OPTIMIZATION OF MALACHITE GREEN DECOLORIZATION USING ACHROMOBACTER AEGRIFACIENS BY RESPONSE SURFACE METHODOLOGY AND CENTRAL COMPOSITE DESIGN

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Abstract

In the present study, optimization of malachite green using Achromobacter aegrifaciens was carried out by response surface methodology and central composite design. Tryptone, glucose, dye concentration and pH were selected for the study based on the results obtained by One-factor-at-a-time and Plackett-Burman design. Optimization by RSM was carried out with 30 runs. Response in terms of percent decolorization was subjected to ANOVA. The results obtained say that the model is significant. All the four factors as individual and a combination of tryptone and dye, tryptone and pH and dye and pH were found to be significant model terms. Decolorization of Malachite green under optimized condition showed that the organism could tolerate higher concentration of the dye. Upto 95% decolorization of the dye was observed at 3500 μg/ml concentration.

Keywords: *Malachite Green Decolorization, Achromobacter Aegrifaciens, Response Surface Methodology, Central Composite Design*

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1. INTRODUCTION

Dyes are natural or synthetic substances used to add color to or change the color of various materials. Among the synthetic dyes, the most widely preferred in the worldwide market are the azo, anthraquinone and triphenylmethane dyes. Approximately 10,000 different dyes and pigments are produced annually worldwide and are used to color various substrates such as synthetic and natural textile fibers, plastics, leather, paper, mineral oils, waxes, foodstuffs and cosmetics. During the industrial application, around 5-50% of the dyestuffs get wasted and enter the effluent due to their improper fixing properties [1]. These effluents when released in the environment, untreated, cause pollution and have negative impact on the terrestrial as well as aquatic ecosystem. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds [2]. The presence of the dyes and the organic metabolites is of concern due to their toxicity as they are mutagenic, carcinogenic as well as cytotoxic in nature [3].

Physiochemical methods such as flocculation, precipitation, coagulation and filtration, etc. used in treatment of wastewater containing dye are not widely preferred as these methods do not facilitate the removal of dyes neither do they reduce the toxicity from the effluent. They are generally expensive and also produces large amount of sludge; disposal of which creates environmental problem. Hence, biological method by means of microbial degradation of effluent containing dyestuff is considered as an alternative as it is eco-friendly, cost-effective, reduces the toxicity of the metabolities and aids in the removal of the toxic dyestuffs from the wastewater [4].

Malachite green, a triphenylmethane dye, is a dark green and crystalline solid prepared by condensing one part benzaldehyde with two parts of dimethylaniline in the presence of concentrated sulfuric acid or zinc chloride. It can exist in two ionic forms- as a dye salt and as the carbinol base or pseudobase. The dye salt is soluble in water and organic solvents such as ethanol, methanol etc. the carbinol base has been found to be less soluble in water than the dye salt. Due to its toxic effects and ability to persist in tissues, ways to remove excess/ residual malachite green from treatment ponds and industrial effluents need to be explored [3].

Biological decolorization of malachite green has been studied using a variety of bacteria. *Bacillus cereus* DC11 [5], *Aeromonas hydrophila* DN322 [6], *Pseudomonas otitidis* W/L3 [7], *Mycobacteria* [8] and *Citrobacter* strain KCTC 18061P [9] have been shown to be effective in decolorization of malachite green.

Response surface methodology is a tool to aid in optimization and evaluation of multiple parameters and their interactions on various chemical and biochemical processes. It used to develop equations and empirical models which analyses the effect of the individual parameters and their interactions on the decolorization efficiency of the organisms. The adequacy of the model developed is tested by analysis of variance (ANOVA). The decolorization of malachite green and optimization using RSM has also been studied using *Pseudomonas putida* wherein the optimization parameters analyzed were temperature, dye concentration, incubation time and incubation volume [10]. In the present

study for optimization of a different set of parameters such as carbon and nitrogen source and pH has been conducted. *Achromobacter aegrifaciens*, having high efficiency for decolorization of malachite green, was selected for the present study. The present investigation was focused toward the examination of an optimization tool for determining optimum process parameters for the treatment of malachite green using *Achromobacter aegrifaciens*. Response surface methodology (RSM) and Central Composite Design (CCD) was adopted to optimize decolorization of malachite green.

