INFLUENCE OF CULTURE CONDITIONS ON DECOLORIZATION OF PATENT BLUE BY PSEUDOMONAS AERUGINOSA USING RESPONSE SURFACE METHODOLOGY

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Abstract: In the present study, Pseudomonas aeruginosa was used to decolorize an acid dye Patent Blue. The bacterial culture exhibited 47.97% decolorization at pH 7.3 and incubation temperature of 49°C whereas 55.89% at 22°C and inoculum size of 40%. Maximum dye-decolorizing efficiency was at 0.005% concentration of the dye. The results were optimized using response surface methodology.

Keywords: Pseudomonas aeruginosa, Patent Blue, Optimization of Culture Conditions, Decolorization assay, Decolorization potential, Response Surface Methodology.

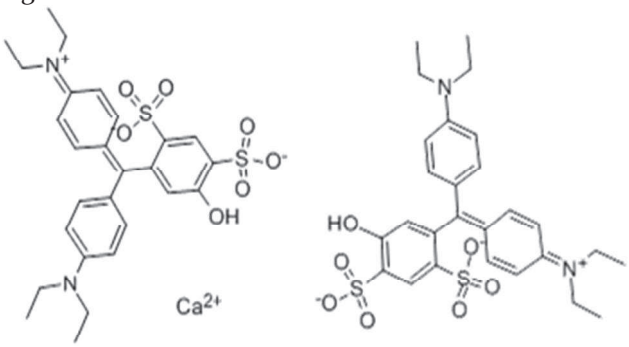
Introduction: The textile industry is one of the industries that generate a high volume of waste water [1, 2 & 3]. Strong color of the textile waste water is the most serious problem of the textile waste effluent [4]. The disposal of these wastes into receiving waters causes damage to the environment. Dyes may significantly affect photosynthetic activity in aquatic life because of reduced light penetration and may also be toxic to some aquatic life due to the presence of aromatics, metals, chlorides etc., [5]. Dye waste water from textile or dye stuff industry is one of the most difficult to treat because dyes have various synthetic, complex aromatic molecular structures, which make them more stable and more difficult to degrade [6 & 7]. The removal of dyes from the textile waste effluent has been carried out by physical, chemical and biological methods such as flocculation, membrane filtration, electrochemical techniques, ozonation, coagulation, adsorption and fungal discoloration [7 & 8]. In recent years, a number of studies have focused on some microorganisms which are able to biodegrade and biosorb dyes in waste waters. A wide variety of microorganisms capable of decolorizing a wide range of dyes include some bacteria, fungi and algae [8, 9 & 10].In general, dye decolorization/degradation using microorganisms depends on the dye structure and the nature and position of substituent's on the chromophore. Dyes with simple structure and low molecular weight exhibits higher rates of color removal in comparison with highly substituted, high molecular weight dyes [11]. There are many variables or factors affecting the enzyme production and decolorization that are expressed by different taxa of microorganisms and culture conditions. These features are important in the process design and optimization of microbial treatment of effluents [8].In view of this, the present study focuses on the process of dye decolorization of Patent blue by *Psuedomonas aeruginosa* under different culture conditions such as pH, temperature, inoculum size and co-substrates using response surface methodology.

Materials and Methods:

Chemicals: Acid dye Patent Blue was obtained from the Dyeing Division, Karnataka Silk Industries Corporation (KSIC), Mysore. All reagents used for the study were of analytical grade obtained from Hi Media laboratories, Mumbai.

Microorganism: The bacterial isolate *Pseudomonas aeruginosa* was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This was maintained on Nutrient Agar plates and refrigerated at 4°C with periodic (30 days) sub-culturing.

Fig-1 Structure of Patent Blue



Preparation of the Dye: A stock solution of the dye (0.005%) was prepared by dissolving 0.05 g of the dye in 1000 ml distilled water.

Measurement of maximum absorbance (λ_{max}) of Patent Blue The maximum absorbance of Patent Blue was estimated by using UV-Visible Spectrophotometer 108 (Systronics) and the λ_{max} was found to be at 415nm.

Inoculum Preparation: The isolate (*Pseudomonas aeruginosa*) was prepared in 1% peptone solution and was used to assess its ability to decolorize the dye.

