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Optimization of process variables for decolorization of Disperse Yellow 211 by *Bacillus subtilis* using Box–Behnken design

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ABSTRACT

Decolorization of textile azo dye Disperse Yellow 211 (DY 211) was carried out from simulated aqueous solution by bacterial strain B*acillus subtilis*. Response surface methodology (RSM), involving Box–Behnken design matrix in three most important operating variables; temperature, pH and initial dye concentration was successfully employed for the study and optimization of decolorization process. The total 17 experiments were conducted in the study towards the construction of a quadratic model. According to analysis of variance (ANOVA) results, the proposed model can be used to navigate the design space. Under optimized conditions the bacterial strain was able to decolorize DY 211 up to 80%. Model indicated that initial dye concentration of 100 mg l^{-1}, pH 7 and a temperature of 32.5 °C were found optimum for maximum % decolorization. Very high regression coefficient between the variables and the response (R^2 = 0.9930) indicated excellent evaluation of experimental data by polynomial regression model. The combination of the three variables predicted through RSM was confirmed through confirmatory experiments, hence the bacterial strain holds a great potential for the treatment of colored textile effluents.

1. Introduction

In textile and paper coloration industries synthetic dyes from residual dye baths are released to waste streams. It is estimated about 10–15% of dyes goes unused in textile effluents [1,2]. Azo dyes, characterized by nitrogen-to-nitrogen double bonds (—N=N—), account for up to 70% of all textile dyestuffs produced, and are the most common chromophores in disperse dyes [3]. The disperse azo dyes containing effluents from these industries have caused serious environment pollution, because the presence of dyes in water is highly visible and affects their transparency and aesthetics even if the concentration of the dyes is low, which is the reason their breakdown is a priority before discharge into environment. Mainly in case of azo dyes, the largest class of synthetic dyes, effluents treatment becomes a serious problem, because of their negative impacts on water ecosystems and human health. Most of these dyes are toxic and potentially carcinogenic and their removal from industrial effluents is a major environmental problem [4–6]. In mammals azo dyes are reduced to aryl amines by Cytochrome p450 and a flavin-dependent cytosolic reductase [7]. Aromatic amines can be mineralized by means of aerobic treatment by non-specific enzymes through hydroxylation and ring-fission of aromatic compounds [8,9]. The anaerobic breakdown of azo dyes can lead to reduction of azo bond producing mutagenic and carcinogenic compounds [10]. Therefore, industrial effluents containing dyes must be treated before their release into the environment [11]. Many physical and chemical methods including adsorption, coagulation, precipitation, and oxidation have been used for the treatment of azo dye-contaminated effluents [11]. Conventional treatments of textile effluents are either ineffective, costly, complicated or have sludge disposal problems [12]. Moreover, their municipal treatment costs are high. Therefore, it may be economical to develop substitute means of dye decolorization, such as bioremediation due to its reputation as on environmentally friendly and widely acceptable treatment technology [11]. In biological removal of color from effluents the use of bacteria constitutes an alternative mode of treatment in aerobic conditions. Some specialized strains of aerobic bacteria have developed the ability to use azo dyes as sole source of carbon and nitrogen [13,14]; others only reduce the azo group by special oxygen-tolerant azo reductases. *Pseudomonas putida mt-2* degrades the azo dyes in two steps: an azo-reduction followed by an oxygen-dependent metabolization [9]. Recently Chen et al. [15] have described bacterial strains which show good growth in aerobic or agitation conditions. Several actinomycete strains have the capability to decolorize industrial effluents containing reactive dyes including anthraquinone, pthalocyanine, azo, and formazan–copper complex through complete aerobic degradation

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[16]. The degradation of azo-dye *p*-aminoazobenzene by *Bacillus subtilis* have been reported through batch experiments. *B. subtilis* cometabolizes *p*-aminoazobenzene in the presence of glucose as carbon source [17].

