Optimization of Enzymatic Decolourization of Amido black by Laccase from Rigidoporus ulmarius using Response Surface Methodology

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Abstract: *In this study laccase mediated decolourization of Amido black, an azo dye was attempted. Laccase was extracted from the solid state fermentation of the white rot fungi Rigidoporus ulmarius on rice bran (50%). The enzyme activity was assayed using guaiacol as substrate and was found to be 0.0145 U/ml. The crude enzyme extract was employed for decolourization experiments. Optimization of the effect of contributing variables; pH, temperature, dye concentration and enzyme volume on decolourization was primarily analysed using OFAT (One Factor At a Time) approach. The optimum operating conditions for maximum decolourization were further statistically optimized by applying the Response surface methodology using Box-Behnken approach. A four factor three level Box-Behnken design generating 29 experimental runs was performed. A quadratic model was fitted and was analyzed using ANOVA at the 95% confidence interval. The determination coefficient R^2 was found to be 0.9616 indicating the significant fit of the model. The optimum operating conditions predicted by the model was found to be at constant incubation time of 24hrs, pH of 4; temperature of 30°C; dye concentration of 150mg/l and enzyme volume of 1.25ml producing 83.39% decolourization which was in good agreement with that of the predicted value, 83.24%.*

Key words*: Amido black, Box-Behnken design, Laccase, Rigidoporus ulmarius, solid state fermentation.*

I. Introduction

Various classes of dyes which are stable to light, chemicals and microbial degradation are used by textile, cosmetic, plastic and printing industries [1]. About 60% of these dyes are azo dyes which are recalcitrant pollutants causing serious environmental and health concerns [2, 3]. They are toxic, mutagenic and carcinogenic in nature and in addition some causes allergy, skin irritation in humans [4, 5]. Amido black is an azo dye; the carcinogenic and mutagenic effects are not well studied.

Large amount of effluents from textile processing industries are liberated with varied composition, various physical/ chemical methods have been used for the removal of dyes from wastewaters [6]. Physio-chemical methods of dye removal are effective only if the effluent volume is small and sometimes the degradation products are toxic [7].

Enzymatic decolourization is an inexpensive eco-friendly alternative to the conventional treatment methods as they can operate over a wide range [6]. Several enzymes (peroxidases, manganese peroxidases, lignin peroxidases, laccases, microperoxidase-11, polyphenol oxidases and azoreductases) have been evaluated for their potential in decolourization and degradation of dyes.

Laccase, copper containing oxidases are mainly produced by white rot basidomycetes and other fungal [8] and bacterial strains and also some plants have been used in various biotechnological and environmental processes. Lack of substrate specificity enabled laccase as an enzyme able to oxidize wide range of chemical compounds such as diphenols, polyphenols, diamines, aromatic amines, benzenethiols, and substituted phenols [9] as well as different groups of colored pollutants. Decolourization of a wide range of synthetic and textile dyes using laccases from basidomycetes has been investigated in recent years.

In this study laccase from a white rot fungi Rigidoporus ulmarius was extracted and utilized for the decolourization of an azo dye, amido black. The decolourization experiments were optimized further using Response surface methodology to obtain the optimum operating conditions for maximum decolourization.

II. Materials And Methods

Materials and reagents

Amido black (λmax-617nm) and all other chemicals of analytical grade was purchased from HI-Media (India). Rigidoporus ulmarius MTCC 1095 was procured from IMTECH, Chandigarh, India. Stock standard solution of the dye (1g/L) was prepared using double distilled water. This solution was diluted to appropriate concentrations before use (100mg/L- 300mg/L).

Enzyme production and recovery

Solid state fermentation of Rigidoporus ulmarius on rice bran was carried out for enzyme production. 10g ground, oven-dried rice bran was sterilized by autoclaving at 121°C for 15mins and inoculated with five 5mm plugs of the fungus. The setup was incubated for 30 days at 30°C in static condition to reach growth confluence maintaining the moisture content at 50% with addition of sterile distilled water supplemented with (0.01% w/v) peptone every 3rd day.

The extracellular enzyme was extracted using 50mM sodium acetate buffer pH 5.5 supplemented with 1% tween 20 at 25°C in static condition. The extraction was carried out in two cycles, medium was incubated with i) 75ml buffer for 5 hrs and filtered with constant stirring ii) 110ml of buffer for 3hrs and filtered. The filtrates were centrifuged at 5000rpm for 15mins. The supernatants collected served as the source of crude enzyme [10].

Enzyme assay (laccase) was carried out using guaiacol as substrate [11-13]. The change in absorbance was monitored at 530nm using UV-Vis spectrophotometer. Enzyme activity was calculated using the following formula (1).

$$\text{Volume activity (U/ml)} = \frac{\Delta A530/min \times 4 \times Vt \times \text{dilution factor}}{e \times Vs} \quad \text{-----------(1)}$$

Where,
Vt = final volume of reaction mixture (ml) = 5.00
Vs = sample volume (ml) = 1
e = extinction co-efficient of guaiacol = 6,740 /M/cm
4 = derived from unit definition & principle
Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml [14].

