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Optimization and decolorization of malachite green using *Pseudomonas putida*

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ABSTRACT

The present study emphasizes on the decolorization of malachite green using Pseudomonas putida. The effect of pH, temperature, dye concentration, inoculum volume and static/agitated condition was studied using One Factor At a Time approach. Malachite green was decolorized by around 90% with this bacterium and the optimal conditions were found to be pH 7, 30°C, 200 mg/L dye concentration, 0.5 mL inoculum under the static condition. Response Surface Methodology using Box Behnken Design was applied to further optimize malachite green decolorization. A quadratic model was obtained for dye decolorization through this design. The optimum values for temperature, dye concentration, incubation time and inoculum volume were found to be 25°C, 200 mg/L, 72 hours and 0.75 mL respectively. The predicted decolorization rate under the optimum conditions determined by Response Surface Methodology was in close agreement with the experimental results and the model was found to be significant. The germination and growth of Triticum aestivum and Vigna radiata seeds and the growth of micro-organisms were not inhibited by the degraded metabolic products of the dye in the toxicity studies. The ability of the strain to tolerate, decolorize and degrade malachite green at high concentration gives it an advantage for the treatment of textile industry wastewater. This approach creates a promising hope for the bioremediation of the environment which is polluted by hazardous dyes.

Keywords: Malachite green, Response Surface Methodology, Box Behnken Design, toxicity studies, bioremediation

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INTRODUCTION

During manufacturing and usage of dyes in the textile industries, approximately 10- 15% of the dye finds its way into the environment as wastewater [1,2]. The textile dyes disturb the marine ecosystem, as they undergo chemical and biological changes [3]. Their breakdown products are also toxic to most aquatic organisms [4,5]. It also greatly affects the photosynthesis of hydrophytes by limiting light penetration, thereby deteriorating gas solubility and water quality [6].

Malachite green, an N-methylated diaminotriphenylmethane dye, has been widely used as the most efficacious antifungal agent in the fish farming industry. It is used extensively for dyeing silk, wool, jute, leather, ceramics, cotton, and used to treat fungal and protozoal infection. Malachite green is highly toxic to mammalian cells; it promotes hepatic tumor formation in rodents and also causes reproductive abnormalities in rabbits and fish. Malachite green and its reduced form, leucomalachite green, may persist in edible fish tissues for extended periods

of time. Therefore, there are both environmental and human health concerns about bioaccumulation of malachite green and leucomalachite green in terrestrial and aquatic ecosystems [21].

Various physical and chemical methods like coagulation or adsorption of dyes, ultra-filtration, ion-exchange, chemical oxidation, electrolysis etc have been developed for the elimination of dyes from the wastewater since many years [7,8]. However these methods are not very much applied because of their high cost, high energy requirements and hazardous by-products [9]. Also these techniques generate a huge volume of sludge and cause secondary pollution due to the formation of sludge and hazardous by-products [10,11].

Biological methods are generally considered environment friendly as they can lead to complete mineralization of organic pollutants at low cost [12]. Bioremediation may be the most effective method for treating industrial dyes wastewater [13].

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables [14]. The application of statistical experimental design techniques in a process results in improvement of yield, reduces process variability, gives closer confirmation of the output response to nominal and reduces overall costs [15]. Box Behnken design is a combination of a two-level (full or fractional) factorial design with an incomplete block design. In each block, a certain number of factors are put through all combinations for the factorial design, while the other factors are kept at the central values [16].

In this paper, the effect of pH, temperature, dye concentration and volume of inoculum was studied using One Factor At a Time (OFAT) approach. Response Surface Methodology (RSM) was applied to optimize the decolorization of malachite green by *Pseudomonas putida*. Box Behnken design using 4 variables (temperature, dye concentration, inoculum volume and incubation time) was used to optimize the effect of these variables on dye decolorization. Microbial toxicity and phytotoxicity studies were carried out to check if the degraded metabolites were non toxic.