The aim of our work was to study the decolorization and degradation of textile azo dye Disperse Yellow 211(DY 211) using *B. subtilis*, because potential of dye to cause toxicity. We used Disperse Yellow 211 in our study, because azo disperse dyes are routinely manufactured by multinational dyes manufacturing industries and used extensively by textile industries throughout the world. The main objective of the present study was to investigate potential of bacterial strain to decolorize the most important textile azo dye DY 211 in liquid system under aerobic conditions. Emphasis was given to the effect of different operating conditions on decolorization and degradation efficiency. There are some vital factors that may significantly influence the degradation process, such as; temperature, pH and initial dye concentration. In the present investigation maximum dye decolorization ability of the bacterium was studied adopting a full range of response surface methodology using Box–Behnken design model to analyze the affectivity of the system under different conditions. The regression model provided an excellent explanation of the relationship among the independent variables and the response.

1.1. Response surface methodology (RSM)

Response surface methodology may be summarized as a compilation of statistical tools and techniques for constructing and exploring an estimated functional relationship between a response variable and a set of design variables [18]. It is a collection of mathematical and numerical techniques that are useful for modeling and analysis of the problems having numerous variables influencing the response and the objective is to optimize the response The most extensive application of response surface methodology can be found in the industrial world, in situations where a number of input variables influence some performance measure, called the response, in away that are not easy or unfeasible to depict with a rigorous mathematical formulation. In these situations, it might be possible to derive an expression for the performance measure based on the response values obtained from experiments at some particular combination of the input variables [19].

RSM is an experimental technique invented to find the optimal response within the specified ranges of the factors. These designs are capable of fitting a second-order prediction equation for the response. RSM is a statistical-based procedure and is a powerful experimental design tool to recognize the performance of composite systems [20–22]. Statistical screening methodology is a powerful and useful tool in searching for the key factors rapidly from a multivariable system. The RSM represents an assemblage of experimental design and multiple regression-based methods that can be applied to evaluate tribulations where several factors might influence a response [23].

In the present study, the three-level, three-factorial Box–Behnken experimental design was applied to investigate and validate process parameters affecting the removal of textile dye Disperse Yellow 211 by bacterial strain *B. subtilis*. Temperature 25–40 °C, pH 7–10 and initial dye concentration 100–250 mg l^{-1} were input variables, the factor levels were coded as −1 (low), 0 (central point) and 1 (high) [24]. Table 1 shows the experimental parameters and the experimental Box–Behnken design levels used. RSM was applied to the experimental data using statistical software, Design-expert (Stat-Ease, trial version). Statistical terms and their definitions used in the Design-expert software are well defined [25]. Linear and second-order polynomials were fixed to the experimental data to obtain the regression equations. The sequential *F*-test, lack-of-fit test and other adequacy measures were used in selecting the finest model [26]. To analyze a process or system mutually with a response, *Y* which depends on the input factors $X_1, X_2, \ldots X_n$, the correlation between the response and the input process parameters are described as

$$Y = f(X_1, X_2, \cdots X_n) \pm \varepsilon \quad (1)$$

where *f* is the real response function its format being unknown, and ε is the residual error which describes the differentiation that can be incorporated by the function *f*. Because the correlation between the response and the input variables can be described as a surface of the $X_1, X_2, \ldots X_n$ coordinates in the graphical sense, so the investigation of these relationships is named as the response surface study. The same statistical software was used to generate the statistical and response plots. A manual regression method was used to fit the second-order polynomial equation (Eq. (2)) to the experimental data and to recognize the relevant model terms. Considering all the linear terms, square terms and by linear interaction items, the quadratic response model can be described as

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_{ii}^2 + \sum \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where β_0 is the constant, β_i the slope or linear effect of the input factor x_i and β_{ij} the linear by linear interaction effect between the input factor x_i and x_j and β_{ij} is the quadratic effect of input factor x_i [27]. RSM is a sequential procedure with an initial objective to lead the experiments rapidly and efficiently along a path of improvement towards the general vicinity of the optimum.

Table 1
Independent factors and their coded levels used for optimization

Factors	Range and levels (coded)		
	−1	0	+1
Temp., *A* (°C)	25	32.5	40
pH, *B*	7	8.5	10
Initial dye conc., *C* (mg l^{-1})	100	175	250

2. Materials and methods

2.1. Chemicals

The textile dye Disperse Yellow 211 was supplied by Nahar Fabrics, Lalru (Punjab) India. Other chemicals used were purchased from Loba-Chemi, Bombay, and were of the highest purity available.

