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Decolourization of the reconstituted dye bath effluent by commercial laccase treatment: Optimization through response surface methodology

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ABSTRACT

This paper aims to study the effect of temperature, pH and enzyme concentration on decolourization of separately two reactive textile dyes (Black Novacron R and Blue Bezaktiv S-GLD 150) used in reconstituted dye bath effluent (textile dye and auxiliary components) and in aqueous dye solutions (dye dissolved in deionised water) by a commercial laccase formulation (DeniLite® IIS). The central composite design (CCD) matrix and response surface methodology (RSM) have been applied to design experiments for the evaluation of the interactive effects of the three most important operating variables: temperature '*T*' (25–45 °C), pH (3.0–7.0), and enzyme concentration 'EC' (80–240 U/L) on the enzymatic decolourization of the different synthetic dyes solutions at initial dye concentration of 40 mg/L. The RSM indicated that the optimum parameter values were respectively for the reconstituted Black Novacron R and the Blue Bezaktiv S-GLD 150 effluents: $T = 43$ °C and 41.44 °C, pH 6 and 6.29, EC = 222 and 226.43 U/L. The maximum colour removal was about 98.9% at 593 nm and 79.9% at 400 nm for reconstituted Black Novacron R effluent and about 98.9% at 620 nm for reconstituted Blue Bezaktiv S-GLD 150 effluent. For aqueous dye solutions, RSM has shown that colour removal obtained were quite similar. However, the optimum parameters were different. Hence, enzyme concentration depends on the effluent component.

1. Introduction

The textile industry plays an important role in the world economy, but at the same time, it consumes a large quantity of water (up to 150 l of water to dye 1 kg of cotton) and generates a huge amount of wastewaters [1,2].

The wastewater from textile processing contains processing bath residues from preparation, dyeing, finishing, slashing and other operations. These residues can cause pollution if they are not properly treated before discharge to the environment [3,4].

Dyes having a synthetic origin with complex aromatic molecular structure are classified as follows: anionic-direct, acid and reactive dyes, cationic-basic dyes, and non-ionic-disperse dyes [5].

Reactive dyes are typically azo-based chromophores combined with different types of reactive groups, e.g., vinyl sulfone, chlorotriazine, trichloropyrimidine and difluorochloropyrimidine [6]. They have poor fixation rates and hence may be hard to remove from wastewaters because of their low biodegradability and their weak absorption into activated sludge [7].

Therefore, innovative treatment technologies need to be investigated. Decolourization of dye wastewater by the action of the enzyme laccase is the subject of many studies [8–10].

Laccase-based decolourization treatments are potentially advantageous to bioremediation technologies since the enzyme is produced in larger amounts. Laccase (p-diphenol oxidase, EC1.10.3.2) [11] catalyzes the oxidation of phenolic compounds and aromatic amines and accepts a broad range of substrates [12,13]. The number of substrates can further be extended by using laccase in combination with mediators [9]. Laccase requires only molecular oxygen as a co-substrate which is concomitantly reduced to water. This makes it very interesting for use in enzyme-based bioreactors [14,15].

The present study aims to determine the ability of commercial laccase in the decolourization of two reactive dyes. However, although a large number of structurally diverse dyes have been successfully oxidized by laccases, decolourizations take place at different rates and to different extents and many dyes are not degraded at all [14].

Generally, temperature, pH and initial enzyme concentration are the most important parameters that significantly influence the enzymatic degradation process. Since the conventional method of optimization, "one factor at a time" approach is laborious, time

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consuming and incomplete, response surface methodology (RSM) using CCD (as factorial experimental design) was applied to model the decolourization process, to identify possible interactions and to determine the optimum operational conditions.

RSM is an advanced tool, commonly applied nowadays as it involves three factorial designs giving number of input (independent) factors and their corresponding relationship between one or more measured dependent responses [16].

RSM has been extensively studied in biotechnology namely for optimization of medium composition [17,18], fermentations [19,20], catalyzed reaction conditions [21], oxidation [22], production [20] and food processes [23]. However, few reports have been 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 [24,25].

In the present investigation, the maximum dye decolourization ability of laccase was studied adopting a full range of response surface methodology (RSM) using central composite design (CCD) model to analyze the efficiency of the system under different conditions. The regression model provides an excellent explanation of the relationship between the independent variables and the response [26].

2. Materials and methods

2.1. Dyes and chemicals

Reactive dyes represent the dyes which are mostly used in the textile industries. The dyes used in this study are reactive Blue Bezactiv S-GLD 150 and Black Novacron R, procured from SARTEX (a textile manufacturing unit in Ksar Helal (Tunisia)) from BEZEMA AG (Montlingen). Other chemicals (sodium hydroxide, sodium carbonate, cibacel DBC, beavin BPA and sodium chlorate) were added to obtain the same composition of the real dye bath and were procured from SARTEX.

2.2. Simulated dye bath effluents

Two synthetics effluents using the chosen dyes were prepared and used in this study.

Separate reconstituted dye bath effluent which can be defined when auxiliary components and only one reactive dye (Reactive Blue Bezactiv S-GLD 150 or Reactive Black Novacron R) were added to deionised water with the following composition:

- As auxiliary components: 80 mg/L sodium hydroxide, 200 mg/L sodium carbonate, 40 mg/L cibacel DBC (detergent), 40 mg/L beavin BPA and 4 g/L sodium chlorate.
- As dye: 40 mg/L of the chosen dye (Reactive Blue Bezaktiv S-GLD 150 or Reactive Black Novacron R).

Aqueous dye solutions using only one reactive dye (Blue Bezactiv S-GLD 150 or Black Novacron R) with concentration of 40 mg/L dissolved in deionised water (same as the above solution without the auxiliary components).

Prior to use, the synthetics dyes effluents were hydrolysed at 60 °C for 2 h and they were then cooled to room temperature.

2.3. Enzyme used

Commercial laccase formulation (DeniLite® IIS; 120 U/g) produced by submerged fermentation of a genetically modified *Aspergillus oryzae* was provided by Novozymes. Laccase activity is determined spectrophotometrically as described by Niku-Paavola et al. [27] with 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (ABTS) as a substrate. One activity unit (1 U) of laccase was defined as the amount of enzyme oxidizing 1 mol of substrate (ABTS) per min. The activities were expressed in U/L [28].

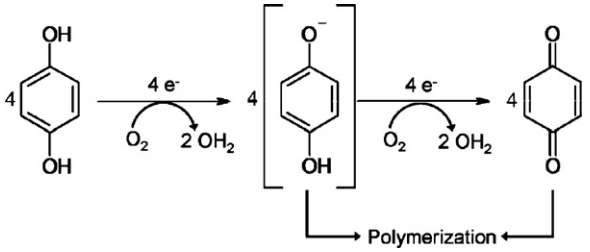

Fig. 1. Schematic representation of laccase reaction (the oxidation of phenolic compounds).

This laccase formulation is usually used for indigo dye decolourization in jean finishing operations and includes a buffer and an enzyme mediator. This enzyme catalyzes the oxidation of phenolic compounds as described in Fig. 1.

2.4. Membrane filtration

Decolourization of the two reconstituted effluent using separately the two reactive dyes was achieved by membrane filtration (ultrafiltration and nanofiltration) using five membranes purchased from Pall Corporation which have molecular weight cut-offs varying from 500 to 5000 Da. These filtrations were made under a pressure of 1 bar by pressurizing with nitrogen on 50 mL of each sample.The dead-end filtration time was variable depending on the membrane cut-off and was between 15 and 30 min.

2.5. Laccase decolourization

Commercial laccase catalysis was conducted in 250 mL Erlenmeyers flasks under slow stirring at 40 rpm during 1 day. 1 g of the commercial laccase formulation (DeniLite® IIS; 120 U/g) was dissolved in 10 mL deionised water and rapidly mixed at 80 rpm for 5 min followed by 10 min of centrifugation at 3500 rpm. Then, supernatant sample was taken for enzymatic decolourization. Reaction medium was about 100 mL and pH values of separate simulated dye solutions (aqueous dye solutions or reconstituted dye bath effluents) were established by adding buffer solution: 50 mM of citrate/di-sodium hydrogen phosphate for pH 3 and 50 mM of di-sodium hydrogen phosphate for pH 5 and pH 7. After 24 h, sample of 5 mL of each solution was taken and dye absorbance was measured at the maximum wavelengths.

2.6. Optimization of biodegradation process using RSM approach

To optimise the enzymatic dye decolourization, a CCD model based on three factors (temperature, pH, and laccase concentration) (Table 1) was used as experimental design model (Table 2). Temperature (25–45 °C), pH (3–7) and laccase concentration (80–240 U/L)

Table 1
Experimental range and levels of independent factors.