EXPERIMENTAL SECTION

2.1. Bacterial strain

Pseudomonas putida MTCC 102 was purchased from IMTECH, Chandigarh. *Escherichia coli* HB101 was purchased from Medox, Chennai. *Bacillus subtilis* and *Bacillus cereus* were purchased from Marina Labs, Chennai.

2.2. Chemicals

Malachite green of analytical grade (dye content 40%) was purchased from HI-Media. All the other chemicals used in our experiments were of analytical grade.

2.3. Equipment

UV spectrophotometer of ELICO, India was used for measuring the decolorization efficiency.

2.4. Decolorization studies

100 mg/L of malachite green dye amended in nutrient broth was inoculated with 1 mL of *Pseudomonas putida* strain and incubated at 37^0C for 72 hours. After the incubation, the reaction mixture was centrifuged at 2000 rpm for 15 minutes and the supernatant was taken to measure the dye decolorization efficiency. Dye decolorization was measured by monitoring the decrease in absorbance of the dye in a double beam UV-Visible spectrophotometer (Elico, India) at 620nm [17]. Dye decolorization efficiency is expressed as follows: equation 1,

$$\text{Decolorization efficiency (\%)} = \frac{[A_0 - A_t]}{[A_0]} * 100 \quad \text{---- (1)}$$

Where A_0 : initial absorbance

A_t : final absorbance

2.5. Physiochemical parameters optimization

The effect of pH (4, 5, 6, 7, 8 and 9), temperature (30, 40, 50, 60, 70^0C), dye concentration (100, 150, 200, 250, 300 mg/L), inoculum volume (0.25, 0.5, 0.75, 1, 1.25 mL), and static/ agitated condition was studied on the decolorization of malachite green by *Pseudomonas putida* using One Factor At a Time (OFAT) approach [18].

2.6. Optimization of process variables

Response Surface Methodology (RSM) is the combination of mathematical and statistical tools to predict the optimal conditions using minimal runs. Three level four factorial Box-Behnken Design was chosen to study the interactions of four variables such as temperature (°C), dye concentration (mg/L), incubation time (hours) and enzyme volume (mL) based upon OFAT approach. Table 1 represents the experimental ranges used in Box-Behnken design.

Table 1. Experimental range and levels of independent variables

Independent variable	Factors	Range levels		
		-1	0	1
Temperature (°C)	X_1	25	30	35
Dye concentration (mg/L)	X_2	150	200	250
Incubation time (hrs)	X_3	24	48	72
Inoculum volume (ml)	X_4	0.5	0.75	1.0

2.7. Toxicity Studies

Microbial toxicity and phytotoxicity studies were performed to determine the degree of toxicity of the dye and its degraded metabolites. The degraded products of 200 mg/L malachite green from the 100 mL media was extracted using equal volume of ethyl acetate, dried and dissolved in distilled water. This solution was then used for the toxicity studies.

2.7.1. Microbial toxicity

Microbial toxicity of the dye malachite green was studied on *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas putida*. The toxicity of the dye and its degradation products were studied using agar well assay. The zone of growth of inhibition of microbes was recorded after 24 hours of incubation at 37^0C [19].

2.7.2. Phytotoxicity

Seed germination and plant growth bioassay are the most common techniques used to evaluate phytotoxicity [20]. Phytotoxicity studies were carried out on *Triticum aestivum* and *Vigna radiata* at room temperature. 20 seeds of each were watered daily with dye and its degraded product separately. Control set was carried out using distilled water. The germination %, length of the plumule and radical were recorded after 3 days [21].

RESULTS AND DISCUSSION

3.1. Physiochemical parameters optimization

pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0 [22, 23, 24].

The optimum pH for malachite green decolorization was found to be 7.

The decolorization rate was found to be maximum at 30°C and found to decrease at higher temperatures. This might have occurred due to adverse effect of high temperature on the enzymatic activities [25]. Hence the optimum temperature was found to be 30°C.

