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Enhanced Biodecolorization of Azo Dye Reactive Orange 16 by Immobilized Phanerochaete Chrysosporium; Optimization of Immobilization Factors

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ABSTRACT

Processes using immobilized growing cells seem to be more promising than those with free cells, since the immobilization allows using the microbial cells repeatedly and continuously. Furthermore, the lignolytic system of Phanerochaete Chrysosporium fungi which is responsible for degradation of industrial dyes can be preserved from shear stress by cell immobilization. In this study, P. Chrysosporium was used for decolorization of azo dye Reactive Orange 16. Calcium alginate biogel was selected as a carrier and Na-alginate concentration (1% and 3% w/v), $CaCl_2$ concentration (10% and 30% w/v), bead diameter (2 and 4 mm) and inoculum size (10^5 and 10^7 spores/l) were considered as the immobilization parameters to be optimized. Full factorial design of experiments was used to estimate each possible independent and interactive effect of mentioned parameters on three responses of biodecolorization percent, lignin peroxidase and manganese peroxidase activities. Under optimum immobilization conditions, lignin and manganese peroxidase activities and decolorization of 324 U/L, 175 U/L and 87% after 2 h were respectively obtained that were at least 8 fold higher than those achieved at center point. The results indicated that decolorization was strongly in accordance with the production of lignolytic enzymes.

Keywords: Biodecolorization, Immobilization, Design of experiments (DOE), Phanerochaete Chrysosporium, Azo dye.

INTRODUCTION

There are about 10,000 different dyes and pigments, produced annually worldwide and used extensively in the dye, textile and printing industries. A necessary criterion for the use of these dyes is that they must be highly stable in light and during washing. They must also be resistant to microbial attacks[1]. Therefore, they are not readily degradable and are typically not removed from water by conventional waste water treatment systems. Azo dyes, characterized by the unsaturated azo bond -N=N-, represent a major proportion of total dye use today [2].

The lignin-degrading system of the white rot fungus is able to degrade a wide range of structurally diverse organic pollutants[2, 3]. Azo dyes are generally considered to be non-biodegradable under aerobic

conditions, for example reactive orange 16 is a commercial azo dye that hard to be degraded by some conventional treatment methods [4]. Nevertheless, the non-specific nature of the ligninolytic enzymes renders white rot fungus suitable for treating azo dyes effectively due to the similarity of structures of azo dyes and lignin [5]. The lignin degradation capability of white rot fungus is due to their extracellular non-specific and nonstereoselective enzyme system composed by laccases, lignin peroxidases (LiP) and manganese peroxidases (MnP), which function together with H_2O_2- producing oxidases and secondary metabolites[6]. These extra cellular peroxidases, are non-specific towards the substrate, can also attack some recalcitrant chemicals of diverse structures, including azo dyes[7, 8]. A majority of the previous studies have focused on the lignin-degrading enzymes of P. chrysosporium, the most well-known strain of white rot fungus and recently, the application of immobilized cells has been receiving increasing attention in the field of wastewater decolorization. Many researchers have studied the effect of immobilized cells on decolorization characteristics, since immobilization provides distinct stability over free cells[8, 9]. Removal of toxic dyestuffs by Ca-alginate beads immobilized MnP showed that this system is also efficient [10]. Although biodegradation of industrial dyes can be employed by immobilized lignolytic enzymes, but this approach is more expensive and difficult than cell immobilization system.

Earlier studies showed that peroxidase activity of P. chrysosporium is inhibited by agitation. Thus, systems which minimize intensive shear both to the organism and the enzyme and also provide a growth support for the controlled growth of the organism are most likely to be successful in the consistent cultivation of P. chrysosporium and the production of lignin peroxidases. Immobilization of fungus cells can provide a suitable low-shear environment for ligninolytic system of the fungus [11-13]. In this study we used calcium alginate biogel as a suitable carrier to immobilize P. chrysosporium spores in order to cope with high shear stress in bioreactors. Dye decolorization ability of the immobilized fungus was studied adopting a full factorial design of experiments to analyze the affectivity of the system under different conditions of Na-alginate concentration, $CaCl_2$ concentration, and inoculum size and bead diameter. The LiP and MnP activities were also determined in order to find the effect of these factors on the enzymatic decolorization of the immobilized fungal cells.

