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Biodegradation Of Reactive Red M5B Dye Using Bacillus subtilis

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Abstract: Azo dyes are widely used in textile industries. Removal of the color from textile waste water is a striking issue. To curb this issue, biological treatment can be employed rather than physico- chemical processes. In this work, *Bacillus subtilis* is used to degrade the reactive dye – RED M5B. It is found that decolorization was due the action of enzyme peroxidase produced by the organisms during its growth. Optimum conditions for the dye degradation were indentified using response surface methodology. Under optimal conditions, 95% degradation was observed within 40 h of inoculation.
Keywords: Azo dyes, biodegradation, reactive dyes, *Bacillus subtilis*, RSM.

Introduction

Textile effluent treatment is of due significance owing to their polluting effects on water bodies[1]. Human health and ecological effects have made this a burning issue. Dye removal from wastewater is a difficult task and expensive[2]. Complete degradation of dye into non-harmful state or form is essential[3]. Different methods employed in dye removal include membrane filtration, coagulation, flocculation, precipitation, floatation, adsorption, ion-exchange, ion-pair extraction, ultrasonic mineralization, electrolysis etc. These operations prove to be expensive, time consuming and not effective to a complete degree[4]. To resolve these limitations biological treatments are employed. These methods use bacteria[5], fungi[6], algae[7], actinomycetes[8] and other aerobic and anaerobic microorganisms. Biological degradation of azo dyes contains two major steps[9]. Firstly, oxidation-reduction reactions cause cleavage of azo bond. The second step involves oxidation of intermediate. The product from this step is less toxic, colorless and in acceptable form in comparison to the initial state. This was found out to be due to the presence of non-specific extracellular enzymes formed during the growth of microorganism[10]. Between aerobic and anaerobic degradation, aerobic degradation is very effective since it oxidizes the reduced components to further lesser toxic state. Hence, aerobic degradation is the safe method over anaerobic degradation. Response surface methodology was used to optimize bacterial decolorization process.

Materials and methods:

Microorganisms and media employed: *Bacillus subtilis* was purchased from MTCC, Chandighar, and was grown on Nutrient agar plates. The mother culture was then stored at 4 °C. The media used for sub-culturing and decolorization contained: 0.5 %

Peptone, 0.1 % beef extract, 0.2% yeast extract, 0.5% NaCl in 1 L of distilled water.

Reactive dye employed: The reactive dye employed was RED M5B. It is a commercial dye widely used by textiles industries near Tirupur, India. It was used in this study without further purification. 100 ml of dye solutions of concentrations 100 ppm, 200 ppm and 300 ppm were prepared to which nutrient media components (as mention above) were added. The flasks were sterilized and were inoculated with the organism under aseptic conditions. Samples were taken at regular time intervals and centrifuged at 6000 rpm for 10 min to separate the cells. Supernatant was taken and analyzed for residual red M5B using Uv-vis spectrophotometer at its λ_{max} (540 nm). When the absorbance exceeded 1.2 the samples were suitably diluted. Decolourization was studied and percent decolourization was found using the formula[2]:

$$\%Dye\ decolorisation = \frac{(initial\ OD - final\ OD)}{initial\ OD} \times 100 \quad \ldots\ldots\ldots\ldots (1)$$

Peroxidase Assay[11]: 1 mL of crude enzyme is extracted from the sample and centrifuged at 6000 rpm for 10 min followed by addition of 1 ml of methylene blue dye solution of 20 ppm and incubated for 10 min at 30 °C. The change in color to green shows the presence of peroxidase enzyme and it is quantitatively measured at 662 nm using UV-Vis spectrophotometer. The amount of enzyme units was calculated using the formula:

$$Enzyme\ units = \frac{(OD_{Control} - OD_{sample})}{reaction\ time} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Results and discussion

Figure 1 shows the degradation effect of *Bacillus subtilis* on red M5B dye of three different concentrations at pH 7 and 30 ºC. Percentage decolorization is in the range of 90 -95%.

Response Surface Methodology:

Optimization of process variables was carried out by employing response surface methodology (RSM). The RSM procedure comprised of variation of parameters such as salt concentration, pH and dye concentration. Several researchers had shown that initial dye concentration and pH influence the biodegradation efficiency. Most of the research reports on biodegradation of textile dyes use synthetic dye solution. However, industrial effluents contains considerable amount of salts like sodium chloride. Thus, this work investigates the biodegradation efficiency in the presence of common salt. Experimental conditions along with the results are shown in table 2. The results were analyzed using Minitab 15. A quadratic model was employed to explain the variation in the response variable percentage color removal with the independent variables namely, pH, initial dye concentration and salt concentration[12-15]. Estimated regression coefficients along with their t- and p-values are given in table 2. From the p-values it was found that the factors 'pH^2', 'pH×salt' and 'dye×salt' were not statistically significant (p-value > 0.05 at 95% confidence level) and hence they were eliminated from the quadratic model and regression was repeated. The model could explain 87.78% (R^2 = 0.8778) variations in the response variable. All the regression coefficients included in the reduced model were found to be statistically significant with p-values less than 0.05. In order to confirm the validity of the model ANOVA was performed and the results are listed in table 3. p-value of lack of fit for the reduced model was found to be 0.036 ($p < 0.05$). Therefore, presence of outliers was tested using residual graphs (graphs not shown here) and it was found that experimental points 34 and 36 could be possible outliers. These two experimental results were removed and regression and ANOVA were performed again. Results are shown in table 2 and table 3 respectively. Both lack-of-fit and R^2 values had increased confirming that the points 34 and 36 were real outliers. R^2 for the reduced model without outlier was 0.9164 indicating that the model could explain 91.64% of variations in the response variable. By substituting the coefficient in the regression model following equation is obtained.

$$\%R = 93.17 + 0.84pH - 2.56C_0 + 1.99S - 6.19C_0^2 + 2.01S^2 + 2.71pH \times C_0 \quad \ldots\ldots\ldots (3)$$

Conclusion

A commercial dye Red M5B was degraded using strain *Bacillus subtilis* in submerged fermentation. The results indicated that the strain used was efficient in degrading the dye and upto 95% color could be removed. Effect of process variables namely initial solution pH, dye concentration and

salt concentration were studied by response surface methodology and it was found that all these variables had influenced percentage color removal. A quadratic model was proposed to explain the variation in the response variable with respect to these process variables and the model was found to be satisfactory with high R^2 value (0.9164). RSM also revealed the interaction between the variables.

