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Bioremediation of Methylene Blue by *Bacillus thuringiensis* 4 G1: Application of Statistical Designs and Surface Plots for Optimization

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Abstract: In this study *Bacillus thuringiensis* 4G1 was used to decolorize Methylene Blue (MB). The decolorization process was optimized by using two sequential experimental designs. Eleven fermentation factors were screened using Plackett-Burman design. Among these factors, the most significant variables influencing MB decolorization were statistically elucidated for optimization and included $MgSO_4.7\ H_2O$, glucose and $(NH_4)_2\ SO_4$. The optimum concentrations of these variables were predicted by using a second order polynomial model fitted to the results obtained by applying the Box-Behnken design. A verification experiment performed under optimal conditions yielded 98.23% of the predicted decolorization% (100%) with an increase by a factor of 1.3 compared with the results obtained under basal conditions.

Key words: *Bacillus thuringiensis*, methylene blue, statistical design-bioremediation

INTRODUCTION

The presence of dyes in effluents is a major concern due to their adverse effect to many forms of life. Colored waters is one of the most important hazard in industrial effluents, which needs to be treated (Garg *et al*., 2004; Forgacs *et al*., 2004; Pearce *et al*., 2003; Pala and Tokat, 2002; Guarantini and Zanoni, 2000; Chun and Yizhong, 1999) because the presence of dyes in water reduces light penetration, precluding the photosynthesis of aqueous flora (Robinson *et al*., 2002). Besides that, some dyes may cause allergy, dermatitis, skin irritation and cancer to humans (Bhatnagar *et al*., 2005) in addition to being mutagenic (Gong *et al*., 2005). Synthetic dyes are extensively employed in textile, paper, photo electrochemical cells, printing, leather, food, cosmetics, etc. industries, which employ these substances to color their final products. The treatment of aqueous water containing soluble dyes thus requires complete removal followed by secure disposal (Webe and Morris, 1964). The most commonly used techniques for color removal include chemical precipitation, ion exchange, reverse osmosis, ozonation and solvent extraction etc. However, these techniques have certain disadvantages such as high capital cost and operational costs or secondary sludge disposal problem (Khattri and Singh, 1998). Microbial decolorization processes offer a complete cleanup of pollutants in a natural way and appear to be an attractive alternative (Radha *et al*., 2005; EL- Sharouny and El- Sersy, 2005; Boer *et al*., 2004; El-Sersy, 2001).

Medium optimization using statistical designs was recently used for the decolorization of dyes (Pavan *et al*., 2005; Ravikumar *et al*., 2005).

In this present study for developing maximum Methylene Blue (MB) decolorization, two experimental designs were sequentially applied as a tool for optimizing bacterial decolorization process. In the first optimization step, Plackett- Burman experimental design (Plackett and Burman, 1946) was applied to test the relative importance of various environmental factors on MB decolorization. In the following step, Box-Behnken design (Box and Behnken, 1960) was applied for further optimization to the most significant variables. This design is a response surface methodology (Montgomery, 2001) employed to find factor settings that produce the best response, find factor settings that satisfy operating or process specifications and model a relationship between the quantitative factors and the response.

MATERIALS AND METHODS

Chemicals: The heterocyclic Methylene blue (MB) dyes ($C_{16}H_{18}Cl\ N_3S.\ H_2O$) was of pure grade provided from BDH, Hseelze-Hanover, Germany. All reagents and medium components used, were obtained from Oxoid Ltd., Basingstoke, Hampshire, England.

Microorganisms and culture conditions: *Bacillus subtilis 168*, *Bacillus lichemniformis* 5A1, *Bacillus thuringiensis* 4G1 and *Bacillus sphaericus* which *are* from Bacillus genetic stock centre Ohio State University, UK, were screened for their ability to decolorize different MB concentrations. Screening test was performed on basal agar plates amended with different MB concentration and incubated at 37°C for two days. Colonies that showed

decolorization clear zones were selected. The basal liquid medium used for decolorization contained in g L^{-1}; glucose, 3; yeast extract, 1; $(NH_4)_2SO_4$, 1; K_2HPO_4, 6; KH_2PO_4, 1; $MgSO_4$. 7 H_2O, 0.1; NaCl, 5; Fe SO_4, 0.001 and MB, 0.3. Laury Broth (LB) medium contained in g L^{-1}: peptone, 10; yeast extract 5 and NaCl, 5.

