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Screening of potential bacteria from soil sample for methylene blue dye degradation: RSM modelling and optimisation

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Abstract: This study aimed to screen the bacteria from the samples collected from textile dye enriched soil. Twenty-one soil bacteria were isolated and examined for their efficacy for methylene blue (MB) decolourisation. *Bacillus cereus* found to be the potential microorganism for MB decolourisation, characterised using 16S rRNA sequence technique. Response surface methodology (RSM) was employed to optimise the three independent variables like dye concentration (mg/L), pH and glucose concentration (g/L) for decolourisation of MB. The experimental run given by central composite design (CCD) with quadratic model was conducted used in this study to optimise the variables and fit the model. The analysis of variance (ANOVA) showed; $R^2 = 0.9992$ and $R^2_{adj} = 0.9985$, which confirmed that experimental and predicted response are in good agreement and the model is highly significant. The optimal values of dye concentration (mg/L), pH and glucose concentration (g/L) proposed by RSM were found to be 70, 7.88 and 3.33, respectively.

Keywords: decolourisation; methylene blue; *Bacillus cereus*; response surface methodology; RSM; central composite design; CCD.

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Y. Pydi Setty is working as an Emeritus Professor at the Department of Chemical Engineering, National Institute of Technology Warangal. He is a pioneer in the field of bioreactors, process engineering and wastewater treatment.

1 Introduction

More than 100,000 commercially available dye are existing and produced annually over 7×10^5 tones (McMullan et al., 2001). 10%–15% of the dyes are passed in the liquid discharge while dyeing, every year 280,000 tons of dyes are fluxed in dyestuff industries globally (Maas and Chaudhari, 2005). Organic and inorganic pollutants of synthetic dyes hinder photosynthesis process, dissolved oxygen (DO) and water transparency of water bodies (Crini, 2006). The wastewater contains xenobiotic compounds, which are recalcitrant and derived from naphthalene, phenol, benzene, toluene (Frankenburg and Baldessarini, 2008; Wainwright and Crossley, 2014). Synthetic dyes have complex aromatic structure and these composite dyes are synthesised to avoid the fading of colour from dyestuff when exposes to soap, sweat, water, light and any oxidising agent (Khan and Husain, 2007).

Methylene blue (MB) ($C_{16}H_{18}N_3SCl$), a heterocyclic cationic azo dye enormously used in textile industries and it is a solid, inodorous, dark green colour powder that converts into a blue solution with maximum absorption of 670 nm in water. MB is most common dye, widely used in dyeing industries, i.e., cotton, wood, silk, leather, paper, plastic, hair, etc. It also be used for staining of cells, as an indicator and antiseptic agent in laboratories and medical therapies (Frankenburg and Baldessarini, 2008; Wainwright and Crossley, 2014). However, it is carcinogenic and mutagenic also, its inhalation causes hemolytic anaemia, hyperbilirubumia, nausea, vomiting, mental disorder, methemoglobinemia and sudden failure of kidney in humans. It can also cause permanently inaction of eyes of humans and animals as well (Tan et al., 2008a, 2008b; Tantiado, 2012). Aquatic flora and fauna life cycles are also affected by synthetic dye wastewater due to inappropriate treatment because MB barricades the DO to reach deep insight the water bodies (Manu, 2002; Singh et al., 2014). It was reported that 50–1,000 mg/dm³ concentration of MB are found in effluents of wastewater (Sarioglu, 2018). By considering the serious causes of discharging MB dye into environment, the research work pertained to MB dye degradation has gained more attention, and numerous approaches have been proposed includes physical, chemical and biological methods. In particular, adsorption (Zhu et al., 2007), photocatalytic degradation (Sohrabi and Ghavami, 2008), sono-chemical degradation (Abbasi and Razzaghi-Asl, 2008), ultrafiltration (Zaghbani et al., 2008), cation exchange membranes (Wu et al., 2008), electrochemical degradation (Fan et al., 2008), integrated chemical-biological degradation (Sudarjanto et al., 2006), fenton-biological treatment (Lodha and Chaudhari, 2007) were used for dye degradation studies. During past three decades, biological treatment have pulled the attention of researcher to discover the potential microorganisms, especially chemical treatment method yielded the toxic intermediate pollutant, which needs further biological treatment (Simi and Azeza, 2010).