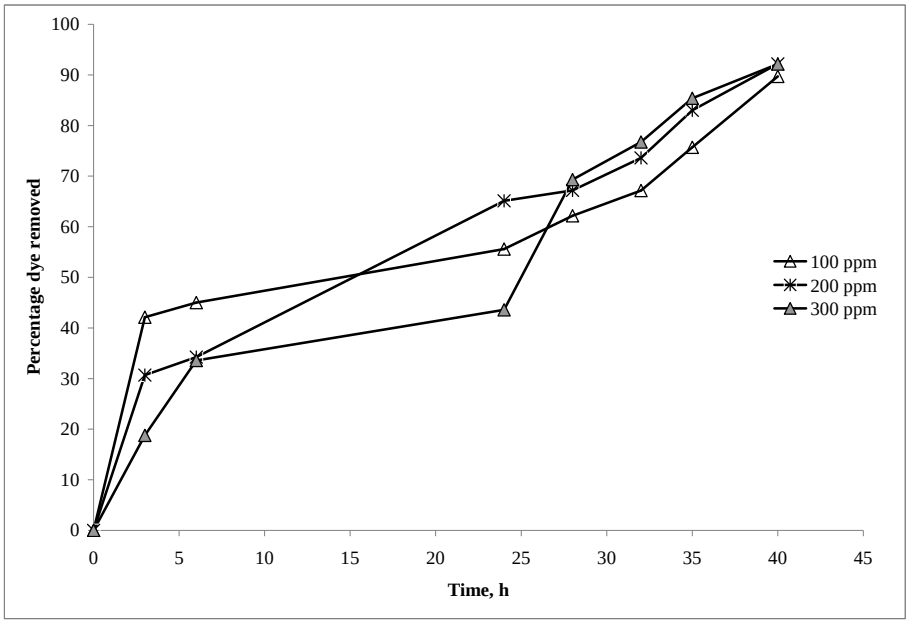

Figure 1. Percentage of red M5B removed by *Bacillus subtilis* at 30 °C and pH 7.

Table 1 -Response Surface Regression: R3 versus Block, pH, Dye conc, salt

RunOrder	pH	Dye conc	salt	Trial 1	Trial 2
1	-1	-1	-1	91.71	89.50
2	1	1	-1	87.73	88.86
3	1	-1	1	91.02	91.92
4	-1	1	1	85.20	86.25
5	0	0	0	92.62	91.38
6	0	0	0	92.62	92.66
7	1	-1	-1	88.08	87.48
8	-1	1	-1	80.42	79.71
9	-1	-1	1	96.42	94.77
10	1	1	1	90.73	93.97
11	0	0	0	92.62	95.67
12	0	0	0	92.62	93.82
13	-1	0	0	92.37	94.07
14	1	0	0	93.32	89.06
15	0	-1	0	88.85	91.75
16	0	1	0	83.06	79.44
17	0	0	-1	93.43	95.51
18	0	0	1	97.28	94.64
19	0	0	0	92.62	93.56
20	0	0	0	92.62	94.07

Table 2- Estimated Regression Coefficients for %R

	Full model				Reduced model without outlier			
Term	Coef	SE Coef	T	P	Coef	SE Coef	T	P
Constant	92.79	0.420	221.052	0.000	93.17	0.317	293.663	0.000
pH	0.59	0.386	1.523	0.138	0.84	0.296	2.823	0.008
Dye	-2.81	0.386	-7.269	0.000	-2.56	0.297	-8.614	0.000
Salt	1.99	0.386	5.151	0.000	1.99	0.288	6.905	0.000
pH^2	-0.16	0.736	-0.223	0.825				
Dye^2	-6.60	0.736	-8.957	0.000	-6.19	0.541	-11.431	0.000
$Salt^2$	2.85	0.736	3.867	0.001	2.01	0.542	3.704	0.001
pH×Dye	2.73	0.432	6.315	0.000	2.73	0.322	8.466	0.000
pH×salt	-0.36	0.432	-0.840	0.408				
Dye ×salt	0.13	0.432	0.300	0.766				

Table 3-ANOVA for degradation of Red M5B

	Reduced model – with outlier						Reduced model – without outlier					
Source	DF	Seq SS	Adj SS	Adj MS	F	P	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	660.65	660.65	110.11	39.5	0.000	6	563.67	563.67	93.946	56.6	0.000
Linear	3	243.57	243.57	81.19	29.1	0.000	3	193.04	215.56	71.853	43.3	0.000
Square	2	298.18	298.18	149.09	53.5	0.000	2	251.73	251.73	125.865	75.9	0.000
Interaction	1	118.9	118.9	118.90	42.7	0.000	1	118.9	118.9	118.902	71.7	0.000
Residual Error	33	91.98	91.98	2.79			31	51.43	51.43	1.659		
Lack-of-Fit	8	41.12	41.12	5.14	2.5	0.036	8	16.19	16.19	2.023	1.3	0.283
Pure Error	25	50.85	50.85	2.03			23	35.25	35.25	1.532		
Total	39	752.62					37	615.11				

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