Decolorization Assay: The decolorization potential is expressed as percentage decolorization [12]. Decolorization process was carried out using static culture by inoculating 30% of the inoculum to 20 ml of the dye solution taken in a test tube, in an anaerobic condition and incubated at 30°C for 24 h.

The decolorized dye solution was then heated on a hot water bath for about 45 minutes, to inactive the centrifuged at 6000 rpm for 30 minutes in Eltek Labspin TC 450 D centrifuge. The cell free supernatant was filtered using sartorius stedium filter disc- grade 292 which was used to determine the percent decolourization of Patent Blue. Decolourisation of the dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Patent Blue (λ_{max} 415 nm) by using UV-Visible spectrophotometer 108 (Systronics). The uninoculated dye was used as control [13]. Decolorization percentage was calculated by the following formula and all assays were done in duplicate.

$$\% \text{ decolorization} = \left(\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \right) \times 100$$

Decolorization of Patent Blue under different culture conditions: The decolorization potential of *Pseudomonas aeruginosa* was assessed under varying culture conditions such as pH, temperature, inoculum size and co-substrates by changing one parameter at a time and keeping the others constant. 20ml of the dye solution (conc. 0.005%) was subjected to a wide range of pH (2-12) by adjusting the pH with 0.01M hydrochloric acid or sodium hydroxide. Dye was subjected to different inoculum size (25% – 50%). The different temperatures ranges were (20°C, 30°C, 37°C , 45°C and 50°C). Two Carbon sources of 0.5 g each – (Starch and Sucrose) and Nitrogen sources of 0.5 g each (Peptone and Ammonium Chloride) were used to check the decolorization potential of Pseudomonas aeruginosa.

Statistical analysis: Data were statistically analyzed using response surface methodology by SAS system. A quadratic response surface was estimated by least square regression. This can be explained by the following formula.

$Y = \exists\cong + \exists_1 X_1 + \exists_2 X_2 + \exists_3 X_1^2 + \exists_4 X_2^2 + \exists_5 X_1 X_2 +$,

Where Y= Predicted Response

$\exists\cong$= Intercept

X_1, X_2 = Factors

$\exists_1, \exists_2, =$ Interaction effects,

= noise or error observed in the response

Results and Discussion: The design opted for the present study was One-Factor-at-a-Time experiment, in which only one factor or variable was changed at a time while keeping others constant [14]. A response surface analysis of percentage decolorization with different factors like pH, temperature, inoculum size and co-substrate were considered to study the factors associated with the response. The results indicated bacterial mass in and to overcome the interferance with the absorbance of the dye. It was then cooled, that all factors to some extent influenced the total process of decolorization.

Effect of pH and Temperature on decolorization: The percentage decolorization of Patent blue by *P.aeruginosa* at pH 2-12 is shown in Table 1. Maximum decolorization was recorded at pH 12 with the mean value of 31.80. Between pH 2-10, the mean value ranged from 28.65 to 30.95, indicating that all the pH ranges had nearly similar effect on percentage decolorization. The percentage decolorization increased at pH 12, indicating that the basic condition was most favourable for dye decolorization. Therefore, pH as one of the culture conditions was found to be influencing the process of dye decolorization.

Table 1: Percentage decolorization at varying pH levels

pH	Min-Max	Mean ± SD	95%CI
2	11-48	29.46±16.35	20.4-38.5
4	3.5-48	28.63±17.39	19.0-38.3
6	11.1-44.3	30.95±15.26	22.5-39.4
8	7.2-44.0	28.71±16.77	19.4-38.0
10	3.3-44.0	28.65±18.29	18.5-38.8
12	**7.4-48**	**31.80±16.77**	**22.5-41.10**

F=0.098; P=0.992 F=0.098; P=0.992:

Table 2 shows the percentage decolorization of Patent Blue by *P.aeruginosa* at varying temperature range (20-50°C). The dye decolorization activity of the strain was found to increase with increasing temperature. Highest percentage decolorization was achieved at 50°C with the mean value of 46.02 and least percentage decolorization was at 20°C with the mean value of 7.98 at the end of 24h incubation period. The probability was found to be highly significant. Therefore, temperature as one of the culture conditions was found to be significantly influencing the process of dye decolorization.