Decolourization studies

The effect of contributing process parameters on decolourization were analyzed using OFAT (One Factor At a Time) approach. The factors include pH, temperature, dye concentration and enzyme volume. The factors were varied one at a time keeping others constant according to the variable analyzed. The absorbance at the absorption maxima at initial hour and after 24 and 48hrs of incubation was recorded and the residual dye concentration was represented in terms of percentage [15, 16]. The decolourization of dye, expressed as dye decolourization (%), was calculated according to the formula:

$$\text{Decolourization (\%)} = [(Ci - Ct)/Ci] \times 100 \quad \text{----------- (2)}$$

Where
Ci: initial concentration of the dye and
Ct: dye concentration along the time [17].

The effect of pH on decolourization was studied by varying the medium pH from 3-9 at room temperature with initial dye concentration of 100mg/l. The temperature in the range of 30°C- 50°C was varied at the optimum pH and initial dye concentration. The dye concentration was increased from 100mg/l to 300mg/l in multiples of 50mg/l at the optimum pH and temperature. The enzyme volume was varied from 0.25ml to 1ml and the effect on decolourization at the optimum pH, temperature and initial dye concentration was recorded. The experiment was carried out in triplicate.

Statistical optimization of process variables

Response surface methodology for the statistical optimization of the influencing process variables was adopted. The influencing parameters considered for optimization includes: pH (X_1), Temperature(X_2), dye concentration(X_3) and enzyme volume(X_4). A four factor three level Box-Behnken design was designed using the software. The 29 run experimental design was performed and the response was calculated in terms of percentage decolourization. A best fit quadratic model was suggested and was further analyzed using ANOVA at 95% confidence interval, the fit quality was evaluated by coefficient of determination R^2 and comparing it with the predicted R^2. The validity of the model was verified by performing the confirmatory trials which produced response which were in good agreement with the predicted response.
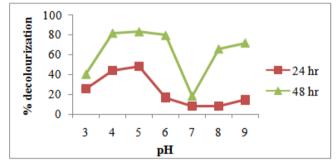
III.   Results And Discussion

Enzyme assay
    The enzyme extracted from solid state fermentation of the fungus was assayed for its activity using guaiacol as substrate in both crude extracts. The enzyme activity was found to remain almost constant in both extractions. The crude enzyme from first extraction cycle showed an activity of 0.0129U/ml and 0.0145U/ml in the second extraction cycle.
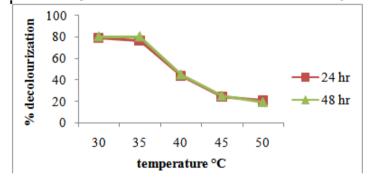
Decolurization using crude enzyme
    The enzyme based decolourization of Amido black was carried out. The influencing process parameters; pH, temperature, dye concentration and enzyme volume were optimized preliminarily using OFAT. The decolourization was studied after 24 and 48hrs of incubation. From the results obtained it was found that increase in incubation time did not cause significant change in decolourization. The dye was maximally decolorized to about 83% in OFAT. The optimum pH for decolourization was at the enzyme optimum pH 5. The optimum temperature for decolourization was 30°C. The decolourization did not vary significantly at the enzyme optimum temperature of 35°C. The decolourization at different dye concentration did not vary significantly; however maximum decolourization was observed at 150mg/l concentration. The optimum enzyme volume was found to be 1ml, though only slight difference was seen in the decolourization produced with 0.75ml of the enzyme. Fig 1(a), 1(b), 1(c) and 1(d) show the effect of pH, temperature, dye concentration and enzyme volume on decolourization of Amido black by the enzyme.

1 (a) Effect of pH on decolourization of Amido black using enzyme
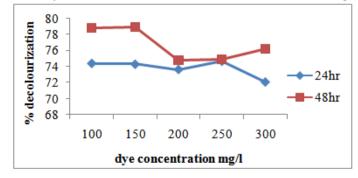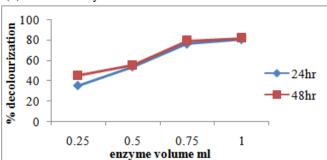

1 (b) Effect of temperature on decolourization of Amido black using enzyme

1 (c) Effect of dye concentration on Amido black decolourization using enzyme

1 (d) Effect of enzyme volume on Amido black decolourization

Statistical Optimization of Amido black decolourization by enzyme

The statistical optimization of the process variables was carried out to obtain the optimum operating conditions for decolourization producing maximum possible decolourization with precision and reproducibility. The response was fitted in terms of % decolourization and a second order quadratic model with effect of linear, quadratic and interaction terms on response was suggested by the software as the best fit. The test of significance of the model, individual coefficients and test for lack of fit was performed.