Decolorization assay: Seed cultures were prepared by inoculating LB liquid medium from single colony and shaken until reached an absorbance (A_{550}) of 1.0. Decolorization cultures were prepared by inoculating 50 mL basal medium containing MB (0.3 g L^{-1}) in 250 mL Erlenmeyer flasks with 0.5 mL of seed cultures.

Cells of 1 mL culture aliquots were pelleted by centrifugation for 10 min at 10,000 rpm. MB concentration was determined by measuring absorbance of clear supernatants at 665 nm (Boer *et al.*, 2004). The medium formula lacking MB was used as a control.

Experimental designs

Plackett-Burman design: The Plackett-Burman experimental design, a fractional factorial design, (Plackett and Burman, 1946) was used to reflect the relative importance of various environmental factors on MB decolorization in liquid cultures. Eleven independent variables were screened in twelve combinations organized according to the Plackett-Burman design matrix (Table 2) for each variable; a high (+) as well as low (-) level was tested. All trials were performed in duplicates and the averages of decolorization observation results were treated as the responses. The main effect of each variable (Table 1) was determined with the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})N^{-1}$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the decolorization percentage in trials where the independent variable (xi) was present in high and low concentrations, respectively and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples (Table 1) were calculated for determination of variable significance.

Box-Behnken design: Experimental design, which is a central composite design (Box and Behnken, 1960), was applied. In this model, the most significant independent variables, designated (X_1), (X_2) and (X_3) were included and each of them was examined at three different levels, low (-), high (+) and central or basal (0). According to the applied design described in the Results section, thirteen dye treatment combinations were tried. For predicting the optimal point, the following second order polynomial model was fitted to correlate relationship between independent variables and response:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where, Y is the dependent variable (MB decolorization %); X_1, X_2 and X_3 are the levels of independent variables; b_0 is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients were calculated using Micrococal Origin 4.1 software and the optimum concentrations were predicted using Microsoft Excel 2000. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The optimal value of MB

Table 1: Factors examined as independent variables affecting methylene blue decolorization and their levels in the Plackett-Burman experiment

		Level				
Variable	Symbol	-1	0	+1	Main effect	[1]t-value
Inoculum size (mL)	IS	0.5	1	1.5	2.80	0.580
Peptone (g L^{-1})	P	0.5	1	1.5	1.85	0.384
Yeast extract (g L^{-1})	YE	0.5	1	1.5	-4.10	-0.880
Glucose (g L^{-1})	G	1.0	3	5	5.50	1.200
$(NH_4)_2SO_4$ (g L^{-1})	NH	0.5	1	1.5	-11.00	-3.300
K_2HPO_4 (g L^{-1})	K2	4.0	6	8	0.80	0.160
KH_2PO_4 (g L^{-1})	KH	0.0	1	2	-3.50	-0.700
$MgSO_4.7H_2O$ (g L^{-1})	Mg	0.05	0.1	0.15	-5.30	-1.166
NaCl (g L^{-1})	Na	2.5	5	7.5	-3.00	-0.640
$FeSO_4$ (g L^{-1})	Fe	0.0	0.001	0.002	2.10	0.440
Methylene blue (g L^{-1})	MB	0.25	0.3	0.35	-0.20	-0.034

[1]Significant t-values at the 1, 5 and 10% levels are 2.764, 1.812 and 1.372, respectively (Chatfield, 1975)

