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Laccase production and dye decolorization by *Trametes versicolor*: application of Taguchi and Box-Behnken Methodologies

[*Trametes versicolor* ile lakkaz üretimi ve renk giderimi: Taguchi ve Box-Behnken yöntemlerinin uygulaması]

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ABSTRACT

Objective: The aim of this study was to investigate the laccase production of *Trametes versicolor* under submerged fermentation condition. Then, dye decolorization by laccase was optimized using Box-Behnken methodology.

Methods: The optimal culture conditions for producing high amount of laccase were determined using Taguchi methodology. The experiments were designed with five factors (glucose, yeast extract, $CuSO_4$, inoculum size and pH) at three levels with orthogonal array layout of L27 (3^5). Then, the optimum conditions for high decolorization activity of Reactive Blue 49 by obtained crude laccase were also investigated using Box-Behnken methodology.

Results: The optimum culture conditions for production of high amounts of laccase were detected as 2 g L^{-1} of glucose, 5 g L^{-1} of yeast extract, 2mM of $CuSO_4$, 4% of inoculum amount and pH 5.5. Yeast extract was the most effective factor, followed by $CuSO_4$, inoculum, glucose and pH. Under these conditions, predicted values were in a good agreement with the actual experimental one. The predicted results showed that the maximum of Reactive Blue 49 decolorization as 98% could be obtained under the optimum conditions of pH 2.95, initial dye concentration 55.6 mg L^{-1}, enzyme amount 0.76 mL and reaction time 46.91 min. The validity and practicability of this statistical optimization strategy was confirmed with the relation between predicted and experimental values.

Conclusion: The results suggested that Taguchi method can be used in the optimization of laccase production process. Production of laccase by *Trametes versicolor* 2008001 can be effectively used for enzymatic decolorization according to the results of decolorization experiments in optimal levels.

Key Words: Laccase, Taguchi Method, Box-Behnken Methodology, dye decolorization.
Conflict of Interest: The authors declare no conflict of interest.

ÖZET

Amaç: Çalışmanın amacı, batık fermentasyon koşulları altında *Trametes versicolor* 2008001 suşu ile lakkaz üretimini araştırmaktır. Daha sonra üretilen lakkaz enzimi ile Box-Behnken metodu kullanılarak boyar madde dekolorizasyonunun optimizasyonu amaçlanmıştır.

Metod: Yüksek aktivitede lakkaz enzimi üretimi için en uygun kültür koşulları, Taguchi yöntemi kullanılarak belirlenmiştir. Deneyler L_{27} (3^5) ortogonal dizi düzeni ile 5 faktör (glukoz, maya özütü, $CuSO_4$, inokulum miktarı ve pH) 3 düzey kullanılarak tasarlanmıştır. Daha sonra, elde edilen ham lakkaz ile Reaktif Mavi 49'un yüksek renk giderimi için en uygun koşullar, Box-Behnken yöntemi kullanılarak incelenmiştir.

Bulgular: Yüksek aktiviteye sahip lakkaz enziminin üretimi için en uygun kültür koşulları, 2 g L^{-1} glukoz, 5 g L^{-1} maya özütü, 2mM $CuSO_4$, %4 inokulum miktarı ve pH 5,5 olarak belirlenmiştir. Maya özütü en etkili faktördür, bunu $CuSO_4$, inokulum miktarı, glukoz ve pH takip etmiştir. Bu koşullar altında tahmini değerler ile deneysel değerler arasında uyum söz konusudur. Önerilen sonuçlar, pH'nın 2,95, başlangıç boya konsantrasyonunun 55,6 mg L-1, enzim miktarının 0,76 mL ve reaksiyon süresinin 46,91 dakika olduğu koşullarda en yüksek Reaktif Mavi 49 renk gideriminin %98 olduğunu göstermiştir. Bu istatistiksel optimizasyon stratejisinin geçerliliği ve uygulanabilirliği, tahmini ve deneysel değerler arasındaki ilişki ile doğrulanmıştır.

Sonuç: Sonuçlar Taguchi yönteminin lakkaz üretim sürecinde optimizasyon için kullanılabileceğini göstermiştir. Ayrıca en uygun koşullardaki renk giderimi deneylerinin sonuçlarına göre, *Trametes versicolor* 2008001 ile üretilen lakkaz enzimi etkin bir şekilde enzimatik renk gideriminde kullanılabilir.

Anahtar Kelimeler: Lakkaz, Taguchi yöntemi, Box-Behnken yöntemi, renk giderimi.
Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Laccase (benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2) is a copper containing enzyme that oxidizes various phenolic and aromatic amine compounds by reducing molecular oxygen to water [1].

Laccases have broad substrate specificity and therefore, they could be used in various biotechnology applications such as pulp delignification [2-5], textile dye bleaching [6,7], detoxification of industrial effluents [2,8], desulphurization of coal [9], xenobiotic detoxification [10-12], biosensor preparation [13], detergent manufacturing, transformation of antibiotics and steroids [6] and catalysts for the manufacture of anti–cancer drugs [14].

Various organisms such as plants, insects, bacteria and fungi could produce this enzyme [15]. However, laccase activity has mainly been demonstrated in Basidiomycetes, Ascomycetes and Deuteromycetes [16]. White rot fungi belong to Basidiomycetes are known as a major producers of this enzyme. *Trametes (Coriolus) versicolor* is an effective laccase producer white rot fungus.