MATERIALS AND METHODS

Microorganism and culture media: The fungus P. chrysosporium (RP78) was inoculated on potato dextrose agar and incubated at 35^{o}C for 3 days, when extensive spore growth occurred. Liquid nitrogen limited culture prepared as described by Tien and kirk[14].

Cell immobilization and biodecolorization: The basic method of immobilization of fungal spores in Ca-alginate beads is to mix the spores with Na-alginate to a uniform suspension and dropping of this suspension to $CaCl_2$ solution. P. chrysosporium were grown into the stationary phase in PDA plates. Spore suspension containing 10^5-10^7 cells L^{-1} was added to 100 mL of 1-3% sodium alginate. The mixture was gently stirred at room temperature to produce a uniform suspension and then dropped into 100 mL of 10-30% calcium chloride solution. Nozzles with different diameters were used to form beads of 1-4 mm diameter size. The beads so obtained were stored in 30% calcium chloride solution at 4^{o}C for 2 h to complete gel formation. Immobilized spores transferred to flasks each of them containing 40 mL nitrogen limited culture medium and incubated on an orbital shaker at 110 rpm and 30^{o}C for 7 days. The azo dye, Reactive Orange 16 (306509-100g Sigma-Aldrich) was added to each flask with concentration of 20 mg L^{-1}, after 7 days of cultivation.

Experimental design: Four factors each at two levels were considered (Table 1); Na-alginate concentration (1% and 3% w/v), $CaCl_2$ concentration (10% and 30% w/v), inoculum size (10^5 and 10^7 spores/L) and bead diameter (2 mm and 4 mm). Each of the variables was studied at two different coded levels (-1, +1) and center-point (0) which is the midpoint of each factor range. Full factorial design of experiments (FFD) containing 16 experiments were employed, four center points were also added which made the number of trials 20 in order to estimate the error and model curvature according to scheme mentioned in table 2. The effect of mentioned variables on three responses consisted of decolorization efficiency and LiP and MnP activity were investigated. All experiments were carried out in duplicates for validation of data. Design expert software (Stat-Ease, 7.0 trial version, USA) was used for regression and graphical analysis of the data obtained.

Table 1. Coded and actual levels of the independent variables

oded values	A: inoculum size (spores/L)	Na-Alginate concentration (%w/v)	C: bead size (mm)	: $CaCl_2$ concentration (%w/v)
1	10^7	3	4	30
0	10^6	2	3	20
-1	10^5	1	2	10

Table 2. The full factorial design matrix for four independent variables along with observed responses

	Coded values of the variables				Mean observed response		
trial	inoculum size (A)	Na-Alginate (B)	ead size (C)	$CaCl_2$ (D)	Decolorization (%)	LiP (U/L)	MnP (U/L)
1	-1	-1	-1	-1	65.0±7.5	177.5±7.5	141.5±8.5
2	1	-1	-1	-1	42.5±3.5	126.0±10.0	112.5±18.5
3	-1	1	-1	-1	7.0±1.2	29.5±3.5	19.5±1.5
4	1	1	-1	-1	87.0±5.0	206.0±4.0	176.0±12.0
5	-1	-1	1	-1	7.0±2.0	24.0±2.1	5.5±1.5
6	1	-1	1	-1	21.0±3.0	53.0±5.3	17.5±0.5
7	-1	1	1	-1	10.5±1.5	26.5±4.0	15.5±2.5
8	1	1	1	-1	84.0±6.0	192±4.3	167.0±13.0
9	-1	-1	-1	1	7.5±2.0	19.5±5.1	4.5±1.5
10	1	-1	-1	1	5.0±1.0	20.0±2.0	6.0±1.0
11	-1	1	-1	1	65.5±8.5	180.0±15.5	133.0±12.0
12	1	1	-1	1	10.5±1.5	31.0±5.2	14.0±3.0
13	-1	-1	1	1	1.0±0.5	10.5±1.5	2.5±0.5
14	1	-1	1	1	11.0±2.0	35.5±6.5	20.0±3.0