Table 1 represents the design matrix with the experimental and predicted value for Amido black decolourization by enzyme. The model was significant showing a Prob> F value of < 0.0001. The linear factors of X_1, X_2 and X_4; quadratic factor of X_1 and interaction terms X_1X_2, X_1X_4 were found to be significant at 95% confidence interval indicating that the model terms are a limiting factor for decolourization. The determination coefficient R^2 was found to be 96.16% and adjusted R^2 was 92.32% indicating the high significance of the model. The multiple correlation coefficient, R and the determination coefficient R^2 are the measure of correlation for estimation of the regression equation. The closer the value of R to 1, the better the prediction of the model is [18]. A higher determination coefficient indicates higher degree of correlation between the experimental and predicted values and it is also a measure of fit of the model. F value for the model was found to be 25.05. The F value is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the F value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. From these observations, it could be concluded that interactive effects are important for true optimization rather than the OFAT method [19]. The insignificant lack of fit explains that the model is adequate to describe the observed data. The lack of fit value was insignificant and was found to be 0.3714. Table 2 shows the ANOVA for Amido black decolourization by enzyme. Equation (3) represents the second order polynomial regression predicted equation for amido black decolourization by enzyme.

$$Y= 69.846 -4.06X_1 -1.57X_2 + 0.1675X_3 + 5.04X_4 + 2.18X_1X_2 +1.05 X_1X_3 -1.945 X_1X_4 -0.22 X_2X_3 -0.46 X_2X_4 -0.14 X_3X_4 +2.79 X_1^2 +0.93 X_2^2 -0.411 X_3^2 -0.438 X_4^2 -----(3)$$

The optimum operating conditions for enzyme based decolourization was found to be at constant incubation time of 24hrs, pH of 4; temperature of 30°C; dye concentration of 150mg/l and enzyme volume of 1.25ml producing 83.39% decolourization which was in good agreement with that of the predicted value, 83.24%.

The 3D and 2D contour plots are graphical representation of the regression equation which aims to showcase the optimum variable interactions such that the response is maximal. Each contour curve represents an infinite number of combinations of two test variables. A circular contour plot of response surfaces suggest that the interaction is negligible between the corresponding variables, while an elliptical or saddle contour plot indicates significance in the interactions between the corresponding variables [20]. Fig 2 (a) and (b) represents the 2D contour plots showing significant two variable interactions for Amido black decolourization by enzyme.

Table 1 Design matrix for Amido black decolourization by enzyme

Std	pH X_1	Temperature (°C) X_2	Dye concentration (mg/l) X_3	Enzyme volume (ml) X_4	Response (%) Decolourization	
					Experimental	Predicted
1	4	25	150	1	81.37	81.39
2	6	25	150	1	70.17	68.89
3	4	35	150	1	72.73	73.87
4	6	35	150	1	70.28	70.12
5	5	30	100	0.75	64.17	63.64
6	5	30	200	0.75	64	64.26
7	5	30	100	1.25	74.4	74.00
8	5	30	200	1.25	73.67	74.06
9	4	30	150	0.75	70.16	69.27
10	6	30	150	0.75	64.48	65.04
11	4	30	150	1.25	83.39	83.24
12	6	30	150	1.25	69.93	71.23
13	5	25	100	1	71.98	71.55
14	5	35	100	1	70	68.84
15	5	25	200	1	70.75	72.32
16	5	35	200	1	67.89	68.74
17	4	30	100	1	75.85	77.17
18	6	30	100	1	65.78	66.94
19	4	30	200	1	76.87	75.40
20	6	30	200	1	71.01	69.38
21	5	25	150	0.75	65.93	66.41
22	5	35	150	0.75	64.1	64.18
23	5	25	150	1.25	77.8	77.41
24	5	35	150	1.25	74.13	73.34
25	5	30	150	1	71.9	69.84
26	5	30	150	1	69.55	69.84
27	5	30	150	1	69.14	69.84
28	5	30	150	1	69.33	69.84
29	5	30	150	1	69.31	69.84

Table 2 ANOVA for Amido black decolourization by enzyme

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	636.78	14	45.48	25.05	< 0.0001*
Residual	25.41	14	1.81		
Lack of Fit	20.05	10	2.00	1.496841	0.3714
Pure Error	5.35	4	1.33		
Cor Total	662.20	28			
R^2- 0.9616 Adj R^2- 0.9232					

* Significant at 95% confidence interval

Figure 2 (a) 2D contour plot showing factor interaction between pH and temperature (b) 2D contour plot showing factor interaction between pH and enzyme volume.

IV. Conclusion

The laccase extracted from Rigidoporus ulmarius showed promising potential for decolourization of amido black. 83% decolourization was achieved by using the crude enzyme at 24hrs of incubation. The enzyme can be applied for low- purity applications like waste treatment. Further purification of the enzyme or induction of enzyme production can provide for complete decolorization solutions. Studies on the degradation pathway of the enzyme can provide an insight for application in treatment of wide range dyes and chemicals.

Acknowledgements

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