Laccase production ability of those fungi could be induced using different inducers and/or different cultivation conditions [17-22]. Traditional procedures require an alteration of one factor at a time and this optimization procedure enables to assess the impact of those particular parameters on the process performance [23]. These techniques are inconvenient, which require more time and experimental data sets, and cannot give any information about the interactions among all of the variables. Taguchi method of orthogonal array (OA) experimental design (DOE) determines the effect of factors, optimal level of conditions [24].

Textile wastewaters contain various dyes which are hardly decolorized by conventional treatment system. These dyes could negatively affect the ecosystem by decreasing the photosynthetic activity and oxygen concentration. Therefore, new and effective methods must be used to decolorize textile dyes [25]. There are many studies on textile dye decolorization activity of laccases from white rot fungi and the strain mentioned above [19, 26-28]. This decolorization activity could be induced by optimizing the conditions. The application of experimental design in treatment process of textile effluents was able to result in improved decolorization, reduced process time and overall costs. Besides, possible synergic interactions between the investigated factors may be evaluated [29].

The aim of this study was to investigate the laccase production of *T. versicolor* under submerged fermentation condition. The optimal culture conditions for producing laccase were determined using Taguchi methodology. Then, dye decolorization by laccase was optimized using Box-Behnken methodology.

Materials and Methods

Microorganism, culture conditions and dye

Trametes versicolor ATCC (200801) originally isolated and cultured by Dr. O Yesilada was used in this study. This fungus was subcultured on Malt Extract Agar (MA) plates of 30 °C and stored at 4 °C.

Laccase production of *T. versicolor* was tested under agitated culture conditions. To this end, firstly this fungus was cultured at 30 ˚C on slant MA for one week. Then, mycelial suspension was prepared and this suspension was inoculated into 250 mL Erlenmeyer flask containing 100 mL Potato Dextrose Broth (PDB). This culture was incubated at 30 °C and 150 rev min^{-1} for 4 days. After that, the culture was homogenized and determined culture of homogenized culture was transferred in 250 mL Erlenmeyer flasks with 100 mL Stock Basal Medium (SBM) that contain K_2PO_4: 0.2 g L^{-1}, $CaCl_2.2H_2O$: 0.01 g L^{-1}, $MgSO_4.7H_2O$: 0.05 g L^{-1}, $NH_4H_2PO_4$: 0.5 g L^{-1}, $FeSO_4.7H_2O$: 0.035 g L^{-1}, glucose:2-5-10 g L^{-1}, yeast extract: 0.5-2-5 g L-1, $CuSO_4$. $5H_2O$: 0-2-4 mM [19] and incubated under agitated conditions at 30 °C and 150 rev min^{-1} for 7 days.

Reactive Blue 49 (RB49) which was kindly provided by Sarar Textile Co., Turkey, was used. The maximum absorbance peak wavelength of RB49 was determined prior to use.

Taguchi Methodology for laccase production

Design of experiment aims to find the relationship between the output and experimental factors in a process. The Taguchi approach is a special type of design of experiment which was developed by Genichi Taguchi to improve the implementation of total quality control in Japan [30]. It is one of the leading approaches to optimize design for performance, quality and cost. The main purpose of this method is to determine the optimal and robust process characteristic that is minimally sensitive to noise [31]. A noise factor is a thing that causes a measurable product or process characteristic to deviate from its target value [32]. Target values are defined as follows:

"The larger the better" quality characteristic is chosen when goal is to maximize the response. The S/N ratio is can be calculated as given below in Eq. (1).

$$S/N = -10*\log_{10}\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{Y_i^2}\right) \qquad (1)$$

In order to get more robust product or process, the best combination of control parameters was searched. The Taguchi experimental design uses standard orthogonal arrays with the help of linear graph, an interaction table, and special techniques. A Taguchi experiment considers only main effects and some pre-determined two-factor in-

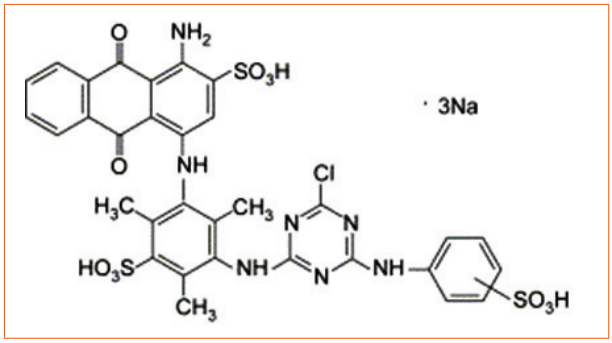

Figure 1. Structure formula of Reactive Blue 49.

teractions, as higher order interactions are assumed to be nonexistent [33].

In this study, optimization of culture conditions by Taguchi methodology was investigated for high amount of laccase production. The experiments were designed with five factors (glucose, yeast extract and inoculum amounts, $CuSO_4$ concentration, and also pH) at three levels with orthogonal array layout of L27 (3^5). All statistical experimental results were analyzed by using Minitab 16 for Windows (Minitab Inc.). All the samples were studied in 250 mL flask with 100 mL growth medium. The agitation rate and temperature were 150 rev min^{-1} and at 30 °C, respectively. The samples were incubated for 7 days and then, laccase activity was determined. All experiments for laccase production were performed in duplicate.

