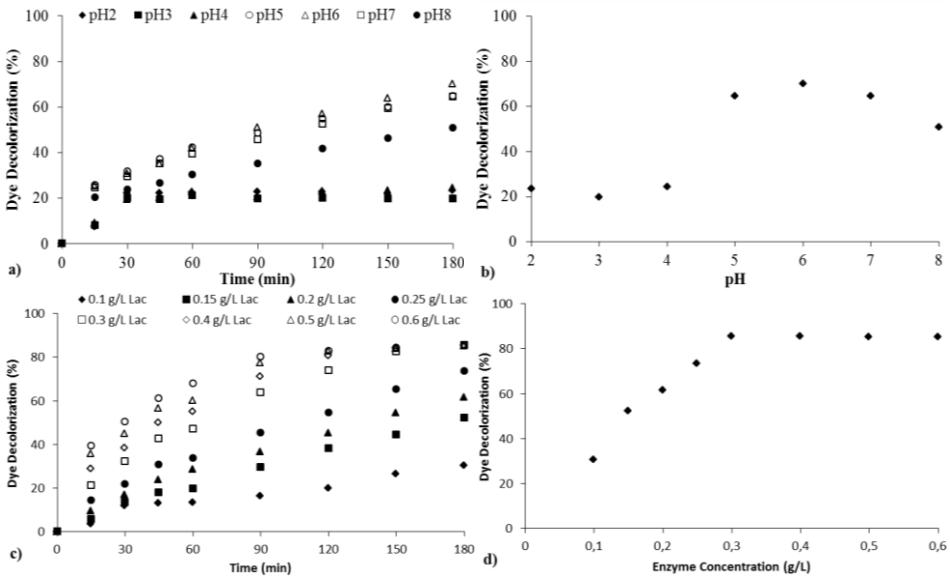

Figure 2. The RB19 decolorization a) changes at different pH values and b) changes at different pH values after 3 h of reaction (RB19 = 200 mg/L, laccase = 0.25 g/L, temperature = 40°C), c) changes at different laccase concentration and d) changes at different laccase concentration after 3 h of reaction (RB19 = 200 mg/L, pH = 6, temperature = 40°C)

The effects of initial RB19 dye concentration and temperature on RB19 dye decolorization are shown in Figure 3. Results showed that the highest dye decolorization for 30°C of temperature could be achieved when the initial RB19 dye concentration was below 100 mg/L. The removal rate and removal efficiency for this temperature gradually decreased after 100 mg/L.

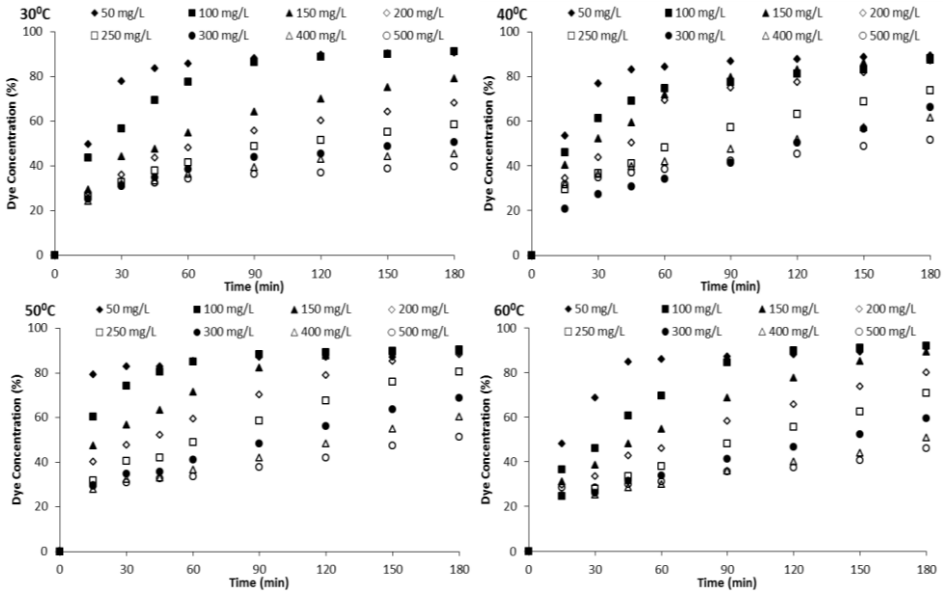

Figure 3. Effect of initial dye concentration and temperature on RB19 decolorization (pH = 6, enzyme concentration = 0.3 g/L)

