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Application of statistical experimental methodology to optimize reactive dye decolourization by commercial laccase

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ABSTRACT

Three-level Box–Behnken factorial design with three factors (pH, temperature and enzyme concentration) combined with response surface methodology (RSM) was applied to optimize the dye degradation of reactive red 239 (RR239), reactive yellow 15 (RY15) and reactive blue 114 (RB114) dyes by commercial laccase. Mathematical models were developed for each dye showing the effect of each factor and their interactions on colour removal. The model predicted for RY15 that a decolourization above 90% (after 24 h) could be obtained when the enzyme concentration, temperature and pH were set at 109.8 U/L, 39.2 °C and 6.6, respectively; whilst for RB114 and RR239 the temperature and enzyme concentration did not affect the decolourization (>90%) in the considered range and optimum pH value was found at 5.5–7.0 and 7.0–7.5, respectively. These predicted values were also experimentally validated. Average final values of responses were in good agreement with calculated values, thus confirming the reliability of the models of RY15, RB114 and RR239 decolourization.

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1. Introduction

Reactive dyes are extensively used in textile industries to colour the cellulosic fibres. These compounds are chemically classified as azo, anthraquinone, formazan, phthalocyanine, oxazine and basic [1,2]. The dyes are first adsorbed on cellulose and then react with the fibres. However, 10–50% (corresponding to a degree of fixation between 50 and 90%) of the initial dye load will be present in the dye bath effluent giving a highly coloured effluent causing serious types of problems in the environment [3].

A number of references related to the applicability of chemical and physical methods such as precipitation, adsorption, filtration, oxidative process, etc. [4,5] for removing reactive dyes is available. Recent studies have shown that fungi or their enzymes are able to decolourize and detoxify industrial dyes [6–8]. However, enzymatic treatments are not still commonly used in the textile industries.

Enzyme methods applied in dye degradation have low energy costs, are easy to control and have low impact on ecosystems. Laccase has been studied in the oxidation of textile dyes. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belongs to the group of oxidative enzymes that catalyse the oxidation of phenolic compounds, polyphenols, and aromatic amines [9]. Studies have shown that the range of substrate specificity of laccases can be extended to non-phenolic substrates by addition of redox mediators [10] such as 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) [11] 1-hydroxybenzotriazole (HBT) [12], polioxometalates [13,14] and violuric acid [15].

The application of experimental design and response surface methodology (RSM) in textile effluent treatment process can result in improved decolourization, reduced process variability, time and overall costs. Additionally the factors that influence the experiments are identified, optimized and possible synergic or antagonistic interactions that may exist between factors can be evaluated [16]. However, only a few recent studies aiming the optimization of textile dye decolourization by enzymatic catalysis are available [12]. The accuracy of the models generated is evaluated by the coefficient of determination R^2. Response surface methodology is a multivariate technique that mathematically fits the experimental domain studied in the theoretical design through a response function. RSM has been extensively studied on biotechnology namely optimization of medium composition [17,18], fermentations [19,20] and food process [21], etc. However, a few reports are presented for dye degradation optimization by enzymatic catalysis with RSM. The most common and efficient design used in response surface modelling is Box–Behnken design. It has three levels per factor, but avoids the corners of the space, and fills in the combinations of centre and extreme levels in which the optimal conditions for an experiment are found [22,23].

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Table 1
Factor levels for a 3^3 Box–Behnken factorial design

Coded factor	Factor	Coded level		
		+1	0	−1
x_1	Temperature (°C)	45	35	25
x_2	pH	7	5	3
x_3	Enzyme (U/L)	192	96	48

In this study a three-level Box–Behnken full factorial design was employed with RSM to maximize the decolourization of three reactive textile dyes, reactive blue 114 (B114), reactive yellow 15 (RY15) and reactive red 239 (RR239) by enzymatic catalysis with a commercial laccase.

2. Materials and methods

2.1. Chemicals and enzyme

2.1.1. Textile dyes

Reactive yellow 15 (Remazol Yellow GR), reactive red 239 (Remazol Brilliant Red 3BS) and reactive blue 114 (Levafix Brilliant Blue E-BRA) were kindly provided by DyStar (Porto, Portugal).

2.1.2. Enzyme

Commercial laccase formulation (DeniLite IIS; 120 U/g and DeniLite Base; 800 U/g) from genetically modified *Aspergillus* was kindly provided by Novozymes. These formulations are used for indigo dye decolourization in denim finishing operations and include a buffer and an enzyme mediator (DeniLite IIS).

