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Full Length Research Paper

Biodegradation of orange G by a novel isolated bacterial strain *Bacillus megaterium* ITBHU01 using response surface methodology

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This research article deals with biodegradation of azo dyes by a newly isolated bacterial strain from soil. Azo dyes are recalcitrant to the conventional modes of treatment due to their complex structure. This article reports decolorization of azo dye by, Gram positive, endospore forming and azo reducing, *Bacillus megaterium* ITBHU01. Response surface methodology was used to optimize the important physical parameters screened by Placket–Burman design. Five physical parameters such as pH, temperature (°C), dye concentration (mg/L), inoculum size% (v/v) and time (h) were tested by using Placket–Burman design criterion and all five parameters showed significant effect ($P < 0.05$) on decolorization of orange G using *B. megaterium* ITBHU01. The values of parameters was optimized by applying central composite design (CCD) and the most suitable values for orange G decolorization by *B. megaterium* ITBHU01, as predicted by the statistical tool, was pH 6.9; temperature 37.0 °C; dye concentration 517 mg/L, inoculum size , v/v, (%) 5.5 % and time 23.7 h. At these optimum levels of parameters, bacterial decolorization of orange G by 94.48% was obtained under static conditions. Biodegradation and decolorization of azo dye, orange G, was confirmed using UV-VIS spectrophotometry, thin layer chromatography (TLC) and fourier transform infrared spectroscopy (FT-IR) and electron spray ionization mass spectrometry (ESI-MS) analysis.

Key words: Azo dye, *Bacillus megaterium* ITBHU01, biodegradation, orange G, response surface methodology.

INTRODUCTION

Rapid urbanization and industrialization has lead to a vast release of waste to the environment adding to the pollution load. Majority of colored effluents contains dyes released from textile, dyestuff and dyeing industries (Resmi and Emilia, 2004). There are over 10,000 commercially available dyes with a production of over 7×10^5 tons per year (Fu and Viraraghavan, 2001). Azo dyes, accounts for almost 60 to 70% of all the synthetic dyes produced globally. They are extensively used in the textile, paper, food, leather, cosmetics and pharmaceutical industries (Telke et al., 2008). Disposal of these dyes into the environment causes serious damage, since they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration (Aksu et al., 2007). Moreover, numerous reports indicate that textile dyes and effluents have toxic effects on the germination rates and biomass of several plant species which have important ecological functions, such as providing a habitat for wildlife, protecting soil from erosion and providing the organic matter that is so significant to soil fertility (Ghodake et al., 2009). In addition, azo dyes also have an adverse impact in terms of total organic carbon (TOC), biological oxygen demand (BOD) and chemical oxygen demand (COD) (Saratale et al., 2009).The presence of unnatural colors is aesthetically unpleasant and tends to

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be associated with contamination. Without adequate treatment, these dyes will remain in the environment for an extended period of time (Olukanni et al., 2006). Dye-house effluent typically contains only 0.6 to 0.8 g L^{-1} dye, but the pollution it causes is mainly due to durability of the dyes in the wastewater (Jadhav et al., 2007). Moreover, there are many reports on the use of physicochemical methods for the color removal from dye containing effluents (dos Santos et al., 2007; Wang et al., 2009). Several methods were adapted for the reduction of azo dyes to achieve decolorization. These include physio-chemical methods (Droste, 2004) such as filtration, specific coagulation, use of activated carbon, chemical flocculation etc. Some of these methods (reverse osmosis, nanofiltration and multiple effect evaporators) are found to be effective but quite expensive (Maier et al., 2004). Microbial or enzymatic decolorization and degradation is an eco-friendly cost-competitive alternative to chemical decomposition process that could help reduce water consumption compared to physicochemical treatment methods (Rai et al., 2005).

A number of microorganisms have been found to be able to decolorize textile dyes including bacteria, fungi, and yeasts (Olukanni et al., 2006; Wesenberg et al., 2003). Keeping in view the importance of biological treatment over conventional modes of treatment of azo dyes, an attempt has been made to study the decolorization abilities of the newly isolated strain of *Bacillus megaterium* ITBHU01 for Orange G, selected as model azo dye. This article describes optimization of parameters for Orange G decolorization by *B. megaterium* ITBHU01. Process optimization by one-factor-at-a-time method involves changing one variable (pH, temperature, dye concentration, inoculum size etc.) while fixing the others at a certain arbitrary levels. The conventional ‘‘one-factor-at-a-time’’ approach is laborious and time consuming, especially for large number of variables. Moreover, it seldom guarantees the determination of optimal conditions (Choudhari and Singhal, 2007). These limitations of a single factor optimization process can be overcome by using statistical methods. In statistical based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization (Fu et al., 2009; Shih et al., 2008). RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions (Kalil et al., 2000). It is a statistically designed experimental protocol in which several factors are simultaneously varied. In this work, we have screened out five most effective parameters such as pH, temperature, dye concentration (mg/L), time (h) and inoculum size % (v/v) for decolorization of Orange G by *B. megaterium* ITBHU01 using response surface methodology (RSM). Placket–Burman design was used to select the factors having significant effect on decolorization of Orange G by *B. megaterium* ITBHU01 and optimization of the selected parameters for the decolorization of Orange G was done by central composite design (CCD).

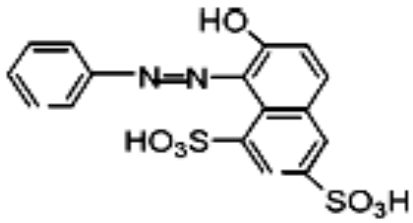
MATERIALS AND METHODS

Media and culture conditions

B. megaterium ITBHU01 (Accession number: HM436653), isolated from soil sample at Banaras Hindu University by the enrichment technique, is stored at 4°C on nutrient agar slants. The composition of mineral medium used for decolorization studies was (g L^{-1}) KH_2PO_4 0.68, K_2HPO_4 1.73, $MgSO_4.7H_2O$ 0.1, NaCl 0.1, $FeSO_4.7H_2O$ 0.03, $CaCl_2.2H_2O$ 0.02, Glucose 5.0, peptone 5.0. The pH was adjusted to 7.0. Inoculum was developed by transferring one loop full of the organism from the slant culture to 50 ml mineral medium in 250 ml Erlenmeyer flask. The flask was incubated in an orbital shaker at 37 ± 1°C and 180 rpm for 24 h for inoculum development.