2.2. Bacterial strain and culture conditions

The bacterial strain was isolated from dye-contaminated soil collected from within the premises of a textile industry and it was designated as *B. subtilis* (MTCC 9023) by the IMTECH, Chandigarh, India. *B. subtilis* colonies were circular, shiny with rhizoid margin. Biochemical characterization showed that Methyl red, Voges proskauer and Indole tests were negative. Nitrate reduction, starch hydrolysis, catalase, Gram's reaction and oxidase tests showed positive results. The bacterial strain was maintained on nutrient agar plates through fortnightly sub-culturing. Further the strain was maintained on nutrient agar slants at 4 °C prior to use. The culture used for inoculation into liquid system was incubated on agar plates for a week under static conditions at 30 °C prior to inoculation into liquid system. Nutrient broth media was used for further color reduction experiments.

Table 2
The Box–Behnken design matrix for coded variables along with actual and predicted responses

Std. order	Temp. (*A*)	pH (*B*)	Initial dye conc. (*C*)	Actual response (*Y* %)	Predicted response (Y_1 %)
1	−1	−1	0	54.21	53.33
2	1	−1	0	53.32	52.27
3	−1	1	0	29.76	30.30
4	1	1	0	30.22	31.09
5	−1	0	−1	42.36	43.15
6	1	0	−1	41.98	42.94
7	−1	0	1	36.56	35.60
8	1	0	1	35.84	35.04
9	0	−1	−1	80.05	80.14
10	0	1	−1	56.78	54.94
11	0	−1	1	67.23	69.07
12	0	1	1	50.64	50.56
13	0	0	0	70.51	70.31
14	0	0	0	67.28	70.31
15	0	0	0	72.68	70.31
16	0	0	0	71.35	70.31
17	0	0	0	69.71	70.31

2.3. Experimental method

A stock solution of dye Disperse Yellow 211 was prepared (1000 mg l^{-1}) and desired concentrations of the dye were obtained by further dilutions. For dye degradation studies 7 days old cultures were used. Erlenmeyer flasks having 100 ml pre-autoclaved simulated dye solution of DY 211 in varying concentrations (100–250 mg l^{-1}) were inoculated with 24 h fresh bacterial broth each (5 ml) inside laminar flow system under extremely hygienic conditions. Experiments were performed by varying the temperature 25–40°C and pH 7–10 keeping other conditions constant to examine the effect of temperature and pH on dye degradation. pH of the aqueous dye solution was adjusted using 0.1 M HCl and 0.1 M NaOH.

Batch studies were performed to determine the equilibrium time required for maximum dye decolorization. Erlenmeyer flasks containing 100 ml dye solution inoculated with *B. subtilis* were shaken in an incubator shaker at 150 rpm for the maximum predetermined time period of 5 days. Samples were withdrawn at fixed time intervals from the flasks, centrifuged (6000 rpm) for 15 min and supernatant was analyzed spectrophotometrically for residual dye concentration in the aqueous solution using a Systronics Spectrophotometer-106 at 496 nm (λ_{max} for DY 211). All the experiments were performed in triplicates and their mean values are reported here. The maximum deviation was found to be ±2.0%. Controls were maintained without bacterial culture. The following formula was used to calculate the percentage decolorization:

$$\text{decolorization}\,(\%) = \frac{C_0 - C_e}{C_0} \times 100 \quad (3)$$

where C_0 is the initial concentration of dye (mg l^{-1}) and C_e is the residual dye concentration (mg l^{-1}) at different time intervals.

3. Results and discussions

In this method, a prior knowledge obtained from previous studies for understanding of the process and the process variables under investigation is necessary for achieving a more realistic model. In the present work, experiments were planned to obtain a quadratic model consisting of 12 trials plus 5-centre points. The range and levels of three independent variables, viz. temperature, pH and initial dye concentration are presented in Table 1. The design matrix of the variables in coded units is given in Table 3 along with the predicted and experimental values of response. Each run was performed in triplicate and mean values for % decolorization of DY 211 are presented in Table 3, while the predicted values of responses were obtained from quadratic model fitting techniques using the software mentioned above.