2.2. Factorial design

A 3^3 Box–Behnken full factorial design, including three replicates at central point, was carried out in order to study the factors (pH, temperature, enzyme concentration) that influence the decolourization by commercial laccase (Tables 1 and 2). The temperature and pH factors were chosen because it is well known that they are the factors affecting more the reaction. The enzyme concentration factor was chosen because the reactions of dyes degradation are unknown and because of the unknown composition of the commercial laccase formulation (DeniLite IIS) that, besides the enzyme, includes a buffer, an enzyme mediator and surfactants.

This design permits to establish both linear and quadratic models, determining their accuracies by comparing lacks of fit of model predictions to experimental points with experimental error estimated from replicates at the central point. The accuracy and general ability of the polynomial model was evaluated by the coefficient of determination R^2. Tables 1 and 2 give the factors, their values, and the experimental design, respectively. The experimental Box–Behnken design, analysis of variance (ANOVA) and 3D response surface were carried out using the software Statistical v.5.1 (Statsoft Inc.). Eq. (1) describes the regression model of the present system, which includes the interaction terms

$$\hat{Y}\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (1)$$

where $\hat{Y}\hat{y}$ is the predicted response, *i.e.* the colour removal; x_1, x_2 and x_3 are the coded levels of the independent factors temperature, pH and enzyme concentration. The regression coefficients are: β_0 the intercept term; β_1, β_2 and β_3 the coefficients for linear effects;

Table 2
Comparison between experimental data and predicted values for RB114, RY15 and RR239 decolourization

Runs	Factors			Dye degradation (%)					
	x_1 (°C)	x_2	x_3 (U/L)	RB114		RY15		RR239	
				Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
1	25	3	48	ND[a]	1.0	3.7	ND	ND	0.4
2	25	3	96	ND	ND	5.8	5.2	ND	ND
3	25	3	192	0.4	ND	ND	0.4	ND	ND
4	25	5	48	87.3	86.9	63.7	70.4	2.0	1.5
5	25	5	96	85.8	84.6	76.0	74.2	ND	0.9
6	25	5	192	75.8	80.8	62.3	66.4	ND	ND
7	25	7	48	94.4	95.3	88.7	89.3	95.8	96.4
8	25	7	96	92.4	91.9	94.3	91.6	97.3	95.6
9	25	7	192	88.4	85.8	83.6	80.7	93.5	94.3
10	35	3	48	ND	0.6	5.2	8.0	ND	ND
11	35	3	96	ND	ND	10.7	14.4	ND	ND
12	35	3	192	0.8	ND	15.9	11.6	ND	ND
13	35	5	48	89.5	86.9	79.3	77.2	1.8	0.8
14	35	5	96	84.7	84.9	83.5	82.0	ND	0.4
15	35	5	192	79.0	81.6	71.9	76.2	ND	ND
16	35	7	48	95.2	95.7	95.1	94.6	94.7	95.3
17	35	7	96	91.7	92.5	94.0	97.9	95.1	94.7
18	35	7	192	87.9	87.0	88.5	89.0	93.7	93.9
19	45	3	48	ND	1.5	ND	8.8	ND	ND
20	45	3	96	ND	0.9	12.2	16.2	ND	ND
21	45	3	192	ND	0.5	16.3	15.4	ND	0.3
22	45	5	48	90.5	88.1	78.7	76.5	1.2	0.4
23	45	5	96	88.4	86.4	83.3	82.3	ND	0.3
24	45	5	192	83.9	83.7	78.2	78.5	ND	0.2
25	45	7	48	96.3	97.3	92.6	92.4	94.0	94.7
26	45	7	96	93.2	94.4	94.0	96.7	94.4	94.3
27	45	7	192	89.9	89.5	91.2	89.8	94.4	93.8
28	35	5	96	81.3	84.9	85.1	82.0	ND	0.4
29	35	5	96	87.4	84.9	85.5	82.0	ND	0.4

[a]ND, not detected.

β_{12}, β_{13}, β_{23} the coefficients for interaction effects and β_{11}, β_{22}, β_{33} the coefficients for quadratic effects. The model evaluates the effect of each independent factor on the response.