Chemicals

The textile dye Orange G was obtained from Himedia, India. The dye content was 80%. All the chemicals were of highest purity available and were of analytical grade. Solvents used for ESI-MS analysis were of HPLC grade. Chemical structure of the selected azo dye, Orange G is as shown:



Acclimatization

The culture was gradually exposed to the increasing concentration of the dye to acclimatize *B. megaterium* ITBHU01. The successive transfer of culture into fresh mineral medium containing 100, 250, 500, 750, 1000 mg L^{-1} of the Orange G dye was done at 37 ± 1°C in static condition. This acclimatized microorganism was used for all studies.

Decolorization studies

The decolorization studies were carried out in 250 ml conical flasks containing 100 ml mineral medium. The medium was inoculated with fully grown culture of *B. megaterium* ITBHU01. Dye solutions of Orange G were filter sterilized as stock solution (1.0% w/v) and added aseptically to the mineral medium to the desired concentration. At first, the effect of five process parameters, including pH, temperature, dye concentration (mg/L), inoculum size/, v/v, (%) and time (h) on decolorization of Orange G by *B. megaterium* ITBHU01 was studied using Placket-Burman design criterion. Decolorization was carried out by adding 5% inoculum of *B. megaterium* ITBHU01 to 100 ml medium in 250 ml conical flask, amended with 500 mg/L Orange G and incubated at 37 ± 1°C at static condition for 72 h. The decolorized medium was then centrifuged at 10,000 g at room temperature for 10 min and the cell free supernatant was used for determination of percentage decolorization of Orange G.

Analytical methods

Decolorization of Orange G was monitored spectrophotometrically at 480 nm, which is an absorbance maximum for Orange G, on a Shimadzu double beam spectrophotometer (UV 1601). Uninoculated controls were used to compare color loss during the experiment. The percentage of decolorization was calculated from the difference between initial and final values. The biodecolorization and biodegradation analysis was done using UV-VIS spectrometry, thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FT-IR). The supernatants obtained after decolorization were extracted with dichloromethane and dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was first examined by thin layer chromatography. It is further subjected to FTIR spectroscopy. Infrared spectra were determined on Thermo NICOLET 5700 Spectrophotometer. The software used in spectrophotometer was OMNIC. Analysis was carried out at room temperature in the mid IR region of 400 to 4000 cm^{-1} at a scan speed of 60.

Electron spray ionization mass spectrometry (ESI-MS) analysis was carried out to find out the degradation products. ESI-MS was carried out on an SL 1200 system (Agilent) with ion trap detection in the positive ion mode. The software used was MassHunter. The culture broth after decolorization in the medium containing azo dye (Orange G) was analyzed using ESI-MS. 50 ml of decolorized samples were centrifuged at 10,000 rpm for 15 min and filtered through 0.45 mm membrane filter. The filtrate was then extracted twice with ethyl acetate and evaporated in a vacuum evaporator at 40 to 45°C and residue was dissolved in 50% acetonitrile in water with 0.1% formic acid and used for ESI- MS analysis.

Response surface methodology

Response surface methodology (RSM) was divided in two stages, first to identify the significant process parameters for decolorization of Orange G, by *B. megaterium* ITBHU01 using Placket–Burman design criterion and later the significant parameters resulted from Placket–Burman design were optimized by using a central composite design (CCD). The experimental design and statistical analysis of the data were done by using statistical software Minitab 15.

Placket–Burman design

Each variable was assigned two levels namely a high level denoted by (+1) and a low level denoted by (-1). The levels of the parameters selected were based on the preliminary experiments and the information available in the literature. pH had a lower limit of 4.0 and an upper limit of 8.0. Temperature was varied between 20 and 40°C. Dye concentration was varied between 550 and 750 mg/L. The lower and upper limits of Inoculum size (%) were 4 and 10%, respectively. Time for decolorization was varied between 16 and 22 h. Five variables were screened by conducting twelve experiments. All experiments were conducted in triplicate and the average value of percentage decolorization of Orange G was used for statistical analysis. The variables, found out to be significant at 5% level ($P < 0.05$) from the regression analysis were considered to have greater impact on decolorization of Orange G and were further optimized using central composite design.

Central composite design

The optimum values, of five most significant process parameters screened from Placket–Burman design criterion, was find out using central composite design (CCD). The effect of the parameters pH, temperature, dye concentration (mg/l), inoculum size (%) and time (h) was studied at five levels: -a, -1, 0, +1 and +a, where a = 2n/4; here n was the number of variables and 0 corresponded to the central point. The levels of factors used for experimental design are given in Table 1. The actual level of each factor was calculated using the following equation (Paul et al., 1992)

$$\text{Coded value} = \frac{\text{Actual level} - (\text{high level} + \text{low level})/2}{(\text{High level} - \text{low level})/2} \quad (1)$$

Regression analysis of Placket–Burman design criterion data was carried out for prediction of significant factors. The response variable was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation used in this study is:

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

Where, Y is the predicted response; x_i and x_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. Analysis of variance (ANOVA) and regression analysis was done and contour plots were drawn by using Statistical Software, Minitab 15.