15	-1	1	1	1	10.7±2.25	26.0±4.6	8.0±1.0
16	1	1	1	1	16.5±3.5	45.0±4.2	23.0±2.0
17	0	0	0	0	10.5±0.5	25.5±0.5	16.0±1.0
18	0	0	0	0	9.6±0.3	23.5±1.2	19.0±2.5
19	0	0	0	0	10.4±0.7	27.4±0.6	15.0±4.0
20	0	0	0	0	10.2±0.5	24.8±0.5	17.0±1.0

Analysis: Dye disappearance was determined by spectrophotometer at 494 nm which is the maximum wavelength absorbance of Reactive Orange 16. LiP activity in the culture medium was measured by the method described by Tien and Kirk, 1983. LiP catalyzes the oxidation of veratryl alcohol to veratryl aldehyde in the presence of hydrogen peroxide. This conversion was monitored by measuring the absorbance of veratryl aldehyde at 310 nm using a UV-visible spectrophotometer (UV- 1601PC Shimadzu, Japan). One unit (U) of enzyme activity is defined as 1 mmol of veratryl alcohol oxidized to veratryl aldehyde in one minute. MnP activity was assayed as described by Paszczynski et al. 1988. Method is based on monitoring the enzymes' oxidation of Mn^{2+} to Mn^{3+} in which Mn^{3+} shows a characteristic absorbance at 238 nm (ε=6500). The reaction mixture contained suitable amount of supernatant as enzyme, 0.1 M sodium tartrate (pH 5.0), 0.1 mM H_2O_2, and 0.1 mM $MnSO_4$. Reactions were initiated by adding of H_2O_2, and the reference cuvette contains all components except Mn^{2+}. Increase in absorbance was monitored during the first 5-30 sec of reaction. One unit of MnP oxidizes 1µmol of Mn^{2+} min^{-1}.

Scanning electron microscopy: The surface morphology of Ca-alginate beads were analyzed using a LEO (model 1455VP) scanning electron microscope (SEM). Samples were fixed with 2.5% glutaraldehyde in phosphate buffer 0.1 M for 2 h. Then, the samples were washed twice with phosphate buffer 0.1 mM for 15 min at 4ºC. After that, the samples were dehydrated through an ethanol series (10%, 25%, 50%, 98%) and critical point dried using CO_2 as the transition liquid. The dried samples were sputter coated with platinum (20 nm). They were observed at a 10 kV accelerating voltage.

RESULTS AND DISCUSSION

Effect of immobilization factors on decolorization and enzyme activity: The objective of FFD is to estimate the interactions between all the parameters as well as giving an idea of the optimum conditions of the parameters to obtain optimum response and calculating a model equation based on the parameters to predict the response [15]. The regression model provides an excellent explanation of the relationship between the independent variables along with all possible interactive effects and the response. The FFD model has the advantage that it permitted to see and calculate the interaction among all the parameters and determining complex response function. The optimal values of the chosen variables were obtained by solving the regression equation and by analyzing the interactive effects [15, 16]. The inconsistency in dependent variable was explained by the multiple coefficient of determination, R^2 and the model equation was used to predict the optimum value and afterward to elucidate the interaction involving the variables within the particular range[15].

The decolorization efficiency and enzyme activity of all experiments have been shown in table 2. The results indicated that there is a strong relation between decolorization efficiency and LiP and MnP activities. For example trials no. 4 and 8 had both maximum decolorization efficiency and enzyme activity. Table 3 presents the effects of the independent variables and their interactions on the responses. Values of prob>F less than 0.05 indicate the model terms are significant at the 95% confidence interval and values

greater than 0.1 show the model terms are not significant. The analysis of variance described that the effects of BC, BD and ABC are neglectable regarding their studentized effects and p-vlaues. The insignificant terms were preferred to be removed from the regression model. With this improvement, the resulted model and thence the calculations will be simplified without any noticebale error in the calculated response.

