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Optimization of decolorization of methylene blue by lignin peroxidase enzyme produced from sewage sludge with *Phanerocheate chrysosporium*

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ABSTRACT

Optimization of decolorization of methylene blue (MB) dye by lignin peroxidase (LiP) enzyme produced by white-rot fungus *Phanerochaete chrysosporium* using sewage treatment plant (STP) sludge as a major substrate was carried out in the laboratory. Optimization by the one-factor-at-a-time (OFAT) and statistical approach was carried out to determine the process conditions on optimum decolorization of MB dye using LiP enzyme in static mode. The OFAT method indicated that the optimum conditions for decolorization of MB dye (removal: 14–40%) was at temperature 55 °C, pH 5.0 with hydrogen peroxide (H_2O_2) concentration 4.0 mM, MB dye concentration 20 mg/L and LiP activity 0.487 U/ml. The addition of veratryl alcohol to the reaction mixtures did not contribute any further increases in decolorization. The initial concentration of MB and the activity of LiP enzyme were further optimized using response surface methodology (RSM). The contour and surface plots suggested that the optimum initial concentration of MB and LiP activity predicted were 15 mg/L and 0.687 U/ml, respectively for the removal of 65%. The validation of the model showed that the decolorization process gave the higher removal of 90% in agitation mode compared to the static mode with 65% for 60 min of incubation time by LiP enzyme.

1. Introduction

The ligninolytic enzymes have wide applications that are currently used in the removal of dyes from industrial effluents [1–4], bio-bleaching [5] and for treating hazardous waste [6–8]. Textile dyeing effluents containing recalcitrant dyes are polluting waters due to their color and by the formation of toxic or carcinogenic intermediates such as aromatic amines from azo dyes that are essential to be eliminated before release into natural water streams. A special problem is encountered in the application of synthetic dyes which have a complex aromatic molecular structure and are designed to be resistant to physical, chemical and microbial fading [9]. In addition, batik industries in Malaysia are one of the pollution contributors in textile wastewater which comes from the dying processes. Therefore, the elimination of dyes from textile dyeing effluents currently represents a major ecological concern. Enzymatic methods used to solve these problems generally have low energy requirements, are easy to control, can operate over a wide range of conditions and have a minimal environmental impact [7].

Currently, the major methods of textile wastewater treatment involve physical and/or chemical processes. Such methods are often very costly and although the dyes are removed, the accumulation of concentrated sludge creates a disposal problem. There is also the possibility that a secondary pollution problem will arise because of excessive chemical use. Other emerging techniques such as ozonation [10], treatment using Fenton's reagent, electrochemical destruction [11] and photocatalysis [12] may have potential for decolorization. However, such technologies usually involve complicated procedures or are economically unfeasible [13].

The interest in new biocatalyst (enzyme) usage has been growing over the last two decades due to the increasing rate of xenobiotics whose degradation is not effective and efficient by means of conventional chemical and biological processes [4]. Therefore, it is an important factor in determining the economic and technical feasibility of application for industrial uses and is also a critical factor in optimizing the commercial production of enzymes. As such, the lignin peroxidase (LiP) produced from renewable resource sewage treatment plant (STP) sludge by *Phanerochaete chrysosporium* would be beneficial for industrial applications due to its low production cost compared to other expensive media-based enzymes [14–16]. Furthermore the decolorization of dyes would be more attractive using this cheaper enzyme under optimum process conditions. Several research works have been reported on the decolorization of various textile dyes with lignocellulolytic enzymes [1,3,4]. But there is no research to our knowledge which encompasses the combined effects of the parameters, i.e. pH,

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temperature, concentration of hydrogen peroxide (H_2O_2) and dyes, and enzyme dosage on the optimum decolorization processes of the dyes.

The optimization by the one-factor-at-a-time (OFAT) method and statistical approach was conducted to determine the optimal conditions for removal of methylene blue (MB) by crude lignin peroxidase enzyme which would be useful to test the effectiveness of the enzyme for industrial applications. Response surface methodology (RSM) is an efficient experimental strategy to determine optimal conditions for a multivariable system rather than optimizing by the conventional method which involves changing one independent variable while keeping the other factors constant. These conventional methods are time-consuming and incapable of detecting the true optimum, especially to the absence of the interactions among the factors [17]. The optimization of operating conditions for the decolorization of MB dye using crude LiP enzyme obtained from the fermentation of STP sludge was determined to evaluate the OFAT and statistical approach.

2. Materials and methods

2.1. Microbial strain and its inoculum preparation

The white-rot fungus *P. chrysosporium* was used for the production of LiP enzyme utilizing STP sludge as a major substrate. The strain *P. chrysosporium* was screened for its potential for higher enzyme production compared to other strains such as *Aspergillus* spp. [18]. The culture of *P. chrysosporium* was grown on the potato dextrose agar (PDA) plate and incubated at 37 °C for mycelial culture production for 5 days of fermentation.

Four plates (petri dishes) of fungal culture for 5 days were washed successively with 100 ml sterile distilled water. With water addition into the plate, the surface of agar was gently rubbed with a sterilized glass rod and poured into a 250-ml sterilized Erlenmeyer flask [19]. The concentration of inoculum as mycelial biomass content determined was 400 mg/L and the flask was kept in the chiller until further use.