RESULTS AND DISCUSSION

Screening of process parameters using Placket–Burman design criterion

Placket–Burman design was used to find out the process parameters that have a significant effect on decolorization of Orange G by *B. megaterium* ITBHU01. Twelve sets of experiments were carried out to study the effect of five parameters on the decolorization of azo dye using *B. megaterium* ITBHU01, which shows maximum percentage decolorization of Orange G in the medium having higher level (+1) of pH, temperature, inoculum size, v/v, (%), time (h) and lower level (-1) of dye concentration. Regression analysis of Placket–Burman design criterion data for prediction of significant factors showed that out of five process parameters studied, all of them including pH, temperature, dye concentration, inoculum size, v/v (%) and time (h) showed significant stimulatory effect on Orange G decolorization as reflected by their P values (<0.05) obtained. The coefficient of determination (R^2) of the model was 0.9538, which indicates the model could explain up to 95.38% variation of the data. Percentage decolorization of Orange G obtained from Placket–Burman design experiments showed wide variation which indicated towards necessity of further optimization.

Optimization of values of process parameters by CCD

Thirty two experiments were conducted according to the CCD as shown in Table 1. By applying multiple regression analysis on the application data, the following second

Table 1. Orange G decolorization by *Bacillus megaterium* ITBHU01 using significant factors based on central composite design criterion.

Run	pH	Temperature (°C)	Dye concentration (mg/L)	Inoculum size	Time (h)	% Decolorization	
						Experimental	Predicted
1	6	45	575.0	6	20	59.1	59.9458
2	6	45	575.0	4	24	55.2	54.4625
3	7	40	537.5	5	22	91.2	91.5875
4	7	40	537.5	5	22	91.4	91.5875
5	7	40	537.5	5	18	86.0	84.8292
6	8	45	500.0	6	20	59.3	61.8875
7	6	35	575.0	4	20	70.1	68.5042
8	6	35	575.0	6	24	77.5	77.1292
9	6	45	500.0	6	24	60.0	62.1542
10	7	40	537.5	5	22	91.7	91.5875
11	8	35	500.0	4	20	59.0	59.1458
12	8	45	575.0	4	20	48.3	47.9958
13	6	45	500.0	4	20	53.2	54.1292
14	8	45	575.0	6	24	54.0	54.9208
15	7	40	537.5	3	22	67.3	69.2958
16	5	40	537.5	5	22	54.0	53.6958
17	7	40	537.5	5	22	91.0	91.5875
18	9	40	537.5	5	22	48.0	46.692
19	6	35	500.0	6	20	72.9	74.1958
20	7	50	537.5	5	22	35.1	32.2125
21	6	35	500.0	4	24	78.9	78.6125
22	8	35	500.0	6	24	75.8	77.1708
23	7	40	612.5	5	22	82.1	84.2625
24	7	40	462.5	5	22	92.0	88.2125
25	7	30	537.5	5	22	62.9	64.1625
26	8	35	575.0	4	24	69.9	68.3792
27	8	35	575.0	6	20	75.8	75.8625
28	7	40	537.5	5	22	91.4	91.5875
29	7	40	537.5	5	22	91.2	91.5875
30	7	40	537.5	5	26	92.0	91.5458
31	8	45	500.0	4	24	54.7	55.7042
32	7	40	537.5	7	22	87.0	83.3792

order polynomial equation was found to explain the Percentage Orange G decolorization by *B. megaterium* ITBHU01.

$$Y = -2012.24 + 133.66X_1 + 38.53X_2 + 1.59\ X_3 + 52.61X_4 + 30.28\ X_5 - 10.35X_1^2 - 0.43X_2^2 - 3.81X_4^2 - 0.21\ X_5^2 + 0.10X_1X_2 + 1.31X_1X_4 - 0.13X_1X_5 - 0.01X_2X_3 - 0.04X_2X_4 - 0.13X_2X_5 - 0.02X_3X_5 - 0.870X_4X_5 \quad (3)$$

Where, Y is the predicted response variable, percentage Orange G decolorization and X_1, X_2, X_3, X_4 and X_5 are the values of independent variables, pH, temperature, dye concentration, inoculum size (%) and time (h) respectively. Regression analysis of the experimental data (Table 2) showed that pH, temperature, dye concentration, inoculum size (%) and time (h) had positive effect on percentage Orange G decolorization as P value of all those factors has a value < 0.05. Analysis of variance for the decolorization of Orange G obtained from this design is given in Table 3. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in Orange G decolorization with the designed parameters level. The closure the value of R^2 (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. In the present study, the value of R^2 (0.9912) revealed that the model could explain up to 99.12% variation of Orange G decolorization by *B. megaterium* ITBHU01. The P value for lack of fit (0.000) indicated that the experimental data obtained fitted well with the model and explained the effect of parameters: pH, temperature, dye concentration (mg/L), inoculum size, v/v,(%) and time (h) on Orange G decolorization by *B. megaterium* ITBHU01.

Table 2. Regression analysis of central composite design criterion data for Orange G decolorization by *B. megaterium* ITBHU01.

Term	Coefficient	SE coefficient	T	P
Constant	-2012.24	222.626	-9.039	0.000
pH	133.66	14.855	8.998	0.000
Temperature	38.53	3.021	12.755	0.000
Dye concentration	1.59	0.461	3.454	0.005
Inoculum size	52.61	14.447	3.641	0.004
Time	30.28	8.009	3.781	0.003
pH×pH	-10.35	0.480	-21.574	0.000
Temperature×temperature	-0.43	0.019	-22.617	0.000
Dye concentration× dye concentration	-0.00	0.000	-2.788	0.018
Inoculum size× Inoculum size	-3.81	0.480	-7.947	0.000
Time × time	-0.21	0.120	-1.772	0.104
pH× temperature	0.10	0.130	0.741	0.474
pH× dye concentration	0.00	0.017	0.221	0.829
pH× inoculum size	1.31	0.650	2.011	0.069
pH × time	-0.13	0.325	-0.414	0.687
Temperature× dye concentration	-0.01	0.003	-1.665	0.124
Temperature ×inoculum size	-0.04	0.130	-0.298	0.771
Temperature× time	-0.13	0.065	-1.953	0.077
Dye Concentration× inoculum size	0.00	0.017	0.067	0.948
Dye Concentration× time	-0.02	0.009	-2.088	0.061
Inoculum size× time	-0.87	0.325	-2.684	0.021

R^2 = 99.12%.