2. MATERIALS AND METHODS

2.1. Dye and Media

The dye (Malachite Green) and all the other chemicals used in the experimental studies were of analytical grade. Mineral salt media (MSM) containing (g/l): NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; NH_4Cl, 1.0 [11] with pH adjusted to 7 using 1 M NaOH or 1 M HCl was used in the present study.

2.2. The Bacterial Isolate

Achromobacter aegrifaciens was capable of showing 90% decolorization of malachite green at 200 µg/ml concentration of dye under unoptimized condition. The decolorization efficiency of the organism was further analyzed by optimizing the process parameters.

2.3. Estimation of Percent Dye Decolorization

Percentage decolorization was quantitatively estimated in the supernatant obtained by centrifugation of the medium at 8000 rpm for 10 min. The absorbance was read using a UV-visible spectrophotometer at maximum wavelength (λ_{max} = 617 nm). Percentage decolorization of Malachite Green (MG) was calculated using the formula given below.

$$\% \text{ Decolorization} = \frac{\text{initial absorbance-final absorbance}}{\text{Initial absorbance}} \times 100$$

2.4. Preparation of Inoculum

Achromobacter aegrifaciens was grown in mineral salts medium with 200 µg/ml of malachite green at 37±2˚C for 24 hrs at 100 rpm of shaking speed. After the incubation period culture medium was subjected to centrifugation at 8000 rpm for 15 min. Supernatant was discarded and the cell pellet of 5% in buffer was taken as inoculum for further studies.

2.5. Optimization of MG Decolorization by RSM

The significant variables identified by Plackett Burman Design were further optimized by RSM employing a central composite design (CCD). A CCD of 30 experiments was used for optimization of process parameters for decolorization of malachite green wherein the chosen variables were evaluated at five different levels: -2, -1, 0, 1, 2 (Table-1). Four independent variables, namely Tryptone (A), Glucose (B), dye concentration (C) and pH (D) were evaluated and the percentage of MG decolorization was the dependent variable (response). Design-Expert version 10.0 (Stat-Ease Inc., Minneapolis, USA) was used for experimental design and statistical analysis. Validation of the optimum decolorization results predicted by the model was done using analysis of variance (ANOVA) and the behavior of the model in terms of mathematical relationship was explained using second order polynomial equation.

Table-1: Experimental range and levels of independent variables selected for Response Surface Methodology for decolorization of Malachite green

Variable	Units	Code	Range and levels				
			-2	-1	0	1	2
Tryptone	µg/ml	A	100	150	200	250	300
Glucose	µg/ml	B	0	50	100	150	200
Dye concentration	µg/ml	C	100	150	200	250	300
pH		D	5	6	7	8	9

2.6. Decolorization of MG Under Optimized Condition

The parameters which showed a positive result in RSM were selected for decolorization of MG. The efficiency of the organism to decolorize malachite green at different concentrations (2000, 2500, 3000 and 3500 µg/ml) under optimized condition was studied. Culture was inoculated into flasks containing mineral salts medium with different concentrations of dye. The flasks were incubated for 24 hr at 37˚C and 100 rpm shaking condition. After the incubation period culture medium was subjected to centrifugation at 8,000 rpm for 10 min to eliminate the bacterial cells. The supernatant was examined by spectrophotometrically at 617 nm. The percentage of decolorization was calculated as given earlier.

3. Results and Discussion

A set of 30 experiments were carried out to evaluate the individual and combined effect of four different parameters (independent variables) on the decolorization efficiency *Achromobacter aegrifaciens*by RSM. CCD was chosen to determine the optimum requirement of tryptone (A), glucose (B), dye concentration (C) and pH (D) for maximum dye decolorization. The experimental and predicted values obtained are given in Table-2.