Coded factor	Factor	Coded level		
		+1	0	−1
X_1	Temperature (°C)	45	35	25
X_2	pH	7	5	3
X_3	Enzyme concentration (U/L)	240	160	80

Table 2
Experimental results of CCD designed experiments for reconstituted effluent.

Run no.	X_1 (°C)	X_2	X_3 (U/L)	Colour removal (Y) (%)		
				Black Novacron R dye		Blue Bezaktiv S-GLD 150 dye
				λ = 593 nm	λ = 400 nm	λ = 620 nm
1	25	3	160	81.61	32.05	95.68
2	25	3	160	81.61	32.05	95.71
3	45	3	160	84.73	72.90	96.00
4	45	3	160	84.73	72.90	96.07
5	25	7	160	94.60	74.74	96.51
6	25	7	160	94.60	74.60	96.55
7	45	7	160	98.93	79.90	98.90
8	45	7	160	98.93	79.90	98.90
9	25	5	80	89.00	0.00	95.70
10	25	5	80	89.00	0.00	95.70
11	45	5	80	95.00	61.40	97.80
12	45	5	80	95.00	61.50	97.80
3	25	5	240	93.00	0.00	97.81
14	25	5	240	93.00	0.00	97.81
15	45	5	240	97.00	59.20	98.20
16	45	5	240	97.00	59.20	98.20
17	35	3	80	88.00	0.00	95.80
18	35	3	80	88.00	0.00	95.80
19	35	7	80	98.70	79.90	98.04
20	35	7	80	98.70	79.85	98.04
21	35	3	240	89.00	6.00	94.70
22	35	3	240	88.00	5.97	94.70
23	35	7	240	98.90	59.30	98.20
24	35	7	240	98.90	59.35	98.20
25	35	5	160	98.40	59.35	98.12
26	35	5	160	98.40	59.30	98.12
27	35	5	160	98.40	59.35	98.12
28	35	5	160	98.40	59.34	98.12
29	35	5	160	98.40	59.33	98.12

were taken as input variables. As usual, the experiments were performed in random order to avoid systematic error. In addition, three central replicates were also added to the experimental design to calculate pure experimental error. In this study a three-level Box–Behnken full factorial design was employed.

The percentage of dye removal (Y) was taken as the response of the design experiments. A total of 29 experiments were performed. Experimental data obtained from the CCD model experiments can be stated in the form of the following equation:

$$Y = \alpha_0 \sum_{i=1}^{k} \alpha_i X_i + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \alpha_{ij} X_i X_j + \sum_{i=1}^{k} \alpha_{ii} X_i^2 + \varepsilon \quad (1)$$

The results of the experimental design, regression and graphical analysis of the data obtained were analyzed and interpreted using NemrodW version 2000-D statistical software.

2.7. Colorimetric analyses

Absorbance measurements were carried out with a UV–visible spectrophotometer (Shimadzu UV 1650 PC). The initial spectra of the two reconstituted reactive dye bath effluents used in the present study are showed in Fig. 2.

These two spectra indicates that the Reactive Blue Bezaktiv S-GLD 150 dye present a maximum absorbance in the visible spectrum at the wavelength of 620 nm (A_{ai} = 0.557). Whereas, the Reactive Black Novacron R dye present two maximum absorbencies

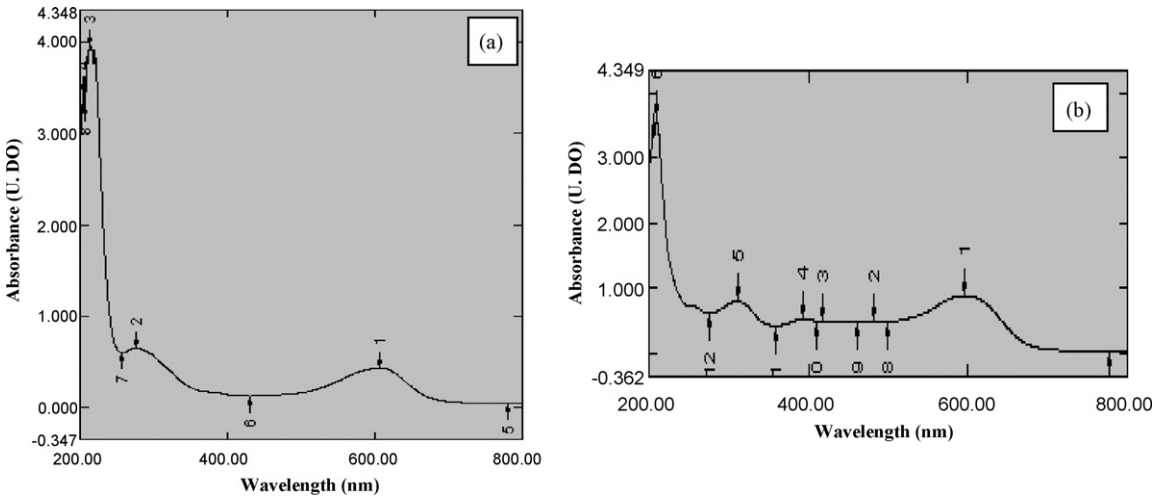

Fig. 2. Absorbance spectra of reconstituted reactive dying effluents using: (a) Blue Bezaktiv S-GLD 150; (b) Black Novacron R at a concentration of 40 mg/L.

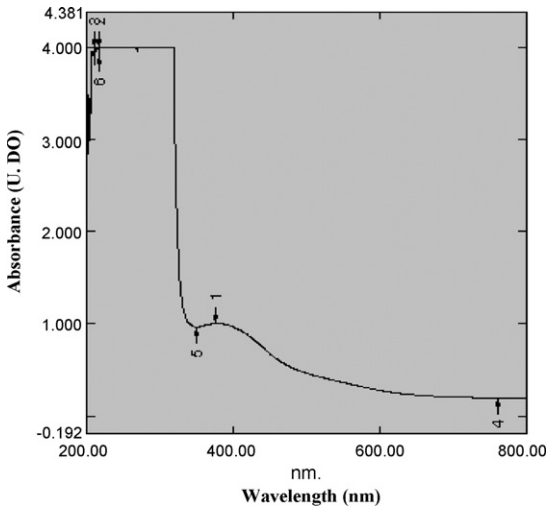

Fig. 3. Absorbance spectra of commercial laccase DeniLite® IIS; 120 U/g.

in the visible spectrum at the wavelengths of 593 nm (A_{bi1} = 0.645) and of 400 nm (A_{bi2} = 0.3).

Decolourization was determined by measuring the optical density of each solution containing 40 mg/L of the appropriate reactive dye (Blue Bezaktiv S-GLD 150 or Black Novacron R) at the absorbance maxima of the respective dyes.

In the other hand, the absorbance spectrum of the commercial laccase used (Fig. 3) indicate that this enzyme has no peak of absorbance in the visible spectrum and so has no interference with the maximum absorbances of the two reactive dyes treated in the visible spectrum.

3. Results and discussion

3.1. Filtration experiments

In the first step decolourization of the two reconstituted dye bath effluents using separately the two reactive dyes at a concentration of 40 mg/L was achieved by filtration using five PALL cell membranes and by enzymatic catalysis using a commercial laccase at a concentration of 160 U/L and at a pH of 6.5 fixed with the use of di-sodium hydrogen phosphate buffer and after incubation during one day under slow stirring at 40 rpm. Results showed

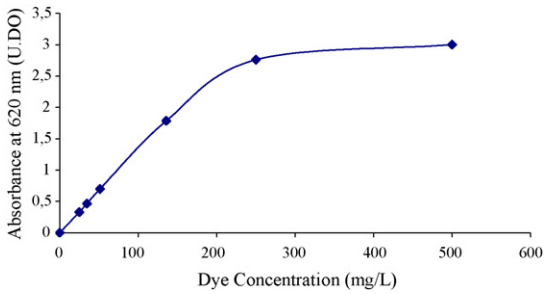

Fig. 4. Variations of absorbance with the concentration of Blue Bezaktiv S-GLD 150 dye in the range of 0–500 mg/L.

that filtration alone is unsatisfactory for colour removal (Table 3). The maximum of decolourization was obtained using a 500 Da cut-off membrane which was 85.5% for the reconstituted effluent with Blue Bezaktiv S-GLD 150 reactive dye and only about 53.7% and 50% for the reconstituted effluent with Black Novacron R reactive dye. Whereas, application of enzymatic treatment using a commercial laccase shows significant colour removal about 94% for the reconstituted Blue Bezaktiv S-GLD 150 reactive dye effluent and about 92% and 73% for the reconstituted Black Novacron R reactive dye effluent (Table 3).

Thus, the enzymatic treatment using a commercial laccase was retained in a further part of our work.