Various microorganisms, i.e., bacteria, fungi, yeast, algae and also enzymes can be used for decolourisation of textile dyes. Several bacteria are being isolated for different dye decolourisation results in two ways: enzymatic process or adsorption on biomass (Kilany, 2017). The abundant availability of microbial source from various collection centres, high biomass growth rate and easy to scale up the technology were encouraged to find out the microbial mediated degradation methods for dyes (El-Sheekh et al., 2009; Anjaneya et al., 2013; Gunasekar et al., 2013). Dye degradation using microorganisms, immobilisation methods, bioreactors are being widely explored. Initially, identification of potential microorganism for specific dye is vital step, subsequently the optimisation and

scaling up the technology using bioreactors are considered (Hu and Reeves, 1997; Veglio and Beolchini, 1997; Vijayaraghavan and Yun, 2007). The understanding from the various dye degradation studies using different microorganisms reiterated that microbial metabolism for the dye intake and degradation is varied with respect to dye and microorganisms. MB dye predominantly used in most of areas such as clinical medicine (ranging from cancer chemotherapy to dementia), textile industries, food industries, dying of cells, etc. (Wainwright and Crossley 2014). Given the details on the rapid use of MB and disadvantages of dye discharge into the environment without treatment, it is warranted to identify the potential microorganism for dye degradation, so that it will be easy to scale up the treatment process using bioprocessing technology.

In the present study, 21 cultures were isolated from the soil sample; these cultures were screened for potential candidature for decolourisation of MB. Identifying the suitable culture and biological method for degradation of MB are quite challenging. The present study was focused on identifying the potential microbe for the MB degradation. The process variables such as dye concentration, pH and glucose concentration were optimised using response surface methodology (RSM). One factor at a time approach (OFACT) is ordinary method for optimisation processes by running one variable at a time and keeping other parameters constant could not able to define the combinatorial effect of all factors (Elibol, 2002). RSM an efficient experimental methodology for multivariable processes and its optimisation reduces the number of experiment compared to full experimental design and immediate interpretation (Shoabegh and Karimi, 2014). It was first reported by Box and Wilson (1951), a mathematical and statistical method for advancement, enhancement and optimisation process used for evaluating the relative significance of many influencing factors in spite of multiple interactions (Subramaniam and Kumar Ponnusamy, 2015). RSM is used to find out the optimum operational condition or region for the designed experiment. The experimental runs proposed by RSM based on the given values of low and higher levels were executed to find out the optimal parameters for higher decolourisation.

2 Materials and method

2.1 Materials

All chemicals were used of analytical grade for the experimental purpose.

2.2 Inoculum and media preparation

All 21 cultures were grown in 100 mL nutrient broth (NB) (composition: beef extract: 10g/L; NaCl: 5g/L; Peptone: 10 g/L) media at 37°C in a 250 mL conical flask individually, was used as inoculum. 10% (v/v) of inoculum was added to mineral salt media (MSM) in composition (g/L) KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1, NH_4Cl 0.8, Na_2HPO_4 1.2, $\text{C}_6\text{H}_{12}\text{O}_6$ 4, yeast 1 containing MB dye prepared for the further experiments.