Analysis of the result was performed using ANOVA and the quadratic regression model obtained indicates the model to be significant. The model F-value of 21.24 implies the model is significant. The values of " Prob->F" were less than 0.05 indicating that the model terms A, B, C, D , AC , AD , CD , A^2,B^2 and $D^2$are significant.

The predicted R^2 of 0.7234 determined by ANOVA is in resonable agreement with the adjusted R^2 of 0.9072. Adequate precision of 16.617 indicated an adequate signal which implies that the predicition of experimental data is quite satisfactory. Comparison of experimental values with

the predicted values leads to the conclusion that the experimental values obtained are found to be much closer to the predicted values as represented in Table-2 and the Figure-1.

3-D plots were obtained to investigate the effect of interactions between two factors on the efficiency of decolorization by *Achromobacter aegrifaciens* and to determine the optimum level of each variable for maximum response. The nature of the curve helps to analyse the type of interaction between the factors wherein the elliptical shape of the curve indicates positive interaction between the two variables and a circular shape implies negative interaction.

The results obtained indicates the magnitude of P and F values which implies that the quadratic terms of tryptone, glucose, dye concentration and pH have positive effect on decolorization of MG. The 3-D plot indicates that Tryptone v/s Dye concentration (Fig-2), Tryptone v/s pH (Fig-3), Dye concentration v/s pH (Fig-4) have positive effect whereas Tryptone v/s Glucose (Fig-5), Glucose v/s Dye concentration (Fig-6) and Glucose v/s pH (Fig-7) have negative effect on the decolorization efficiency of the organism. Therefore, the optimum conditions obtained from RSM after analysis by ANOVA are temperature 37˚C, pH 8, tryptone 200 μg/ml and glucose 100 μg/ml.

Optimization studies have been conducted by several authors using various microbial species wherein RSM was used to evaluate the effect of interactions of the parameters on efficiency of dye decolorization. Optimization studies were carried out by Rajeshkannan *et al* [12] for removal of malachite green from the aqueous solution using *Hydrilla verticillata* wherin the parameters selected were temperature, adsorbent size, contact time, agitation speed and adsorbent dosage. According to their results temperature and sorbent dosage, sorbent dosage and contact time, sorbent dosage and stirring speed and contact time and particle size were found to be significant modal terms. Similar kind of study was also conducted by Rajeshkannan *et al* [13] for removal of malachite green by tamarind seed, wherein temperature and sorbent dosage, temperature and stirring speed and sorbent dosage and stirring speed were found to be significant modal terms. Adsorption studies and the optimization of process parameters by RSM was also explored by Ahmad *et al* [14] for malachite green removal using lime peel. According to their results activation temperature and impregnation ratio was found to be significant modal term. Study of activity of the organism in dye removal and determining the effect of the study parameters have been done by using strains of *Pseudomonas putida* [10], *Pseudomonas aeruginosa* BCH [15] and *Pseudomonas aeruginosa* [16] wherein the usual parameters evaluated include temperature, pH, dye concentration, inoculum load etc. Sneha *et al* [10] have reported that dye concentration v/s temperature, inoculum volume v/s temperature and inoculum volume v/s dye concentration are significant interactions. Jadhav *et al* [15] have shown that interactions between pH v/s cell mass and temperature v/s cell mass are positively influencing dye decolorization activity by the organism. Temperature v/s pH, temperature v/s dye concentration and pH v/s dye concentration have been found to be enhancing the decolorizing ability of the organism by Hafshejani *et al* [16].

The decolorization of malachite green by *Achromobacter aegrifaciens* was observed at increasing concentrations of the dye from 2000 μg/ml to 3500 μg/ml wherein the results indicated that the efficiency of the organism for decolorization decreased with increase in the dye concentration and the maximum of 95% was obtained at 3500 μg/ml dye concentration (Fig-8). Similar results have been obtained during study of decolorizing activity using *Kocuria rosea* MTCC 1532 [1] and *Exiguobacterium* sp. MG2 [17].