F=699.98; P<0.001**

Response Surface Analysis: Response surface methodology is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, one can optimize a response (output variable) which is influenced by several independent variables (input variables). It is used to test the significance of an individual factor [14].

Table 2: Percentage decolorization at different temperature levels

Parameter	Estimate	Standard Error	t Value	Pr > |t|	Parameter Estimate from Coded Data
Intercept	-26.2414	9.1632	-2.86	0.0053	27.8770
ph	-0.8384	1.0840	-0.77	0.4414	0.6190
Temp	1.8049	0.4782	3.77	0.0003	21.4565
ph*ph	0.0464	0.0621	0.75	0.4571	1.1614
Temp*ph	**0.0089**	**0.0170**	**0.52**	**0.6022**	**0.6681**
Temp* Temp	-0.0062	0.0065	-0.95	0.3436	-1.4042

Table 3: Response Surface Analysis for maximizing the percentage decolorization

Temp	Min-Max	Mean ± SD	95%CI
20	3.3-15.0	7.98±4.16	5.9-10.0
30	11.0-18.5	12.83±2.88	11.4-14.3
37	33.0-40.8	37.68±2.62	36.4-39.0
45	40.0-48.0	43.98±2.21	42.9-45.1
50	**44.0-48.0**	**46.02±2.04**	**45.0-47.0**

Table 3 shows the response surface analysis for maximizing the percentage decolorization by *P.aeruginosa*. Even though, both pH and temperature were significant, the combined effect of both the factors on the percentage decolorization was to be assessed. Therefore, response surface analysis was used to assess the effects of both pH and temperature on percentage decolorization. The combined analysis indicated that both the factors were not significant, although individually they influenced the process of decolorization.

Ridge Analysis**:** The method of ridge analysis computes the estimated ridge of optimum response for increasing radii from the centre of the original design. It helps in computing ridge of maximum or minimum response [15].The ridge analysis in Table 4 indicates that a maximum response of 47.97% can be obtained by *P.aeruginosa*. at pH 7.3 and temperature 49.9 ℃

Table 4: Ridge analysis for maximizing the response of percentage decolorization

Estimated Response	Standard Error	Uncoded Factor Values	
		pH	Temp
27.8770	1.2215	7.0000	35.0000
30.0097	1.2104	7.0163	36.4991
32.1149	1.1881	7.0368	37.9979
34.1926	1.1572	7.0616	39.4962
36.2430	1.1226	7.0911	40.9937
38.2662	1.0923	7.1257	42.4905
40.2623	1.0773	7.1659	43.9862
42.2315	1.0913	7.2119	45.4807
44.1740	1.1477	7.2644	46.9737
46.0900	1.2561	7.3238	48.4650
47.9797	**1.4197**	**7.3907**	**49.9541**

Effect of Inoculum Size and Co-substrate on decolorization: The decolorization potential of *P.aeruginosa* at inoculum size ranging from 25-50% is shown in Table 5. Percent decolorization was lowest when inoculum size was 30% with mean value of 45.11 and highest percent decolorization was at 50% inoculum size with mean value of 49.42.There was gradual increase in percent decolorization when inoculum size was increased from 30-50%, indicating that higher the inoculum size, greater the percent

decolorization.

Table 5: Percentage decolorization with varying Inoculum size

Inoculum size (%)	Min-Max	Mean ± SD	95%CI
25	5.0-59.2	46.49±12.37	39.6-53.3
30	6.0-59.2	45.11±11.92	38.5-51.7
35	7.0-59.2	45.87±12.64	38.9-52.9
40	8.0-66.6	47.7±14.24	39.8-55.6
45	9.0-66.0	49.2±15.63	40.5-57.9
50	**10.0-66.0**	**49.42±14.24**	**41.5-57.3**

F=0.257; (Fischer's co-efficient) P=0.935 (Probability) The F value is the ratio of the mean regression sum of squares divided by the mean error sum of squares. Its value will range from zero to an arbitrarily large number. By rule of thumb, F-value > 4.00 is usually statistically significant.

Effect of Inoculum Size and Temperature on decolorization : Table 6 shows the percentage decolorization at different temperature levels. The decolorization potential of *P.aeruginosa* was compared across a temperature range ranging from 20-50°C. The dye decolorization activity of the strain was found to decrease with increasing incubation temperature with the exception at 37°C and 50°C. Highest percentage decolorization was achieved at 20°C and least percentage decolorization was at 45°C at the end of 24h incubation period. The probability was found to be highly significant. Therefore, temperature as one of the culture conditions was found to be significantly influencing the process of dye decolorization.