Table 3. Analysis of variance for Orange G decolorization by *B. megaterium* ITBHU01 using central composite design criterion.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	8410.15	8410.15	420.51	62.29	0.000
Linear	5	1993.64	1507.26	301.45	44.65	0.000
Square	5	6260.85	6260.85	1252.17	185.48	0.000
Interaction	10	155.67	155.67	15.57	2.31	0.093
Residual error	11	74.26	74.26	6.75	*	*
Lack-of-fit	6	73.97	73.97	12.33	213.79	0.000
Pure error	5	0.29	0.29	0.06	*	*
Total	31	8484.41	*	*	*	*

DF, Degrees of freedom; Seq SS, sequential sums of squares; Adj SS, adjusted sums of squares; Adj MS, adjusted mean square.

Interpretation of parameters interaction

Figure 1a to j shows the 2D contours plots of percentage decolorization of Orange G by *B. megaterium* ITBHU01 for each pair of process parameters value, keeping the other three parameters constant. The main goal of response surface is to efficiently look out for the optimum values of the variables such that the response is maximized. Contour plots are 2-D plots, which are a useful tool to analyze the interactive effects of factors on the response (Hasan et al., 2009). The numbers in the contour line inside the contour plots indicate percentage dye decolorization at various decolorization conditions. Figure 1a, portrays the interactive effect of time and inoculum size on percentage dye decolorization. We can understand from the plot that as the time increases, the dye decolorization increases, while increase in inoculum size up to 5.3%(v/v) increases the percentage dye decolorization and further increase in inoculum size has negative effect on percentage dye decolorization. Figure 1b depicts the interactive effect of decolorization time and dye concentration on percentage dye decolorization. We can infer from the plot that the percentage dye decolorization increases as the time increases while decrease in dye concentration increases the percentage decolorization. The optimal point for maximum decolorization dye

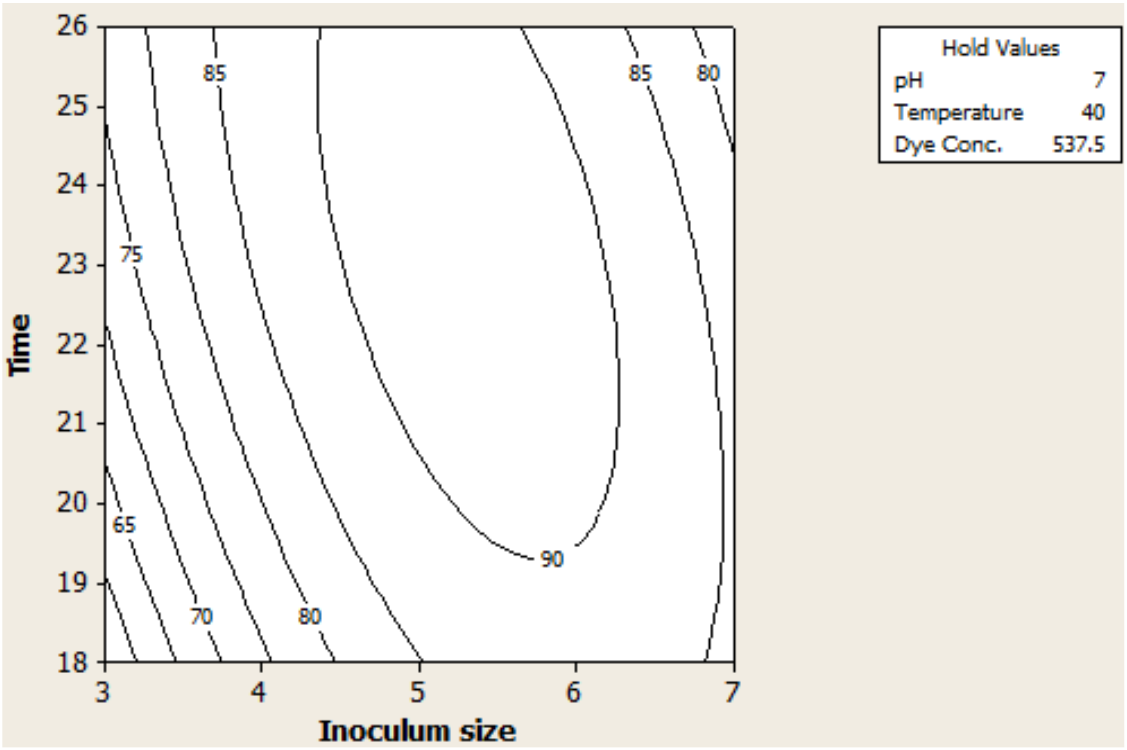


Figure 1a. Contour plot of Percentage decolorization: Effect of time and Inoculum size on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping pH, temperature, and dye concentration at constant values.