Laccase activity was measured spectrophotometrically at 465 nm by monitoring the oxidation of guaicol at 37 °C for 15 min. The assay mixture contained 0.1 mL of culture supernatant and 4.9 mL sodium acetate buffer (50 mM, pH 4.5) containing 1 mM guaiacol as substrate. 1 U of enzyme activity was defined as the amount of enzyme that elicited an increase in A465 of 1 absorbance unit per minute [18].

Box-Behnken Methodology for Dye Decolorization

Structural formula of Reactive Blue 49 was given in Figure 1. The optimum conditions for dye decolorization were determined by means of Box-Behnken methodol-

Table 1. L27 (3^5) orthogonal array of Taguchi experimental design

Experiment No	Glucose (g L^{-1})	Yeast extract (g L^{-1})	$CuSO_4$ (mM)	Inoculum amount (%)	pH	Laccase activity (U ml^{-1})	
	(A)	(B)	(C)	(D)	(E)	Repetition 1	Repetition 2
1	2.0	0.5	0	2.0	4.5	0.832	3.687
2	2.0	0.5	0	2.0	5.0	0.520	0.602
3	2.0	0.5	0	2.0	5.5	0.529	0.727
4	2.0	2.0	2.0	4.0	4.5	6.148	6.628
5	2.0	2.0	2.0	4.0	5.0	16.033	18.185
6	2.0	2.0	2.0	4.0	5.5	19.917	18.029
7	2.0	5.0	4.0	6.0	4.5	13.285	12.640
8	2.0	5.0	4.0	6.0	5.0	20.365	18.9
9	2.0	5.0	4.0	6.0	5.5	20.008	20.151
10	5.0	0.5	2.0	6.0	4.5	0.370	4.149
11	5.0	0.5	2.0	6.0	5.0	0.380	0.374
12	5.0	0.5	2.0	6.0	5.5	0.374	0.409
13	5.0	2.0	4.0	2.0	4.5	0.393	0.536
14	5.0	2.0	4.0	2.0	5.0	0.398	0.396
15	5.0	2.0	4.0	2.0	5.5	0.352	0.384
16	5.0	5.0	0	4.0	4.5	19.244	19.859
17	5.0	5.0	0	4.0	5.0	18.131	20.580
18	5.0	5.0	0	4.0	5.5	18.040	18.493
19	10.0	0.5	4.0	4.0	4.5	0.391	0.4514
20	10.0	0.5	4.0	4.0	5.0	0.383	0.504
21	10.0	0.5	4.0	4.0	5.5	0.376	0.452
22	10.0	2.0	0	6.0	4.5	2.287	3.59
23	10.0	2.0	0	6.0	5.0	2.398	2.782
24	10.0	2.0	0	6.0	5.5	3.409	4.185
25	10.0	5.0	2.0	2.0	4.5	18.027	15.098
26	10.0	5.0	2.0	2.0	5.0	22.272	21.015
27	10.0	5.0	2.0	2.0	5.5	22.078	19.243

ogy, one of the most principal of Response Surface Methodology (RSM). The RSM consist of a group of empirical techniques devoted to the evaluation of the relationship existing between the independent variables and measured responses [34].

In this study, decolorization studies were conducted using the dye solution in 10 mL of tubes at 30°C under static conditions. For minimizing the number of experiments required, Box-Behnken design matrix (BBM) has been used. Design-Expert statistical software package was used to analyze the data. By using four factors at three levels BBM with 29 runs, the effects of independent variables (pH, temperature, incubation period and inoculum amount) coded as x_1, x_2, x_3, x_4 were selected for optimization. Design-Expert statistical software package has been used to analyze the data. pHs of the tubes were adjusted to the values 2.5, 4.5 and 6.5 suggested by the used experimental design. For pH 2.5 and 4.5, sodium acetate buffer adjusted pH with acetic acid; for pH 6.5 Na_2HPO_4–NaH_2PO_4 buffer were used. Decolorization tubes included various enzyme amounts (0.05-0.525-1 mL), and 9 mL of several initial dye concentrations (25-62.5-100 mg L^{-1}) for different incubation times (5-62.5-120 min). All experiments were performed in triplicate. Dye decolorization was monitored using the maximum wavelength of this dye (587 nm). The reaction mixture containing dye solution with heat denaturated enzyme was used as control. The absorbances of the solutions were measured using a UV/VIS spectrophotometer (Schimadzu-UV2550) and they were compared with a standard curve plotted using different concentrations of the dye value.

Results and Discussion

Taguchi Methodology for laccase production

Several studies have been carried out to determine the most effective laccase producer; to choose the most suitable culture medium; to develop suitable, reproducible, and cheap isolation procedures and to optimize enzyme production [15,22,35].

The interaction effects of the parameters were not taken into account. The validity of this assumption was checked by confirmation experiments conducted at the optimum conditions.