2.2. Fermentation media for lignin peroxidase production

Sewage treatment plant sludge with 1% (w/w) of total suspended solids (TSSs) was used as a major substrate and wheat flour (3%, w/w) was used as supplementary nutrients for initial growth [14,18]. No other macro- or micro-nutrients were added to the major substrate. The pH of the fermentation broth was adjusted to pH 4.0 before being sterilized at 121 °C for 30 min.

2.3. Enzyme production in stirred tank bioreactor

The lignin peroxidase production by *P. chrysosporium* was carried out in a 2-L Biostat® B2 (Sartorius BBI Systems GmbH) fermenter with a working volume of 1.5 L. The optimized fermentation medium and process conditions were used from the previous study [14,18]. The sample was sterilized at 121 °C for 30 min and inoculated with 3% of inoculums. The fermentation was carried out at 30 °C for 5 days with the initial pH of 4. The impeller speed was set to 200 rpm and air was sparged (aeration rate) into the medium at 2.0 vvm. Lignin peroxidase activity was determined spectrophotometrically according to Tien and Kirk [20]. The reaction mixture contained 10 mM veratryl alcohol diluted with 1.5 ml distilled water, 50 μl enzyme sample, 0.25 M sodium tartrate buffer (pH 2.5). The reaction was starting by adding 5 mM H_2O_2. One unit (U) was defined as 1 μmol of veratryl alcohol oxidized in 1 min, and the activity was reported as U/L.

2.4. Selection of a dye for effective decolorization by lignin peroxidase enzyme

Methylene blue, malachite green and methyl orange dyes were first tested to evaluate the maximum decolorization by the LiP enzyme. The effect of incubation time was carried out to determine the equilibrium time of dye decolorization. The general reaction mixtures (4 ml) contained 2 ml of individual dye (methylene blue, malachite green or methyl orange), 1 ml of buffer, 200 μl of LiP enzyme and 800 μl of H_2O_2 solution noted as the 'sample' in this experiment. The reaction was started by the addition of the enzyme and H_2O_2 solution. After each period of incubation, the sample mixture was immediately cooled in an ice bath and the remaining color was determined using the spectrophotometer. The initial absorbance of dye with buffer and distilled water was used as a reference for dye change and decolorization noted as 0% removal. The effect of the enzyme and H_2O_2 alone in the reaction mixtures was also determined to show the effect of both on the decolorization of dye. The mixture which contained dye, buffer, and H_2O_2 alone was noted as 'without enzyme' and mixture which contained dye, buffer, and enzyme alone was noted as 'without H_2O_2' in this experiment. The effect of the enzyme and H_2O_2 alone was measured separately for each dye in a reaction mixture where enzyme or H_2O_2 was replaced by distilled water. Absorbance at the maximum absorption wavelength according to the individual dyes: 670 nm for methylene blue [4], 615 nm for malachite green [2] and 468 nm for methyl orange [21] was used as a measure of the decolorization ability of the enzyme.

All the reaction mixtures were incubated for 60 min and samples were taken every 10 min. Reaction mixtures which contained 2 ml of 30 mg/L individual dye, 1 ml of 50 mM sodium acetate buffer (pH 5.0), 200 μl of LiP enzyme (0.887 U/ml) and 800 μl of 0.75 mM H_2O_2 solution were incubated in 55 °C based on the LiP stability test conducted earlier [14]. The individual dye that gives the highest decolorization was chosen and its equilibrium time was used for the rest of the experiments to study the effect of temperature, pH, H_2O_2 concentration, dye concentration, LiP activity and veratryl alcohol concentration.

Table 1
Effect of process parameters for decolorization of methylene blue using one-factor at-a-time (OFAT)

Study	Dye concentration (mg/L)	Type of buffer	H_2O_2 concentration (mM)	LiP activity (U/ml)	Temperature (°C)	Veratryl alcohol concentration
Effect of temperature	30	pH 5.0	0.75	0.887	35–55	Nil
Effect of pH	30	pH 5–11	0.75	0.887	55	Nil
Effect of dye	10–50	pH 5.0	0.75	0.887	55	Nil
Effect of LiP activity	30	pH 5.0	0.75	0–0.887	55	Nil
Effect of H_2O_2	30	pH 5.0	0–1.25	0.887	55	Nil
	20	pH 5.0	1.0–5.0	0.487		
Effect of veratryl alcohol	20	pH 5.0	1.25	0.487	55	0–1.5 mM

Nil: Not included.

2.5. Optimization of process parameters for the decolorization of methylene blue dye by LiP enzyme using the one-factor-at-a-time method

The variation of the MB dye concentration, types of buffers: 50 mM glycine/HCl (pH 3); 50 mM sodium acetate/HCl buffer (pH 5); 50 mM Tris/HCl–NaOH buffer (pH 7); 50 mM Tris/NaOH buffer (pH 9); 50 mM glycine/NaOH buffer (pH 11), H_2O_2 concentration, veratryl alcohol concentration, LiP activity and temperature used for each study is shown in Table 1. For the first five experimental studies, the 4 ml general mixtures (sample) were used to observe the effect of temperature, pH, dye concentration, H_2O_2 concentration and LiP activity without adding any veratryl alcohol into the mixtures. The concentration of H_2O_2 solution had very much effect in the decolorizing of dye. The effect of H_2O_2 concentration was once again studied with high concentration of H_2O_2 varying from 1.0 to 5.0 mM at 20 mg/L MB concentration.