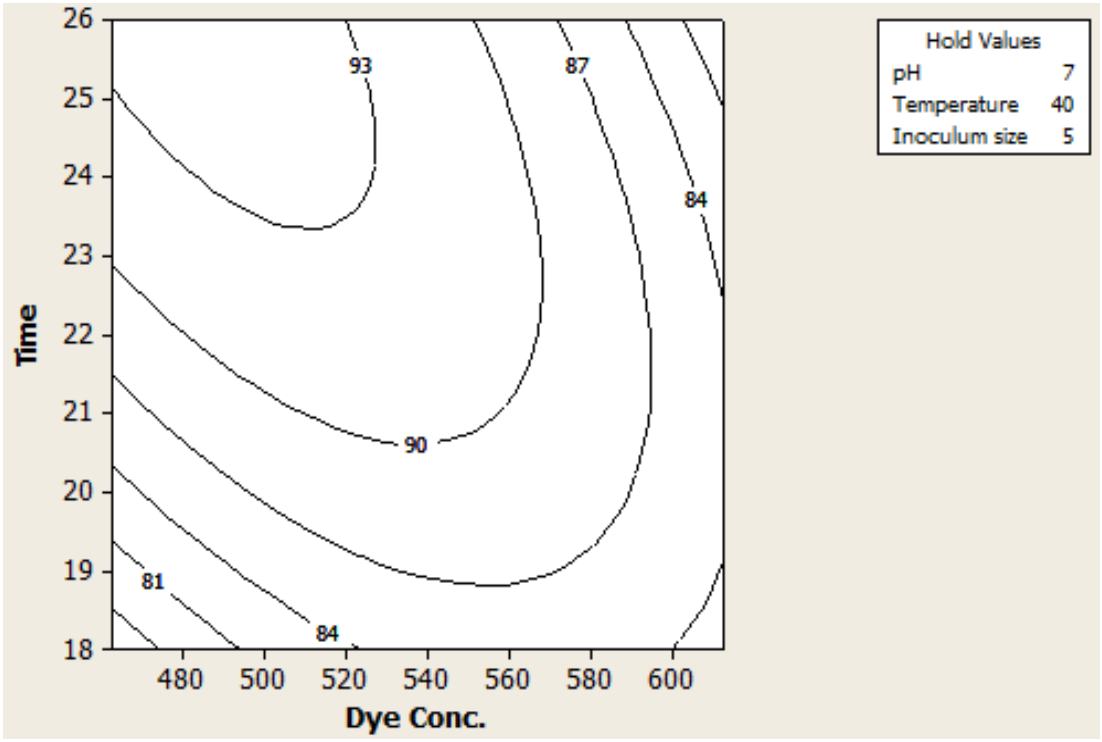


Figure 1b. Contour plot of percentage decolorization: Effect of time and dye concentration on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping pH, temperature and inoculum size at constant values.

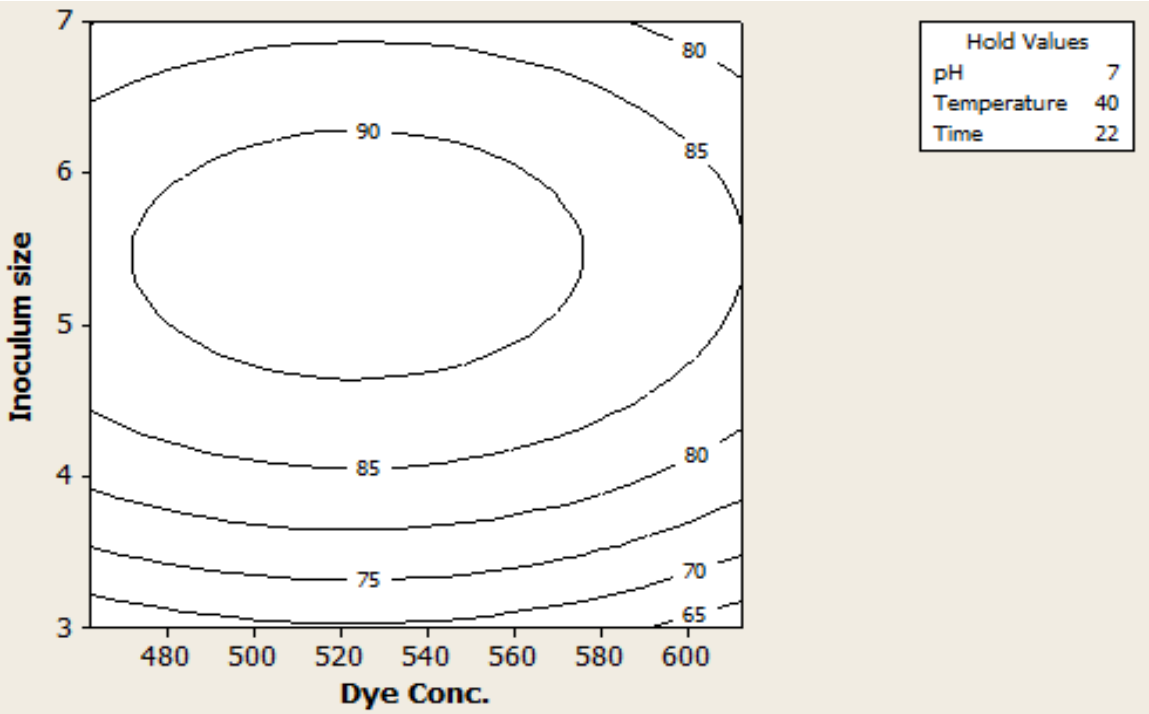


Figure 1c. Contour plot of percentage decolorization: Effect of inoculum size and dye concentration on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping pH, temperature and time at constant values.