Table 3. Analysis of variance results for all possible effects

Term	Studentized effects			p-value		
	Decolorization	LiP	MnP	Decolorization	LiP	MnP
A: Inoculum size	12.91	43.88	25.75	<0.0001	<0.0001	<0.0001
B: Na-alginate concentration	16.47	50	30.75	<0.0001	<0.0001	<0.0001
C: Bead size	-16.03	-72.63	-43.5	<0.0001	<0.0001	<0.0001
D: $CaCl_2$ concentration	-24.53	-97.13	-55.5	<0.0001	<0.0001	<0.0001
AB	13.16	50.75	25.25	<0.0001	<0.0001	<0.0001
AC	12.91	51.87	23.25	<0.0001	<0.0001	<0.0001
AD	-23.34	-86.38	-47	<0.0001	<0.0001	<0.0001
BC	3.97	9.75	11.25	0.0188	0.0556	0.0045
BD	3.22	15.75	5.5	0.0518	0.0007	0.1364
CD	3.72	6.38	17.5	0.0266	0.0020	<0.0001
ABC	0.66	2.5	9	0.6790	0.1159	0.0191
ABD	-27.34	-98.75	-56	<0.0001	<0.0001	<0.0001
ACD	5.41	13.63	14.25	0.0023	0.007	0.0006
BCD	-16.03	-62	-43.25	<0.0001	<0.0001	<0.0001
ABCD	11.41	41.5	20.5	<0.0001	<0.0001	<0.0001

The experimental results were evaluated and the approximating functions of dye decolorization percent, LiP activity and MnP activity were obtained in the form of quadratic equations (Eq's. 1-3). The improved models (after neglecting the insignificant effects) are as below:

$$R1 = 28.23 + 6.45\times A + 8.23\times B - 8.02\times C - 12.27\times D + 6.58\times A\times B + 6.45\times A\times C - 11.67\times A\times D + 1.86\times C\times D - 13.67\times A\times B\times D + 2.70\times A\times C\times D - 8.02\times B\times C\times D + 5.70\ A\times B\times C\times D \quad \text{Eq. 1}$$

$$R2 = 75.09 + 13.47\times A + 16.87\times B - 23.56\times C - 29.22\times D + 13.07\times A\times B + 16.37\times A\times C - 26.47\times A\times D + 7.68\times B\times D + 6.88\times C\times D - 32.50\times A\times B\times D + 7.68\times A\times C\times D - 22.22\times B\times C\times D + 14.56\times A\times B\times C\times D \quad \text{Eq. 2}$$

$$R3 = 54.13 + 12.87\times A + 15.38\times B - 21.75\times C - 27.75\times D + 12.63\times A\times B + 11.63\times A\times C - 23.50\times A\times D + 5.62\times B\times C + 8.75\times C\times D + 4.50\times A\times B\times C - 28.00\times A\times B\times D + 7.12\times A\times C\times D - 21.63\times B\times C\times D + 10.25\times A\times B\times C\times D \quad \text{Eq. 3}$$

Which R1, R2 and R3 are as three responses of the decolorization percent of Reactive Orange 16 after 2 h at day 7, LiP activity (U/l) and MnP activity (U/l), respectively.

Analysis of variance (ANOVA) results have been presented in Table 4. The model F-value of 144.55 for decolorization response implies the model is significant. There is only a 0.01% chance that a model F-value this large could occur due to noise. The curvature F-value of 130.85 implies there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points in the design space). The value adjusted R^2 is close to 1.0, which is advocated a high correlation between the observed values and the predicted ones. This means that regression model provides an excellent explanation of the relationship between the independent variables and the response (% decolorization). The predicted R^2 of 0.93 is in reasonable agreement with the adjusted R^2 of 0.97. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. So the ratio of 37 indicates an adequate signal. This model can be used to navigate the design space for the decolorization response. For other two models for LiP and MnP, analysis are similar to decolorization model and they are also able to navigate the design space.

Table 4. Analysis of variance for the calculated models

model	Sum of squares	Degree of freedom	Mean squares	F value	p-value	R-Squared	Adj R-squared	Pred R-Squared	Adeq recision	Curvature F-value
Decolorization	6872.03	12	2239.34	44.55	<0.0001	0.9841	0.9773	09326	37.040	130.85
LiP activity	158200	13	12169.83	25.26	<0.0001	0.9831	0.9752	0.9341	34.430	162.00
MnP activity	128900	14	9206.82	15.42	<0.0001	0.9830	0.9744	0.9319	31.261	113.58

Residuals show how well the model satisfies the assumptions of the analysis of variance. The model adequacy check is an important part of the data analysis procedure, as the approximating model would give poor or misleading results fit were an inadequate fit. This would be done by looking at the residual plot, which is examined for the approximating model. The studentized residual and normal probability plot of decolorization of azo dye Reactive Orange 16 showed that neither response transformation was needed for the model nor there was any apparent problem with its normality.