By applying multiple regression analysis methods, the predicted response, *Y* for can be obtained and given as

Table 3
ANOVA results of quadratic model for decolorization of Disperse Yellow 211 using *Bacillus subtilis*

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*	
Model	4247.266	9	471.9185	110.4639	<0.0001	Significant
A	0.29645	1	0.29645	0.069391	0.7998	
B	955.2821	1	955.2821	223.6067	<0.0001	
C	119.3513	1	119.3513	27.93703	0.0011	
A^2	2948.034	1	2948.034	690.0582	<0.0001	
B^2	16.34893	1	16.34893	3.826859	0.0913	
C^2	91.45373	1	91.45373	21.40694	0.0024	
AB	0.4489	1	0.4489	0.105076	0.7553	
AC	0.0289	1	0.0289	0.006765	0.9368	
BC	11.1556	1	11.1556	2.611236	0.1501	
Residual	29.90507	7	4.272153			
Lack-of-fit	13.62575	3	4.541917	1.115997	0.4413	Not significant
Pure error	16.27932	4	4.06983			
Cor. total	4277.171	16				

R^2 = 0.9930; Pred. R^2 = 0.9431; Adj-R^2 = 0.9840; CV = 3.78 and PRESS = 243.45.

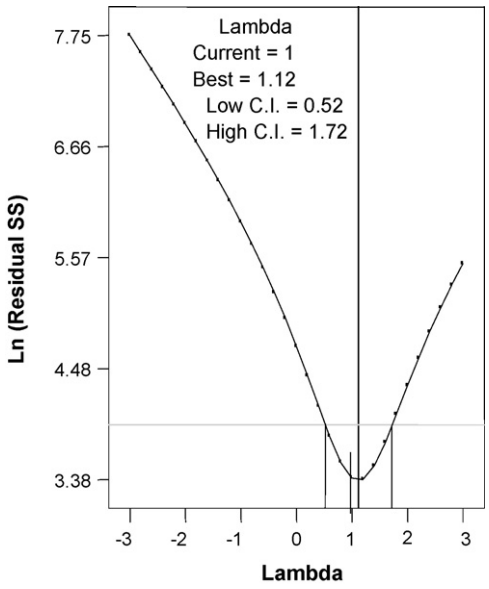

Fig. 1. Box–Cox plot of model transformation for decolorization of Disperse Yellow 211 by *Bacillus subtilis*.

$$Y = +70.31 - 0.19 \times A - 10.93 \times B - 3.86 \times C - 26.46 \times A^2 - 1.97 \times B^2 - 4.66 \times C^2 + 0.33 \times A \times B - 0.085 \times A \times C + 1.67 \times B \times C \quad (4)$$

where Y is the predicted response and A, B and C are the coded values of the test variables, temperature (°C), pH and initial dye concentration (mg l^{-1}), respectively. The statistical significance of Eq. (2) was checked by F-test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 2.

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]. The goodness of the model can be checked by the determination coefficient R^2 and the multiple correlation coefficients R. The value of adjusted R^2 (0.9840) suggests that the total variation of 98% for the dye degradation to the independent variables and only about 2% of the total variation cannot be explained by the model. The closer the values of R (multiple correlation coefficient) to 1, better the correlation between the experimental and predicted values [28]. Here, the value of R^2 (0.9930) indicates good relation between the experimental and predicted values of the response. 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) revealed that the quadratic model is statistically significant for the response and therefore it can be used for further analysis.