As shown in Table 1, the effects of five factors on laccase production named as glucose amount, yeast extract amount, $CuSO_4$ concentration, inoculum amount and pH were studied. The appropriate experimental design was determined and the analysis of data was performed by the Minitab 16 for Windows (Minitab Inc.). The experiments were designed with five factors at three levels with orthogonal array layout of L27 (3^5). Table 1 shows the results of laccase production determined with the Taguchi experimental design.

Analysis of variance (ANOVA) is a most widely used

Table 2. Results of the ANOVA for laccase production

ANOVA Table for S/N ratio

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Glucose (g L^{-1})	2	571.24	571.24	285.62	45.67	0.000
Yeast extract (g L^{-1})	2	4416.75	4416.75	2208.38	353.14	0.000
CuSO4 (mM)	2	521.28	521.28	260.64	41.68	0.000
Inoculum amount (%)	2	313.49	313.49	156.74	25.07	0.000
pH	2	3.01	3.01	1.51	0.24	0.789
Error	16	100.06	100.06	6.25		
Total	26	5925.83				

S = 2.50 R-Sq = 98.31% R-Sq(adj) = 97.26%

ANOVA Table for means

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Glucose (g L^{-1})	2	84.95	84.95	42.48	5.78	0.013
Yeast extract (g L^{-1})	2	1531.16	1531.16	765.58	104.21	0.000
$CuSO_4$ (mM)	2	141.53	141.53	70.77	9.63	0.002
Inoculum amount (%)	2	99.54	99.54	49.77	6.78	0.007
pH	2	26.96	26.96	13.48	1.84	0.192
Error	16	117.54	117.54	7.35		
Total	26	2001.69				

S = 2.71 R-Sq = 94.13% R-Sq(adj) = 90.46%

Table 3. Response table for means and S/N ratios for laccase production

	Means					S/N Ratio				
	Glucose (g L^{-1})	Yeast extract (g L^{-1})	$CuSO_4$ (mM)	Inoculum Amount (%)	pH	Glucose (g L^{-1})	Yeast extract (g L^{-1})	$CuSO_4$ (mM)	Inoculum Amount (%)	pH
Level										
1	14.679	-5.913	10.76	5.028	8.682	10.9548	0.8617	7.7719	7.0605	7.0897
2	3.414	7.877	13.46	13.368	9.126	6.8257	5.8917	11.5961	11.2136	9.1232
3	9.214	25.344	3.087	8.911	9.499	7.719	18.7461	6.1314	7.2253	9.2864
Delta	11.265	31.257	10.373	8.34	0.817	4.1291	17.8844	5.4646	4.1531	2.1967
Rank	2	1	3	4	5	4	1	2	3	5

method to determine the significant parameters on the response. The ANOVA results for means and S/N Ratio were shown in Table 2. The ratio between the variance of process parameters and F-test determine whether the parameter has a significant effect on the performance characteristics. The F-test value of the parameter is compared with the standard F Table value with corresponding degrees of freedom v_1, v_2, ($F_{0.05}$) at the 5% significance level. If F-test value is greater than 0.05, the process parameter is considered as significant [36]. In this study, it could be concluded that all parameters except pH have a significant effect on laccase activity.

The optimum level of process parameter depends on the level with highest S/N value. The response table of S/N ratios and means were displayed in Table 3. According to these findings, laccase activity is maximum at the first level of glucose, third level of yeast extract, second level of $CuSO_4$, second level of inoculum amount, third level of pH. The amount of yeast extract was found to be the most effective factor for laccase production, and pH was the least significant one. Maria and coworkers found that fungal laccase production was induced up to four times through the addition of yeast extract [37].

One of the important parameters for fungal cultivation is pH of the medium. The optimum pH value for high laccase production was detected as 5.5. This value was reported as 5.0-5.5 for *Pleurotus ostreatus* 180 cultivated under submerged culture conditions with the best glucose concentration as 2.0 g L^{-1} [23,38]. Periasamy and Palvannan found that glucose had a higher effect at Level 3 (2.0 g L^{-1}) for laccase production [39]. In our study, copper positively affected the laccase production of *T. versicolor*. Although high amounts of copper may have toxic effect, it is also an important metal for laccase production by white rot fungi [21]. This metal may play an important role in laccase genes regulation at transcription level as showed in *T. versicolor* [40].

Optimization in the Taguchi experiment involves finding the factor level combination that gives the optimal response. In this study, the larger is the indication of better performance. Therefore, "the larger the better" performance characteristics were selected to obtain the suitable levels. The optimum conditions for each factor in terms of achieving higher laccase activity were summarized as shown in Table 4.

The confirmation experiment is conducted to verify the conclusions based on Taguchi's parameter design approach. The confirmation experiment is performed by conducting a test with a specific combination of the optimum levels. Based on Table 4, the level of a parameter with highest value of S/N ratio is the best combination level. In this study, three additional experiments were carried out at the optimum levels. The confirmation experiment levels for the laccase activity were shown in Table 5. The results of the three confirmation experiments were close to the experimental findings. These data, confirmation test in triplicate and % error were presented in Table 5. The responses for optimal point fell into the prediction interval (20.98; 34.61). The experimental results

Table 4. Optimum conditions for *T. versicolor* fermentation

Factors	Levels	
	Actual	Coded
Glucose (g L^{-1})	2.0	1
Yeast extract (g L^{-1})	5.0	3
$CuSO_4$ (mM)	2.0	2
Inoculum amount (%)	4.0	2
pH	5.5	3