2.3. Dye decolourization experiments

To study the decolourization of the three textile dyes, solutions with concentration of 50 mg/L of each dye were incubated in 25 mL Erlenmeyer flasks under stirring during 1 day. Dye degradation conditions are presented in Table 1 according to the experimental design (Table 2): laccase concentrations (192, 96 and 48 U/L), temperature (25, 35 and 45 °C) and pH (3, 5 and 7). The pH values of dye solutions were established by preparation of the buffers: 50 mM of citrate/di-sodium hydrogen phosphate for pH 3.0; 50 mM of di-sodium hydrogen phosphate for pH 5.0 and pH 7.0.

2.4. Determination of dye degradation

Dye decolourization by laccase was determined by monitoring the decrease in the absorbance peak at the maximum wavelength (peak) for each dye: reactive yellow 15 (416 nm), reactive red 239 (542 nm) and reactive blue 114 (593 nm) or calculating (by integration of absorbance between 350 and 750 nm) the total area (area) under the plot. The first approach considers that absorbance reduction at maximum wavelength is totally due to dye oxidation, not taking into account the dye fraction eventually converted to other compounds. The second one takes into account the conversion of the dye molecules to other compounds absorbing at different wavelengths and then the ratio of the area under the visible spectrum is always equal or lower than the ratio of the absorbances at the peak. UV–vis spectrophotometer (Thermo, model UV1) was used in all experiments. Decolourization is reported as: % decolourization = $(A_i - A_f)/A_i \times 100$, where A_i is the initial absorbance or total area from the initial spectrum and A_f is the final absorbance or total area from the final spectrum.

3. Results and discussion

In this study a commercial laccase formulation containing a specific mediator (DeniLite IIS) and pure laccase (DeniLite Base) was used for degradation of RB114, RR239 and RY15. Preliminary results had shown that pure laccase did not decolourize the three reactive dyes studied (data not shown), indicating that the presence of mediator is required. Similarly to this study, reports from literature show that laccase alone does not decolourize some types of textile dyes [24,25]. Thus the further experiments for the experimental design were carried out with DeniLite IIS.

3.1. Experimental design

In order to optimize the dye degradation, Box–Behnken full factorial design with three factors (enzyme concentration, pH and temperature) was chosen. The levels of the factors and the results from the 29 experiments for each dye are presented in Tables 1 and 2, respectively. Using the experimental data, the second order polynomial model was fitted to decolourization results (peak) of RR239, RY15 and RB114 and obtained in terms of coded factors:

$$\hat{Y}\hat{y}\ (\text{RY15}) = 58.66 + 4.53x_1 + 40.98x_2 - 0.491x_3 + 1.859x_1^2 + 12.932x_2^2 + 2.59x_3^2 - 1.48x_1x_2 + 1.509x_1x_3 - 2.29x_2x_3 \quad (2)$$

$$\hat{Y}\hat{y}\ (\text{RB114}) = 58.76 + 1.02x_1 + 45.82x_2 - 2.62x_3 - 0.312x_1^2 + 19.35x_2^2 - 0.131x_3^2 + 0.381x_1x_2 + 0.402x_1x_3 - 1.71x_2x_3 \quad (3)$$

$$\hat{Y}\hat{y}\ (\text{RR239}) = 31.7 - 0.218x_1 + 47.35x_2 - 0.442x_3 - 0.095x_1^2 - 23.45x_2^2 - 0.048\,x_3^2 - 0.317x_1x_2 + 0.308x_1x_3 - 0.300x_2x_3 \quad (4)$$

The dye decolourization results predicted by the models presented above, at each experimental point, are presented in Table 2.

The statistical significance of the polynomial model for the experimental responses (Table 2) was evaluated by ANOVA. Accord-

Table 4
Comparison between dye decolourization (%) based on the absorbance reduction at maximum wavelength and on the area under all dye spectrum range

Dye	Decolourization (%)	
	Peak	Area
RR239	96	70
RB114	90	25
RY15	93	84

Table 3
Analysis of variance (ANOVA) for the fitted quadratic polynomial models of RB114, RY15 and RR239 decolourization