Temperature is an important parameter affecting both reaction rate and laccase stability [25]. RB19 decolorization rate was improved by increasing the temperature up to 40°C. When the temperature is increased to 40°C, it can be seen that the decolorization was approximately 88% up to 150 mg/L of RB19 dye concentration. On the other hand the color removal at 50°C did not change significanlty compared to the 40°C temperature. However, it appears that the color removal is beginning to decrease when the temperature is increased to 60°C. The reason of this could be that the high temperature caused the breakdown of the laccase structure [26]. As it is explained above, all enzymes have specific pH and temperature and the suitable temperature was 40-50°C for the *Cerrena unicolor* laccase enzyme used in this study. This value is consistent with the literature, the optimal temperature range for enzymatic degradation of RB19 dye using *Funalia trogii, Trametes trogii, Armillaria* is given in the range of 40-60°C [25,27-28]. Furthermore, the maximum laccase activity temperature for *Cerrena species* laccases is reported to be in the range of 45-50°C [18,29].

3.2. Effect of metal salts on RB19 decolorization

Cu^{2+} and Mn^{2+} metals increased the laccase activity and therefore RB19 decolorization (up to 90%) was improved at 0.25 mM concentration compared to the control sample (88.3%). However, further increase in the Cu^{2+} concentration decreased the RB19 decolorization i.e for 4 mM decolarization 376epolarization was 56.9% . Many researchers have observed that Cu^{2+} and Mn^{2+} metals enhanced the laccase activity [18,26,29-31]. On the other hand, RB19 decolorization was reduced at 10 mM of Cu^{2+} by the laccase from *Trametes trogii* [4] and at 1 mM of Cu^{2+} by the laccase from *Ganoderma lucidum* [12].

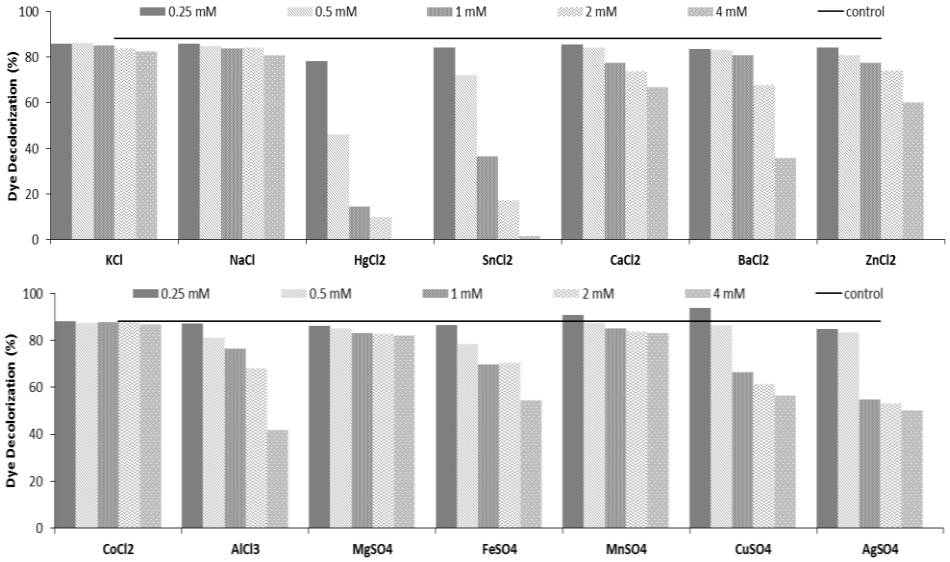

Figure 4. Effect of metal salts on RB19 decolorization (RB19 concentration = 200 mg/L, pH = 6, enzyme concentration = 0.3 g/L, temperature = 40°C, time = 3 hours)