Table 2: Plackett-Burman experimental design for 11 factors

	Independent variables[1]											MB[2] decolorization
Trial	G	NH	K2	KH	Mg	Na	Fe	MB	IS	P	YE	(%)
1	+	-	+	-	-	-	+	+	+	-	+	83.30
2	+	+	-	+	-	-	-	+	+	+	-	71.80
3	-	+	+	-	+	-	-	-	+	+	+	61.32
4	+	-	+	+	-	+	-	-	-	+	+	73.88
5	+	+	-	+	+	-	+	-	-	-	+	60.00
6	+	+	+	-	+	+	-	+	-	-	-	63.10
7	-	+	+	+	-	+	+	-	+	-	-	64.50
8	-	-	+	+	+	-	+	+	-	+	-	72.20
9	-	-	-	+	+	+	-	+	+	-	+	63.10
10	+	-	-	-	+	+	+	-	+	+	-	80.30
11	-	+	-	-	-	+	+	+	-	+	+	62.00
12	-	-	-	-	-	-	-	-	-	-	-	76.40
13	0	0	0	0	0	0	0	0	0	0	0	76.10

[1]See Table 1 for clarification of factor symbols, [2]Results were taken after 24 h, + = High, - = Low

decolorization was estimated using the solver function of Microsoft Excel tool.

Statistica 6.1 software was used to illustrate the quadratic responses plots.

RESULTS

Screening for MB decolorization ability: From the four tested bacillus strains (*Bacillus subtilis* 168, *Bacillus lichemniformis* 5A1, *Bacillus thuringiensis* 4G1 and *Bacillus sphaericus*) on basal agar plates amended with 0.2, 0.3, 0.4 and 0.5 g L^{-1}, the largest decolorization zone (5.6 mm) was detected around *Bacillus thuringiensis* 4G1 amended with 0.3 g L^{-1} MB. According to this result, this strain was chosen to complete the work with on a concentration of 0.3 g L^{-1} MB.

Factors affecting MB decolorization (Plackett Burman design): For elucidation of medium components affecting MB decolorization, the independent variables examined in the Plackett Burman experiment and their settings are shown in Table 1. The main effect of each variable was calculated according to MB decolorization % results (Table 2). The data indicated that, the presence of high levels of Inoculum size, Peptone, glucose, K_2HPO_4 and Fe SO_4, in the growth medium affects MB decolorization positively. On the other hand, the presence of KH_2PO_4, $MgSO_4$. 7 H_2O, NaCl, $(NH_4)_2SO_4$ and MB at their lowest levels would result in high decolorization %. According to this results it can be predicted that the near optimum medium for MB decolorization by *Bacillus thuringiensis* 4G1 is (g L^{-1}): Peptone, 1.5; glucose, 5; yeast extract, 0.5; $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 8; $MgSO_4$. 7 H_2O, 0.05; NaCl, 2.5; Fe SO_4, 0.002 and MB, 0.25; inoculum size, 1.5 mL (50 mL)$^{-1}$ medium.

In order to evaluate the accuracy of the applied Plackett Burman screening test, a verification experiment was carried out in triplicate. The predicted near optimum levels of independent variables were examined and compared to the basal condition setting except for MB concentration was fixed to be at the basal setting (0.3 g L^{-1}). The average of MB decolorization % was recorded. MB decolorization reached about 89% which is approximately 1.2 times higher than that obtained from basal medium (76.1%). On the basis of the calculated t-values (Table 1), $MgSO_4$.7 H_2O; Glucose and $(NH_4)_2$ SO_4 were chosen for further optimization, since these factors had the most significant effects on the MB decolorization process.

Optimization of MB decolorization by Box-Behnken design: In this second optimization step the levels of the three significant independent variables $MgSO_4$.7 H_2O (X_1); Glucose (X_2); and $(NH_4)_2$ SO_4 (X_3) were further investigated each at three different levels (Table 3). Near optimum levels of the other factors, suggested by the Plackett-Burman experimental results were used in all trials. All cultures were performed in duplicate and the averages of the observations (MB decolorization %) were used. Presenting experimental results in the form of surface plots (Fig. 1a-c) showed that high $MgSO_4$.7H_2O concentration together with high level of glucose (Fig. 1a) slightly inhibit MB decolorization, while high $MgSO_4$.7 H_2O concentration together with high level of $(NH_4)_2$ SO_4 appeared to be positive with respect to decolorization process (Fig. 1b). On the other hand the interaction between $(NH_4)_2SO_4$ and glucose concentration (Fig. 1c) illustrated that low Glucose concentration together with high $(NH_4)_2SO_4$ level greatly supports MB decolorization. For predicting the optimal point, a second-order polynomial function was fitted to the experimental results of MB decolorization (Y):

$$Y = 176.28 + 414.36X_1 - 43.32X_2 - 69.7X_3 - 247.8X_1X_2 - 64.01X_1X_3 + 0.322X_2X_3 + 7213X_1^2 + 5.5X_2^2 + 24X_3^2$$