3.2. Choice of the dye concentration

The dye concentration of 40 mg/L was chosen from determination of absorbance of real effluents samples. It was found that the absorbance (U.D.O.) of the real effluent containing the reactive Blue Bezaktiv S-GLD 150 dye varied between 0.092 and 0.416 at 620 nm (Table 4).

The variation of the absorbance of an aqueous dye solution with the Blue Bezaktiv S-GLD 150 concentration dye in the range of 0–500 mg/L shows that for low concentration (in the range of 0–200 mg/L) the absorbance at 620 nm increases with concentration and then becomes independent of concentration at the high values (Fig. 4). From this figure we can plot a linear relationship between absorbance and dye concentration in the range of 0–150 mg/L.

At the same way, for the Black Novacron R in aqueous solution with the same range of concentrations (0–500 mg/L), the varia-

Table 3
Decolourization of reconstituted dying effluents at 40 mg/L of dyes by the two treatment methods: membrane separation and laccase treatment.

Treatment way		Blue Bezaktiv S-GLD 150	Black Novacron R	
	Wavelength (nm)	620	593	400
Membrane separation	Cut off membrane	Colour removal (%)		
	10 kDa	45.7	16.6	10
	5000 Da	56.9	25.92	26.6
	4000 Da	58.8	27.77	30
	1000 Da	66.6	46.29	33.3
	500 Da	85.45	53.7	50
Enzymatic treatment with laccase	Laccase concentration (U/L)	Colour removal (%)		
	160	93.7	92.2	73.3

Table 4
Determination of dye concentration of the real textile effluents produced when the Blue Bezaktiv S-GLD 150 dye was used in the dying processes of SARTEX.

Samples	S1 (minimum value)	S2	S3	S4	S5 (maximum value)
Absorbance (U.D.O.)	0.092	0.337	0.106	0.180	0.416
Dye concentration (mg/L)	6.5	25	7.5	13	31

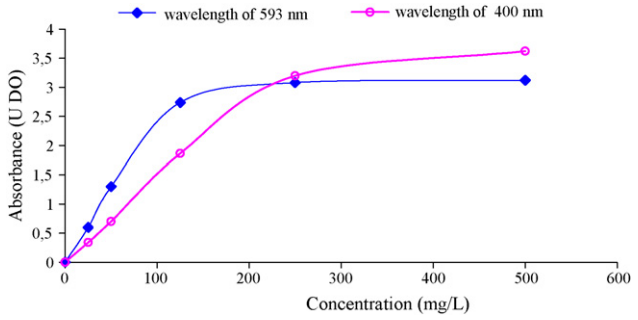

Fig. 5. Variations of absorbance with the concentration of Black Novacron R dye in the range of 0–500 mg/L.

tion of absorbance shows that whatever the concentration used the absorbance at 593 nm is proportional to the concentration of the dye up to a maximum concentration of 150 mg/L, while at the wavelength of 400 nm the absorbance is proportional to the concentration of the dye up to a maximum concentration of 200 mg/L (Fig. 5).

As SARTEX uses always the same formula (recipe) of reactive dye bath whatever the reactive dye used is that of Black Novacron R or Blue Bezaktiv S-GLD 150 (thus the same amount of dye added) and also uses the same number of rinsing baths, the dye concentrations of 40 mg/L were chosen for the remainder of this study.

3.3. Central composite design (CCD) and response surface methodology (RSM)

The results of a 29-run CCD in three variables: temperature, pH and laccase concentration, chosen for optimization of enzymatic dye decolourization process are shown in Table 2. It shows percent dye decolourization corresponding to combined effect of three components in their specified ranges. The response equations (2)–(4) were obtained for the percentage decolourization. The colour removal efficiencies obtained from experiments were influenced by the all investigated factors, i.e., temperature, pH and laccase concentration, and their effects were either individual or interactive. To investigate the interactive effect of two factors on the percentage decolourization, the RSM was used and two-dimensional graphs were drawn. The inferences so obtained are discussed in Section 3.6.

3.3.1. Decolourization of reconstituted Black Novacron R dye solution

Decolourization varied markedly with the conditions tested, in the range of 81.61–98.93% at λ_{max} = 593 nm and in the range of 32.05–79.9% at λ_{max} = 400 nm (Table 2). At the two maximum wavelengths 593 and 400 nm, lowest decolourization was observed when temperature and pH were minimum and laccase concentration was at the medium (160 U/L) (runs 1 and 2). Maximum decolourization values above 98.93% and 79.9% were observed when high temperature and pH and the medium concentration of laccase were used (runs 7 and 8).

3.3.2. Decolourization of reconstituted Blue Bezaktiv S-GLD 150 dye effluent

In Table 2, duplicate runs 7 and 8 gave the best colour removal, 98.9%. This maximum decolourization values were observed with high temperature and pH and with medium concentration of laccase (160 U/L). Lowest decolourization was observed at minimum pH, with maximum laccase concentration and at the medium temperature (runs 12 and 22).

The experimental results suggest that these variables (*T*, pH and EC) are strongly affecting the decolourization process.

3.4. Statistical analysis

Further RSM steps, the statistical analysis of the CCD experimental results, response surface modelling and optimization of process variables were carried out using NemrodW software. The statistical analysis employed Fisher's '*F*' test and Student's '*t*' test (Table 5).

Analysis of variance (NemrodW) for percentage decolourization shows that fitted second order response surface model is highly significant with $F_{statistics} > F_{Fischer}$ (2.42) ($p < 0.01$) as shown in Table 5. The Student's '*t*' test was used to determine the significance of the regression coefficients of the variables.

A *p* value is the indicator of the significance of the test, whose value below 0.05 indicates that test parameter is significant at 5% level of significance. In general, the larger magnitude of '*t*' and the smaller value of '*p*', more significant is the corresponding coefficient term [29].

3.5. Response surface methodological approach for optimization of process variables

In using the RSM approach, the runs were conducted in CCD model-designed experiments to visualize the effects of independent factors on the response and the results along with the experimental conditions. According to the sequential model sum of squares, the models were selected based on the highest-order polynomials where the additional terms were significant. The experimental results were evaluated and approximating function of dye degradation percent was obtained in the form of the following Eqs. (2)–(4), respectively:

$$Y_{(a)} = 98.40 + 2.181X_1 + 6.036X_2 + 0.838X_3 - 4.229X_1^2 - 4.204X_2^2 - 0.671X_3^2 + 0.303X_1X_2 - 0.5X_1X_3 \quad (2)$$

$$Y_{(b)} = 59.334 + 20.841X_1 + 22.854X_2 - 2.102X_3 - 0.294X_1^2 + 5.84X_2^2 - 28.878X_3^2 - 8.905X_1X_2 - 0.562X_1X_3 - 6.634X_2X_3 \quad (3)$$

$$Y_{(c)} = 98.12 + 0.65X_1 + 1.18X_2 + 0.196X_3 - 0.319X_1^2 - 1.011X_2^2 - 0.424X_3^2 + 0.508X_1X_2 - 0.427X_1X_3 + 0.315X_2X_3 \quad (4)$$

In Eqs. (2)–(4) $Y_{(a)}$, $Y_{(b)}$ and $Y_{(c)}$ correspond to response of colour removal percents of reactive dyes Black Novacron R at 593 nm, Black Novacron R at 400 nm and Blue Bezaktiv S-GLD 150 at 620 nm respectively; X_1, X_2 and X_3 correspond to independent variables of temperature, pH and enzyme concentration respectively.

Table 5
Comparison $F_{Fischer}$ and $F_{statistics}$.

Sources of variation	Degree of freedom (DF)	$F_{statistics}$ value			F_{Fisher} value
		Black Novacron R		Blue Bezaktiv S-GLD 150	
λ (nm)		593	400	620	
Regression	9	102.29	15.19	21.91	2.42
Residual	19				

Table 6
Optimum values of factors at the highest desirability for the decolourization of RDE and ADS.

Dyes	Blue S-GLD 150 (at 620 nm)		Black Novacron R (at 593 nm)	
Samples	ADS	RDE	ADS	RDE
(X_1): Temperature (°C)	35	43	40	41.9
(X_2): pH	7	6	6,5	5.91
(X_3): Enzyme dosing (U/L)	80	222	160	220.65

ADS: aqueous dye solutions; RDE: reconstituted reactive dying effluents.

Positive sign in front of the terms indicates synergistic effect, whereas negative sign indicates antagonistic effect. The quality of the model developed was evaluated based on the correlation coefficient value.

The statistical significance of the polynomial model for the experimental responses (Table 6) was evaluated by NemrodW. According to the NemrodW results, the models present different correlation coefficients (R^2).