To increase the percentage removal of dye, 1 ml of veratryl alcohol solution (0–1.5 mM) was added to 4 ml general mixtures to see whether veratryl alcohol can enhance the activity of LiP in decolorizing the dye. The mixture contents for 'without enzyme' and 'without H_2O_2' also varied as did the 'sample' mixtures according to the parameters studied. The incubation time for all studies in the OFAT method was 60 min.

2.6. Optimization of dye concentration and LiP activity on the decolorization of methylene blue dye using response surface methodology

A RSM with five levels for two factors (MB concentration and LiP activity) was conducted to optimize the decolorization of MB dye and to explore the interaction effects of the factors (variables). Other parameters such as temperature 55 °C, sodium acetate buffer (pH 5.0) and H_2O_2 solution 4.0 mM were kept constant throughout the investigations with the incubation time of 60 min. A total of 13 experiments were generated by the centre composite design (CCD) under RSM with five center points. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Table 2.

2.7. Decolorization of methylene blue dye by LiP enzyme with optimum conditions in static and agitation mode: validation of the model

The validation of the statistical approach through methylene blue removal by LiP enzyme was carried out in two different modes. The static mode was carried out in the water bath and the agitation mode carried out in an incubator shaker. For removal of methylene blue in the static mode, the initial concentration of methylene blue was set at 15 mg/L while other conditions were kept the same: 50 mM sodium acetate buffer (pH 5.0); 4.0 mM H_2O_2 solution and 0.687 U/ml LiP enzyme. The mixture solutions was incubated in 55 °C for 2 h. Samples were withdrawn for the determination of the decolorization at 15, 30, 45, 60, 90 and 120 min of incubation time.

The experiment for the removal of methylene blue in the agitation mode, the sodium acetate buffer was not used but the initial pH of the methylene blue solution was adjusted to pH 5.0 with HCl or NaOH. Total 25 ml solution was shaken in the incubator shaker at speed 150 rpm. The mixtures contained 18.75 ml methylene blue dye (15 mg/L), 6.25 ml H_2O_2 solution (4.0 mM) and 1.25 ml LiP enzyme (0.687 U/ml) incubated at 55 °C for 2 h. Samples were taken at the same interval as in the static mode.

2.8. Statistical analysis

The response variable (decolorization) obtained by the design of experiment was attempted to fit in a quadratic model in order to correlate the response variable with the independent variables during the optimization studies. The second order quadratic coefficients were calculated using the software Minitab Release 14. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included Fisher's *F*-test (for overall model significance), its associated probability $p(F)$, determination coefficient, R^2 which measures the goodness of fit of the regression model. It also includes the Student's *t*-value for the estimated coefficients and the associated probabilities $p(t)$. The 3D response surface and 2D contour plot were presented to evaluate the parameters tested within the surface of response.

3. Results and discussion

3.1. Selection of a dye to be suitable for decolorization using LiP enzyme

The 'sample', 'without enzyme', 'without H_2O_2' reaction mixtures (refer to the methods) for all dyes had an initial concentration 30 mg/L. The decolorization of the dyes was tested every 10 min until 60 min of incubation period. Fig. 1 shows the percentage removal (%) of MB dye by lignin peroxidase in the reaction mixture. In 10 min, the decolorization of MB was found to be about 6.5% by the LiP from its initial concentration. The removal of color increased with the increasing of the incubation period at 55 °C.

The equilibrium time was about 60 min where the percentage removal of dye was almost constant at that time. The maximum color removal was about 14–16% at 40–60 min of incubation time. Decolorization of MB with either LiP enzyme or H_2O_2 alone resulted

Table 2
Experimental and predicted values of methylene blue (MB) percentage removal by central composite design

MB concentrations, X_1 (mg/L)	LiP activity, X_2 (U/ml)	Percentage removal (%) of MB	
		Observed	Predicted
30(+1)	0.587(+1)	55.27	55.81
20(0)	0.487(0)	60.32	60.23
5(−2)	0.487(0)	53.68	59.22
30(+1)	0.387(−1)	50.88	50.65
35(+2)	0.487(0)	49.50	49.50
10(−1)	0.387(−1)	60.82	54.45
20(0)	0.487(0)	60.61	60.23
10(−1)	0.587(+1)	65.52	64.97
20(0)	0.687(+2)	63.39	63.47
20(0)	0.487(0)	59.27	60.23
20(0)	0.287(−2)	47.03	47.79
20(0)	0.487(0)	59.27	60.23
20(0)	0.487(0)	60.19	60.23

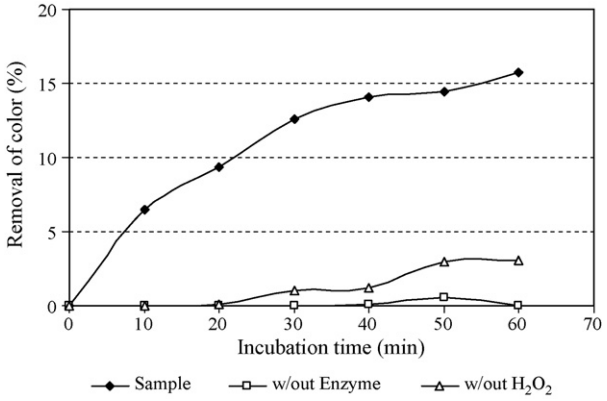

Fig. 1. Effect of incubation time on the removal of methylene blue dye by LiP enzyme. Solution incubated for 60 min in 30 mg/L of methylene blue; 50 mM sodium acetate buffer (pH 5.0); 0.75 mM of H_2O_2 and 0.887 U/ml at 55 °C.