Cerrena unicolor is resistant to metal salts such as KCl, NaCl, $CoCl_2$, and $MgSO_4$ and for this reason small changes in RB19 decolorization was observed in the presence of these metals. Increasing the concentration of metal salts such as $BaCl_2$, $CaCl_2$, $ZnCl_2$, $AlCl_3$, $FeSO_4$, $AgSO_4$ caused gradual decrease in the RB19 dye decolorization. Severe inhibition was observed in the presence of $HgCl_2$ and $SnCl_2$ metal salts and therefore no color removal for RB19 was observed at 4 mM concentration. Hg^{2+} generally showed the strongest inhibition effect on the laccase activity of white rot fungus [12,19,26,30,32] and the negative effect of Hg^{2+} is indicated to be due to the binding of Hg^{2+} to the sulfhydryl (-SH) groups which causes the distortion of enzyme structure [26,30]. Also, 1 mM Sn^{2+} inhibited the laccase activity of *Lentinula edodes* by 99% [31].

3.3. Effect of carbon and nitrogen source on RB19 decolorization

Figure 5 shows the effect of carbon and nitrogen source on RB19 decolorization. There is no significant difference in RB19 decolorization between the carbon sources, as well as the nitrogen sources. Das et al. (2016) observed that the laccase activity of *Pleurotus ostreatus* was close to each other when glucose, lactose and starch were used as a carbon source [26]. Among the nitrogen source, KNO_3, peptone and $NaNO_3$ did not markedly affect the RB19 removal while the strongest negative effect was observed using NH_4Cl. Increasing urea concentration gradually decreased the RB19 decolorization from in case of 88.3% to 49.0%. Dhakar et al. (2014) found that the laccase activity of *Penicillium pinophilum* with KNO_3 is higher than NH_4Cl and urea [33]. Mikiashvili et al. (2006) observed that peptone as a nitrogen source increased the laccase activity while $NaNO_3$ did not significantly change the laccase activity of *Pleurotus ostreatus* [34].

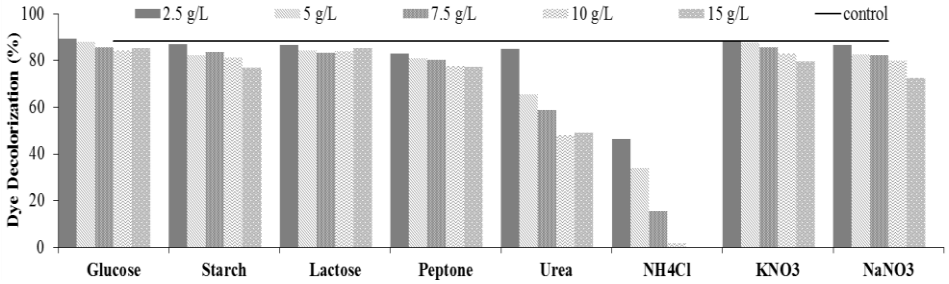

Figure 5. Effect of carbon and nitrogen source on RB19 decolorization (RB19 concentration = 200 mg/L, pH = 6, enzyme concentration = 0.3 g/L, temperature = 40°C, time = 3 hours)

3.4. Effect of solvent on RB19 decolorization

To determine the effect of solvents, between 2.5-20% (v/v) of acetone, isopropanol, ethanol and methanol was used as solvent. According to Figure 6, acetone, isopropanol and methanol were not merely affected the RB19 removal up to 5% solvent addition, but when the 20% acetone, isopropanol or methanol was added, the RB19 decolorization decreased to 46.7, 53.2 and 53.7%, respectively. Besides, ethanol severely affected the enzyme activity and the RB19 dye decolorization was reduced to 37.3% with the addition of 20% ethanol. Yang et al. (2014) found that the relative laccase (from Cerrena sp. HYB07) activity was decreased to 57.3% and 70.9% when 25% of methanol or ethanol was used, respectively [18]. The order of the negative effect of solvent was determined as ethanol > acetone > isopropanol > methanol for 20% of solvent addition.