The R^2 value for Eq. (2) was 0.98, 0.892 for Eq. (3) and (0).912 for Eq. (4), however the R^2-value of 0.892 for Eq. (3) was considered moderate to validate the fit of the predicted model. The R^2-value of 0.98 for Eq. (2) and the R^2 of 0.912 for Eq. (4) were considered relatively high, indicating that there was a good agreement between the experimental and the predicted decolourization uptake from this model.

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. These results indicate that the accuracy of the polynomial models was well adapted. The regression coefficients and the interaction

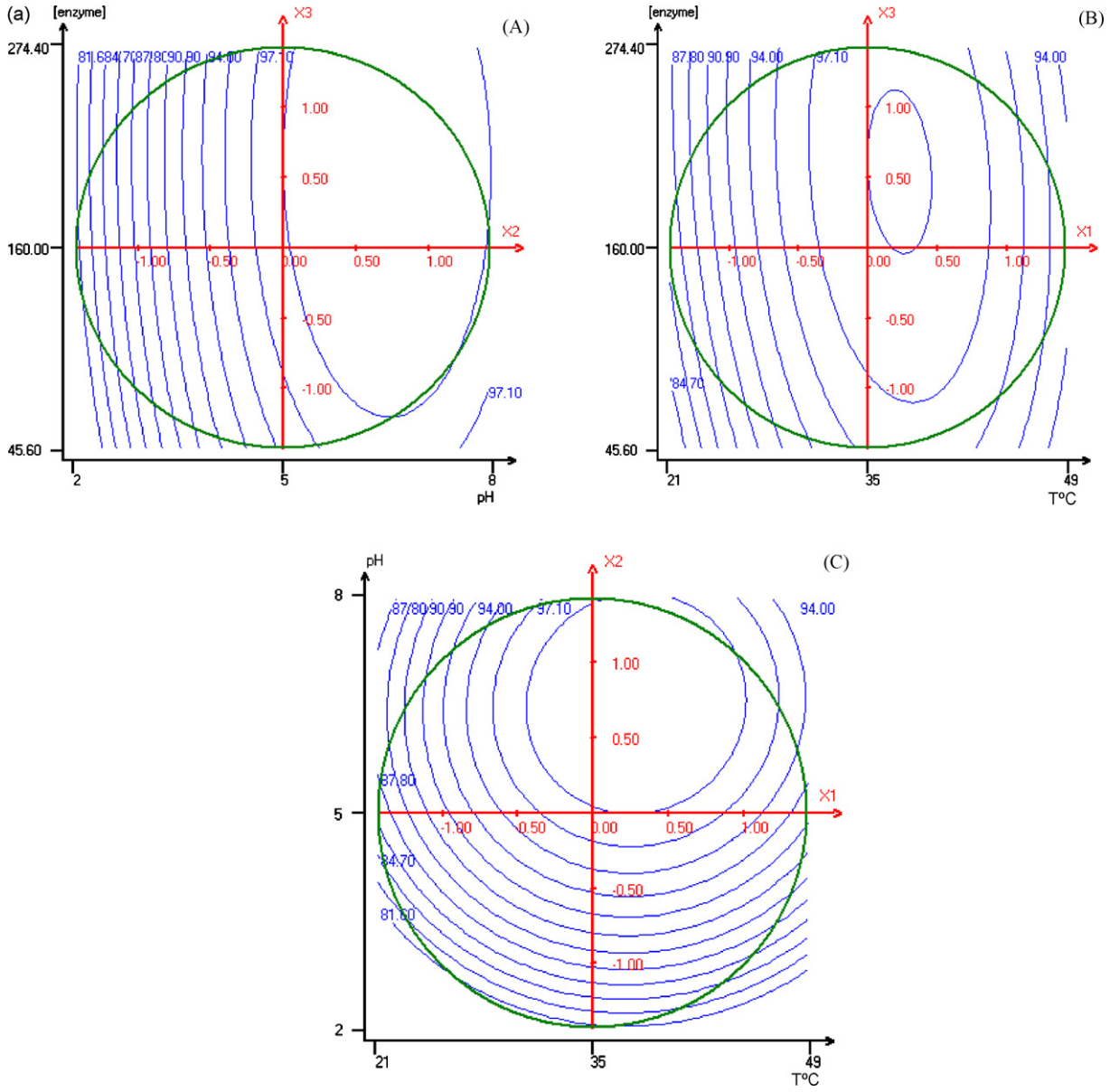

Fig. 6. (a and b) Two-dimensional and three-dimensional response surface plots for the removal of Reactive Black Novacron R dye at 593 nm by commercial laccase as a function of: (A) laccase concentration and pH (temperature = 35 °C), (B) laccase concentration and temperature (pH 5), (C) temperature and pH (laccase concentration = 160 U/L).

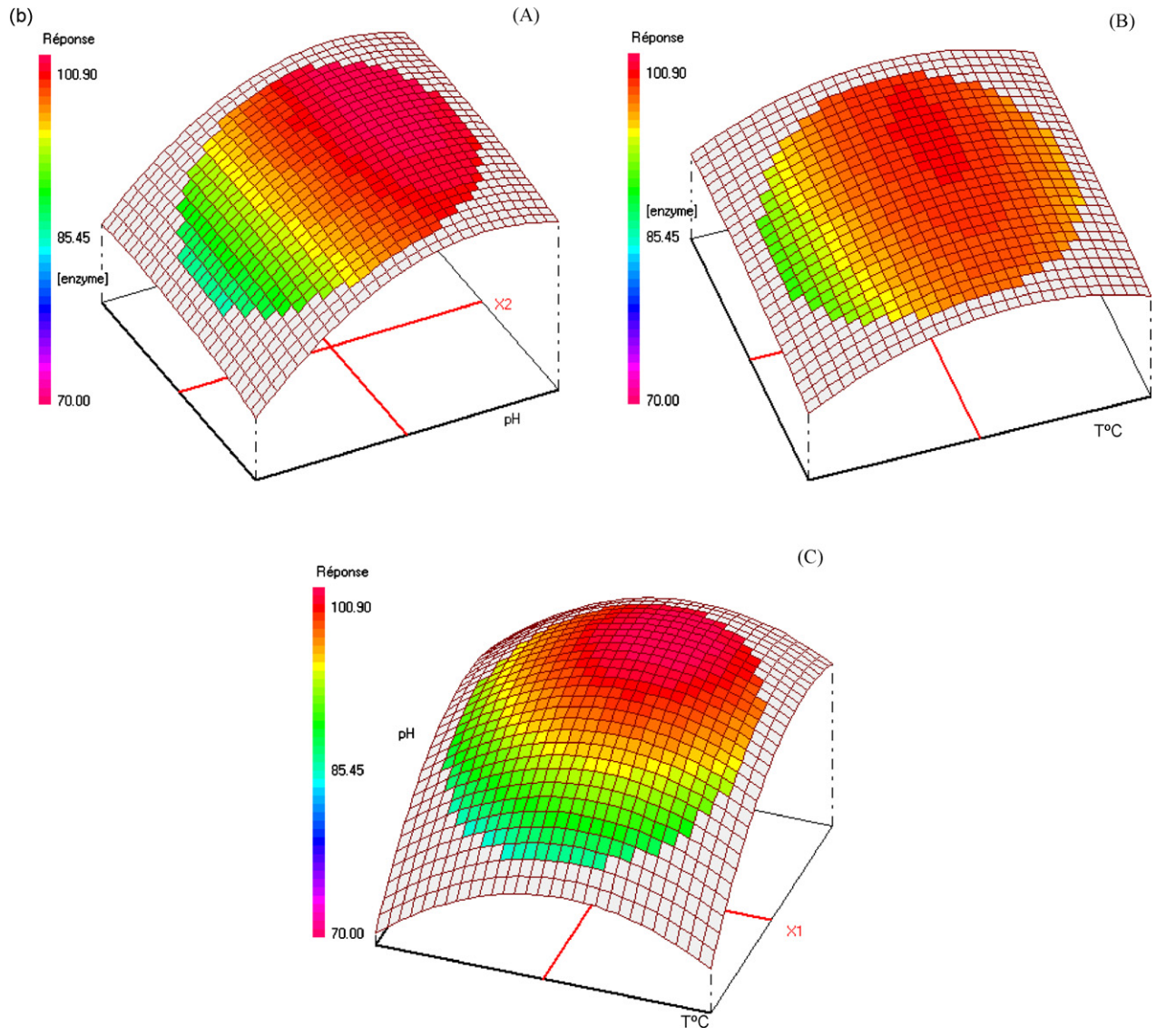

Fig. 6. (*Continued*).

between each independent factor can be considered statistically significant.