Source	Sum of squares (SS)			d.f.[a]	Mean square (MS)			*F*-value			*p*-Value		
	RY15	RB114	RR239		RY15	RB114	RR239	RY15	RB114	RR239	RY15	RB114	RR239
(1) x_1 (L[b])	363	18.5	0.84	1	363	18.5	0.84	29.6	3.69	1.73	0.0000	0.0697	0.2037
x_1 (Q[c])	91.9	2.58	0.24	1	91.9	2.58	0.24	7.49	0.51	0.50	0.0131	0.4818	0.4886
(2) x_2 (L)	29.7	37.1	39.7	1	29.7	37.1	39.7	2421	7405	81.8	0.0000	0.0000	0.0000
x_2 (Q)	4447	9965	14.3	1	4447	9965	14,630	362	1986	30.2	0.0000	0.0000	0.0000
(3) x_3 (L)	4.35	123	3.51	1	4.35	123	3.51	0.35	24.6	7.25	0.5588	0.0001	0.0144
x_3 (Q)	171	0.44	0.06	1	171	0.44	0.06	13.9	0.09	0.12	0.0014	0.7707	0.7307
x_1 L by x_2 L	26.5	1.74	1.20	1	26.5	1.74	1.20	2.16	0.35	2.48	0.1584	0.5628	0.1316
x_1 L by x_3 L	28.3	2.02	1.18	1	28.3	2.02	1.18	2.31	0.40	2.44	0.1452	0.5337	0.1347
x_2 L by x_3 L	65.1	36.5	1.12	1	65.1	36.5	1.12	5.31	7.27	2.31	0.0327	0.0143	0.1446
Error	233	95.3	9.21	19	12.3	5.02	0.48						
Total SS	36.6	48.6	55.5	28									

RY15: $R^2 = 0.99363$, adj $R^2 = 0.99061$; RB114: $R^2 = 0.99804$, adj $R^2 = 0.99711$; RR239: $R^2 = 0.99983$, adj $R^2 = 0.99976$.

[a] df, degrees of freedom.

[b] L, linear.

[c] Q, quadratic.

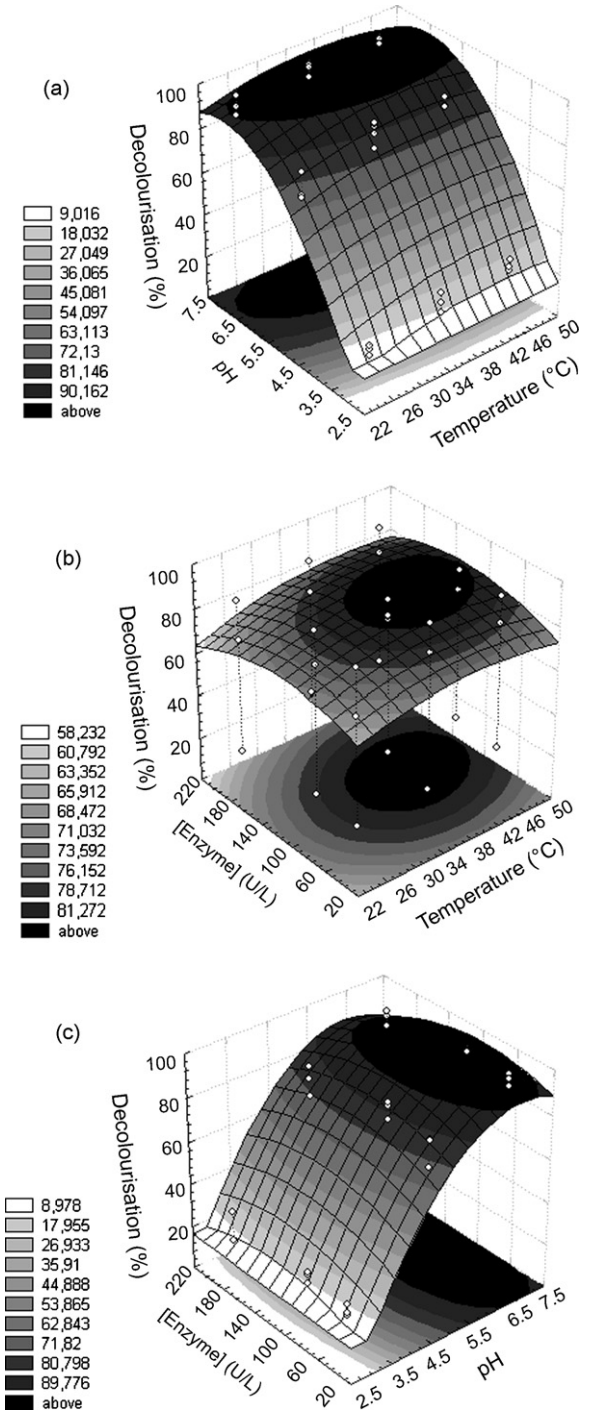

Fig. 1. Response surface plots for decolourization of RY15 as a function of: (a) pH and temperature at 96 U/L; (b) enzyme concentration and temperature at pH 5; (c) pH and enzyme concentration at 35 °C.