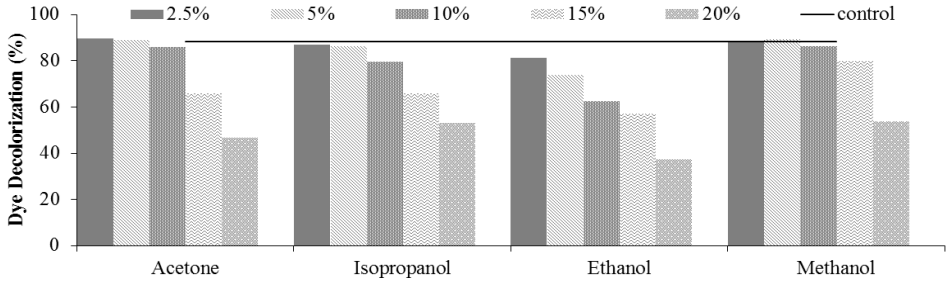

Figure 6. Effect of solvents on RB19 decolorization (RB19 concentration = 200 mg/L, pH = 6, enzyme concentration = 0.3 g/L, temperature = 40°C, time = 3 hours)

3.5. Evaluation of kinetics

The kinetic parameters of the commercial laccase enzyme were calculated according to the Michaelis–Menten model by Lineweaver–Burk plot. The Lineweaver–Burk plot of RB19 dye decolorization is shown in Fig. 7. According to the Lineweaver–Burk plots, when the temperature was increased from 30°C to 40°C, the V_{max} value increased (Table 1). It is observed that the value of V_{max} decreases with rising the temperature above 40°C. Although a slight decrease in V_{max} was observed when the temperature was 50°C, the lower K_m value was obtained comparing to 40°C which means that the lower K_m values has higher affinity of laccase enzyme to the substrate [35].

K_m and V_{max} values were 214 mg/L (0.342 mM) and 6.61 mg/L.min (0.0105 mM/min) at 40°C, respectively for the RB19 dye decolorization in this study. K_m and V_{max} values at 30°C for

RB19 dye decolorization were determined as 145.8 mg/L and 24.9 mg/L.min, respectively using *Pleurotus florida* laccase in the presence of 0.85 mM concentration of HBT as a redox mediator [13]. Ashrafi et al. (2013) reported that the K_m and V_{max} values of *Paraconiothyrium variabile* laccase were 38.3 µM and 50 mmol/min.mg at 40°C for RB19 dye decolorization, respectively [36]. K_m and V_{max} were obtained as 1090-1446 µM/sec and 300-132 µM at 25 °C for the RB19 dye decolorization by two laccases obtained from *Pycnoporus sanguineus* using violuric acid and N-hydroxyphthalimide as laccase mediators, respectively [21].