2.3 Isolation and screening of potential bacteria

Twenty-one bacterial strains were isolated from dye contaminated soil samples by serial dilution method (Benson, 2002). Which are isolated from textile dye enriched soil

Samples collected near dye contaminated water bodies situated near dye industry, Warangal, India. 5 g soil was mixed in 100 ml distilled water in different test tube, then 1ml of each soil suspension samples were serially diluted in distilled water. Volume of 0.1 ml of each soil suspension samples were spread onto nutrient agar plates and incubated at 37°C for 24 hours. Twenty-one different colonies were selected and subculture on the individual slants and stored at 4°C. All 21 strains were inoculated in 150 mL of Erlenmeyer flask containing 50 ml nutrient broth media and MB dye at a concentration of 25 mg/L, incubated at 37°C for 24 hours. At different time intervals 3 mL of culture was withdrawn, centrifuged at 8,000 rpm/min for ten minutes to separate the bacterial cells and collected supernatant was recorded for absorbance at 664 nm using UV-Visible spectrophotometer.

2.4 Sequencing report of selected isolate

Molecular identification of selected strain was carried out using 16S rRNA gene sequencing by National collection of Industrial microorganism (NCIM), Pune. DNA was isolated by using commercial DNA isolation kit (for bacteria). Universal primers were used for PCR amplification for 16S rRNA gene sequencing. Basic local alignment search tool (BLAST) algorithm was used to identify the member of gene family. BLAST report revealed that the strain showed the closest homology with *Bacillus* sp. (closer to *Bacillus cereus*).

2.5 Decolourisation experiment

Decolourisation experiment was conducted according to the design elaborated by the central composite design (CCD) surface methodology. All experiments were conducted in 250 mL Erlenmeyer flasks in 100 mL of total aqueous MSM. 10% NB inoculum were added in MSM and rest conditions were maintained as per the designed experiments. Dye concentration of synthetic wastewater was maintained from the stock solution of dye and pH was adjusted using 0.1 M NaOH or HCl as stated by CCD. The flask was incubated at 37°C for 24 hours at 130 rpm in orbital shaking incubator (LABLINE 05124C). Samples were collected at different time intervals and centrifuged (REMI, RM-12C) at 10,000 rpm for 10 min. Dye concentration were calculated using UV-Visible spectrophotometer at 664 nm (Lovibond XD7500). All experimental runs were conducted in triplicate mode and average values were fitted into the designed runs.

MB decolourisation percentage is consider as response of the experimental design and calculated as follows:

$$\% \text{MB decolourisation} = \frac{\text{initial concentration} - \text{final concentration}}{\text{initial concentration}} \times 100$$

2.6 Experimental design and optimisation of parameters

Dye concentration (X_1), pH (X_2) and glucose concentration (X_3) were chosen as the independent input variables and dye removal efficiency as the dependent output variable and each variables were studied at five different coded level (-1.68, -1, 0, 1, 1.68). CCD consisting three-level and three-factor with 20 experimental runs were performed for

optimisation of design variables. Independent design variables with its range and levels for MB removal are represented in Table 1.

Table 1 Range and levels of design variables for MB removal

Design variables/independent variables	Units	Symbols	Range and levels				
			-1.68	-1	0	1	1.68
Dye Con.	mg/l	X ₁	66.59	70	75	80	83.40
pH		X ₂	6.31	7	8	9	9.68
Glucose	g/l	X ₃	0.63	2	4	6	7.36

The statistical calculation for independent variables (X_i) was calculated according to the following equation (Shoaebargh and Karimi, 2014):

$$X_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where X_i denotes the independent variable coded value, X_i and X_0 are independent variable real value; X_0 is the centre point of the domain and ΔX_i is the step change. The experimental design, effect of variables on optimisation process and its statistical analysis were studied in the Design expert version 7.0.0. Analysis of variance (ANOVA) is the best way to estimate the quality of the fitted model. The removal percentage of MB dye was estimated using a second order polynomial equation in terms of linear, quadratic and cross product for correlating effect of variables. The general quadratic equation is as follow (Shoaebargh and Karimi, 2014),

$$Y = b_0 + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n b_{ii} x_i^2 + \sum_{j>1}^n \sum_{i=1}^n b_{ij} x_i x_j \quad (2)$$

where b_0 is a constant term, x_i are the coded variables, b_i are the linear term coefficients, b_{ii} are the coefficients for the quadratic terms, b_{ij} are the coefficients for the cross product and n is the number of variables. Table 2 illustrate the appropriate model suggested by the RSM, F-value is calculated for each model and highly significant model is chosen for the optimisation of variables.