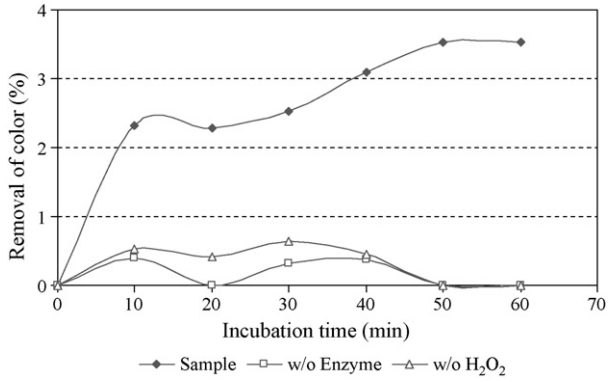

Fig. 3. Effect of incubation time on the removal of methyl orange dye by LiP enzyme. Solution incubated for 60 min in 30 mg/L of methyl orange; 50 mM sodium acetate buffer (pH 5.0); 0.75 mM of H_2O_2 and 0.887 U/ml at 55 °C.

in small percentage removal (2–3% removal with LiP and 0–1% with H_2O_2) compared to the combined effect of them in the reaction mixture (Fig. 1). The maximum removal of MB dye was found by using LiP enzyme with certain amount of such oxidizing agent (H_2O_2) in the reaction mixture which had the influence of the decolorization. The greater removal of dye in solution mixture with the enzyme alone ('without H_2O_2') compared to solution mixture with H_2O_2 alone ('without enzyme') verify that the removal of dye may be due to the enzyme reaction and H_2O_2 may act as an accelerator of enzyme activity [3]. The mechanisms of the decolorization of dyes using the combined effect of theses peroxidases were studied by several authors [1,7,22]. They reported that hamoenzymes especially LiP and MnP use hydrogen peroxidase or other peroxidases as electron acceptors to catalyze the oxidation of a wide range of substrates.

For the mixture solution which contained malachite green (MG) dye, the reaction was begun by adding 0.8 ml H_2O_2 solution which was incubated at 55 °C for 1 h. The maximum color removal was only 8% in 60 min of incubation time (Fig. 2). The result (color removal rate) observed in the MG was lower than the color removal of MB in reaction mixture which removed about 14% dye within 40 min of incubation time.

Another dye, methyl orange (MO) was tested to evaluate its decolorization by LiP compared with the other two dyes (MB and MG). The percentage removal of MO using LiP enzyme was much lower than the other two dyes. The removal was only 3.5% in 50 min incubation time (Fig. 3). With these results, MB showed the maximum removal rate compared to MG and MO thus MB was chosen for further optimization with others parameters. Mohorčič et al. [3] found that the enzyme is able to decolorize some dyes while leaving others almost untouched which the decolorization depends on the chemical structures of the dyes.

3.2. Optimization of process parameters for decolorization of methylene blue dye by LiP enzyme using the one-factor-at-a-time method

From the selection of dyes with the effect of incubation time, MB gave a higher percentage of color removal (%) compared to MG and MO. The equilibrium time was 60 min which was applied in the rest of these experiments to observe the effect of temperature, pH, H_2O_2 concentration, MB concentration and LiP activity. The effect of temperature on the decolorization of MB by LiP enzyme was shown in Fig. 4. Maximum color removal of MB was observed at 45 and 55 °C with about 12% but temperature 55 °C showed a slightly higher percentage color removal. After 65 °C, the percentage removal decreased to 8% and the same goes for the reaction mixture incubated at 35 °C.

Fig. 5 shows the effect of pH on the removal of MB dye. The MB was decolorized by LiP enzyme at pH 5.0 with about 14%. After pH 7.0, the percentage of color removal decreased to 2% indicating that the enzyme may not work well under alkaline condition in removing the MB dye. Young and Yu [1] agreed that most of the dyes need acidic conditions (pH 3.5–5). The results from Figs. 4 and 5

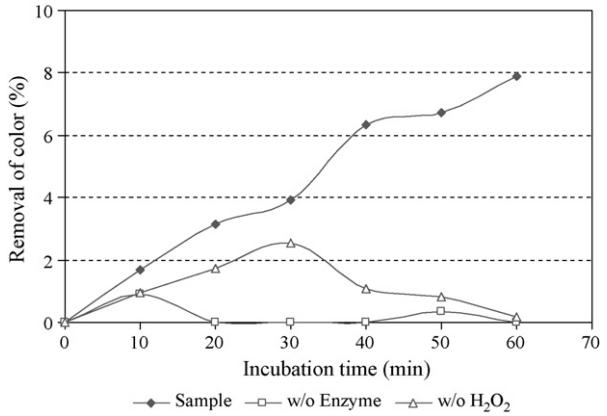

Fig. 2. Effect of incubation time on the removal of malachite green dye by LiP enzyme. Solution incubated for 60 min in 30 mg/L of malachite green; 50 mM sodium acetate buffer (pH 5.0); 0.75 mM of H_2O_2 and 0.887 U/ml at 55 °C.