Logarithm (Ln) of the residual SS (sum of square) against λ (lambda) should dip fairly steeply with a minimum in the region of the optimum value [29]. Typically, most of the parameters estimate might appear to be significant outside the region of the optimum λ, but near it only a few will be highly significant. The model showed the minimum confidence interval value is 0.52 and maximum value is 1.72 (Fig. 1). The current point of confidence interval (λ = 1), lies very close to model design value (1.12), indicating no transformation of the model required. If the value of current lambda is outside from low-confidence interval (CI) and high-confidence interval (CI) values, in that case there is need of model transformation. In ideal conditions value of current lambda must be in between low-CI and high-CI The shape of the standard error plot not only fit on the design points but also polynomial being fit showed low and flat error exhibiting circular contours and symmetrical shape around the centroid which is the ideal condition (Fig. 2). A base standard deviation of 1.0 is used to generate the standard error plot for design evaluation. The actual magnitude of the plot will be a function of the standard deviation, which depends on the response data. The standard error value around the centroid is 0.441, which is the best value. Standard error value increases at the centroid as well as away from optimization point.

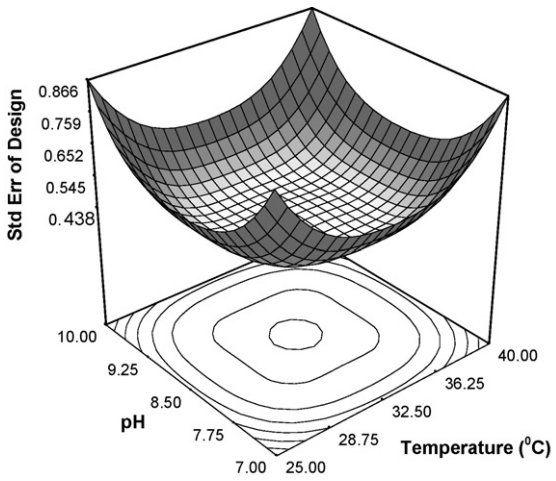

Fig. 2. Three-dimensional standard error plot for decolorization of Disperse Yellow 211 by *B. subtilis*.

The three-dimensional response surface plots are the graphical representations of the regression equation. These plots are presented in Figs. 3–5. The main goal of response surface is to track efficiently for the optimum values of the variables such that the response is maximized. By analyzing the plots, the best response range can be calculated. From Fig. 3 it is clearly evident, as the temperature increased from 25 °C, up to temperature range of 32.5 °C, an increase in decolorization rate was observed with increasing temperature after that there was a decrease in the % decolorization rate as the temperature was further increased. However, there was a sharp decline in decolorization rate as pH was increased from 7.0 (Fig. 4), this shows bacterial species is best working in the neutral environment. The decolorization rate decreases with increase in the

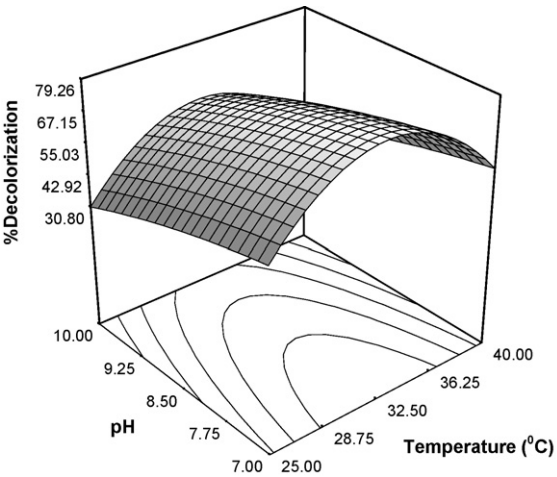

Fig. 3. Three-dimensional response surface plot for the effect of temperature and pH on decolorization of Disperse Yellow 211 by *B. subtilis*.