Table-2: Full factorial Central Composite Design matrix and their observed responses for Malachite green decolorization using Response Surface Methodology

Standard	Run	Factor 1 (tryptone)	Factor 2 (glucose)	Factor 3 (dye concentration)	Factor 4 (pH)	Percent decolourization	
						Actual	Predicted
4	1	1	1	-1	-1	83	81.58
10	2	1	-1	-1	1	96	98.66
2	3	1	-1	-1	-1	93	93
24	4	0	0	0	2	99	96.54
5	5	-1	-1	1	- 1	89	89.63
11	6	-1	1	-1	1	86	85.93
15	7	-1	1	1	1	89.6	89.60
23	8	0	0	0	-2	84.5	85.29
28	9	0	0	0	0	97.3	97.30
30	10	0	0	0	0	97.3	97.30
18	11	2	0	0	0	89	89.94
14	12	1	-1	1	1	95	92.58
20	13	0	2	0	0	71.9	73.15
7	14	-1	1	1	-1	85	84.01

19	15	0	-2	0	0	94	91.07
8	16	1	1	1	- 1	90	87.95
27	17	0	0	0	0	97.3	97.30
13	18	-1	-1	1	1	93	96.10
26	19	0	0	0	0	97.3	97.30
16	20	1	1	1	1	83	84.91
21	21	0	0	-2	0	93	91.45
25	22	0	0	0	0	97.3	97.30
29	23	0	0	0	0	97.3	97.30
9	24	-1	-1	-1	1	95	97.05
22	25	0	0	2	0	97	96.87
6	26	1	-1	1	-1	93	94.75
17	27	-2	0	0	0	87	84.39
1	28	-1	-1	-1	-1	83	82.76
12	29	1	1	-1	1	87	86.37
3	30	-1	1	-1	-1	70.1	72.58

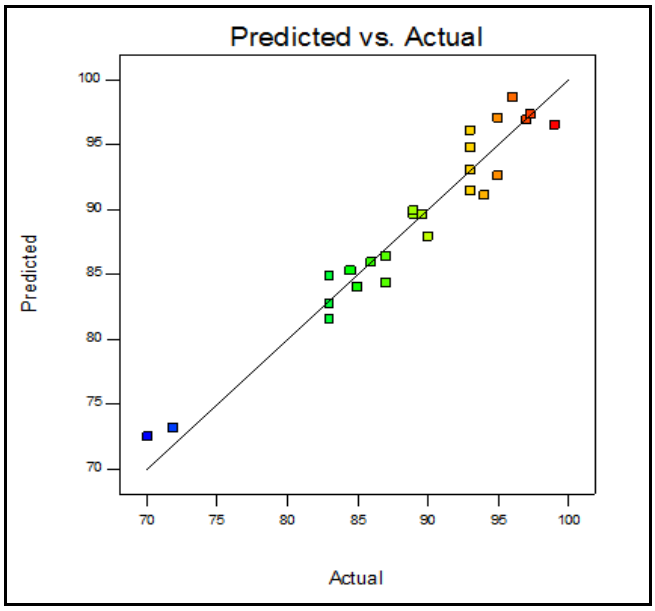

Fig-1: Predicted and actual experimental response for the dye decolourization
Note: R1 = Percent decolorization

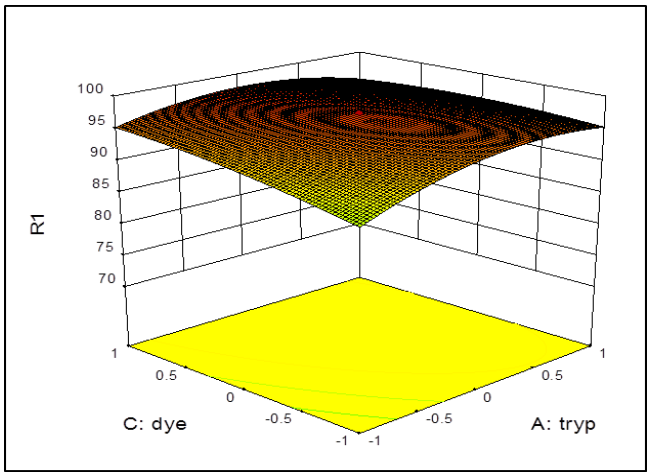

Fig-2: Three dimensional response surface plot for the effect of A: Tryptone C: Dye conc.
Note: R1 = Percent decolorization