Table 6: Percentage decolorization at different temperature levels

Temp (°C)	Min-Max	Mean ± SD	95%CI
20	48.0-66.0	54.70±7.29	51.1-58.3
30	5.0-66.6	43.89±26.64	30.6-57.1
37	47.0-56.1	52.02±3.32	50.4-53.7
45	37.0-44.0	41.18±2.47	40.0-42.4
50	42.4-48.4	44.70±1.70	43.9-45.5

F=3.820; P=0.007

Response Surface Analysis for maximizing the percentage decolorization: Even though, both inoculum size and temperature were independently influencing the process of decolorization, it was necessary to assess the combined effect of both the factors. Therefore, response surface analysis was carried out. The combined analysis indicated that both the factors were significantly influencing the process of decolorization as shown in Table 7.

Ridge analysis for maximizing the response of percentage decolorization: The ridge analysis in Table 8 indicates that maximum decolorization potential(55.89%) of *P.aeruginosa* was at temperature 22°C and inoculum size of 40%.

Table 7: Response Surface Analysis for maximising the percentage decolorization

Parameter	Estimate	Standard Error	t Value	Pr > |t|	Parameter Estimate from Coded Data
Intercept	35.1858	39.3566	0.89	0.37	46.0843
IS	3.0554	8.5806	0.36	0.72	2.5501
Temp2	0.0752	1.1494	0.07	0.94	-4.8865
IS*IS	0.1958	0.5405	0.36	0.71	1.2239
Temp2*IS	**-0.1420**	**0.0740**	**-1.92**	**0.05**	**-5.3280**
Temp2* Temp2	0.0094	0.0142	0.67	0.50	2.1362

Table 8: Ridge analysis for maximising the response of percentage decolorization

Estimated Ridge of Maximum Response for Variable decolorisation2: decolorisation2

Estimated Response	Standard Error	Uncoded Factor Values	
		IS	Temp2
46.0843	2.6544	7.5000	35.0000
46.6772	2.6512	7.6234	33.6955
47.3546	2.6242	7.7579	32.4301
48.1176	2.5786	7.8994	31.1915
48.9670	2.5233	8.0455	29.9716
49.9032	2.4708	8.1948	28.7653
50.9263	2.4388	8.3463	27.5693
52.0366	2.4487	8.4994	26.3810
53.2341	2.5237	8.6539	25.1988
54.5190	2.6839	8.8094	24.0215
55.8913	**2.9415**	**8.9656**	**22.8482**

Decolorization activity of *P.aeruginosa* with addition of Co-substrates: With p value of less than 0.001 and high F value of 3256.34, all co-substrates were significant. Starch, Sucrose and Ammonium Chloride indicated 37% decolorization whereas peptone showed poor response with 11% decolorization. Further, experimental design is required to optimize the process and to study the interaction within co-substrates and with other factors like pH, temperature etc.,

Table 9: Decolorization activity of *P.aeruginosa* with addition of Co-substrates

Co-substrate	Starch	Sucrose	Peptone	NH_4Cl
Min-Max	37- 37	37- 37.8	11- 11.5	37 - 38
Mean± SD	37.00±0.00	37.27±0.46	11.17±0.29	37.67±0.58
95 % CI	37.00-37.00	36.11-38.41	10.44-11.88	36.23-39.10
F value	3256.34			
P value	<0.001**			

Conclusion;The present study confirms the ability of bacterial culture *Pseudomonas aeruginosa to* decolorize the synthetic acid dye Patent Blue with decolorization efficiency of 47.97%, thus suggesting its application for decolorization of textile wastewaters. Presence of co-substrates (Starch, Sucrose & Ammonium Chloride) are essential for

attaining greater decolorization efficiency. It is important to further experiment with different range of culture conditions to enhance the decolorization potential of the bacterial strain. Structural complexity of the dye, Patent Blue has also contributed towards the decolorization process. The application of statistical programs such as response surface methodology and ridge analysis have further contributed to get more precise results and also to optimize the process parameters.

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