Table 5. Comparative results for confirmation tests for laccase production

Test	Experimental results	Error %
1	26.42	-3.5
2	27.23	-0.5
3	26.88	-1.8
Average % error		-1.93

Note: (actual-pred)/actual*100=error formulation was used

Table 6. Box–Behnken design matrix for variables of RB49 decolorization

Run no.	Independent values								Response
	Initial dye concentration (mg L^{-1})		Enzyme amount		pH (ml)		Reaction time (min)		Avarage % dec.
	X_1 (coded)	X_1 (uncoded)	X_2 (coded)	X_2 (uncoded)	X_3 (coded)	X_3 (uncoded)	X_4 (coded)	X_4 (uncoded)	Y(observed)[a]
1	-1	25	-1	0.05	0	4.5	0	62.5	53.82
2	1	100	-1	0.05	0	4.5	0	62.5	45.81
3	-1	25	1	1	0	4.5	0	62.5	78.13
4	1	100	1	1	0	4.5	0	62.5	77.92
5	0	62.5	0	0.525	-1	2.5	-1	5	80.82
6	0	62.5	0	0.525	1	6.5	-1	5	0.32
7	0	62.5	0	0.525	-1	2.5	1	120	92.9
8	0	62.5	0	0.525	1	6.5	1	120	9.35
9	-1	25	0	0.525	0	4.5	-1	5	39.07
10	1	100	0	0.525	0	4.5	-1	5	66.95
11	-1	25	0	0.525	0	4.5	1	120	89.63
12	1	100	0	0.525	0	4.5	1	120	90.57
13	0	62.5	-1	0.05	-1	2.5	0	62.5	79.69
14	0	62.5	1	1	-1	2.5	0	62.5	86.97
15	0	62.5	-1	0.05	1	6.5	0	62.5	0.32
16	0	62.5	1	1	1	6.5	0	62.5	7.94
17	-1	25	0	0.525	-1	2.5	0	62.5	95.17
18	1	100	0	0.525	-1	2.5	0	62.5	91
19	-1	25	0	0.525	1	6.5	0	62.5	1.46
20	1	100	0	0.525	1	6.5	0	62.5	6.96
21	0	62.5	-1	0.05	0	4.5	-1	5	10.44
22	0	62.5	1	1	0	4.5	-1	5	59.38
23	0	62.5	-1	0.05	0	4.5	1	120	67.02
24	0	62.5	1	1	0	4.5	1	120	85.44
25	0	62.5	0	0.525	0	4.5	0	62.5	82.98
26	0	62.5	0	0.525	0	4.5	0	62.5	85.87
27	0	62.5	0	0.525	0	4.5	0	62.5	83.67
28	0	62.5	0	0.525	0	4.5	0	62.5	92.71
29	0	62.5	0	0.525	0	4.5	0	62.5	91.42

Yoa indicates the average % decolorizaiton of triplicate experiments (n=2).

Table 7. Analysis of variance (ANOVA) for RB49 decolorization

Source	Sum of Squares	df	Mean Square	F	p
Model	31786.25	8	3973.281	43.39058	<0.0001
x_1	40.07708	1	40.07708	0.437665	0.5158
x_2	1602.679	1	1602.679	17.5022	0.0005
x_3	20850	1	20850	227.6944	<0.0001
x_4	2638.257	1	2638.257	28.81133	<0.0001
x_1^2	272.5453	1	272.5453	2.976356	0.0999
x_2^2	1859.921	1	1859.921	20.31144	0.0002
x_3^2	5618.426	1	5618.426	61.35653	<0.0001
x_4^2	942.9617	1	942.9617	10.2977	0.0044
Residual	1831.403	20	91.57013		
Lack of Fit	1751.28	16	109.455	5.464404	0.0561
Pure Error	80.1222	4	20.03055		
Cor Total	33617.65	28			