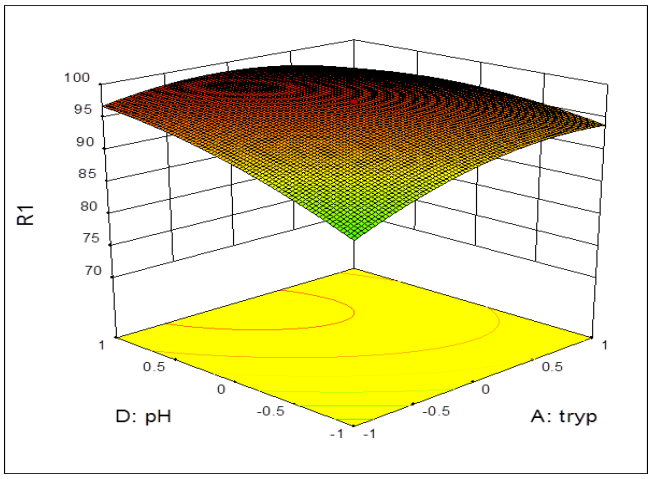

Fig-3: Three dimensional response surface plot for the effect of A: Tryptone D: pH
Note: R1 = Percent decolorization

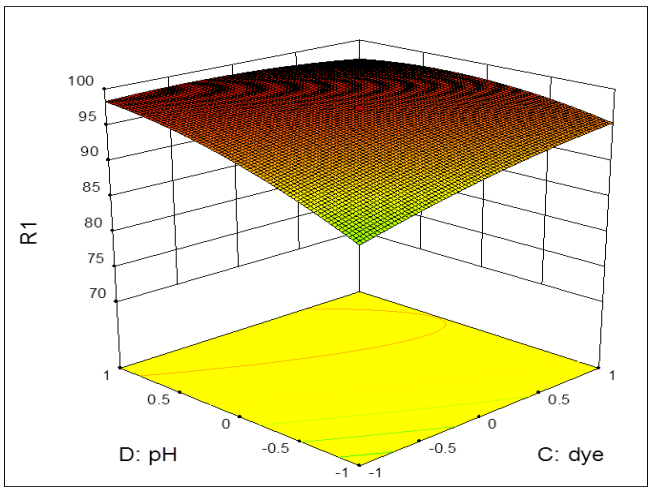

Fig-4: Three dimensional response surface plot for the effect of C: Dye conc. D: pH
Note: R1 = Percent decolorization

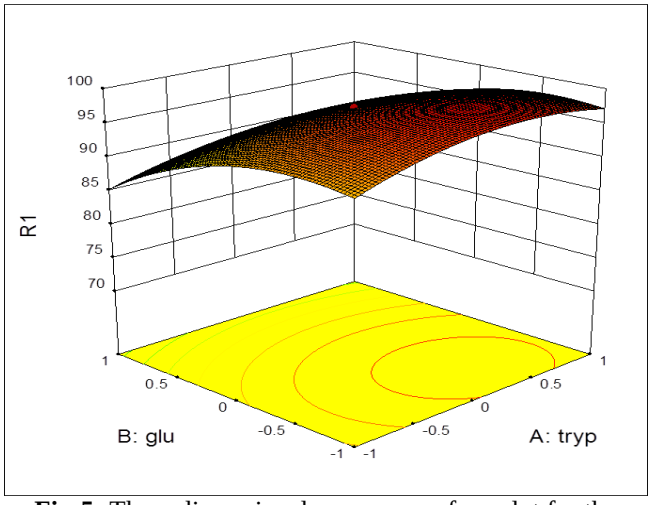

Fig-5: Three dimensional response surface plot for the effect of A: Tryptone B: Glucose
Note: R1 = Percent decolorization

