Vol.53, n. 6: pp. 1503-1510, November-December 2010
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Dye decolorizing potential of a novel fungus *Coriolus versicolor* ML04 in the medium optimized by response surface methodology

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ABSTRACT

The potential of the white rot fungus, Coriolus versicolor *ML04 to decolorize the widely used textile dye Blue BB was tested by employing statistical optimization. Response surface methodology (RSM) involving a central composite design (CCD) was applied to evaluate the interactive effects of four significant factors in different ranges i.e., glucose (0.5 – 2.5 g/L), yeast extract (0.4 –1.2 g/L), dye concentration (100 – 500 ppm) and inoculum size (5 – 20 % v/v) to decolorize the Blue BB. The results demonstrated the effectiveness of the statistical experimental design and the ability of* C. versicolor *ML04 for maximum dye decolorization (>96%) at the optimum conditions of the significant factors.*

Keywords: Blue BB; *Coriolus versicolor* ML04; Plackett – Burman design; central composite design

INTRODUCTION

Synthetic dyes are extensively used in textile, leather, food, cosmetics, pharmaceutical and paper industries and more than 7 x 10^5 tons of these dyes are produced annually worldwide (Keck *et al.*, 1997). As most of the synthetic dyes are of chemical contents, discharge of such dyes into environment poses significant environmental problems. One of the environmental problems is the contamination of water sources by dyestuff effluents discharged by industries, which results in immense health predicament to all life forms. Various methods of treatment of such dye contaminated water sources are now being attempted to prevent the deterioration of our ecosystem

A number of physicochemical methods, such as adsorption, coagulation, precipitation, filtration, and oxidation, have been used to treat the dyestuff effluents, but these methods have many limitations. Alternately, the biological methods are of great value as such methods are inexpensive, ecofriendly and have less sludge producing properties. Currently, extensive research is focused to find optimal microbial biomass, which is as cheap as possible for the removal of contaminating dyes from large volumes of polluted water (Jadhav and Govindwar, 2006). For bioremediation of synthetic dye effluents, several microorganisms, including bacteria and fungi can be employed.

Many microorganisms have been reported for their ability to decolorize the dyes (Chang *et al.*, 2001; Khehra *et al.*, 2005). Anaerobic and

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microaerophilic microorganisms have been found to reduce the dye bonds, non specifically in anaerobic conditions leading to dye decolorization (Coughlin *et al.,* 1999). Among the microorganisms, white-rot fungi are the most intensively studied dye decolorizing microorganisms (Wesenberg *et al.,* 2003). Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites and for their capacities to adapt to severe environmental constraints. Beyond the production of such relevant metabolites, fungi have been attracting a growing interest for the biotreatment of waste water. Fungi with their ligninolytic enzyme systems are also being applied in the biological decolorization of textile dyestuff effluents (Lacina *et al.,* 2003). *Coriolus versicolor* showed varying decolorizing capacity to remove dyes from industrial effluents (Wesenberg *et al.,* 2003).

The objectives of this study were to screen a potent fungus capable of degrading the dye Blue BB and optimize the medium conditions and factors through response surface methodology for maximum dye decolorization.

MATERIALS AND METHODS

Dyes and chemicals

The dye Blue BB, a commercial grade dye was locally procured and utilized.

Organisms and culture conditions

The fungal strain was isolated from the textile dye contaminated soil collected within the premises of a textile industry and it was identified as *Coriolus versicolor* ML04. The culture was grown at 30 ^{o}C in Sabouraud's dextrose broth (g/L: Peptone, 10; Dextrose, 40) and was subjected as a biological agent to test the decolorization of the dye Blue BB.

Decolorization experiments

Preliminary experiments for dye decolorization were carried out in triplicate in 100 ml Erlenmeyer flasks. In each flask, a dye (Blue BB) concentration of 50 mg/L was taken as standard concentration at 30 ^{o}C and pH 5.0. Four millimeter of fungal mycelium was inoculated using cork borer into each flask. A control flask with all the components, except the fungal mat was maintained in parallel to obtain the abiotic decolorization, if any. All the experiments as well as controls were run in triplicate. The flasks were incubated at 28 ^{o}C for five days. Four milliliter of mixed liquor was drawn from the flask at 24 h interval and was centrifuged at 10,000 rpm for 10 min to separate the fungal biomass. Dye (Blue BB) clearance from the culture fluid (supernatant) was monitored by assaying at A_{600}.