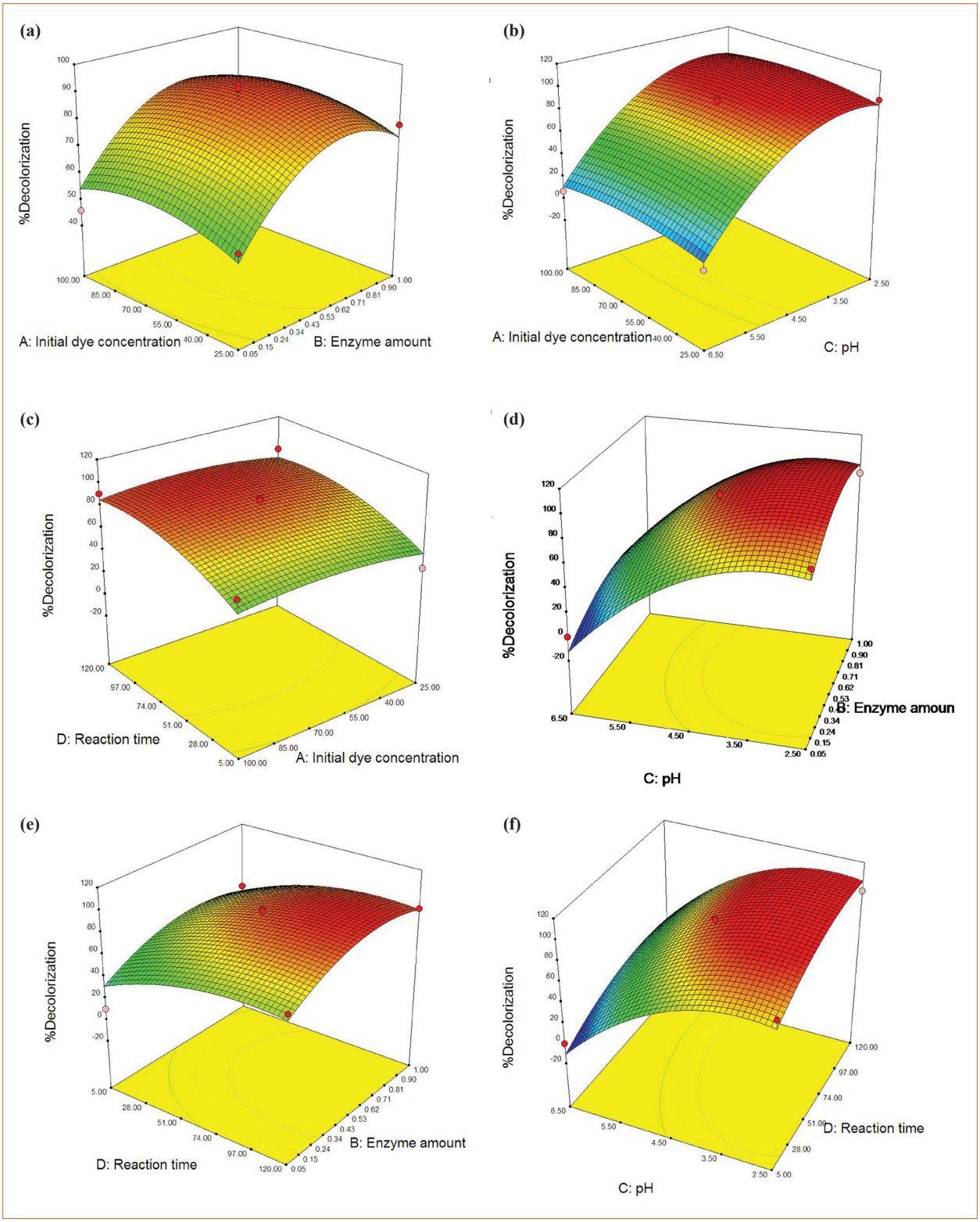

Figure 2. Response surface curves for RB49 decolorization showing the interactions between **(a)** initial dye concentration and enzyme amount, **(b)** initial dye concentration and pH, **(c)** reaction time and initial dye concentration, **(d)** pH and enzyme amount **(e)** reaction time and enzyme amount **(f)** reaction time and pH.

confirmed the validity of the applied technique to find the optimal point for laccase production.

Box-Behnken Methodology for dye decolorization

By using Box-Behnken design, 29 experiments contained the effects of independent variables: initial dye concentration (x_1), enzyme amount (x_2), pH (x_3), and reaction time (x_4) were systematically investigated. The levels of factors used in this study for the responses of decolorization of RB49 were shown in Table 6.

The first degree effects of factors, except initial dye concentration, and the second degree effects of all factors were significant. However, x_1 factor was not omitted from the model. By eliminating the insignificant model terms, the reduced second-order polynomial function for dye decolorization (Y) in terms of coded independent variables was presented in Eq. (2):

$$Y_{(\%dye\ decolorization)} = 87.33 + 1.82\ x_1 + 11.55\ x_2 - 41.68\ x3 + 14.82\ x_4 - 6.48\ x_1^2 - 16.93\ x_2^2 - 29.43\ x_3^2 - 12.05\ x_4^2 \quad (2)$$

where x_1, x_2, x_3, x_4 are initial dye concentration, enzyme amount, pH and reaction time, respectively.

The quadratic model was suggested given in Eq. (2) with the value of R^2 (0.9455). The experimental data of the predicted R^2=0.8790 was in a good agreement with the adjusted R^2=0.9237. According to ANOVA given in Table 7, a high F-value (43.39) and a very low probability ($p<0.0001$) indicates that the model adequately predicts the experimental results. The ANOVA results confirmed a satisfactory adjustment of the reduced quadratic model to the experimental data.

The three dimensional surface plots are for the graphical representation of regression equations. Figure 2 (a)-(f) showed the interactions between variables, while the other two were kept constant at their central values. For all the interaction of variables, actual factors were enzyme amount 0.53 mL, pH 4.5, 62.5 min of reaction time and 62.5 mg L^{-1} of initial dye concentration. Figure 2 (a) showed that decolorization increased with the increase in enzyme amount up to one point. Surface plot of 1 (b) showed that dye decolorization did not change with increase of dye concentration while decolorization increased with decreasing of pH value considerably. An analysis of Figure 2 (c) indicated that decolorization increases with increasing of reaction time and dye decolorization did not change with increase of dye concentration. Figure 2 (d) represented the effect of pH and enzyme amount on the dye decolorization and its surface plot showed the increase in dye decolorization with decrease on pH value while decolorization increased with increase in enzyme amount. Figure 2 (e) illustrated that increase in both reaction time and enzyme amount provided an increase of decolorization in the response surface. It could be seen from Figure 2 (f) that decreasing the pH and increasing the reaction time increased decolorization.