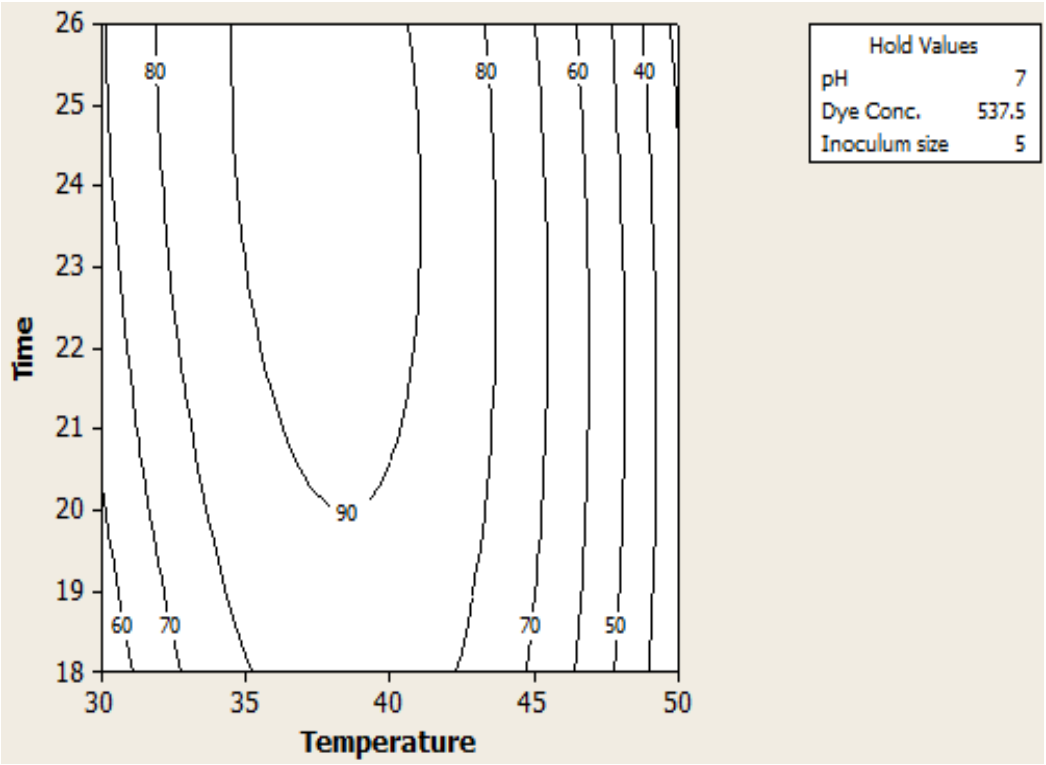


Figure 1d. Contour plot of percentage decolorization: Effect of time and temperature on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping pH, dye concentration and inoculum size at constant values.

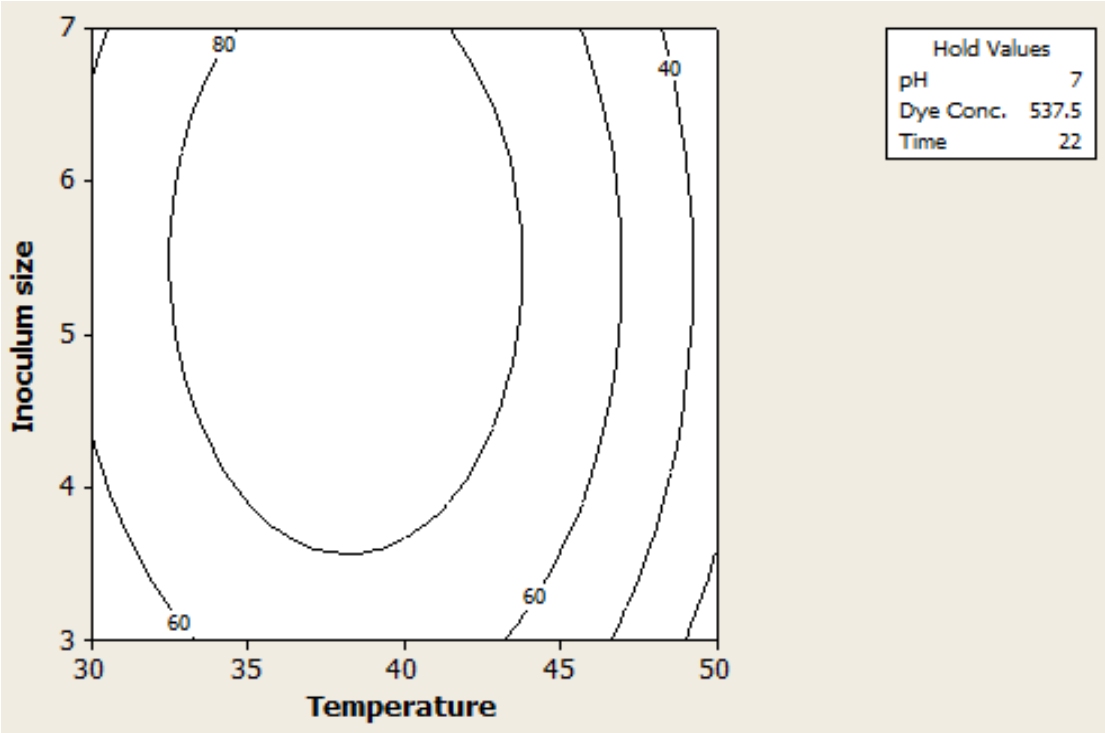


Figure 1e. Contour plot of percentage decolorization: Effect of inoculum size and temperature on the percentage decolorization of Orange G by *Bacillus megaterium* ITBHU01, while keeping pH, dye concentration and time at constant values.