The data obtained for both other responses i.e. enzyme activities showed the same results that mentioned for the decolorization efficiency. It can be concluded that the dye decolorization is an enzymatic process caused by ligninases. Consequently, these factors have a significant effect on LiP and MnP activities of immobilized P. chrysosporium influence on dye decolorization as well. As shown in Eq's 1-3, all four variables affected the responses and the behaviors of three responses to each factor were similar, meaning that the change in ligninolytic activity of P. chrysosporium would directly affect the biodecolorization activity. In the other word, decolorization is a biological reaction which conducted by ligninolytic enzymes.

The optimum conditions calculated from these models for maximum MnP and LiP activity and maximum biodecolorization were inoculum size of 10^7 spores L^{-1}, Na-alginate concentration of 3%, $CaCl_2$ concentration of 10% and the bead diameter of 2 mm.Interactive effect of inoculum size and Na-Alginate concentration: Fig. 1 shows the interaction of inoculum size (A) and Na-alginate concentration (B) on the responses. The maximum decolorization occurs at the maximum inoculum size and maximum Na-alginate concentration which is in accordance with the model. The decolorization rate decline by decreasing in Na-alginate, this might be due to the rigidity of the beads which decreases by reducing the Na-alginate concentration and might result in the release of entrapped spores. The stability and rigidity of the beads decreases with decreasing Na-alginate concentration. The release of entrapped spores to the medium inhibits the growth of microorganism, more important it narrows the ligninolytic system of the microorganism. As it can be seen from data resulted and the models, decreasing in Na-alginate

concentration declined enzymatic activity of the microorganism for both MnP and LiP, and sequentially decreased the biodecolorization rate.

The inoculum size of 10^7 and Na-alginate concentration of 3% (w/v) found to be optimum for maximum decolorization.