One of the important parameters for dye decolorization is pH of the decolorization reaction. The optimum pH value for dye decolorization was detected as 2.95. Nyanhongo et al. [41] found that pH 4.5 for Acid Blue 225 and Reactive Black 5 and pH 3.0–4.0 for Acid Violet 17; pH 3.0–4.5 for Basic Red 9 for decolorization [41]. Enzyme concentration was an important parameter for optimal decolorization. In our study, the optimum enzyme amount was obtained as 0.76 mL. Li et al. found that enzyme amount as 0.8 mL for enzymatic decolorization of Congo red by manganese peroxidase [42]. The optimal initial dye concentration for dye decolorization was determined as 55.6 mg L^{-1}. Lu and coworkers reported that initial dye concentration for decolorization was obtained as 100 mg L^{-1} [43]. In this study, the maximum decolorization percentage was observed 46.91 min.

Under the optimal conditions, 97.53% of decolorization was experimentally achieved for RB49, which is in good agreement with the decolorization predicted by the model.

Conclusion

According to the statistical analysis, Taguchi's technique revealed that yeast extract is the most significant factor and pH has the lowest impact on the laccase production. The optimum conditions for laccase production were obtained on the first level of glucose (2.0 g L^{-1}), the third level of yeast extract (5.0 g L^{-1}), the second level of $CuSO_4$ (2.0 mM), the second level of inoculum amount (4.0%) and third level of pH (5.5). The results suggested that Taguchi method can be used in the optimization of laccase production process.

The predicted results showed that the maximum removal efficiency (98.03%) of Reactive Blue 49 could be obtained under the optimum conditions of pH 2.95, initial dye concentration 55.6 mg L^{-1}, enzyme amount 0.76 mL, reaction time 46.91 min. Experimentally achieved decolorization of 97.53 %, was within the confidence interval (89.83; 106.24) which was within the predicted optimum of maximum removal efficiency. According to the verification experiments conducted in optimal levels, actual and predicted responses were in a good agreement.

Conflict of Interest

There are no conflicts of interest among the authors.

References

[1] Thurston CF. The structure and function of fungal laccases. Microbiology 1994; 140:19-26.

[2] Eriksson KEL, Blanchette RA, Ander P. Microbial and enzymatic degradation of wood and wood components 1990, Springer-Verlag, Berlin.

[3] Duran N, Esposito E. Potential application of oxidative enzymes and phenoloxidases like compounds in wastewater and soil treatment: a review. Appl Catal B Environ 2000; 28:83-99.

[4] Cohen R, Persky L, Hadar Y. Biotechnological applications and potential of wood degrading mushrooms of the genus *Pleurotus*. Appl Microbiol Biotechnol 2002; 58:582-594.

[5] Bajpai P. Application of enzymes in the pulp and paper industry. Biotechnol Prog 1999; 15:147-157.

[6] Shah V, Nerud F. Lignin degrading system of white-rot fungi and its exploitation of dye decolorization. Can J Microbio. 2002; 48:857-870.

[7] Yesilada O, Asma D, Cing S. Decolorisation of textile dyes by fungal pellets. Process Biochem 2003; 38:933-938.

[8] Karam J, Nicell JA. Potential applications of enzymes in waste treatment. J Chem Tech Biotechnol 1997; 69:141-153.

[9] Aytar P, Gedikli S, Şam M, Ünal A, Çabuk A, et al. Desulphurization of Some Low-rank Turkish Lignites with Crude Laccase Produced from *Trametes versicolor* ATCC 200801. Fuel Process

Technol 2011; 92:71-76.

[10] Paszczynski A, Crawford RL. Potential for bioremediation of xenobiotic compounds by the white-rot fungus *Phanerochaete chrysosporium.* Biotechnol Prog 1995; 11:368-379.

[11] Reddy CA. The potential for white-rot fungi in the treatment of pollutants. Curr Opin Biotechnol 1995; 6:320-328.

[12] Çabuk A, Sıdır YG, Aytar P, Gedikli S, Sıdır I. Dechlorination of some chlorinated compounds by *Trametes versicolor* ATCC 200801 crude laccase and quantitative structure-activity relationships analysis on toxicity. J Environ Sci Heal A 2012; 47:1938-1947.

[13] Rodriguez Couto S, Herrera JLT. Industrial and biotechnological applications of laccases: A review. Biotechnol Adv 2006; 24:500-513.

[14] Unyayar A, Demirbilek M, Turkoglu M, Celik A, Mazmancı MA, et al. Evaluation of cytotoxic and mutagenic effects of *Coriolus versicolor* and *Funalia trogii* extracts on mammalian cells. Drug Chem Toxicol 2006; 29:69-83.

[15] Gianfreda L, Xu F, Bollag J. Laccase: A useful group of oxidoreductive enzymes. Bioremed J 1999; 3:1–26.

[16] Sadhasivam S, Savitha S, Swaminathan K, Lin F. Production, purification and characterization of mid–redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochem 2008; 43:736-742.