The decolorization rate was found to increase with dye concentration upto 200 mg/mL beyond which the rate is lowered. The optimum dye concentration was hence found to be 200 mg/L. Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases [21]. The decrease in decolorization efficiency might be due to the toxic effect of dyes [26].

The decolorization rate was found to increase with increase in inoculum volume. Beyond 0.5 mL the increase was not significant. Thus the optimum inoculum was found to be 0.5 mL.

3.2. Optimization of process variables

The effect and nature of interactions of the four process variables on dye decolorization was explored by Box-Behnken design of RSM. Table 2 represents the Box-Behnken design for malachite green decolorization. Multiple

regression analysis represented effect of variables on the dye decolorization in a form of second order polynomial mathematical equation as given in (3),

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12} X_1X_2 + \beta_{13}X_1X_3 + \beta_{14} X_1X_4 + \beta_{23} X_2X_3 + \beta_{24} X_2X_4 + \beta_{34} X_3X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \varepsilon \quad \text{---------- (2)}$$

where Y is the response (decolorization); X_1, X_2, X_3 and X_4 are the coded variables; X_{12}, X_{22}, X_{32} are the square effects; X_1X_2, X_1X_3 and X_2X_3 are the interaction effects; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the squared coefficients; β_{12}, β_{13}, β_{23} are the interaction coefficients; β_0 and ε are the constant and the random error, respectively.

$$Y = 87.72 - 4.76 X_1 - 0.97 X_2 + 4.04 X_3 - 0.55 X_4 - 2.25 X_1X_2 - 0.29 X_1X_3 - 2.5 X_1X_4 - 0.32 X_2X_3 - 2.38 X_2X_4 - 0.23 X_3X_4 - 1.71 X_1^2 - 3.89 X_2^2 + 0.94 X_3^2 + 0.89 X_4^2 \quad \text{---------- (3)}$$

is the predicted polynomial equation for malachite green decolorization using *Pseudomonas putida*.

Table 2. Box-Behnken Design towards malachite green decolorization using *Pseudomonas putida*

Run	Temperature (°C)	Dye concentration (mg/L)	Incubation time (hours)	Inoculum volume (mL)	Actual response (%)	Predicted response (%)
1	30	200	24	0.5	85.85	85.83
2	30	200	48	0.75	89.07	87.72
3	30	200	48	0.75	89.14	87.72
4	35	150	48	0.75	78.59	80.56
5	35	200	72	0.75	86.78	85.93
6	30	200	72	0.5	93.34	94.37
7	25	200	72	0.75	95.87	96.04
8	25	200	48	0.5	87.91	89.7
9	30	150	48	1	85.56	87.52
10	30	200	48	0.75	87.82	87.72
11	30	250	24	0.75	80.21	80.07
12	35	200	24	0.75	78.06	78.44
13	30	200	72	1	92.45	92.82
14	30	150	24	0.75	82.32	81.38
15	30	200	48	0.75	87.97	87.72
16	25	150	48	0.75	86.97	85.59
17	30	150	48	0.5	84.7	83.85
18	35	250	48	0.75	72.4	74.13
19	30	150	72	0.75	90.85	90.09
20	30	250	48	1	79.42	80.82
21	25	250	48	0.75	89.78	88.16
22	30	250	48	0.5	88.09	86.68
23	25	200	48	1	93.98	93.62
24	30	200	48	0.75	84.59	87.72
25	25	200	24	0.75	85.98	87.38
26	35	200	48	1	81.77	79.08
27	30	250	72	0.75	87.47	87.52
28	30	200	24	1	85.87	85.19
29	35	200	48	0.5	85.72	85.18

Table 3 represents the relationship between the independent variables and dependent response in the form Analysis Of Variance (ANOVA). The analysis of variance (ANOVA) of regression model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test with a very low probability value [(P model > *F*) = 0.0001]. Linear factors such as X_1 and X_3 were found to be contributing variable towards malachite green decolorization.