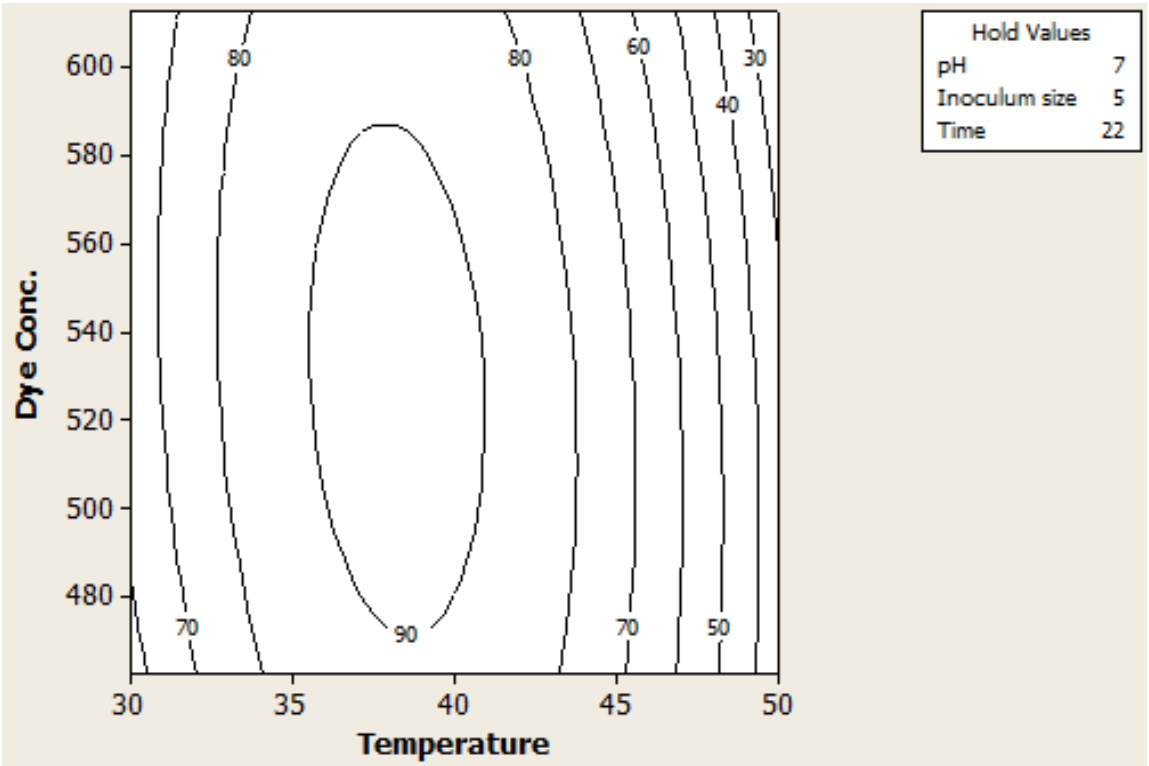


Figure 1f. Contour plot of percentage decolorization: Effect of dye concentration and temperature on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping pH, inoculum size and time at constant values.

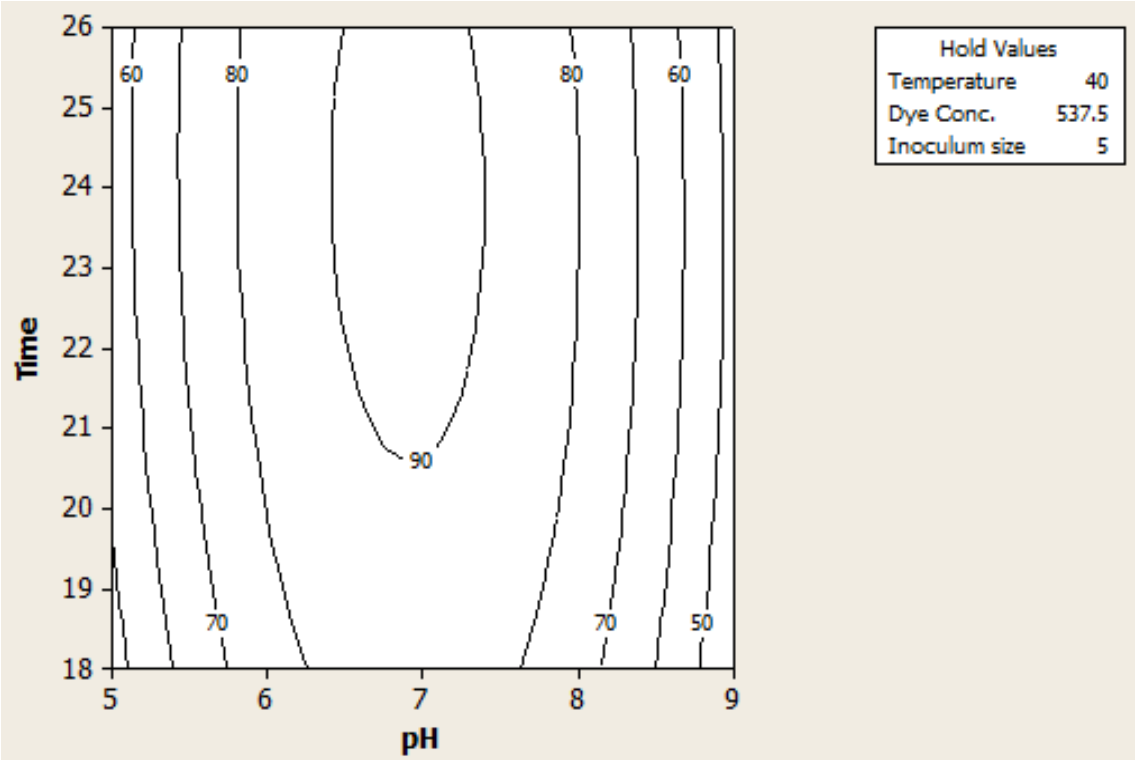


Figure 1g. Contour plot of percentage decolorization: Effect of time and pH on the Percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping temperature, dye concentration and inoculum size and at constant values.