Experimental design

Screening of important nutrient components using Plackett – Burman design

The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-) (Plackett and Burman, 1946). The experimental design and levels of each variable are shown in Table 1. The medium was formulated as per the design and the flask culture experiments for dye decolorization were assayed as described earlier (2.3). Response was calculated as the rate of dye decolorization and expressed as % decolorization. All the experiments were performed in triplicate and the average of the rate of the decolorization was considered as the response.

The effect of each variable was calculated using the following equation:

$$E = (\sum M_{+} - \sum M_{-}) / N$$

Optimizaion of the screened medium components using response surface methodology

The screened medium components affecting the dye decolorization were optimized using central composite design (CCD) (Box & Wilson, 1951; Box & Hunter, 1957). Four important parameters, i.e., concentration of glucose (X1), yeast extract (X2), dye (X3) and inoculum size (X4), were screened from Plackett – Burman design as the independent variables and percentage of decolorization was the dependent response variable. Each of the four independent variables was studied at five different levels as per CCD in a total of 30 experiments. The percentage of dye decolorization corresponding to combined effects of four variables was studied in their specified ranges: glucose: 0.5 – 2.5 (g/L), yeast extract: 0.4 –1.2 g/L, dye concentration: 100 – 500 ppm and inoculum size: 5 – 20 (% v/v). The other two process variables, pH and temperature were kept constant at 6 and 30 ^{o}C, respectively throughout the 30 experiments. The plan of CCD in coded

levels of the four independent variables is as shown in Table 3.

According to this design, the total number of treatment combinations was $2^k + 2k + n0$ where '*k*' was the number of independent variables and *n*0 the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i were coded as x_i according to the following transformation:

$$x_i = X_i - X_0 / \delta X$$

Validation of the experimental model

The statistical model was validated with respect to dye decolorization under the conditions predicted by the model in flask conditions. The samples were withdrawn at the desired intervals and dye decolorization assay was determined as described above.

RESULTS AND DISCUSSION

The new isolate *C. versicolor* ML04 was able to decolorize the dye Blue BB using it as the sole source of carbon and energy. While performing the medium optimization studies for obtaining maximum decolorization, glucose was the best carbon source amongst various carbon sources tested, supporting the maximum decolorization (Mohana *et al.*, 2007).

The interaction of eleven medium factors namely lactose, glucose, $(NH_4)_2SO_4$, NH_4NO_3, tryptone, yeast extract, dye concentration, inoculum size, incubation period, $CaCl_2$ and $MnSO_4$ in dye decolorization investigated in 12 runs using Plackett – Burman design is presented in Table 1. The data indicated a wide variation in the dye decolorization, ranging from 31.40 to 79.36 %. This variation reflected the effect of the interaction among the variables in the dye decolorization. Among the variables screened, the most effective factors with high significance level were in the order of glucose, yeast extract, dye concentration and inoculum size. They were selected for further optimization.

The statistical analysis of the Plackett – Burman design (Table 2) demonstrated that the model F – value of 0.71 was significant. The *p*-value < 0.05 indicated that the model terms were significant.

Table 1- Plackett – Burman experimental design for evaluating factors influencing dye degradation by *Coriolus versicolor* ML04

Run	Lactose (g/L)	Glucose (g/L)	$(NH_4)_2SO_4$ (g/L)	NH_4NO_3 (g/L)	Tryptone (g/L)	Yeast (g/L)	Dye conc (ppm)	Inoculum size (% v/v)	Incubation period (h)	$CaCl_2$ (g/L)	$MnSO_4$ (g/L)	Decolorization (%)
1	3	0.5	2	2	2	0.25	100	5	96	0	0.1	78.32
2	0.5	3	0.25	2	2	0.25	500	20	96	0	0	45.54
3	3	0.5	0.25	0.25	2	0.25	500	20	24	0.1	0.1	38.93
4	0.5	0.5	2	0.25	2	2	100	20	96	0.1	0	68.22
5	3	3	0.25	2	2	2	100	5	24	0.1	0	79.36
6	3	3	0.25	0.25	0.25	2	100	20	96	0	0.1	64.53
7	3	3	2	0.25	0.25	0.25	500	5	96	0.1	0	31.40
8	0.5	3	2	2	0.25	0.25	100	20	24	0.1	0.1	61.03
9	0.5	0.5	0.25	0.25	0.25	0.25	100	5	24	0	0	74.92
10	3	0.5	2	2	0.25	2	500	20	24	0	0	33.76
11	0.5	0.5	0.25	2	0.25	2	500	5	96	0.1	0.1	49.32
12	0.5	3	2	0.25	2	2	500	5	24	0	0.1	47.05

Table 2 - Analysis of variance for dye decolorization by *Coriolus versicolor* ML04.