Table 2 Sequential model for the experimental design

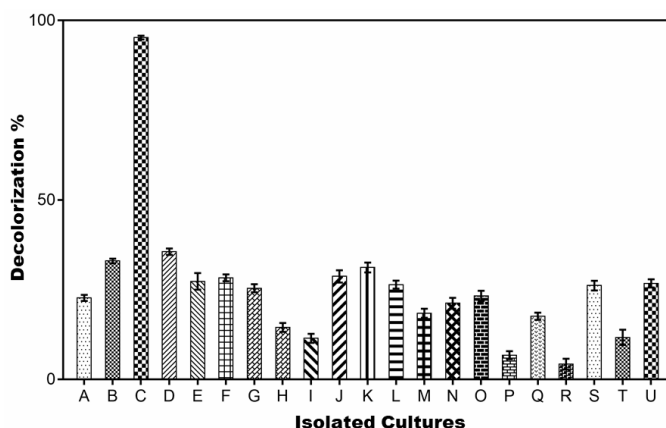
Source	Sum of square	Degree of freedom	Adj. mean square	F	P	
Mean vs. total	76,723.04	1	76,723.84			
Linear vs. mean	7,431.80	3	2,477.27	17.97	< 0.0001	
2FI vs. linear	235.61	3	78.54	0.52	0.6771	
Quadratic vs. 2FI	1,963.15	3	654.38	879.33	< 0.0001	Suggested
Cubic vs. quadra	2.41	4	0.60	0.72	0.6103	Aliased
Residual	5.04	6	0.84			
Total	86,361.84	20	4,318.09			

3 Result and discussion

3.1 Screening and identification of potential bacteria for MB removal

The dye decolourisation of MB results using 21 bacterial isolates, which are isolated from textile dye enriched soil are depicted in Figure 1. The bacterial isolates are represented as alphabet letter, i.e., A to U. Only one bacterial isolates (C in Figure 1) out of 21 showed the maximum decolourisation activity towards MB. Although bacterial isolate C showed maximum decolourisation, B and D bacterial isolates were also showed a significant decolourisation compared over other bacterial isolates. However, MB decolourisation by B and D bacterial isolates is insignificant compared to bacterial isolate C (Figure 1). All experiments were conducted by maintaining uniform conditions for all bacterial isolates, only isolate C has showed maximum decolourisation. Thus, it is very important to identify the bacterial isolate 'C' and also enumerate the efficiency of this bacteria for MB dye decolourisation. 16S ribosomal RNA sequencing has employed to identify the 'C' bacterial isolate, the sequencing results confirmed that isolated bacteria are assigned to *Bacillus cereus*. Thus, isolate C in Figure 1 represents *Bacillus cereus*, remaining bacterial isolates have not been considered for sequencing due to the research findings of this work has confined to screen and identify the potential bacteria for MB decolourisation. The experimental results illustrated that *Bacillus cereus* is a potential bacterium for MB dye decolourisation and also may have ability to use for other textile decolourisation. However, we observed 95.25% of MB decolourisation by *Bacillus cereus*. The dye removal efficiency could be enhanced by providing optimal conditions for bacterial growth and also to improve its biological interaction. Hence, optimisation of process parameters for maximising decolourisation using RSM is an important aspect of the present study.

Figure 1 Representation of 21 bacterial isolates efficiency towards MB decolourisation



Note: All experiments were conducted at dye concentration of 25 mg/L, temperature-37°C, pH-7 and 130 rpm.