[17] Revankar MS, Lele SS. Enhanced production of laccase using a new isolate of white rot fungus WR-1. Process Biochem 2006; 41:581–588.

[18] Gedikli S, Aytar P, Ünal A, Yamaç M, Çabuk A, Kolankaya N. Enhancement with inducers of laccase production by some strains and application of enzyme to dechlorination of 2,4,5–trichlorophenol. Elect J Biotech 2010; 13(6):1-15.

[19] Birhanli E, Yesilada O. Increased production of laccase by pellets of Funalia trogii ATCC 200800 and *Trametes versicolor* ATCC 200801 in repeated-batch mode. Enzyme Microb Tech 2006; 39:1286-1293.

[20] Apohan E, Yesilada O. Enhancement of laccase production of pregrown fungal pellets in wastewater of olive oil mills. Fresen Environ Bull 2011; 20:1216-1224.

[21] Pazarlıoglu NK, Sarisik M, Telefoncu, A. Laccase: production by *Trametes versicolor* and application to denim washing. Process Biochem 2005; 40:1673-1678.

[22] Arora DS, Gill PK. Effects of various media and supplements on laccase production by some white rot fungi. Bioresour Techno 2001; 77:89-91.

[23] Prasad, KK, Mohan SV, Pati BR, Sarma PN. Laccase production by immobilized *Pleurotus ostreatus* 1840: effect of process parameters and its purification. Commun Proc Biochem 2005; 24:17-26.

[24] Joseph J, Piganatiells JR. An overview of the strategy and tactics of Taguchi. IIE Trans 1988; 20:247-53.

[25] Willmott N, Guthrie J, Nelson G. The biotechnology approach to colour removal from textile effluent. J Soc Dyers Colour 1998; 114:38-41.

[26] Yesilada O, Sik S, Sam M. Biodegradation of olive oil mill wastewater by *Coriolus versicolor* and *Funalia trogii:* Effects of agitation, initial COD concentration, inoculum size and immobilization. World J Microbiol Biotechnol 1998; 14:37-42.

[27] Birhanli E, Yesilada O. Enhanced production of laccase in repeated-batch cultures of *Funalia trogii* and *Trametes versicolor*. Biochem Eng J 2010; 52:33-37.

[28] Sasmaz S, Gedikli S, Aytar P, Güngörmedi G, Çabuk, A, et al. Decolorization potential of some reactive dyes with crude laccase and laccase mediated system. Appl Biochem Biotech 2011; 163:346-361.

[29] Tavares APM, Coelho MAZ, Coutinho JAP, Agapito MSM, Xavier AMRB. Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. Appl Biochem Biotech 2006; 134:233-248.

[30] Taguchi G, Yokoyama Y, Wu Y. Taguchi Methods/Design of Experiments 1993, American Supplier Institute (ASI) Press, Tokyo.

[31] Montgomery DC. Design and Analysis of Experiments. 2005; pp 394-395, 6th ed, New York: John Wiley & Sons.

[32] William YF, Creveling MC. Engineering methods for robust product design 1995, MA: Addison-Wesley.

[33] Yang K, El-Haik BS. Design for six sigma: A roadmap for product development 2009, 2nd. The McGraw-Hill Companies, Inc.

[34] Amini M, Younesi H. Biosorption of Cd(II), Ni(II) and Pb(II) from aqueous solution by dried biomass of *Aspergillus niger*: Application of response surface methodology to the optimization of process parameter. Clean 2009; 37:776-786.

[35] Mikiashvili N, Wasser SP, Nevo E, Eliashvili V. Effects of carbon and nitrogen sources on *Pleureotus ostreaus* ligninolytic enzyme activity. World J Microb Biot 2006; 22:999-1002.

[36] Acherjee B, Kuar AS, Mitra S, Misra D. Application of grey-based Taguchi method for simultaneous optimization of multiple quality characteristics in laser transmission welding process of thermoplastics. Int J Adv Manuf Technol 2011; 56:995-1006.

[37] Maria AMV, Paolo S, Burla G, Buonocore V. Differently induced extracellular phenol oxidase from *P. ostreatus*. Phytochem 1992; 31:3685–3690.

[38] Ardon O, Karem Z, Hadar Y. Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. J Biotechnol 1996; 51:201-207.

[39] Periasamy R, Palvannan T. Optimization of laccase production by Pleurotus ostreatus IMI 395545 using the Taguchi DOE methodology. J Basic Microb 2010; 50:548-556.

[40] Collins P, Dobson A. Regulation of laccase gene transcription in *Trametes versicolor.* Appl Environ Microbiol 1997; 63:3444–3450.

[41] Nyanhongo GS, Gomes J, Gubitz GM, Zvauya R, Read J, Steinera W. Decolorization of textile dyes by laccases froma newly isolated strain of *Trametes modesta.* Water Res 2002; 36:1449-1456.

[42] Li X, Jia R, Li P, Ang S. Response surface analysis for enzymatic decolorization of Congo red by manganese peroxidase. J Mol Catal B: Enzym 2009; 56:1–6.

[43] Lu L, Zhao M, Zhang BB, Yu SY, Bian XJ, et al. Purification and characterization of laccase from *Pycnoporus sanguineus* and decolorization of an anthraquinone dye by the enzyme. Appl Microbiol Biotechnol 2007; 74:1232-1239.