Source	Sum of square	Degree of freedom	Mean square	F – Value	*p* - Value	
Model	8451.06	7	1334.44	0.7183	0.0029	significant
B-Glucose	232.856	1	287.856	6.53482	0.0589	
F-Yeast extract	5863.55	1	5728.55	135.365	0.0003	
G-Dye concentration	1325.89	1	1275.89	31.52324	0.0047	
H-Inoculum size	7.2383	1	7.23753	0.17001	0.7012	
Residual	130.307	4	52.5768			
Cor Total	8531.36	11				

CV – 6.72; R^2 – 0.98

The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of R^2 (0.98) closer to 1 denoted better correlation between the observed and predicted responses. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low CV (6.72) denoted that the experiments performed were highly reliable.

From the analysis, it was inferred that the dye decolorization was supported by glucose, yeast extract, dye concentration and inoculum size as shown by their F-values and *p*-values. Therefore, these variables were considered as highly significant for dye decolorization by *C. versicolor* ML04 and were further investigated with central composite design to find the optimal range of these variables.

Yeast extract has been the most commonly used nitrogen source for dye decolorization process. Many microbial cultures have exhibited effective decolorization of different dyes in presence of yeast extract (He *et al.*, 2004; Moosvi *et al.*, 2005; Khehra *et al.*, 2005). The temperature required to produce the maximum rate of color removal tends to correspond with the optimum cell culture growth temperature of 35 – 45^{o}C (Pearce *et al.*, 2003). On the basis of these reports, SDB medium containing glucose (0.15 %) and yeast extract (0.80 %) as cosubstrates with growth at 30 ^{o}C were used. These culture conditions resulted in 78.32 % decolorization of the dye within 48 h. Further incubation did not enhance the decolorization. These results led to plan and carry out a systematic study of dye decolorization process, hence, proceeded with central composite design. The main objective was to determine the optimum operational conditions for the variables or to determine a region that satisfied the operating specifications (Ravikumar *et al.*, 2006).

Central composite design

The result of 30 run CCD for four variables, glucose concentration, yeast extract concentration, dye concentration and inoculum size chosen for optimization of dye decolorization process are shown in Table 3. It showed the percent of dye decolorization corresponding to combined effect of four components in their specific ranges. The decolorization varied markedly ranging from 32.82 – 96.21 % in the conditions tested. At high concentration of yeast extract and dye, and low concentration of glucose and inoculum size (run 9), lowest % of decolorization was observed. The decolorization values above 95 % were observed when high concentration of glucose and inoculum size, and low concentration of dye and yeast extract were used (run 30). The experimental results suggested that these variables strongly affected the decolorization process.

The results obtained from the central composite design were fitted to a second order polynomial equation to explain the dependence of decolorization on the medium components.

$$Y = +91.33 + 1.08\ A + 1.08\ B - 2.67\ C + 1.25\ D + 5.75\ AB + 2.63\ AC - 2.00\ AD - 0.62\ BC - 1.75\ BD - 2.37\ CD - 3.79\ A^2 - 1.04\ B^2 - 12.79\ C^2 - 17.29\ D^2$$

Where Y is the predicted response (% decolorization), A, B, C and D are the coded values of glucose concentration, yeast extract concentration, dye concentration and inoculum size respectively.

The analysis of variance of the quadratic regression model suggested that the model was very significant as was evident from the Fisher's F – test (Table 4). The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of R^2 (0.79) closer to 1 denoted better correlation between the observed and predicted responses. The low CV (3.45) denoted that the experiments performed were highly reliable. The *p*-values denoted the significance of the coefficients and also the importance in understanding the pattern of the mutual interactions between the variables. The choices for level combinations of the four variables; glucose, yeast extract, inoculum size and dye concentration could be made easily from contour plots and response surface curves (Fig. 1).

Table 3 - Central composite design for decolorization of Blue BB by *Coriolus versicolor* ML04.