3.2 CCD analysis

The experimental setup considered three variables X_1 (dye concentration), X_2 (pH) and X_3 (glucose concentration) and response of dye removal (%). CCD has proposed 20 experimental run with respective to the range and levels of variables given in Table 1. All 20 experimental runs composed of 14 trials and six centre points including predicted and actual responses are showed in Table 3. All experiments suggested by CCD were conducted in accordance to Table 3; ANOVA was performed.

Table 3 CCD values for experimental and predicted response

Run	Coded levels			Actual levels			Dye removal (%)	
	X_1	X_2	X_3	X_1 (Dye concentration)	X_2 (pH)	X_3 (Glucose concentration)	Expt.	Pred.
1	0	0	0	75	8	4	76.33	76.74
2	0	1.682	0	75	9.68	4	56.21	56.92
3	0	0	0	75	8	4	75.63	76.74
4	-1	1	1	70	9	6	70.85	70.98
5	0	-1.682	0	75	6.32	4	47.01	46.51
6	0	0	0	75	8	4	77.23	76.74
7	1	1	1	80	9	6	37.11	36.64
8	-1.682	0	0	66.59	8	4	94.89	94.95
9	0	0	0	75	8	4	76.96	76.74
10	-1	1	-1	70	9	2	81.02	80.27
11	1	1	-1	80	9	2	44.98	44.74
12	1	-1	1	80	7	6	20.56	21.16
13	-1	-1	-1	70	7	2	83.05	83.36
14	1.682	0	0	83.41	8	4	17.98	18.14
15	0	0	1.682	75	8	7.36	54.84	54.56
16	0	0	-1.682	75	8	0.64	66.24	66.74
17	-1	-1	-1	70	7	6	76.89	76.98
18	1	-1	-1	80	7	2	26.64	26.36
19	0	0	0	75	8	4	75.96	76.74
20	0	0	0	75	8	4	78.36	76.74

The design of experiments administered to monitor the dye removal efficiency by changing dye concentration, pH and glucose concentration. All actual responses entered in Table 3 are the average of triplicates, while quadratic fitting model provided the predicted values. ANOVA analysis has checked for model fitting, and confirmed that response of dye removal was mostly fitted with quadratic polynomial model. The individual and interactive effect of process variable on the response of dye removal was evaluated using ANOVA and the corresponding equation generated after model fitting described that change in response is relatively occurs with respective alter in variable ranges. The response of dye removal by *Bacillus cereus* can be predicted using following equation:

$$Y = 76.74 - 22.84X_1 + 3.10X_2 - 6.62X_3 + 5.37X_1X_2 + 0.30X_1X_3 - 0.72X_2X_3 - 7.14X_1^2 - 8.85X_2^2 - 5.69X_3^2 \quad (3)$$

where X_1 , X_2 and X_3 are coded variables, represents dye concentration (mg/L), pH and glucose concentration (g/L) respectively. The ANOVA for response surface quadratic model showed that model is highly significant ($p < 0.0001$). p value less than 0.05 indicates the model terms are significant. The interactive and individual effect of variables was found to be significant on dye removal of MB by *Bacillus cereus*. X_1 , X_2 , X_3 , X_1X_2 , and X_2X_3 variables attributed for significant dye removal ($p < 0.05$). The lack of fit is non-significant, which confirms that model was well fitted, wherein pure error value is less than residual error value (Table 4). This clearly indicates that predicted and experimental data 99.92% can be explained by this model because R^2 value of the quadratic model is 0.9992 shows that model is highly significant. The adjusted R^2 value 0.9985 also closest to R^2 value, which authenticated the model is highly significant. Several studies were reported that R^2 value should be in between 0.75 to 0.99 to validate the model significant with higher correlation, and generally it should be between 0 to 1 (Masoumi et al., 2011; Priya and Kanmani, 2011). The coefficient of variation represents model reproducibility, it has been reported that the recommended value should be below than 10% (Priya and Kanmani, 2011). In this study, coefficient of variation (CV) was 1.39% attributed to the higher validity of experimental data with model. It was reported that lower CV value denotes the higher degree of precision, and the signal to noise ratio should be greater than 4 is desirable (Sohrabi et al., 2016). The results of the current study also in line with previous reports signal to noise ratio was 125.92 represents adequate precision.