The fit of the model is expressed by coefficient of determination, R^2, which was calculated to be 0.938. The closer the R^2 value to 1.00 the stronger the model is and the better it predicts response. Accordingly, our calculated R^2 value indicates that the model could explain 93.8% of the variability in the response. The predicted optimal concentrations of the three components as obtained from the solver function of Microsoft Excel tool were calculated to be (g L^{-1}): $MgSO_4$.7 H_2O (0.096), glucose (3) and $(NH_4)_2$ SO_4 (0.5) with a predicted MB decolorization % of 100.

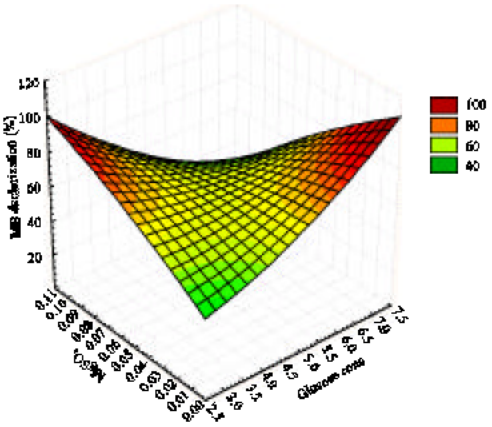
Table 3: Examined concentrations of the key variables and results of the Box-Behnken experiment

Trial	$MgSO_4$.7 H_2O *(X_1)	Glucose *(X_2)	$(NH_4)_2$ SO_4 *(X_3)	MB**decolorization (%)
1	0.10	7	1.5	20.6
2	0.10	3	1.5	68.3
3	0.01	7	1.5	92.3
4	0.01	3	1.5	50.3
5	0.10	5	3.0	90.0
6	0.10	5	0.5	63.3
7	0.01	5	3.0	98.3
8	0.01	5	0.5	50.0
9	0.05	7	3.0	98.3
10	0.05	7	0.5	67.3
11	0.05	3	3.0	98.2
12	0.05	3	0.5	71.3
13	0.05	5	1.5	22.0

*All variables were in g L^{-1}, **Results were taken after 24 h

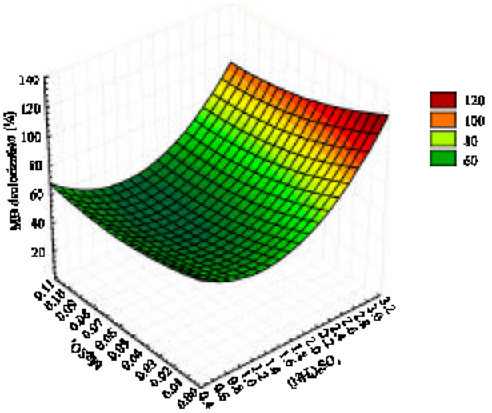
(a)

MB decolorization% = 40.2269-0.4149*x+1245.4485*y+1.283x*x-247.9141*x*y-1283.2341*y*y



(b)

MB decolorization% = 89.5889-45.9028*x-300.221*y+17.9088*x*x-66.4426*x*y+2481.0216*y*y



(c)

MB decolorization% = 186.5088-41.8622*x-59.8222*y+4.058*x*x+0.4196*x*y+20.2898*y*y