Run	Glucose (g/L)	Yeast (g/L)	Dye conc (ppm)	Inoculum size (% v/v)	Decolorization (%)	
					Experimental	Predicted
1	0	0	0	0	95.43	91.33333
2	0	0	2	0	43.23	34.83333
3	-1	-1	-1	-1	56.64	57.29167
4	-2	0	0	0	78.02	74
5	-1	-1	-1	1	63.54	72.04167
6	1	1	1	1	54.12	58.79167
7	0	0	0	0	89.35	91.33333
8	1	1	-1	-1	42.98	46.70833
9	-1	1	1	-1	32.82	45.625
10	-1	1	-1	-1	68.81	52.70833
11	1	-1	1	-1	49.02	52.625
12	2	0	0	0	76.26	78.33333
13	-1	-1	1	-1	68.02	52.70833
14	0	0	-2	0	39.05	45.5
15	1	1	1	-1	92.32	88.54167
16	-1	-1	1	1	45.91	57.95833
17	0	0	0	0	94.94	91.33333
18	1	-1	1	1	49.81	49.875
19	-1	1	1	1	43.03	43.875
20	0	0	0	0	94.09	91.33333
21	0	0	0	0	90.33	91.33333
22	0	2	0	0	87.38	89.33333
23	1	-1	-1	-1	62.3	65.125
24	0	0	0	-2	0	0
25	0	-2	0	0	89.73	85
26	1	1	-1	1	64.89	64.875
27	0	0	0	2	46.32	44.66667
28	-1	1	-1	1	58.01	60.45833
29	1	-1	-1	1	51.05	53.45833
30	0	0	0	0	96.21	91.33333

Table 4 - ANOVA of % decolorization for Blue BB: effect of glucose, yeast extract, dye concentration and inoculum size.

Source	Sum of square	Degree of freedom	Mean square	F – Value	P- Value	
Model	12510.8	14	893.627	4.04427447	0.0055	significant
A-Glucose	28.1667	1	28.1667	0.12747341	0.7260	
B-Yeast	28.1667	1	28.1667	0.12747341	0.7260	
C-Dye concentration	170.667	1	170.667	0.77238328	0.3933	
D-Inoculum size	37.5	1	37.5	0.16971312	0.6862	
AB	529	1	529	2.39408644	0.1426	
AC	110.25	1	110.25	0.49895658	0.4908	
AD	64	1	64	0.28964373	0.5983	
BC	6.25	1	6.25	0.02828552	0.8687	
BD	49	1	49	0.22175848	0.6445	
CD	90.25	1	90.25	0.40844291	0.5324	
A^2	394.333	1	394.333	1.78462776	0.2015	
B^2	29.7619	1	29.7619	0.13469295	0.7187	
C^2	4488.05	1	4488.05	20.3114819	0.0004	
D^2	8201.19	1	8201.19	37.1159904	< 0.0001	
Residual	3314.42	15	220.961			
Lack of Fit	3111.08	10	311.108	7.65020492	0.0183	significant
Pure Error	203.333	5	40.6667			
Cor Total	15825.2	29				

CV – 3.45; R^2 – 0.79.