Table 4 ANOVA for response surface quadratic model

Source of variation	Sum of square	Degree of freedom	Adj. mean square	F	P	
Regression	9,630.56	9	1,070.06	1,437.90	< 0.0001	Significant
X_1	7,121.76	1	7,121.76	9,569.90	< 0.0001	
X_2	130.97	1	130.97	175.99	< 0.0001	
X_3	179.07	1	179.07	240.63	< 0.0001	
X_{12}	734.84	1	734.84	987.45	< 0.0001	
X_{22}	1,127.88	1	1,127.88	1,515.59	< 0.0001	
X_{32}	466.49	1	466.49	626.85	< 0.0001	
$X_1 X_2$	230.70	1	230.70	310.00	< 0.0001	
$X_1 X_3$	0.71	1	0.71	0.95	0.3524	
$X_2 X_3$	4.20	1	4.20	5.65	0.0388	
Residuals	7.44	10	0.74			Not significant
Lack-of-fit	2.52	5	0.50	0.51	0.7598	
Pure error	4.92	5	0.98			
Total	9,638.01	19				

$R^2 = 0.9992$ $R^2_{adj} = 0.9985$, Pred. $R^2 = 0.9972$

3.3 Three-dimensional response surface plots

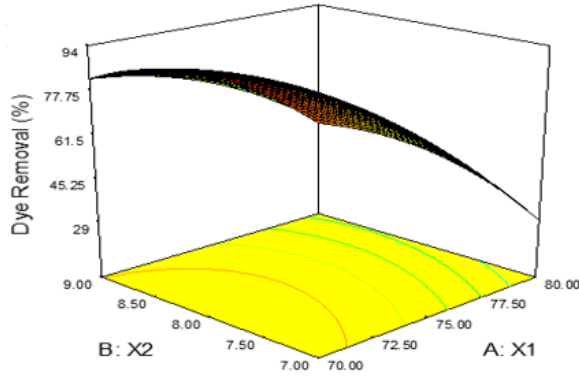
Three-dimensional (3D) response surface plots were used to illustrate the combined effect of influencing variables on dye removal. The 3D plots were showed in Figure 2 demonstrate the response dye removal with respective to change in two variables, while third variable kept as a constant. Numerical optimisation has suggested the optimal values, which are considered by software automatically to keep maintaining optimal value as a constant. The interactive effect of dye concentration (mg/L) and pH on dye removal was explained in Figure 2(a), while glucose concentration maintained at constant level of 3.33 g/L. The increase in dye concentration and pH resulted in decrease of dye removal by *Bacillus cereus*. However, slight increase in pH and dye concentration showed maximum dye decolourisation of 93.22%. Dye concentration and pH beyond the range of 70 mg/L and 8 respectively would have inhibited the bacterial growth and activity resulted in poor dye removal. The higher dye concentration could have introduced the substrate inhibition, which leads to poor biological interaction of bacteria with dye resulted in less decolourisation. Bacterial growth is highly influenced by the excess limit of dye, toxic for microorganism (Abu Talha et al., 2018). pH is an important factor for the decolourisation since, it provides the sustainable environment for the growth of culture and pH 7-8 is the favourable condition for decolourisation of azo dyes (Aksu and Dönmez, 2003).

Dye and glucose concentrations effect synergistically on dye decolourisation was depicted in Figure 2(b). These two variables are changing while pH maintained at constant level of 7.89 was used to describe the effect on dye removal efficiency. It was already confirmed that individual effect of these two variables on decolourisation is highly significant ($p < 0.0001$) shown in Table 4. The synergistic effect of these variables on dye removal was demonstrated that the increase in glucose concentration and lower concentration of dye is desirable to maximise the decolourisation. The increase in glucose concentration up to 4 g/L could have potentially used by bacteria accounts for improved decolourisation and decreasing trend was noticed at beyond 4 g/L concentration of glucose. At higher glucose concentration, bacteria tend to utilise the glucose instead of dye as a substrate, the additional carbon source is required for the microbial growth and even the higher glucose concentration leads to immediate consumption by microorganism than metabolising the dye of our interest. It was reported that the minimal glucose/carbon sources is essential for microbial mediated dye degradation process (Tan et al., 2014).