ing to the ANOVA results (Table 3), the models present high correlation coefficients (R^2): 0.99363, 0.99983 and 0.99804 for the degradation of RY15, RR239 and RB114, respectively. These results indicate that the accuracy of the polynomial models was good. The regression coefficients and the interaction between each independent factor can be considered statistically significant for *p-values* below 0.05, with 95% of confidence interval. For RY15, the regression coefficients of all the linear and quadratic terms were significant, except for x_3 and for the interactions x_1x_2 and x_1x_3. RB114 presented linear and quadratic coefficients of pH, linear coefficient of enzyme and the interaction x_2x_3 as significant coefficients. For RR239 the significant coefficients were only linear and quadratic terms of pH and linear term of enzyme.

3.1.1. Response surface methodology

Using RSM, the effects of the independent factors (pH, temperature and enzyme concentration) and their interaction on the dyes decolourization are represented, the response can be predicted and the optimum values of decolourization can be determined. The response surface plots (Figs. 1–3) show the decolourization of RY15, RR239 and RB114 as function of two factors, whilst the third was kept at a constant level.

Fig. 1 represents the response surface for RY15 decolourization. Surface plots show the increase on dye degradation with increasing on pH values. Plots 1a and 1c clearly show that the decolourization by commercial laccase was sensitive even to small alterations of the pH. As it can be seen from plots 1a and 1c, the temperature slightly influenced the decolourization. On the other hand the enzyme concentration did not affect the decolourization in the treated range.

The response surface plots of RB114 and RR239 degradation are presented in Figs. 2 and 3, respectively. From the results it can be observed that pH was the only influential factor.

The results presented above showed that pH was the more relevant factor for the decolourization of the three dyes. At acidic conditions (pH <4) a little or no decolourization was observed for all dyes. Similar results were observed in other studies [26,27] with

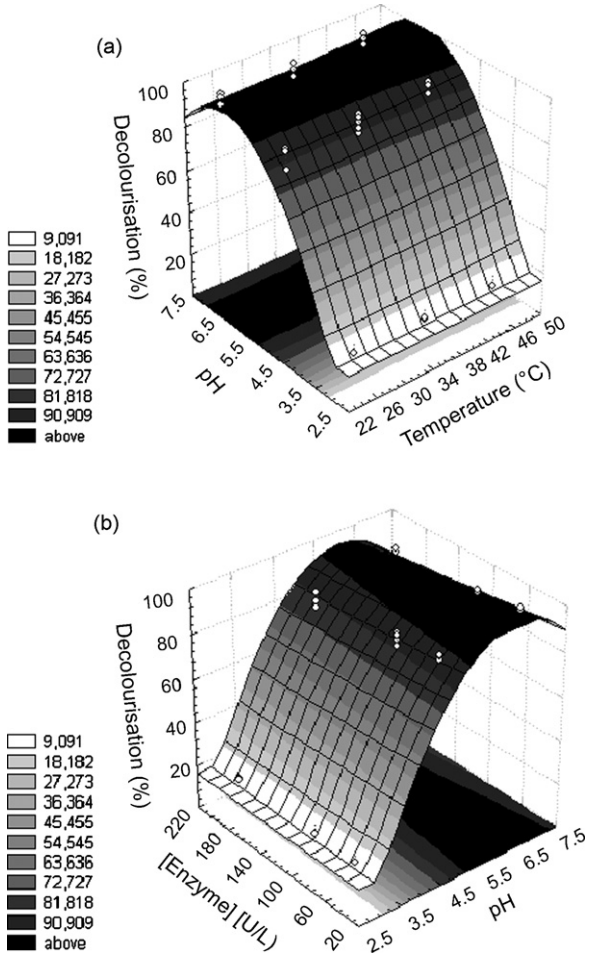

Fig. 2. Response surface plots for decolourization of RB114 as function of: (a) pH and temperature at 96 U/L; (b) pH and enzyme concentration at 35 °C.

dyes or similar substrates. These results suggest that acidic pH values may influence the stability of the enzyme causing denaturation. According to Tavares et al. [19] laccase loses stability at pH of 3.0 whilst for pH of 5.0 no loss of enzyme activity is observed.