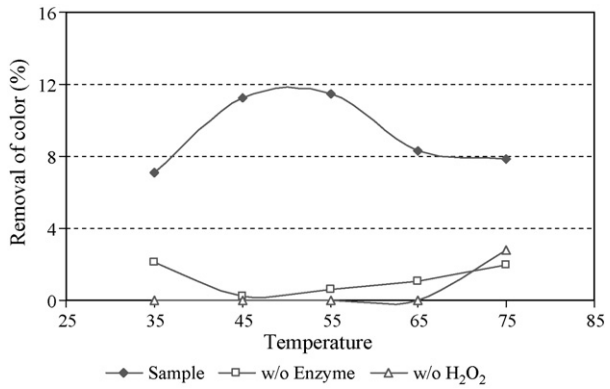

Fig. 4. Effect of temperature on the decolorization of methylene blue. Temperature varying from 35 to 75 °C in 30 mg/L of methylene blue; 50 mM sodium acetate buffer (pH 5.0); 0.75 mM of H_2O_2 and 0.887 U/ml of LiP.

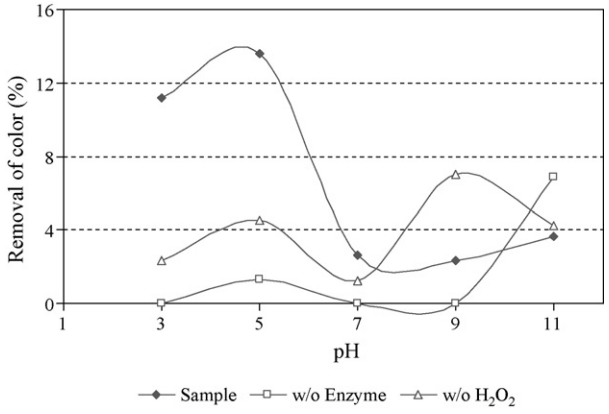

Fig. 5. Effect of pH on the decolorization of methylene blue. pH varying from 3 to 11 in 30 mg/L of methylene blue; 0.75 mM of H_2O_2 and 0.887 U/ml of LiP; at 55 °C.

also confirmed the stability test done earlier that the LiP enzyme is favorable at temperature 55 °C and at pH 5.0 [14].

For the effect of MB concentration on its removal, a low concentration of methylene blue showed higher percentage of color removal compared to a higher concentration of MB (Fig. 6). At 20 mg/L of MB concentration, the percentage removal was high with about 28%, followed by the concentration 10 and 30 mg/L with about 23% and 12%, respectively. The same observation found by Young and Yu [1] that the dye concentration had a negative effect on the decolorization rate. A general tendency is that high dye concentrations will cause a slower decolorization rate. High dye concentration implies less average attacks of LiP to each dye molecule, and hence a slower color removal rate [1].

Fig. 7 shows the effect of enzyme activity on the removal of MB dye. Low activity of LiP enzyme (0.287 U/ml) gave lower dye removal (about 17%) but as the activity increased to 0.487 U/ml the decolorization increased to 37% and the removal decreased again when the LiP activity increased. The result indicated that the removal of MB dye may not be favorable with the high activity of lignin peroxidase enzyme. However, these are the important parameters to evaluate its feasibility to be used in the industrial applications that need to be studied severely in statistical optimization to observe the interaction of LiP activity with other parameters.

The effect of increasing concentration of H_2O_2 (0–1.25 mM) on the decolorization of MB by LiP enzyme was shown in Fig. 8. The percentage removal of MB was continuously increased with increasing concentrations of H_2O_2. The removal was maximum (about 22%) in the presence of 1.25 mM H_2O_2. Fig. 8 also clearly shows the color

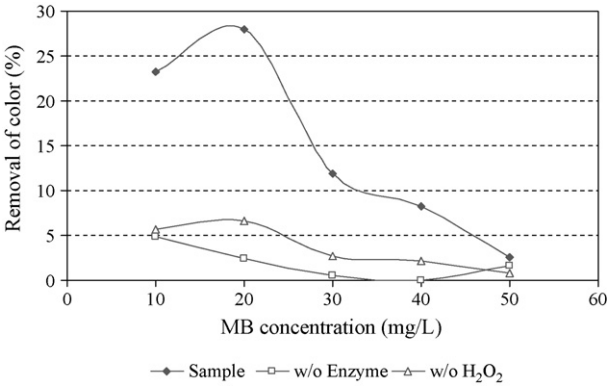

Fig. 6. Effect of initial concentration of the methylene blue on decolorization. Concentration varying from 10 to 50 mg/L in 50 mM sodium acetate buffer (pH 5.0); 0.75 mM of H_2O_2 and 0.887 U/ml of LiP at 55 °C.