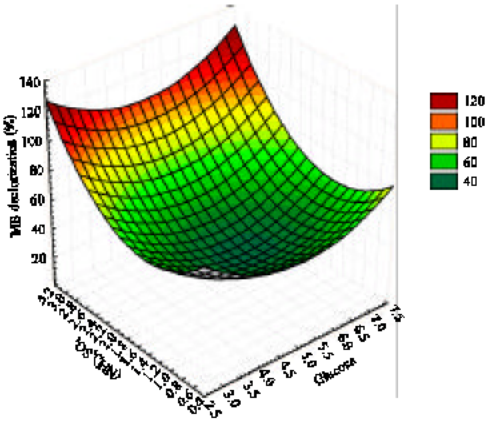


Fig. 1: The response of MB decolorization as function of $MgSO_4.7H_2O$ and glucose concentrations (a), $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$ concentrations (b), $(NH_4)_2SO_4$ and glucose concentrations (c)

Verification of the model: In order to determine the accuracy of the of the model and to verify the optimization results, an experiment was performed under basal and predicted optimal conditions where MB decolorization was monitored after 24 h. MB concentration were fixed at a concentration of 0.3 g L^{-1} in both cultures. Under the optimized condition, a 98.23% decolorization was reached after 18 h, while the basal control medium reached 76.2% after 24 h. These results indicate that the optimized condition accelerated the reaction rate with decolorization % of 1.3 fold increase.

Matching the predicted degradation (100%) and the observed degradation (98.23%) under optimal condition also proves the accuracy and validity of the model. According to this results it can be predicted that the optimum medium for MB decolorization by Bacillus *thuringiensis* 4G1 is (g L^{-1}): Peptone, 1.5; glucose, 3; yeast extract, 0.5; $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 8; $MgSO_4$. 7 H_2O, 0.096; NaCl, 2.5; $FeSO_4$, 0.002 and MB, 0.25; inoculum size, 1.5 mL (50 mL)$^{-1}$ medium.

DISCUSSION

Several studies have demonstrated the ability of fungi to decolorize synthetic dyes (Radha *et al*., 2005; Ravikumar *et al*., 2005; Pavan *et al*., 2005; Boer *et al*., 2004) while other studies reveled bacterial dye decolorization (El-Sersy, 2001; Hu, 1994, 1996). In the present study, four Bacillus strains were screened for their ability to decolorize MB dye. *Bacillus thuringiensis* 4G1, had a relatively high ability to decolorize MB.

Synthetic dye removal in a batch system usually depends on several factors. The optimization of all those variables using the univariate procedure is very tedious, because any variable (factor) is optimized, by varying just one factor by the time and fixing the others. Then, the best value achieved by this procedure is fixed and other factors will be varied by the time. The disadvantage of this univariant procedure is that the best condition could not be attained, because the interactions among all the factors are disregarded and also it is not known if the set of other fixed variables was fixed at other levels, the results would lead to the same optimization. In order to overcome these disadvantages, statistical design of experiments can be carried out to achieve the best optimization of any possible system (Brasil *et al*., 2005). Environmental studies were carried out in this work by applying a Plackett-Burman multifactorial experiment which reflected the influence of various fermentation factors on MB decolorization. The results showed that the presence of inoculum size, peptone, glucose, K_2HPO_4 and $FeSO_4$ in their high level appear to promote MB decolorization process, while the presence of yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$ and NaCl in their low level enhance the process as well. However, glucose, $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ were significantly effective factors. This agree with Radha (2005) who stated that dye decolorization process is mainly an extracellular enzymatic process which is promoted by the presence of glucose and $(NH_4)_2SO_4$ as a simple carbon and nitrogen sources respectively, while $MgSO_4.7H_2O$ is a growth promoter factor.

The optimal response region of these significant factors was predicted by using a second-order polynomial model fitted to the results obtained by applying Box-Behnken statistical design.

Substantial differences in MB decolorization appeared as a result of reducing glucose concentration to 3 g L^{-1} and increasing $MgSO_4.7\ H_2O$ levels to 0.096 g L^{-1}. Although these results were deduced by Plackett-Burman experiment, this unexpected result could be due to the new combination among the medium components in the optimized condition. This is one of the advantages of applying multifactorial experiments that consider the interaction of independent variables and provide a basis for model to search for the non linear nature of the response in short-term experiment (Ravikumar *et al*., 2005; Pavan *et al*., 2005). The great similarity observed between the predicted and experimental results reflects the high accuracy and applicability of the model.

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