The lack-of-fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression. The non-significant value of lack of fit (>0.05) reveals that the quadratic model is statistically significant for the response and hence it can be used for further analysis.

The two-dimensional contour plots are the graphical representations of the regression equation. These plots are presented in figure 1 for malachite green decolorization. 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 [27]. By analyzing the plots, the best response range can be calculated.

3.3. Adequacy of the model

The fitted model must be assessed to ensure that it gives sufficient approximation of the results obtained in the experimental conditions. A check of the normality assumption can be made by constructing a normal probability plot of the residuals as given in figure 2. The normality assumption is satisfied if the residuals lie approximately along a straight line [28]. The coefficient of multiple regression R^2, is a global statistic parameter to assess the fitness of a model [14]. R^2 was found to be 0.93 which is close to 1 and hence denotes that the model is fit. For further validation of the model, adjusted R^2 was used for confirming the model adequacy. The adjusted R^2 value was 0.86 which confirms that model is fit.

Table 3. Analysis Of Variance for malachite green decolorization using *Pseudomonas putida*

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	696.5511	14	49.75365	13.79605	< 0.0001*
X_1-Temperature	272.3674	1	272.3674	75.52402	< 0.0001*
X_2-Dye concentration	11.25203	1	11.25203	3.120046	0.0991
X_3-Incubation time	195.7784	1	195.7784	54.28686	< 0.0001*
X_4-Inoculum volume	3.586133	1	3.586133	0.994389	0.3356
X_1X_2	20.25	1	20.25	5.615067	0.0327*
X_1X_3	0.342225	1	0.342225	0.094895	0.7626
X_1X_4	25.1001	1	25.1001	6.959938	0.0195*
X_2X_3	0.403225	1	0.403225	0.111809	0.7431
X_2X_4	22.70523	1	22.70523	6.29587	0.0250*
X_3X_4	0.207025	1	0.207025	0.057405	0.8141
X_1^2	19.04671	1	19.04671	5.28141	0.0375*
X_2^2	98.39823	1	98.39823	27.28458	0.0001*
X_3^2	5.748748	1	5.748748	1.594055	0.2274
X_4^2	5.168781	1	5.168781	1.433237	0.2511
Residual	50.48916	14	3.606368		
Lack of Fit	36.78088	10	3.678088	1.073246	0.5163
Pure Error	13.70828	4	3.42707		
Cor Total	747.0403	28			

** significant at 95% interval*

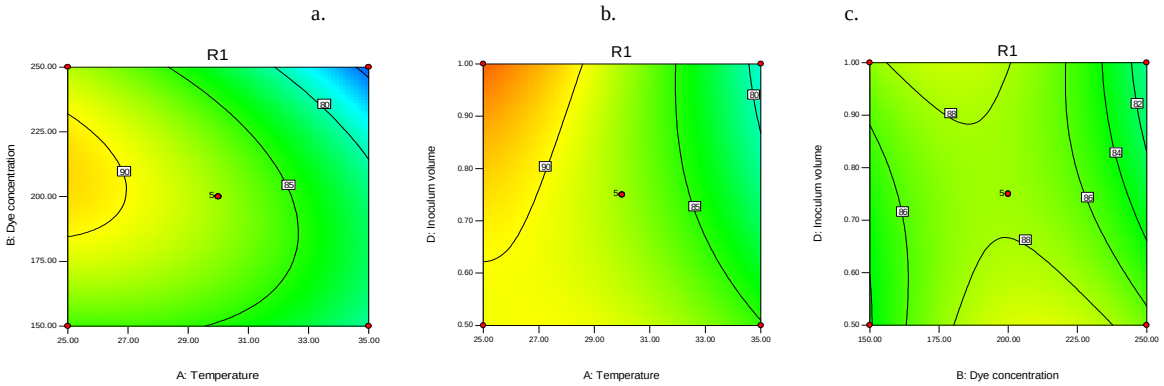

Figure 1. Significant 2D Contour plots for decolorization of malachite green by *Pseudomonas putida.* a. Dye concentration vs temperature, b. Inoculum volume vs temperature, c. Inoculum volume vs dye concentration