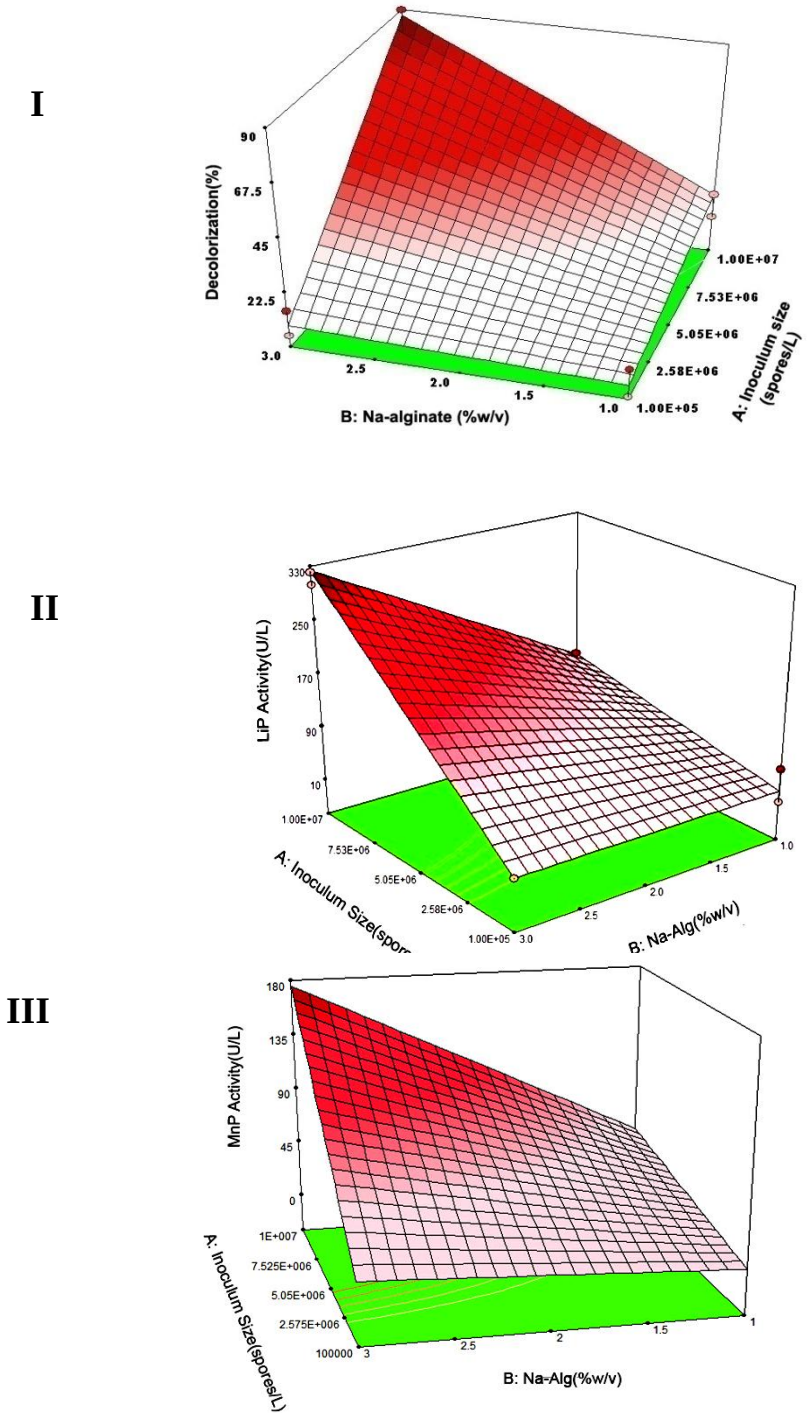
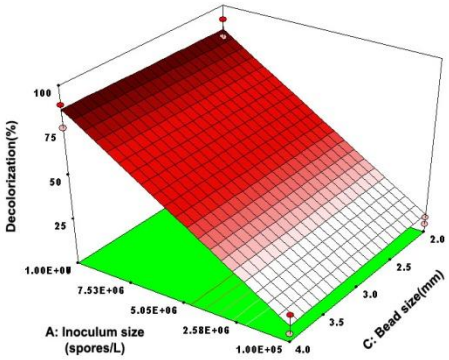


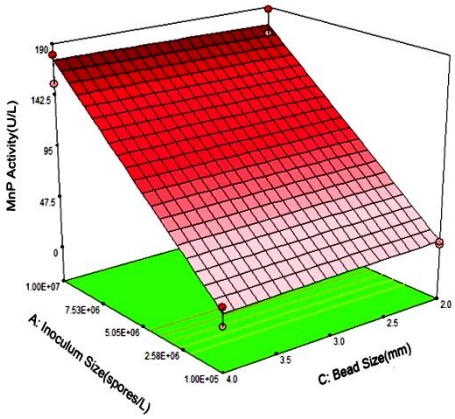
Fig. 1. 3D surface plot for the removal of azo dye Reactive Orange 16 (I), LiP activity (II) and MnP activity (III) by immobilized P. chrysosporium as a function of inoculum size and Na-alginate concentration.

Interactive effect of inoculum size and bead diameter: Mutual effect of inoculum size (A) and bead diameter (C) on the responses has been analyzed as shown in fig. 2. It is estimated that the point of maximum decolorization was achieved using high value of inoculum size as 10^7 spores L^{-1}. Te behavior of MnP and LiP activity is similar to decolorization.

I



II



III

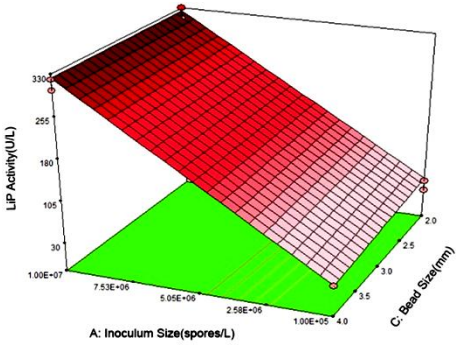


Fig. 2. 3D surface plot for the removal of azo dye Reactive Orange 16 (I), LiP activity (II) and MnP activity (III) by immobilized P. chrysosporium as a function of inoculum size and bead diameter.