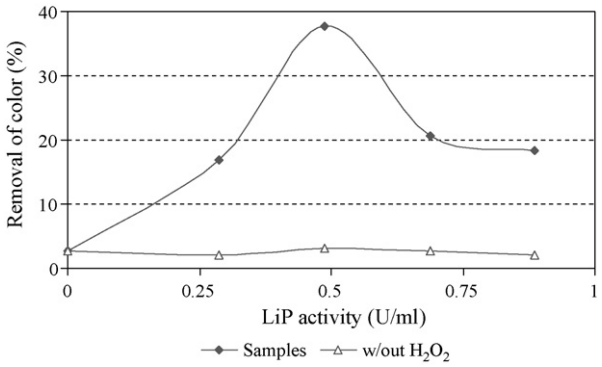

Fig. 7. Effect of LiP activity on the decolorization of methylene blue. LiP activity varying from 0 to 0.887 U/ml in 30 mg/L of methylene blue; 50 mM sodium acetate buffer (pH 5.0) and 0.75 mM of H_2O_2 at 55 °C.

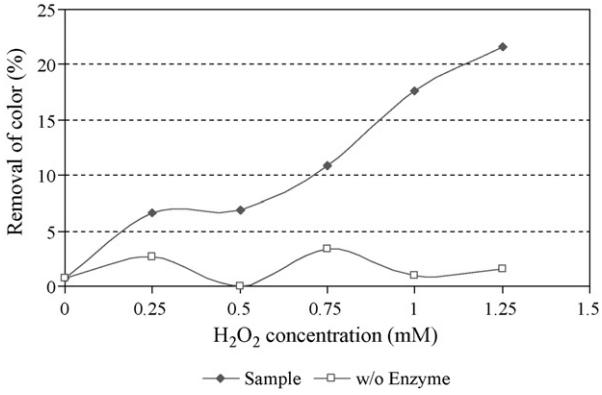

Fig. 8. Effect of H_2O_2 on the decolorization of methylene blue by LiP enzyme. Concentration of H_2O_2 varying from 0 to 1.25 mM in methylene blue dye (30 mg/L), 50 mM sodium acetate buffer (pH 5.0) and 0.487 U/ml of LiP; at 55 °C.

removal of MB in the presence of H_2O_2 alone which was lower even though increasing the concentration of H_2O_2. The results shown in Fig. 8 indicate that the percentage removal of color still increased at H_2O_2 concentration of 1.25 mM. Thus, the effect of higher concentration of H_2O_2 was studied to find the optimum concentration of H_2O_2 for the removal of MB dye. The effect of increasing the concentration of H_2O_2 (1–5 mM) on the removal of methylene blue dye by LiP enzyme is shown in Fig. 9 with an initial concentration 20 mg/L based on the optimum concentration obtained. The color removal of MB was continuously increased with the increasing of H_2O_2 concentrations. The decolorization was maximum in the presence of 4 mM H_2O_2 and it remained almost unchanged to 5 mM H_2O_2. Fur-

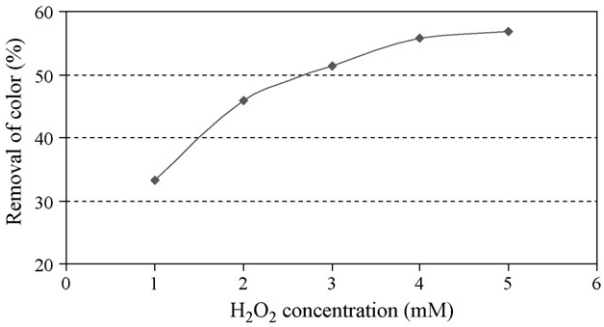

Fig. 9. Effect of H_2O_2 on the decolorization of methylene blue by LiP enzyme. Concentration of H_2O_2 varying from 1 to 5 mM in methylene blue dye (20 mg/L), 50 mM sodium acetate buffer (pH 5.0) and 0.487 U/ml of LiP; at 55 °C in 45 min.

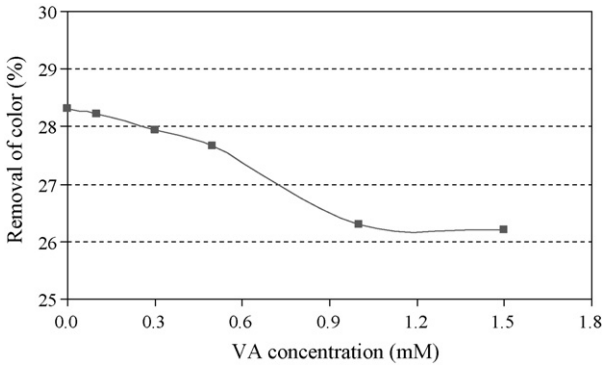

Fig. 10. Effect of veratryl alcohol (VA) on the decolorization of methylene blue. Concentration of veratryl alcohol varying from 0 to 1.5 mM in 20 mg/L of methylene blue; 50 mM sodium acetate buffer (pH 5.0); 1.25 mM of H_2O_2; 0.487 U/ml of LiP; at 55 °C.

ther studies on H_2O_2 concentration may find the decline of the decolorization of MB. At this moment, 4 mM H_2O_2 concentration was considered as the optimum concentration in removing the MB dye at about 55% from its initial concentration. Young and Yu [1] reported that two types of H_2O_2 effect were observed in that some dyes such as Acid orange-74 (AO-74) and Reactive blue-15 (RB-15) could be degraded under high H_2O_2 concentrations. However, other dyes such as Acid violet-7 (AV-7) and Acid blue-25 (AB-25) had an optimum in low H_2O_2 concentrations depending on the dye structures.