The literature studies show that laccase-catalyzed dye oxidation is affected by the temperature [27,28]. From the results presented in Figs. 1–3 it can be concluded that the temperature did not seem to play an important role on decolourization of RY15, RR239 and RB114 in the range 25–45 °C. Temperatures above 45 °C were not studied since previous results (data not presented) have shown that the increase from 40 to 50 °C did not promote an increase on dye decolourization. As temperature, the laccase concentration did not influence the dye degradation for the three dyes.

3.2. Model validation

The adequacy of the proposed model (Eq. (1)) for dye decolourization by commercial laccase was evaluated using the optimum conditions for each dye. For this purpose new experiments were conducted in triplicate to verify the optimum conditions. According to the models the optimum conditions for the RY15 dye degradation were: temperature 39.2 °C, enzyme concentration 109.8 U/L and pH 6.6, predicting above 90% decolourization. For RR239 and RB114 it was not possible to calculate the optimum of temperature and enzyme concentration as these factors did not affect the response (Figs. 2 and 3). In these cases, according to the response surface, it is possible to determine an optimal region for dye decolourization: pH of 5.5–7.0 for RB114 and pH of 7.0–7.5 for RR239. At these conditions, the highest RB114 and RR239 decolourization (>90%) was obtained. Under the optimal conditions, a decolourization of 96%, 90% and 93% was experimentally achieved for RR239, RB114 and RY15, respectively, which is in good agreement with the decolourization predicted by the model. For the entire range of the tested factors, the experimental results are very close to the predicted values obtained from the models.

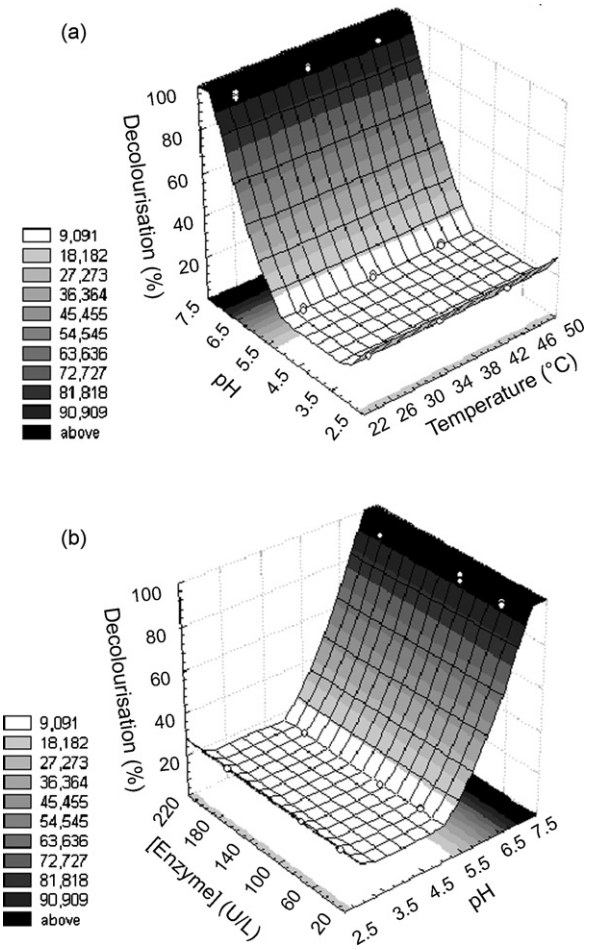

Fig. 3. Response surface plots for decolourization of RR239 as function of: (a) pH and temperature at 96 U/L enzyme concentration; (b) pH and enzyme concentration at 35 °C.