3.6. Effect of interactive variables

The experimental design model (CCD) and RSM were used with three process variables (pH, *T* and EC) to evaluate their effect and their interaction on the dye decolourization process. The response equations ((2), (3) and (4)) was obtained for the percentage decolourization and has been used to visualize the effects of experimental factors on responses under optimized conditions in two-dimensional graphs (Figs. 6–8). The response surface plots show the decolourization of the reconstituted reactive Black Novacron R dye and Blue Bezaktiv S-GLD 150 dye effluents as function of two factors, while the third was kept at a constant level.

3.6.1. Effect of interactive variables on the decolourization of the reconstituted Black Novacron R dye effluent

The analysis of the Black Novacron R dye spectra shows that it displays three absorption peaks at 300, 400 nm and at 593 nm in the UV–vis spectra (including two peaks in the visible spectrum).

The Black Novacron R dye chemical structure shows that it is a mixture of three azo dyes: (I): the "4-amino-5-hydroxy-3,6-bis [[4-[[2-(sulfooxy) ethyl] sulfonyl] phenyl] azo]-2.7 sodium-naphthalènedisulfonate" at the rate of 70-80%; (II): the disodium-3,5-diamino-2-[(2-sulfophenyl) azo] benzoate, reaction products with diazotized 2-[(4-aminophenyl) sulfonyl] ethyl hydrogen sulfate, sodium salts "at the rate of 10–15% and (III)": the "2-naphthalenesulfonic acid, 7-amino-4-hydroxy-8-[[2-sulfo-4-[[2-(sulfooxy) ethyl] sulfonyl] phenyl] azo]-, potassium sodium salt, coupled with diazotized 2-[(4-amino-5-methoxy-2-methylphenyl) sulfonyl] ethyl hydrogen sulphate" at a rate of 8–12%.

This heterogeneous composition of the Black Novacron R dye can explain the presence of more than one peak in the visible spectrum. The observation of only two peaks although the dye is made up of 3 compounds suggests that one of these 3 compounds is characterized by a peak which does not belong to the visible spectrum (the one at 300 nm).

Indeed, analysis of the peaks area on the spectrum of the Black Novacron R dye shows that the compound having a wavelength of 593 nm represents the major component of the dye; its peak area represents approximately 75% of the total surface of the three peaks. The analysis of the technical certificate of the Black Novacron R dye indicates that this compound is that (I). The compound is a minor one with an absorbance at 400 nm with a peak area of about

10%, this compound is (III) from the same technical certificate. In the end the compound with an absorbance at 300 nm is (I).

In conclusion, since the reactive Black Novacron R dye contains two components with theirs absorbencies are in the visible spectrum at the wavelengths of 593 and 400 nm, the study of the commercial laccase catalysis must be made on the two components of this dye. Therefore the absorbance should be carried out for the two wavelengths 593 and 400 nm.

3.6.1.1. Effect of interactive variables on the decolourization of the reconstituted Black Novacron R dye effluent at 593 nm. To investigate the combined effect of temperature of the system, pH and enzyme concentration on the percent decolourization of the reconstituted Black Novacron R dye effluent, the RSM was used and results were shown in the form of two-dimensional plots (Fig. 6).

Based on the NemrodW results obtained from the decolourization of the reconstituted Reactive Black Novacron R dye effluent at 593 nm (Fig. 6), both pH and temperature were found to have significant effects on the Black Novacron R dye uptake, with pH imposing the greatest effect on colour removal of the reconstituted dye bath effluent. On the other hand enzyme concentration imposed the least effect on the response. However, the interaction effects between X_2 (pH) and X_3 (laccase concentration) were considered not significant.

In fact, Fig. 4 shows the two-dimensional response surfaces which were constructed to show the iso-responses of the three variables on the colour removal of the reconstituted Black Novacron R dye effluent at 593 nm (Y_{593nm}). When the temperature was fixed at zero level (35 °C), as can be seen from the two-dimensional surface plot in Fig. 6A, colour uptake increases with the increase in pH independent of enzyme concentration. The highest colour removal was obtained when pH was at the maximum point within the range studied (3–7).

Fig. 6B shows the two-dimensional response surface when pH was fixed at 5, the highest colour removal was obtained when temperature is between 30 and 43 °C independent of enzyme concentration in the range studied. In Fig. 6C, when enzyme concentration was fixed at zero level (160 U/L), as can be seen from this two-dimensional surface plot, colour uptake increases with the increase of both pH (pH >5) and temperature (30–43 °C).

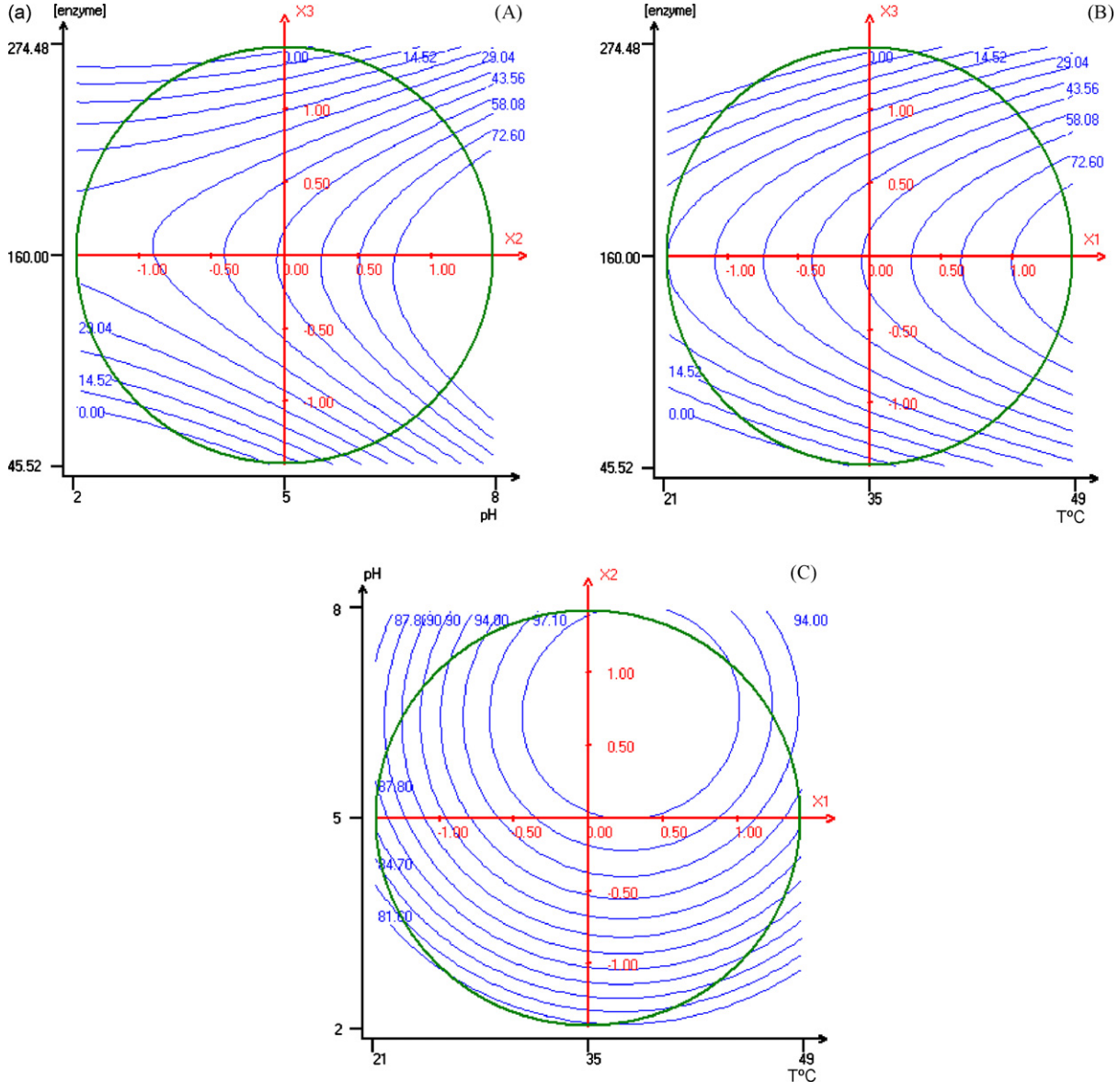

Fig. 7. (a and b) Two-dimensional and three-dimensional response surface plots for the removal of Reactive Black Novacron R dye at 400 nm by commercial laccase as a function of: (A) laccase concentration and pH (temperature = 35 °C), (B) laccase concentration and temperature (pH 5), (C) temperature and pH (laccase concentration = 160 U/L).