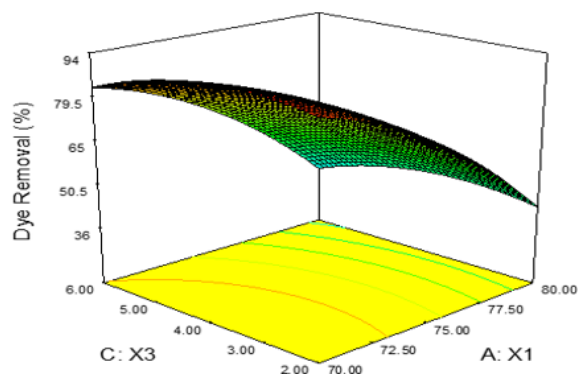
In Figure 2(c), the combined effect of pH and glucose concentration on dye removal was explained precisely using 3D surface plot. These two variables pH and glucose concentration were varied in the range of 7 to 9 and 2 to 6 g/L, while dye concentration kept at 70 mg/L. *Bacillus Cereus* was differently acted for dye decolourisation at the same dye concentration of 70 mg/L with respective to change in pH and glucose concentration was clearly confirmed in Figure 2(c). The individual effect of pH and glucose concentration on dye removal was showed highly significant ($p < 0.001$) and synergistically these two variables significantly monitoring the efficiency of decolourisation. The dye removal efficiency was increased by increasing the glucose concentration, whereas increase of pH till 8 contributed for maximising decolourisation. The response surface plots were confirmed the significance of interactive effect of variables, and numerical optimisation suggested that optimal values of dye concentration, pH and glucose concentration were 70 mg/L, 7.89, and 3.33 g/L respectively. The

validation experiment was conducted at suggested optimal values in triplicates, 95% of dye removal by *Bacillus cereus* was achieved by using RSM.

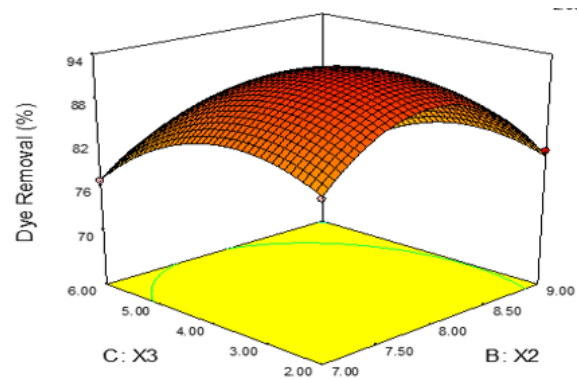
Figure 2 The combined effect of variables on dye removal was explained using response surface plots, (a) dye concentration (X_1) and pH (X_2) (b) dye concentration (X_1) and glucose concentration (X_3) (c) pH (X_2) and glucose concentration (X_3) (see online version for colours)



(a)



(b)



(c)

4 Conclusions

In this study, we have checked for the potential bacterial isolate from textile dye enriched soil for MB decolourisation. The maximum decolourisation was obtained by one bacterial isolate among 21 isolates. 16S rRNA sequencing results was confirmed that bacterial isolate was *Bacillus cereus*. RSM was used to enhance the dye removal efficiency using *Bacillus cereus*. The design of experiment proposed runs were conducted and the model was significant with a 99.92% confidence level. This concludes that the conditions optimised using RSM was improved the dye removal efficiency by *Bacillus cereus* and optimal values of dye concentration, pH and glucose concentration were found to be 70 mg/L, 7.89, 3.33 g/L.

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