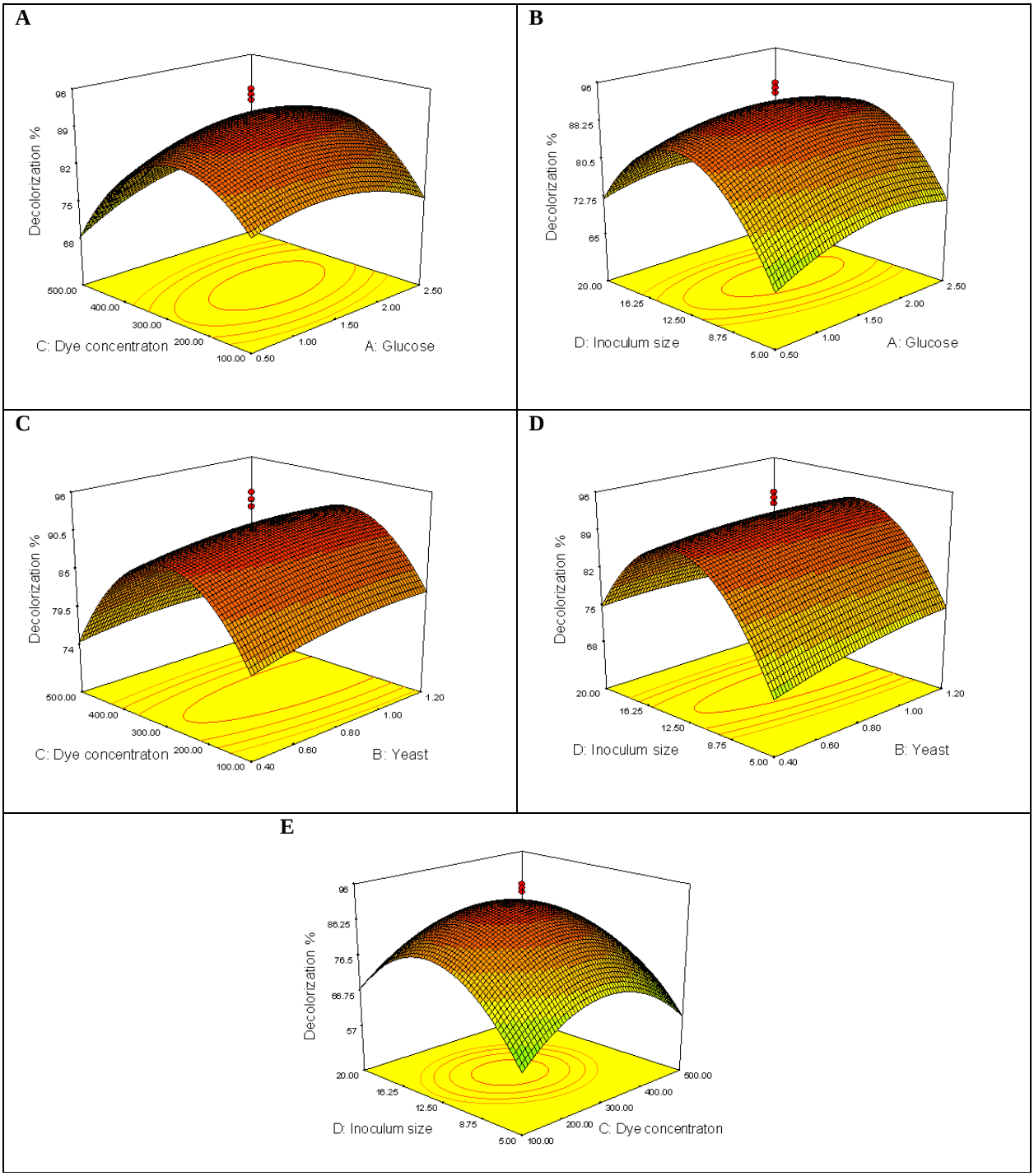

Figure 1 - Response surface and contour plot of the combined effect of (A) glucose, dye concentration; (B) glucose, inoculum size; (C) yeast extract, dye concentration; (D) yeast extract, inoculum size; (E) dye concentration, inoculum size on dye decolorization by *Coriolus versicolor* ML04.

Pearce *et al.* (2003) reported that the dye concentration could influence the efficiency of decolorization through a combination of factors, including the toxicity of the dye. Low decolorization efficiency at high dye stuff concentration has been reported for different cultures such as *Kruthia* sp. (Sani & Banerjee, 1999) and *Pseudomonas aeruginosa* NBAR12 (Bhatt *et al.*, 2005). Both, glucose and the inoculum size had synergistic as well as antagonistic effect on maximizing the decolorization. Initially with increase in concentration of glucose and inoculum size, there was increase in the decolorization, but higher concentrations were found to be inhibitory. Knapp and Newby (1995) and Chen *et al.* (2003) have also reported decrease in dye decolorization efficiency at high glucose concentrations. Therefore, the maximum decolorization was achieved at the optimum concentration of the two variables (glucose and inoculum size). Bhatt *et al.* (2005) observed decrease in dye decolorization efficiency at high concentration of yeast extract. Hence, a proper choice of level combination of glucose and yeast extract is desirable for maximizing the decolorization. The nutrient requirement for optimum decolorization depends on the nature of the microbial species employed.

This study demonstrated the potential of the fungus *C. versicolor ML04* to be exploited for the dye decolorization and degradation and effect of statistical optimization design of the medium factors for this process.

CONCLUSIONS

The application of *C. versicolor* ML04 to decolorize the synthetic dye Blue BB seemed to be one of a pragmatic approach. This study showed that the response surface methodology was an appropriate method to optimize the culture conditions for obtaining the maximum decolorization of the dye. By applying the central composite design and RSM to the optimization experiments, the process variables were investigated to achieve the maximum decolorization of 96.21 %. The experimental and predicted values were very close, which reflected the accuracy and the applicability of RSM. Moreover, the ability of *C. versicolor* ML04 to decolorize Blue BB of more than 96 % indicated its potential for decolorizing the dyeing effluents.

ACKNOWLEDGEMENTS

The authors are thankful to the authorities of Bharathiar University, Coimbatore, Tamil Nadu, India for providing the infrastructure facilities for this study.

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Received: May 18, 2009;
Revised: January 06, 2010;
Accepted: April 14, 2010.