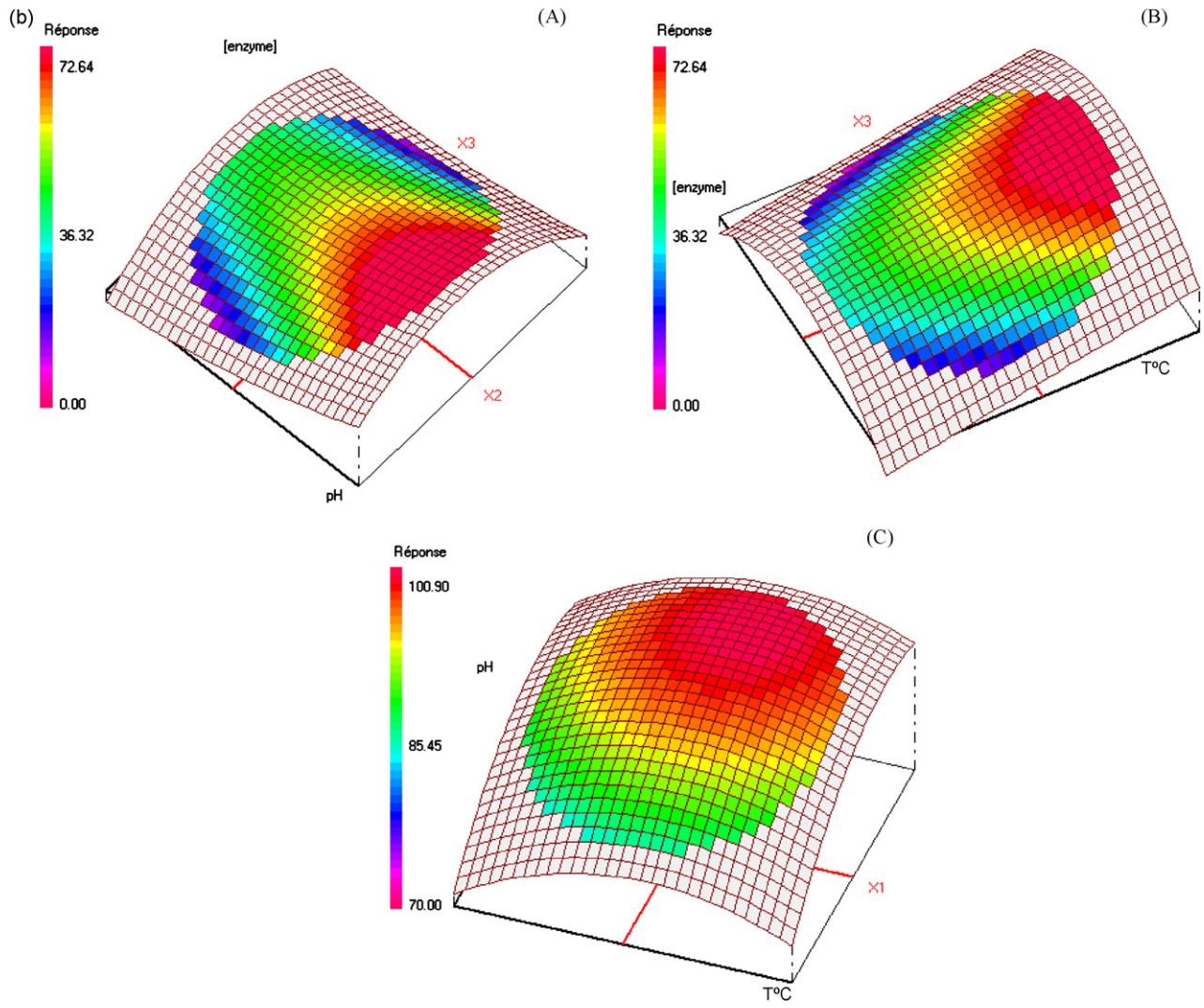

Fig. 7. (*Continued*).

3.6.1.2. Effect of interactive variables on the decolourization of the reconstituted Black Novacron R dye effluent at 400 nm. Eq. (3) has been used to visualize the effects of experimental factors on colour removal of the Black Novacron R dye at 400 nm under optimized conditions in two-dimensional graphs of Fig. 7.

Fig. 7A shows that under a pH increase, the dye decolourization rate increased independent with laccase concentration when the temperature was fixed at 35 °C. When pH was fixed at 5, Fig. 7B shows that under higher temperature, the dye decolourization rate increased to an optimum temperature. In Fig. 7C, colour removal percent increased with temperature and pH above 41 °C and 6, respectively, and more than 72.64% colour removal was realized when enzymatic concentration was fixed at 160 U/L.

3.6.2. Effect of interactive variables on the decolourization of the reconstituted Blue Bezaktiv S-GLD 150 dye effluent at 620 nm

The analysis of spectra shows that the reactive Blue Bezaktiv SGL-D 150 dye has only one peak absorbance at the visible spectrum corresponding to the wavelength of 620 nm. The chemical structure of this reactive dye (presented on the technical certificate) shows that it is composed of the 4-amino-5-hydroxy-3,6-bis ((4-((2-(sulfonatooxy) ethyl) sulfonyl) phenyl) azo)-naphthalene-2,7-disulfonate.

Based on the NemrodW results obtained from the decolourization of the reconstituted Reactive Blue Bezaktiv S-GLD 150 dye effluent at 620 nm, similarly to the decolourization of the reconstituted Black Novacron R dye effluent studied below, both pH and temperature have significant effects on the reactive Blue dye uptake and with pH imposing the greatest effect on colour removal of the reconstituted dye bath effluent. On the other hand enzymatic concentration imposed the least effect on the response. However, the interaction effects between X_2 (pH) and X_3 (laccase concentration) are significant and have an effect on the colour removal.

Fig. 8 shows the two-dimensional response surfaces which were constructed to show the iso-responses of the three variables on the colour removal (Y_{620nm}). Fig. 8A and B shows the same results as obtained for decolourization of the reconstituted reactive Black Novacron R at 593 nm (Fig. 6). In the case of enzymatic concentration being fixed at the zero level (160 U/L), as can be seen from this two-dimensional surface plot, colour uptake raises with the increase of both pH and temperature. The highest colour removal was obtained when pH and temperature were at the maximum point within the range studied.

3.6.3. Discussion

The results presented above show that pH was the more relevant factor for the decolourization of the two reactive dyes studied. A similar result has been reported by Tavares et al. [30] using the

same commercial laccase for decolourization of three reactive dyes (yellow15, red239 and blue114).

At acidic conditions (pH <5), little or no decolourization was observed for the two dyes. Similar results were observed in other studies [30–32] with 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 while for pH of 5.0 no loss of enzyme activity is observed.

On the other hand, results obtained above showed that temperature affects decolourization of the two dyes. This result agrees with the literature studies [32,33].

Nevertheless, laccase concentration in the range studied (80–240 U/L) did not influence the dye degradation for the two dyes studied.

The experimental conditions with the highest desirability were selected at about 98.93% at 593 nm and about 79.9% at 400 nm for the reconstituted reactive Black Novacron R dye effluent and about 98.9% at 620 nm for the reconstituted reactive Blue Bezaktiv S-GLD 150 dye effluent. The optimum values predicted from the models (2), (3) and (4) for colour removals are registered in Table 6.

Preliminary results for application of RSM to optimize the decolourization of aqueous dye solutions (in the absence of auxiliary components) using separately the same two reactive dyes (Blue Bezaktiv S-GLD 150 or Black Novacron R) by the commercial laccase had shown that the same maximum colour removal was obtained compared to that of reconstituted dye bath effluents (data not shown). However, the optimum parameters were different with the two reactive dyes used: T = 35 and 40 °C, pH 7 and 6.5 and EC = 80 and 160 U/L, respectively for the Blue Bezaktiv S-GLD 150 and the Black Novacron R dyes (Table 6).