The influence of veratryl alcohol on the decolorization of MB was investigated within the ranges 0–1.5 mM. Parameters used in this experiment were based on the results obtained before where all the optimum conditions were applied. The mixture was then incubated for 30 min. Harvey et al. [23] proposed that veratryl alcohol acts as a redox mediator between oxidized LiP and substrates such as lignin and dyes. By this mechanism, veratryl alcohol is first oxidized by a single electron to form the cation radical then oxidizes other substrates. This mechanism was proposed based on the observation that some chemicals which were not oxidized by LiP could be oxidizing after veratryl alcohol was added, a phenomenon observed in dye decolorization of their study.

However, if veratryl alcohol just served as a redox mediator between LiP cations and dye molecules, the decolorization of the dye would not depend on the veratryl alcohol as shown in Fig. 10. Without an addition of veratryl alcohol the removal was at about 28–29% similar with the addition of 0.1 mM of veratryl alcohol in the mixtures and the rate of removal decreased with the increasing of the veratryl alcohol concentration. In contrast, Young and Yu [1] showed that veratryl alcohol played an important role in the decolorization of several dyes: Reactive blue-15 (RB-15); Acid violet-7 (AV-7); Acid green-27 (AG-27) and Acid orange-74 (AO-74). Otherwise, the decolorization of these dyes was less than 10%. The enhancement of dye decolorization by veratryl alcohol depends on the dye's individual structure. The results obtained in this experiment showed that the addition of veratryl alcohol in the reaction mixture would not be effective in the decolorization of MB dye.

3.3. Optimization of dye concentration and LiP activity on the decolorization of methylene blue using response surface methodology

The optimization using the OFAT method showed that the two main factors affecting the decolorization of MB dye were the initial concentration of the MB dye and LiP activity. Thus, the initial concentration of MB and LiP activity were analyzed in order to determine the optimum conditions to increase the removal rate of MB dye in aqueous solution using CCD. In the optimization study, it was determined that the levels of the factors were from 5 to 30 mg/L and 0.287 to 0.687 U/ml for the initial concentration of MB and LiP activity, respectively. Other parameters such as pH 5.0 (in 50 mM sodium acetate buffer) and H_2O_2 concentration 4 mM were kept constant throughout the studies. The mixture content was incubated for 60 min in water bath at temperature 55 °C.

The results obtained were then analyzed by ANOVA, which gave the regression equation as follows:

$$Y_{\%\text{removal}} = -5.48 + 1.39X_1 + 181X_2 - 0.0261X_1^2 - 115X_2^2 - 1.34X_1X_2 \quad (1)$$

where $Y_{\%\text{removal}}$ is the removal percentage of MB dye, X_1 is MB initial concentration and X_2 is the LiP enzyme activity. The predicted response (% removal) obtained by the Eq. (1) and experimental results are presented in Table 2.

For testing the goodness of fit of the regression equation, the determination coefficient, R^2 was evaluated. The R^2 value (Table 3) implied that the sample variation of 98.2% for MB removal was attributed to the independent variables: dye concentration and LiP activity. The value of the adjusted determination coefficient (adj R^2) was also very high to indicate a high significance of the model (0.97). The corresponding ANOVA is presented in Table 3. The *F*-value is a measure of variation of the data about the mean. Generally, the calculated *F*-value should be several times greater than the tabulated value if the model is a good prediction of the experimental results and the estimated factor effects are real [24]. Here, the high *F*-value and a very low probability ($p > F = 0.000$) shows that the present model is in good prediction of the experimental results.

The *t*-distribution and the corresponding *p*-values of each coefficient are listed in Table 4. The *p*-value serves as a tool for checking the significance of each of the coefficients. The pattern of interactions between the variables is indicated by these coefficients. The variables with low probability levels contribute to the model, whereas the others can be neglected and eliminated from the model [24]. The *p*-values suggest that the coefficients for all linear and quadratic effects of MB concentration and LiP activity are highly significance. With the *p*-values less than 0.01, the null hypothesis, H_0 can be rejected at a 1% significance level. Meanwhile, since the interactive effect of MB concentration and LiP enzyme (X_1X_2) had a value 0.025, the H_0 can be rejected at a 5% significance level.

The 2D contour plot, the graphical representation of the regression equation, is presented in Fig. 11. The main goal of response surface is to efficiently hunt for the optimum values of the variables so that the response is maximized [24]. The contour plots are not perfectly elliptical and the *p*-value (0.025) indicates that there are

Table 3
Analysis of variance (ANOVA) for the quadratic model

Source	Degree of freedom	Sum of squares	Mean squares	*F*-value	$p > F$
Regression	5	348.915	69.783	77.39	0.000
Residual error	7	6.312	0.902		
Total	12	355.227			

R^2: 0.982; R^2 (adj): 0.97.

Table 4
Estimation of the least-squares fit and parameters (significance of regression coefficient)

Predictor	Coefficient	Standard error coefficient	*t*-Value	*p*-Value
Constant	−5.481	7.178	−0.76	0.470
MB concentration (mg/L), X_1	1.3912	0.2748	5.06	0.001[a]
LiP activity (U/ml), X_2	180.51	22.05	8.18	0.000[a]
${X_1}^2$	−0.026127	0.003363	−7.77	0.000[a]
${X_2}^2$	−114.55	19.72	−5.81	0.001[a]
X_1X_2	−1.3442	0.4748	−2.83	0.025[b]

[a] $p < 0.01$.
[b] $p < 0.05$.

fewer interactions between the independent variable corresponding to the response surface. From the quadratic model, ANOVA, *p*-values, *t*-test, response surface and contour plot, the optimum initial concentration of MB and LiP activity can be predicted as approximately 15 mg/L and 0.687 U/ml, respectively.