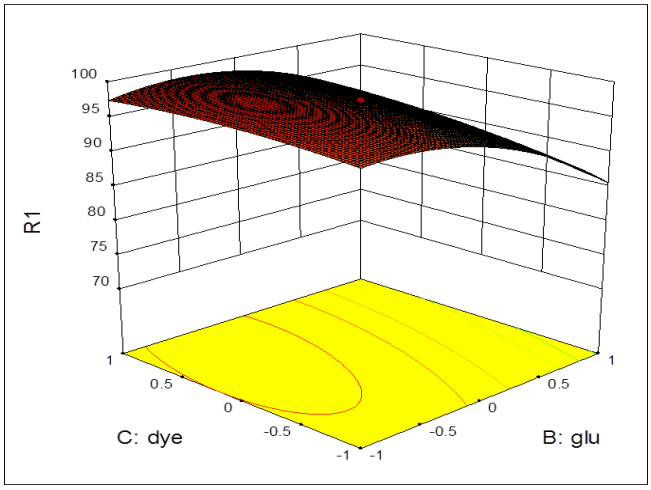

Fig-6: Three dimensional response surface plot for the effect of B: Glucose C: Dye conc.
Note: R1 = Percent decolorization

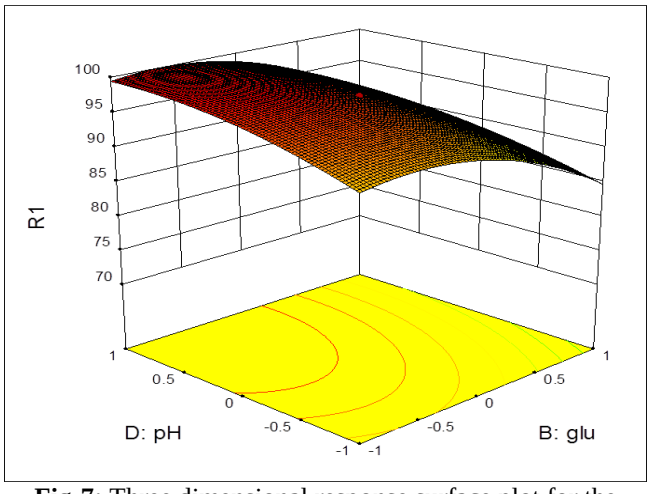

Fig-7: Three dimensional response surface plot for the effect of B: Glucose D: pH
Note: R1 = Percent decolorization

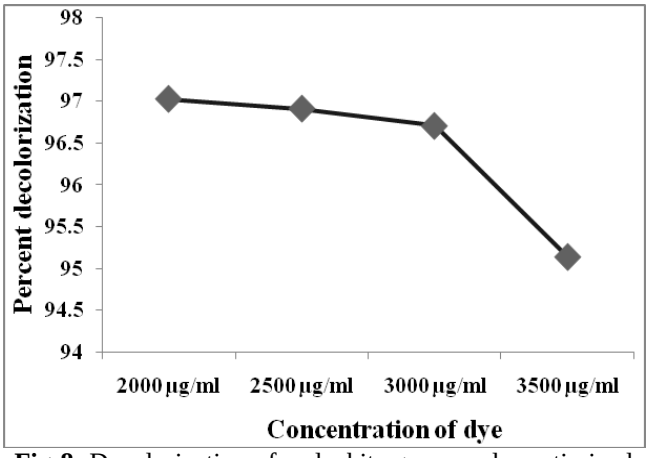

Fig-8: Decolorization of malachite green under optimized condiions

4. CONCLUSION

The isolate, *Achromobacter aegrifaciens* is efficient in decolorization of malachite green. Optimization process indicates that concentration of tryptone, glucose, dye and pH are influenzing factors for malachite green decolorization. Under optimized conditions of glucose, tryptone, glucose and shaking speed the isolate can be used to decolorize malachite green upto 3000 µg/ml concentration.

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