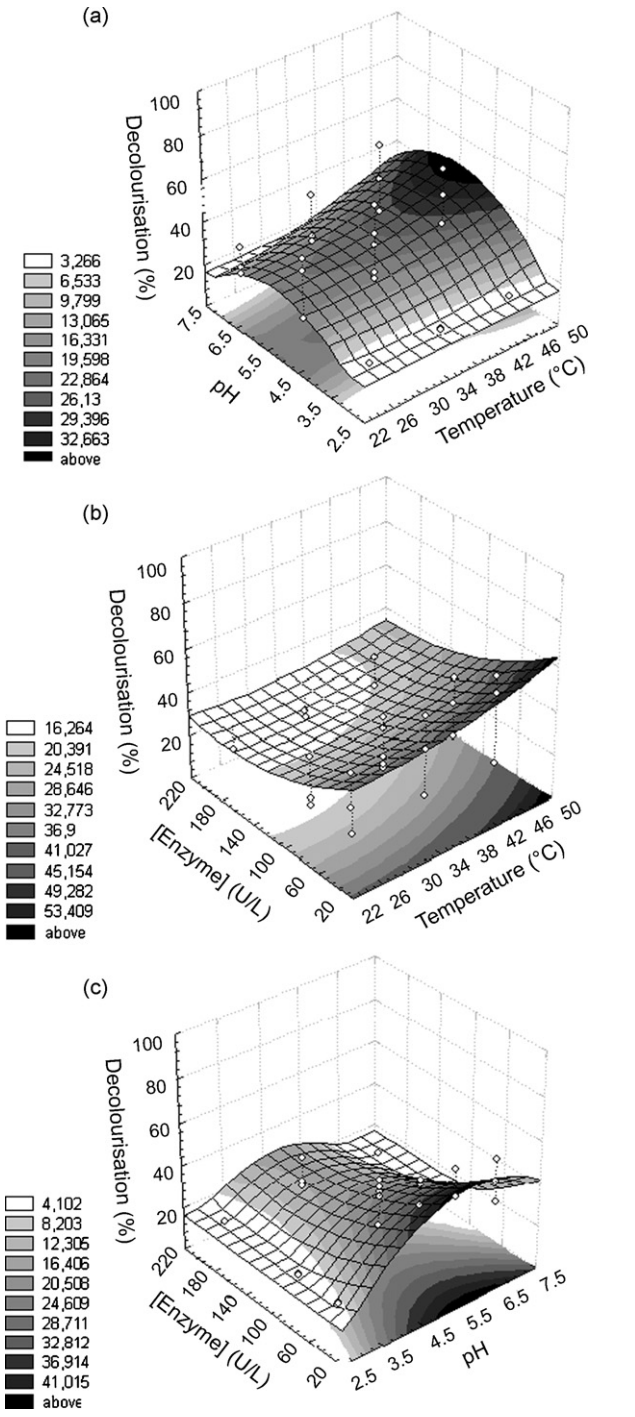

Fig. 4. Response surface plots for decolourization of RB114 based on the area under all dye spectrum range as function of: (a) pH and temperature at 96 U/L enzyme concentration; (b) enzyme concentration and temperature at pH 5; (c) pH and enzyme concentration at 35 °C.

3.3. Decolourization at the optimum conditions

For a better performance in dye wastewater treatment, no absorbance in the visible spectrum must be detected after decolourization. Thus, the decolourization at the optimum conditions was followed by measuring the absorbance reduction over all the visible spectrum. The results presented in Table 4 show a lower decolourization based on the entire visible spectrum when compared with the absorbance reduction at the maximum wavelength, particularly for RB114 which presents a difference of 65% in the decolourization. This result can be explained by the new peak of absorbance that emerged between 400 and 420 nm during the degradation of RB114, corresponding to a light orange colour formed probably due to the coloured intermediates. Sugano et al. [29] showed the generation of light red-brown reaction products during the decolourization of reactive black 5 by versatile peroxidase. RR239 and RY15 did not show any colour change and no additional peaks of absorbance were observed in the entire visible spectrum (data not shown). The application of RSM to RB114 degradation results based on the area under all dye spectrum range are presented in Fig. 4. Although the area based decolourization is lower when compared to the peak absorbance reduction, which is the classical method to calculate decolourization, pH continues to be the factor that affects more and presents practically the same optimal region (pH 5.0–6.5). However, in this case the temperature and enzyme concentration also present a small influence in RB114 decolourization.

4. Conclusions

Box–Behnken statistical experimental design and response surface methodology are important tools to optimize the conditions for textile dye wastewater treatment as well as to reduce the number of experiments and provide useful information about the effect of the factors and the possible interactions. The decolourization of RY15, RR239 and RB114 by enzymatic catalysis using commercial laccase was optimized leading to dye degradation above 90%. pH proved to be the principal factor that affects the decolourization of the three reactive dyes whilst temperature and enzyme concentration presented a low or none effect on dye degradation. The decolourization based on all the visible spectrum showed to be lower when compared with the degradation at the maximum wavelength for all dyes studied. In the case of RB114 this difference is high (65%) due to the formation of another peak, corresponding to a formed light orange colour during the degradation.

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