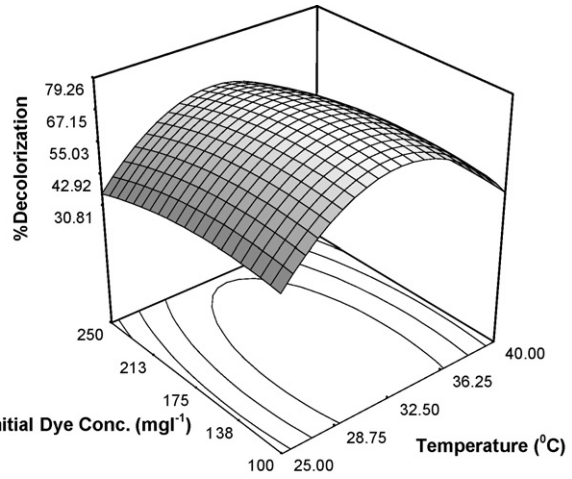

Fig. 4. Three-dimensional response surface plot for the effect of temperature and initial dye dosage on decolorization of Disperse Yellow 211 by *B. subtilis*.

initial dye concentrations (Fig. 5), which might be due to inhibitory effects caused due to toxicity of dye [30]. The optimum values of the variables can be analyzed by saddle point or by checking the maxima formed by the *X* and *Y* coordinates. The conditions obtained at the saddle point for best response. Responses were temperature range of 32.5 °C, pH 7 and initial dye concentration 100 mg l^{-1}, up to 80% color degradation was achieved under optimum growth conditions. These points were located within the experimental ranges, implying that the analytical techniques could be used to identify the optimal conditions.

3.1. Model validation and confirmation

To validate the optimum combination of the variables, confirmatory experiments were carried out. Verification experiments performed at the predicted conditions, indicating the validity and adequacy of the predicted models. Moreover, the verification experiments also proved that when values of pH and initial dye concentration were changed from their minimum levels a decrease in % decolorization was observed (Table 4). The observed results were well accorded with the predicted results. As a result, the model developed was considered to be accurate and reliable.

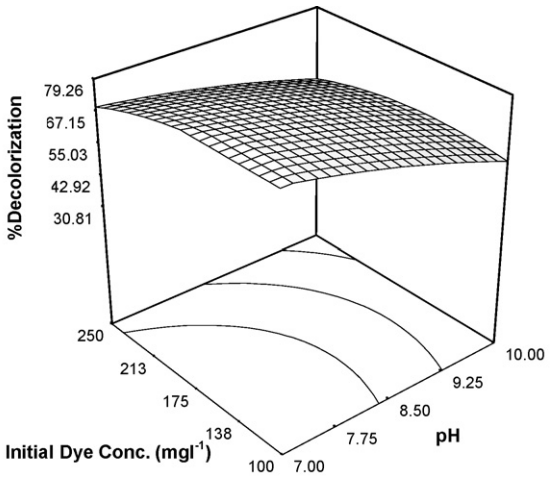

Fig. 5. Three-dimensional response surface plot for the effect of pH and initial dye dosage on decolorization of Disperse Yellow 211 by *B. subtilis*.

Table 4
The Box–Behnken design matrix for model validation and confirmation

Solution	Temp. (°C)	pH	Initial dye conc. (mg l^{-1})	Observed response (%)
1	34.84	6	75	67.43
2	32.75	6	75	71.29
3	31.01	6	75	70.93
4	31.61	6	75	71.81
5	32.5	6	100	72.58
6	32.43	7	119	78.76
7	32.61	7	120	76.63
8	33.08	7.84	117	75.47
9	25.24	6	75	56.76
10	26.19	6	75	59.52

4. Conclusions

Applications of the bacterial strain *B. subtilis* for decolorization of textile disperse azo dye Disperse Yellow 211 seems to be a practical approach. This study showed that response surface methodology was a suitable approach to optimize the best culture conditions for achieving maximum decolorization of the dye. The experimental and the predicted values were very close which reflected the correctness and the applicability of RSM. In this case, the value of the determination coefficient ($R^2 = 0.9930$) indicated that near about negligible of total variations were not explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9840$) was near to 1, showing a high significance of the model. By applying Box–Behnken design to the optimization experiments, we could investigate the process variables completely and achieved decolorization values up to 80%. The use of an experimental design permitted the rapid screening of a large experimental domain for optimization of the % decolorization ability of bacterial isolate *B. subtilis*. Moreover, the ability of the bacterial strain to decolorize dye indicated its potential application for decolorizing textile-dyeing effluents. Further pilot scale studies are necessary with this strain for real industrial applications and detailed study is desirable to explore the mechanism involved.

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