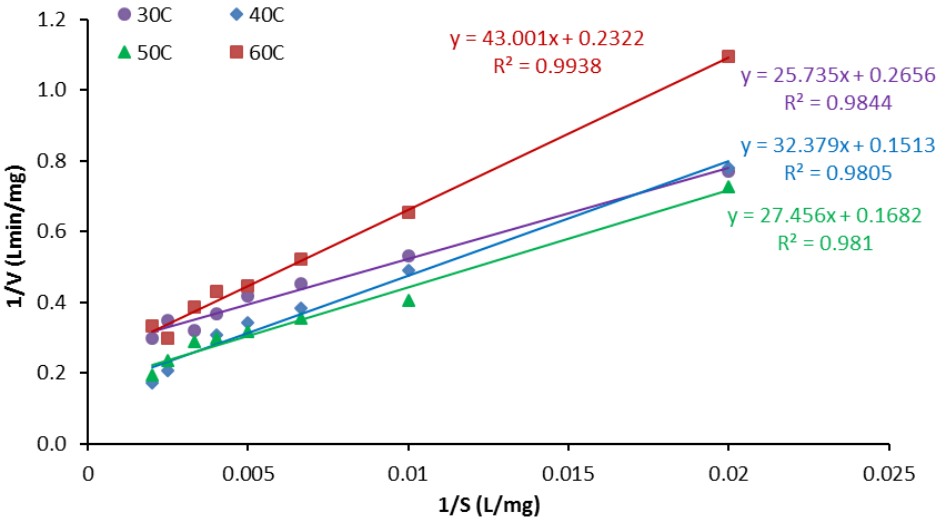

Figure 7. Lineweaver–Burk plot of RB19 dye decolorization kinetics by *Cerrena unicolor* at different temperatures (pH = 6, enzyme concentration = 0.3 g/L, time = 3 hours)

Table 1. Kinetic constants of laccase from *Cerrena unicolor* for RB19 dye decolorization

Temperature (°C)	V_{max}		K_m		
	(mg/L.min)	(mM/min)	(mg/L)	(mM)	R^2
30	3.77	0.0060	96.9	0.155	0.9844
40	6.61	0.0105	214.0	0.342	0.9805
50	5.95	0.0095	163.2	0.261	0.9810
60	4.31	0.0069	185.2	0.296	0.9938

Studies had shown the RB19 dye removal pathway with free or immobilised laccase enzymes such as *Pleurotus ostreatus, Aspergillus flavus and Trametes pubescens* [37-38]. In the studies, it was stated that as a result of enzymatic degradation of RB19 dye, RB19 dye was firstly broken into two sub-products with less toxic effect [37-39]. The rings of these two sub-products are opened as a result of reduction, hydroxylation, deamination, and oxidation reactions [38-39].

These by-products obtained in the treatment of RB19 dye with laccase are less toxic than pure RB19 dye [13, 36]. Sathishkumar et al. (2013) investigated the phytotoxicity of laccase treated RB19 dye by *Pleurotus florida* [13]. Although RB19 degradation products were reported to be moderate toxic according to the Germination Index of seeds (ryegrass), they are less toxic than RB19 dye. Similarly, Ashrafi et al. (2013) showed that the percentage of growth inhibition of six bacterial strains (*E. coli, P. aeruginosa, S. typhi, B. subtilis, S. aureus, M. luteus*) was decreased

in the laccase treated RB19 dye by *Paraconiothyrium variabile* compared to the untreated RB19 dye [36].

4. CONCLUSION

The decolorization of RB19 using commercial laccase (*Cerrena unicolor*) was investigated in this study. The maximum RB19 dye decolorization was obtained as 86% at 0.3 g/L enzyme concentration, pH 6 and 40°C after 3 h of reaction. Cu^{2+} and Mn^{2+} metals increased the decolorization of RB19 however, RB19 dye removal is significantly reduced in the presence of Hg^{2+} and Sn^{2+} metals. While urea and NH_4Cl as nitrogen sources reduced the RB19 decolorization, there was no significant change in the removal of RB19 by laccase in the presence of nitrate derived nitrogen such as KNO_3, $NaNO_3$ and in the presence of carbon source. RB19 decolorization was nearly same in the presence of solvents (up to 5%) such as acetone, isopropanol and methanol. However, RB19 decolorization decreased gradually with increasing the solvent amount by up to 20%. According to the Michaelis–Menten kinetics, the highest V_{max} was obtained as 6.61 mg/L.min at 40°C. The results obtained in this study showed that the use of the laccase enzyme (*Cerrena unicolor*) in color removal of reactive dyes such as RB19 could be a very convenient method.

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