Results had shown that for optimal decolourization (above 99%) by the commercial laccase, the optimum pH was between 6 and 7 independent on the dye treated. However, the optimum tem-

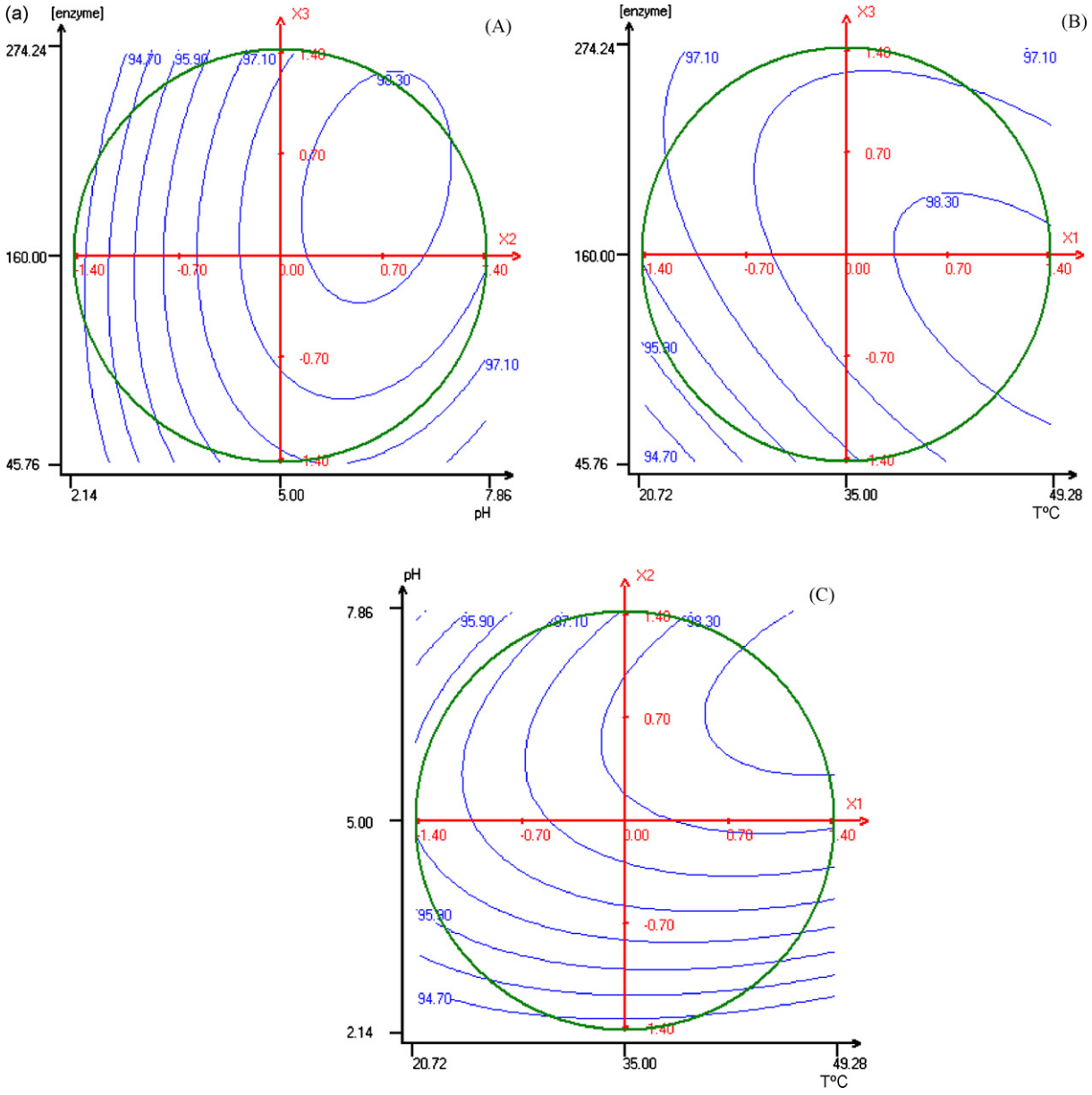

Fig. 8. (a and b) Two-dimensional and three-dimensional response surface plots for the removal of Reactive Blue Bezaktiv S-GLD 150 dye at 620 nm by commercial laccase as a function of: (A) laccase concentration and pH (temperature = 35 °C), (B) laccase concentration and temperature (pH 5), (C) temperature and pH (laccase concentration = 160 U/L).

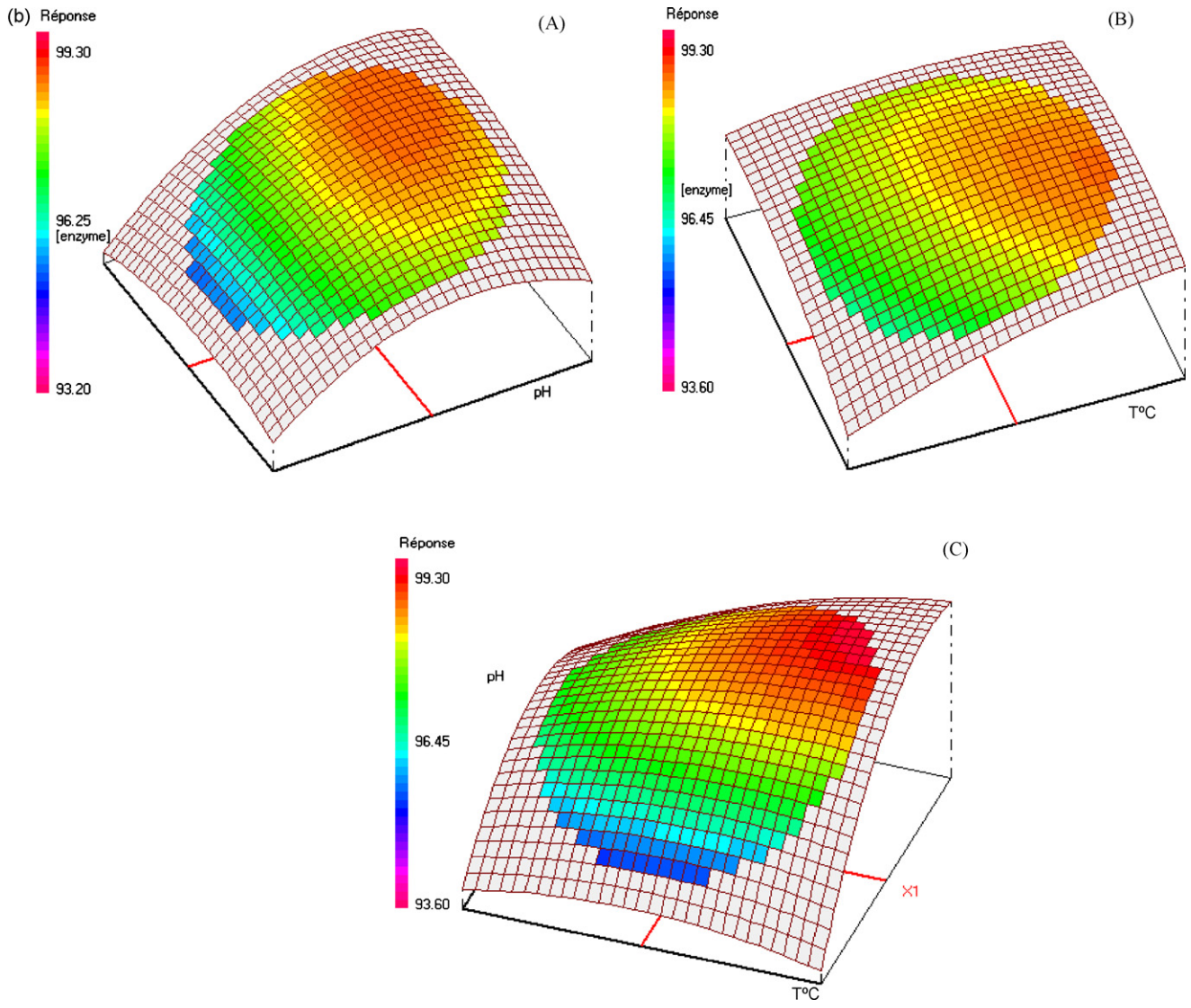

Fig. 8. (*Continued*).

perature was variable. For the aqueous Blue Bezaktiv S-GLD 150 solution, it was about 35 °C against 41.9 °C for the reconstituted dye bath effluent. For the aqueous Black Novacron R solution, the optimum temperature was between 40 and 43 °C.

At last, enzyme concentration used for optimal decolourization was important when we used the reconstituted dye bath effluents are compared to aqueous dye solutions. This result shows that substantially higher enzyme concentration is needed in the presence of the auxiliary components.

In fact, real textile effluents are extremely variable in composition since they contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products and surfactants that can inhibit enzyme activity and then the percent of decolourization [34]. Therefore, decolourization of textile effluents requires an appropriate choice of the type of enzyme as well as of reactor environment [35].

In our study, laccase catalysis at optimum conditions leads to the appearance of light pink-orange colour when the sample is based on the Black Novacron R dye (either in aqueous solution or in reconstituted effluent) while when the Blue Bezaktiv S-GLD 150 dye was used, a total decolourization was observed and no visual colour change was emerged (Fig. 9).

Similar observations have been reported in other studies. Sure enough, Kumarasamy et al. [36] have observed a strong dark brown colour formation for RB-5 (Azo dye Remazol Black-5 (RB-5) at 50 mg/L) mixture after the laccase decolourization reaction. Moreover, Sugano et al. [37] showed the generation of light red-brown reaction products during the decolourization of RB-5 (reactive black 5) by versatile peroxidase. Else,

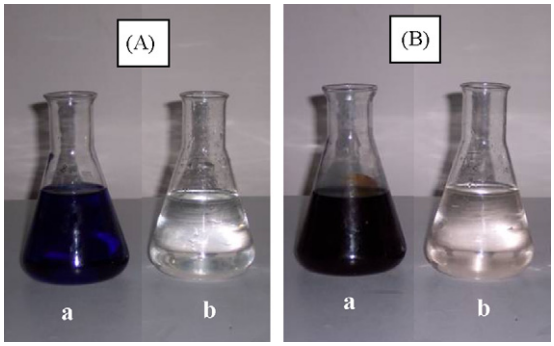

Fig. 9. Photo of reconstituted Blue Bezaktiv S-GLD 150 effluent (A) and reconstituted Black Novacron R effluent (B) before (a) and after (b) laccase treatment.