3.4. Decolorization of methylene blue dye by LiP enzyme with optimum conditions in static and agitation mode: validation of the model equation

In order to determine the accuracy of the model, a set of experiment was performed by selecting an initial concentration of methylene blue: 15 mg/L and incubation period: 120 min. The remaining factors were constant: sodium acetate buffer (pH 5); 4.0 mM H_2O_2 solution and 0.687 U/ml LiP enzyme. Fig. 12 shows the experimental and predicted removal (%) of MB in the reaction mixture with an initial concentration 15 mg/L at 15–120 min of incubation in water bath. The predicted removal was slightly higher than the percentage removal obtained from the experiments for both cases. As the difference was small it can be concluded that the quadratic model obtained was acceptable for the determination of optimum conditions with maximum decolorization of MB within the variables (factors) tested. The maximum removal of MB dye observed reached the equilibrium time within 90 min of incubation time with the removal of about 75% for initial concentration of 15 mg/L.

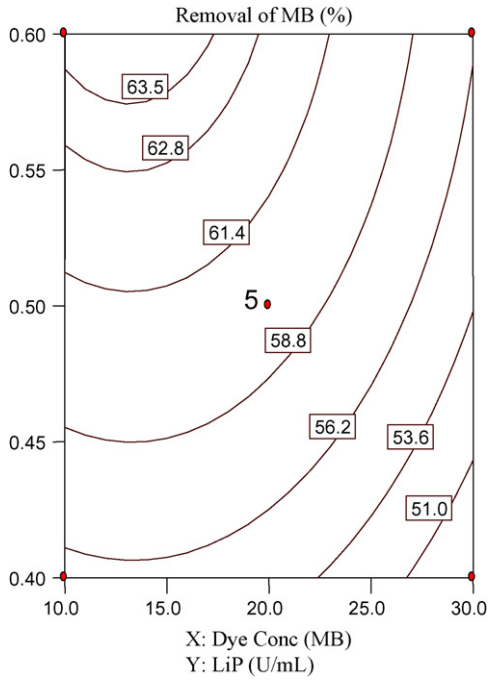

Fig. 11. 2D contour plot for the effect of initial concentration of methylene blue and LiP activity on the removal of MB dye.

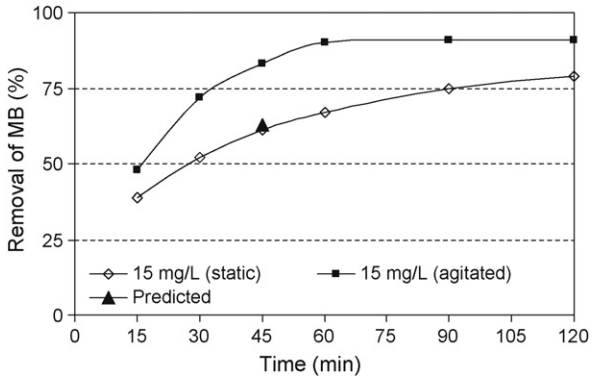

Fig. 12. Removal of methylene blue dye in static and agitate mode. Methylene blue initial concentration 15 mg/L, LiP enzyme (0.687 U/ml) and 4 mM of H_2O_2 in pH 5.0 at 55 °C.

The mixing effect on the decolorization of MB by LiP enzyme was observed to evaluate its feasibility for large-scale applications. Hence, removal of MB dye was carried out in a 100 ml shake flask with 25 ml of MB with 15 mg/L. During 60 min of operation, the maximum decolorization of MB was achieved with about 90% (Fig. 12). This showed that agitation might have effects in removing MB dye from the aqueous solution using LiP enzyme. Agitation gave a higher removal of dye due to uniform mixtures achieved throughout the incubation times so that the electron transfer was faster compared to that just incubated in the static mode. Thus, the decolorization in agitated mode reached the equilibrium time faster than that incubated in the static mode. The equilibrium time reached in the static mode was 90 min for the color removal of 75% while it was 90% at 60 min of operation by LiP. This would suggest the large-scale applications in treating the textile effluent in the agitated mode. Sani et al. [25] suggested that the agitated cultures have often been found more efficient in decolorization of various dyes, compared to the static ones, presumably because of an increased mass and oxygen transfer.

4. Conclusions

The LiP enzyme obtained from the fermentation of STP sludge showed the potential to be applied in the treatment of textile effluents (decolorization of dyes). The results from the selection of dyes such as MB, MG and MO showed that the LiP enzyme was able to remove a higher content of methylene blue (14%) compared to the other two dyes (3–8%). The optimization with the OFAT method determined the operating conditions of the decolorization of MB dye at temperature 55 °C, pH 5.0 (in 50 mM sodium acetate buffer) with H_2O_2 concentration 4.0 mM. The addition of veratryl alcohol to the reaction mixture had no affect on decolorization of dye. The statistical optimization approach showed the higher removal of MB

dye (65%) with the initial concentration of MB 15 mg/L and LiP activity of 0.687 U/ml with 60 min of reaction time in the static mode, while the MB removal was 90% with the same operation time in the agitated mode.

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