Interactive effect of inoculum size and $CaCl_2$ concentration: As shown in fig. 3, using 10^7 spores L^{-1} of inoculum size (A) and 10% (w/v) of $CaCl_2$ (D) gives the maximum decolorization percent. It can be said that it is because of the influence of $CaCl_2$ concentration on rigidity and porous sizes of the beads. Regarding Fig. 4, the structure of beads is so packed at high concentrations of $CaCl_2$ and the porous sizes are low. It could limit the microorganism growth by limiting access of microorganism to fresh nutrients and also it could narrow the mycelium to grow adequately. These results are in complete accordance with the model. In case of LiP and MnP, observations were similar to decolorization, high inoculum size in the presence of high $CaCl_2$ concentration resulted less growth because the access of these spores to nutrients is so difficult due to the rigidity of the beads and its very low porous sizes. Hence, by limited growth, reduced enzyme activity would appear.

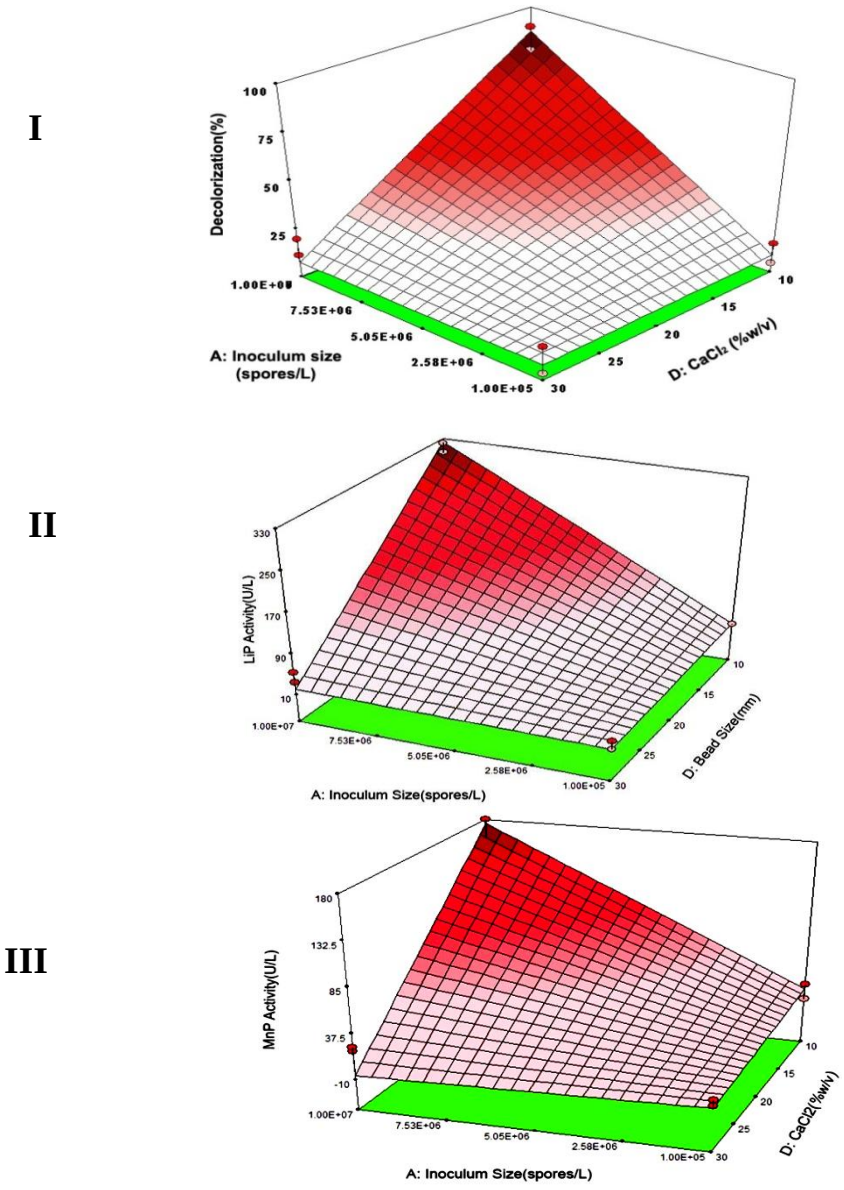


Fig. 3. 3D surface plot for the removal of azo dye Reactive Orange 16 (I), LiP activity (II) and MnP activity (III) by immobilized P. chrysosporium as a function of inoculum size and $CaCl_2$ concentration.