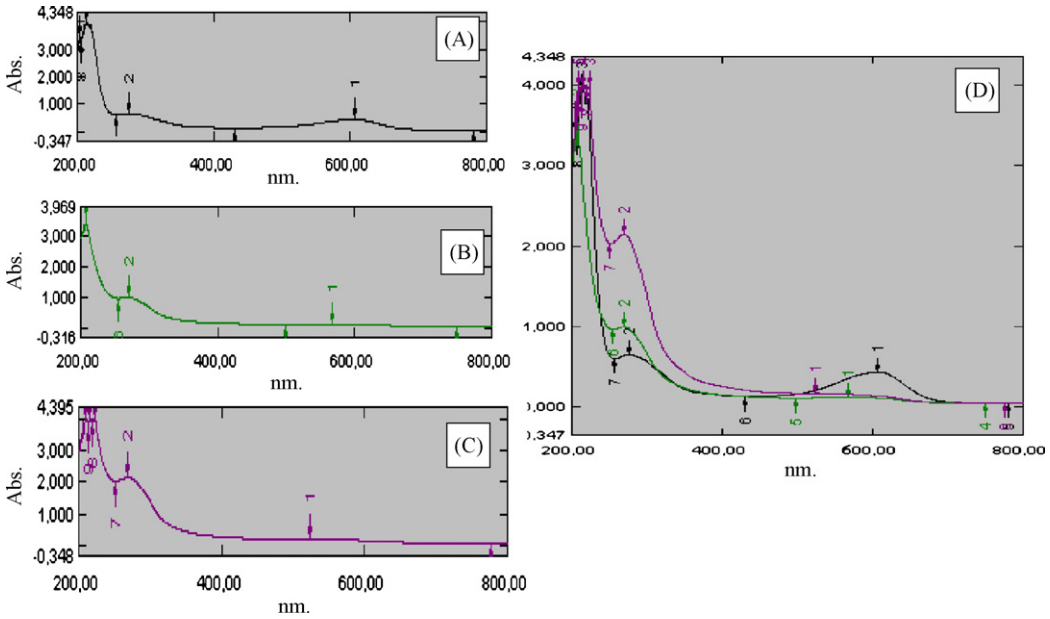

Fig. 10. UV–vis absorbance spectra of reconstituted Blue Bezaktiv S-GLD 150 decolourization under different Cl concentration (pH and *T* were fixed at optimum values); (A) initial spectra of the dye at a concentration of 40 mg/L; (B) spectra of the dye after decolourization by a laccase concentration of 80 U/L; (C) spectra of the dye after decolourization by a laccase concentration of 240 U/L; (D) summary spectra.

Tavares et al. [19] has observed after commercial laccase decolourization of aqueous RB114 dye (reactive blue114) solution, a generation of light orange colour witch can be explained by a new peak of absorbance that emerged between 400 and 420 nm. Whereas, for the two dyes: RR239 (reactive red239) and RY15 (reactive yellow15), neither colour change nor additional peaks of absorbance were observed in the entire visible spectrum.

According to Tavares et al. [19] for better performance in dye wastewater treatment, no absorbance in the visible spectrum must be detected after decolourization. Hence, the decolourization of the reconstituted Black Novacron R and Blue Bezaktiv S-GLD 150 effluents at the optimum values of pH and Temperature was achieved when enzyme concentration was fixed at minimum and at maximum levels (80 and 240 U/L) by measuring the absorbance reduction over the entire visible spectrum (Figs. 10 and 11).

From Fig. 10, a higher decolourization based on the absorbance reduction at the maximum wavelength of the reconstituted Blue Bezaktiv S-GLD 150 (620 nm) was observed and no additional peaks of absorbance were emerged in the entire visible spectrum.

For the reconstituted Black Novacron R, Fig. 11 shows a higher decolourization based on the absorbance reduction at the maximum wavelength of 593 nm. However a lower decolourization based on the absorbance reduction at the wavelength of 400 nm

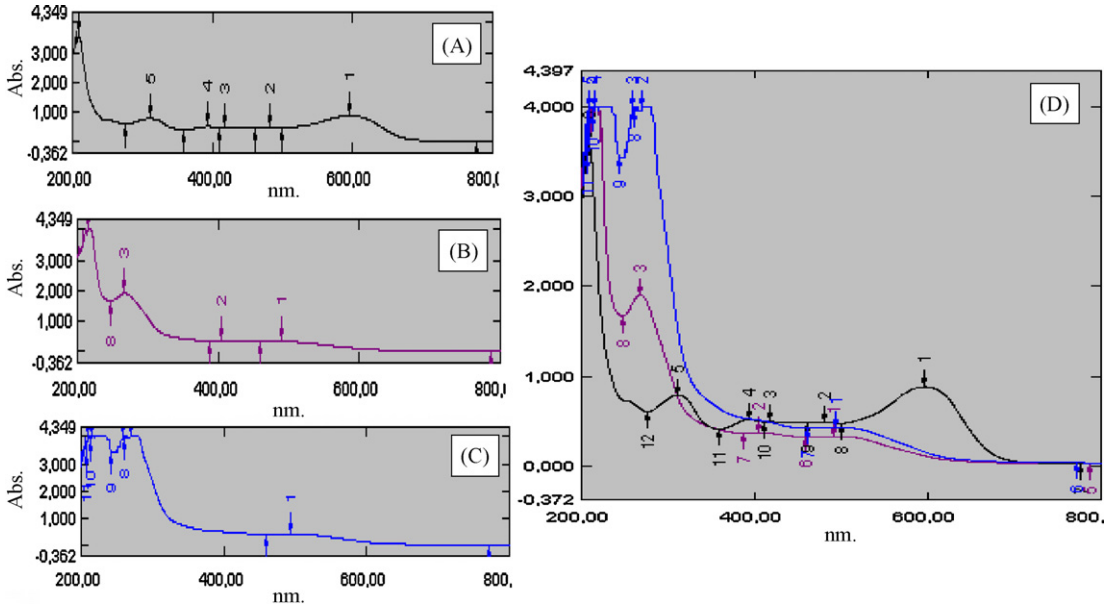

Fig. 11. UV–vis absorbance spectra of reconstituted Black Novacron R decolourization under different Cl concentration (pH and *T* were fixed at optimum values); (A) initial spectra of the dye at a concentration of 40 mg/L; (B) spectra of the dye after decolourization by a laccase concentration of 80 U/L; (C) spectra of the dye after decolourization by a laccase concentration of 240 U/L; (D) summary spectra.

was observed in the same figure and also no additional peaks of absorbance were observed in the entire visible spectrum.

These observations are in accordance with the results obtained by the application of RSM approach. Laccase colour removal based on the absorbance reductions at the maximum wavelengths were about 98.9%, 98.93% and 79.9% respectively for the reconstituted Blue Bezaktiv S-GLD 150 at 620 nm and for the reconstituted Black Novacron R at 593 nm and at 400 nm.

Hence, the generation of the light pink-orange colour observed for the Black Novacron R dye (either in aqueous solution or in reconstituted effluent) after laccase decolourization (Fig. 9) can be explained by the fraction of the Black Novacron R dye witch characterised by an absorption peak at 400 nm (this compound is (III) from the technical certificate of the dye) that cannot be totally catalyzed by the enzyme (only 79.9% colour removal) since no additional peaks of absorbance were observed in the entire visible spectrum for this dye.

4. Conclusion

The optimization of the decolourization of the reconstituted dye bath effluent by commercial laccase treatment was achieved through the response surface methodology. The highest colour removal was achieved as 98.93%, 79.9%, and 98.9% respectively for the reconstituted Black Novacron R dye effluent at 593 and at 400 nm and for the reconstituted Blue Bezaktiv S-GLD 150 dye effluent at 620 nm at initial dye concentration of 40 mg/L. Nevertheless, the enzyme concentration used depends on type of dye and of components contained in the reconstituted dyeing effluents. The obtained results demonstrate that the use of the laccase has an enormous potential to degrade the textile dyes. So, this enzyme can be used for treating textile wastewaters, particularly for water recycling.

Further pilot scale studies are required and detailed study is needed to explore the enzymatic catalysis mechanism.

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