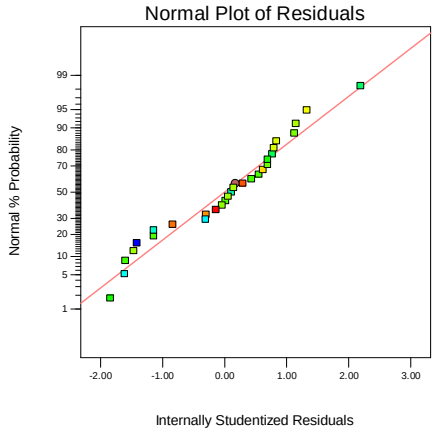

Figure 2. Normal plot for decolorization of malachite green by *Pseudomonas putida*

3.4. Toxicity studies

3.4.1. Microbial toxicity

The zone of inhibition for *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli and Pseudomonas putida* against malachite green and its degraded metabolites was observed after 24 hours and recorded (Table 4). The micro-organisms did not show any zone of inhibition against the degraded metabolite, thereby indicating that the degraded metabolite was not toxic to the micro- organisms.

Table 4: Microbial toxicity study of malachite green and its decolorized product

Micro organism	Diameter of zone of inhibition (mm)	
	Dye	Decolorized product
Escherichia coli	25	0
Bacillus subtilis	17	0
Bacillus cereus	15	0
Pseudomonas putida	10	0

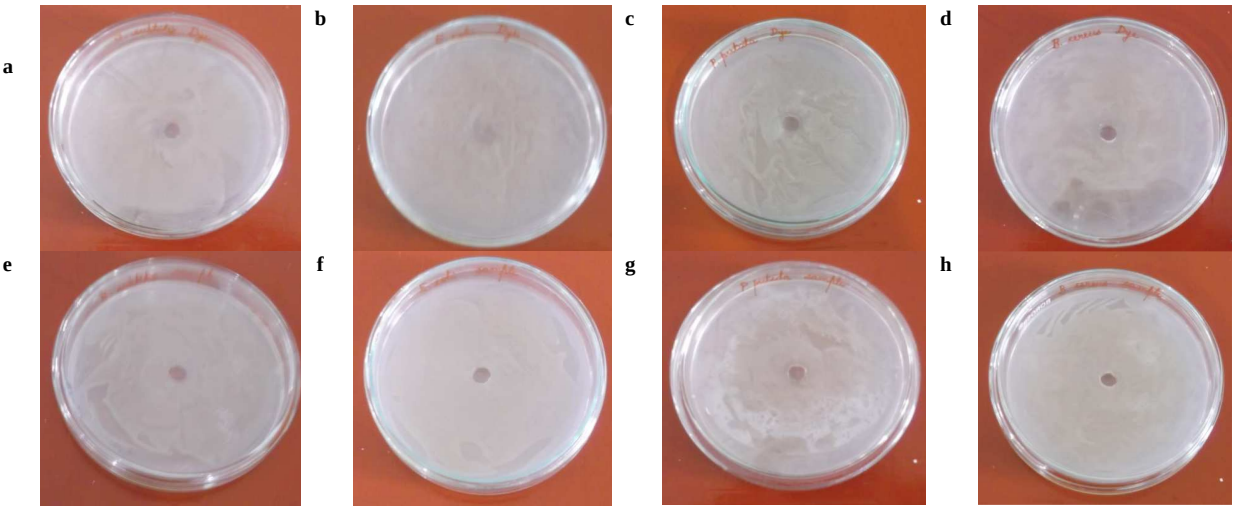

Plate 1: Zone of inhibition by malachite green (200mg/L) on a: *Bacillus subtilis*, b: *Escherichia coli*, c: *Pseudomonas putida*, d: *Bacillus cereus*

Zone of inhibition by the decolorized metabolites of malachite green e: *Bacillus subtilis*, f: *Escherichia coli*, g: *Pseudomonas putida*, h: *Bacillus cereus*

3.4.2. Phytotoxicity studies

The seedling germination rate and growth for both the plants (Table 5) was good in the presence of treated dyes as compared to the untreated dyes. In fact, the seedling germination and growth in the presence of treated dyes was almost equivalent to that of the control which was grown in the presence of distilled water.