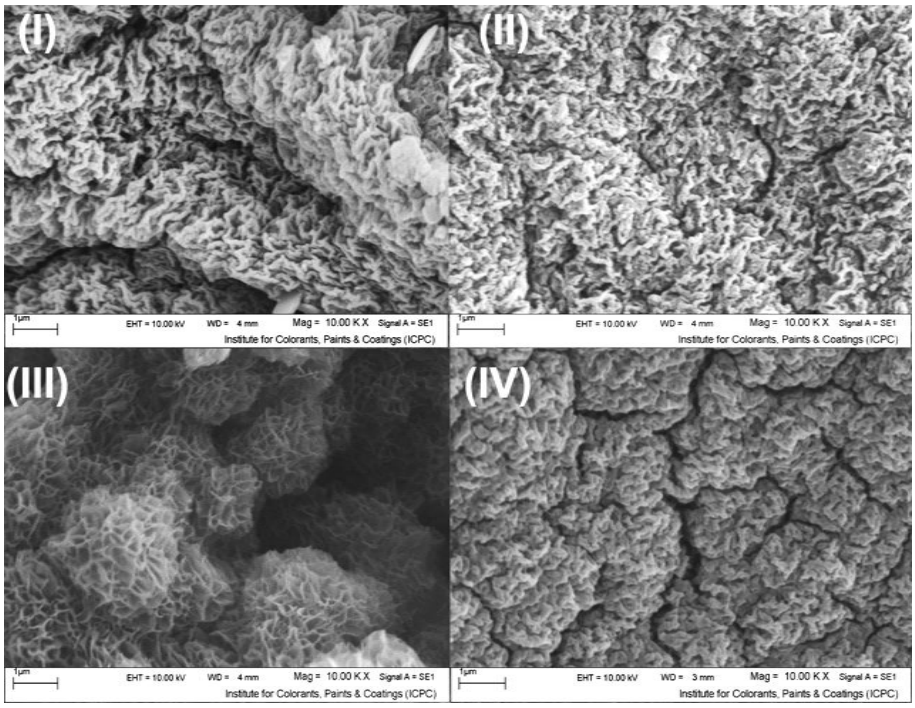


Fig 4. Micrograph of the calcium alginate beads; (I) Na-alginate (3% w/v) & $CaCl_2$ (10% w/v), (II) Na-alginate (1% w/v) & $CaCl_2$ (30% w/v), (III) Na-alginate (1% w/v) & $CaCl_2$ (10% w/v), (IV) Na-alginate (3% w/v) & $CaCl_2$ (30% w/v).

CONCLUSIONS

The use of an experimental design permitted the rapid screening of a large experimental domain for optimization of the decolorization ability of fungal strain P. chrysosporium immobilized on calcium alginate beads. The fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2>0.9) was high, showing a high significance of the model. The optimized conditions of P. chrysosporium immobilization on Ca-alginate beads for highest decolorization (87.5%) of Reactive Orange 16 in 2 h were at Na-alginate concentration of 3% w/v, $CaCl_2$ concentration of 10% w/v, inoculum size of 10^7 spores/L and beads diameter of 4 mm. The immobilization of fungus spores has a great impact on peroxidase activity of the microorganism, the ligninolytic system of P. chrysosporium is sensitive to shear stress and it can inhibit MnP and specially LiP activity. Immobilization with optimum conditions can protect microorganism from shear stress and increase its growth. Both of them would increase the peroxidase activity of P. chrysosporium.

The results showed that the immobilized P. chrysosporium on Ca-alginate beads with these optimum conditions has an enormous potential to degrade the textile dyes and resolve the problem of unnecessary dyes present in the effluents of textile industries. Further pilot scale studies are required for actual industrial applications, and detailed study is needed to explore the mechanism involved.

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