Table 5: Phytotoxicity study of malachite green and its decolorized product

Seed	Germination rate (%)	Plumule length (cm)	Radicle length (cm)
Vigna radiata			
Control	100	7	5
Decolorized sample	100	6.5	4.8
Dye	85	2.5	1
Triticum aestivum			
Control	90	8	6
Decolorized sample	85	7.4	5.7
Dye	70	4.5	2.3

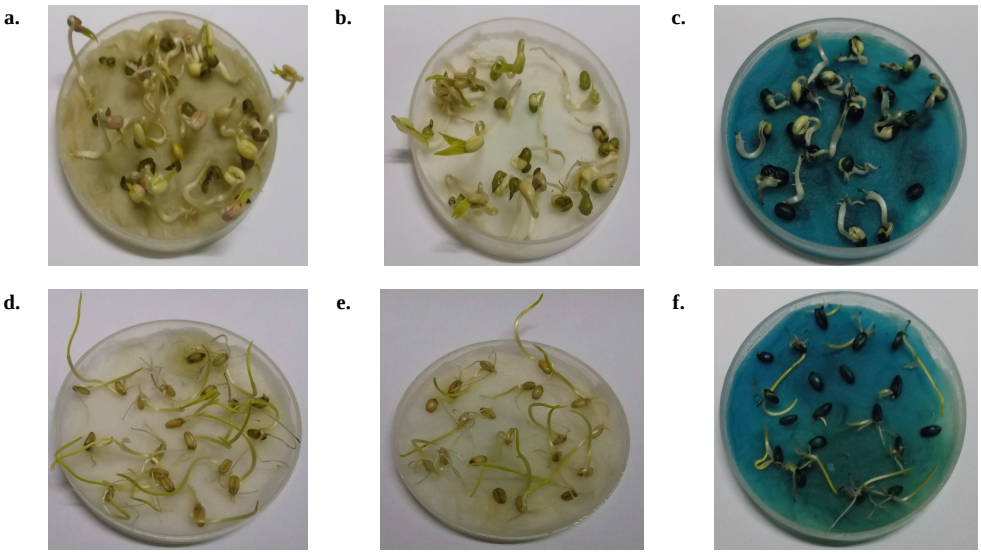

Plate 2: Phytotoxicity studies on *Vigna radiata* and *Triticum aestivum.*
***Vigna radiata* after 3 days of incubation- a. control b. decolorized metabolites c. dye.**
***Triticum aestivum* after 3 days of incubation- d. control e. decolorized metabolites f. dye.**

CONCLUSION

Bioremediation has proved to be a very effective method in encountering the textile dye pollution in an eco-friendly manner. This approach creates a promising hope for remediation of the environment which is polluted by hazardous dyes. The present study confirms the ability of *Pseudomonas putida* to decolorize malachite green dye with an efficiency of 95%. The decolorized metabolites of the dye showed no toxic effect on microbial growth and plant germination. Thus the hazardous dye was converted into non toxic metabolites.

The ability of the strain to tolerate, decolorize and degrade malachite green at high concentration gives it an advantage for the treatment of textile industry wastewater. However the potential of the bacteria needs to be demonstrated for its application in the treatment of dye containing industrial effluents using appropriate bioreactors. This study further recommends the identification and purification of enzymes in *Pseudomonas putida* and their kinetics involved in the degradation of malachite green. To understand the mechanism behind the degradation of the dyes by the bacteria LC-MS, FTIR can be performed. Further research on this bacterial strain could explore new tools and techniques to evolve viable and eco friendly solutions for the treatment of dyes in the industrial effluents.

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