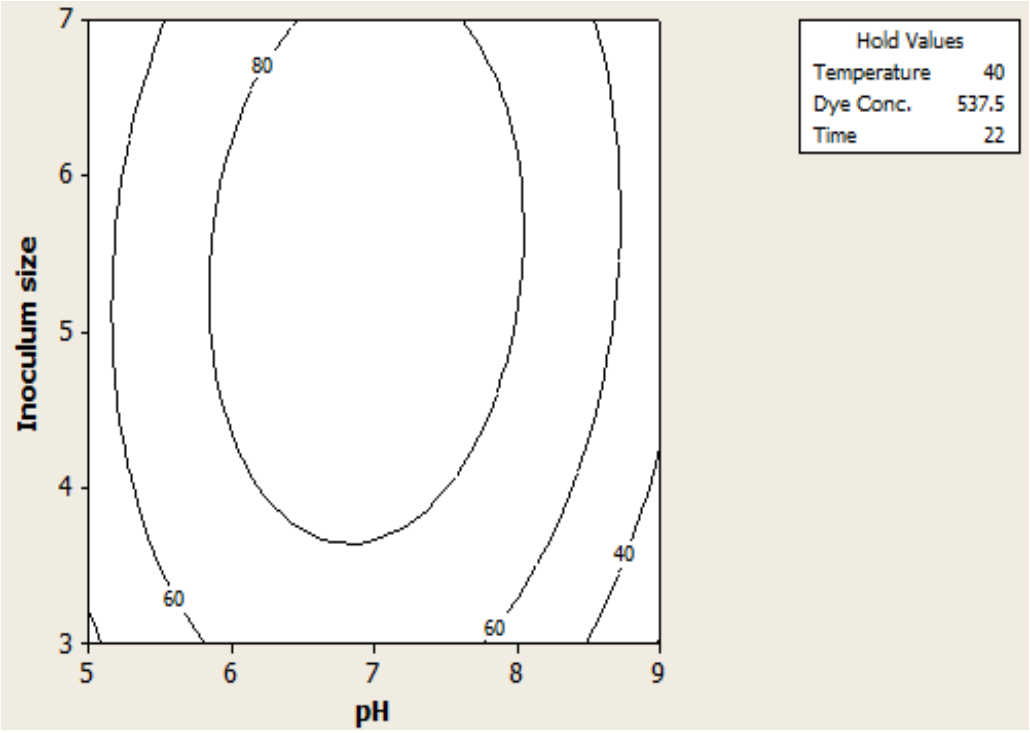


Figure 1h. Contour plot of percentage decolorization: Effect of Inoculum size and pH on the Percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping temperature, dye concentration and time at constant values.

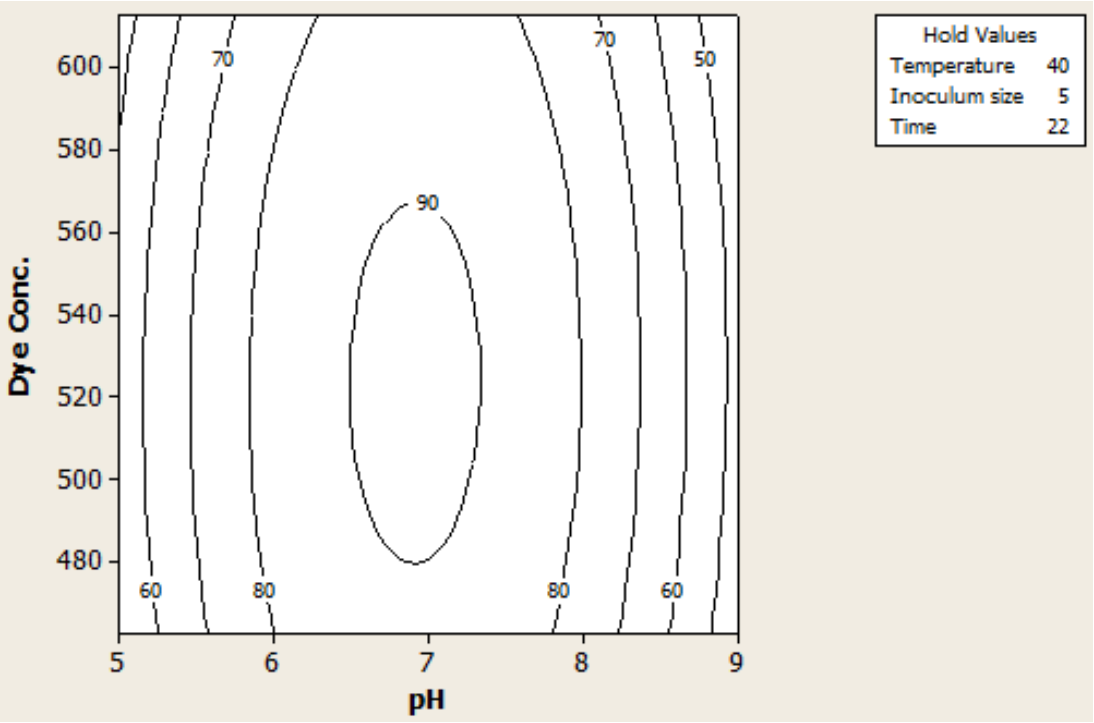


Figure 1i. Contour plot of percentage decolorization: Effect of dye concentration and pH on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping temperature, inoculum size and time at constant values.

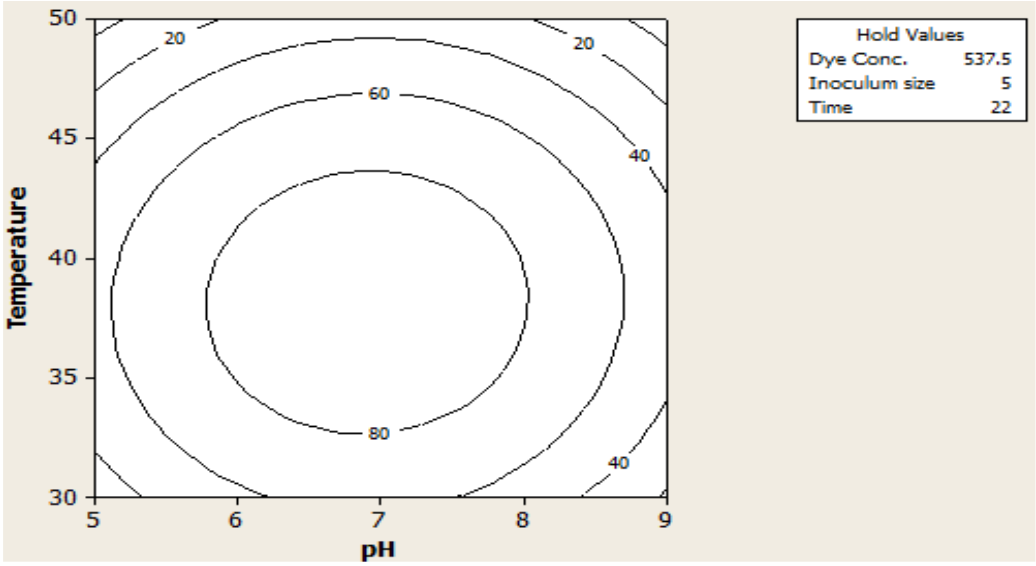


Figure 1j. Contour plot of percentage decolorization: Effect of temperature and pH on the percentage decolorization of Orange G by *B. megaterium* ITBHU01, while keeping dye concentration, inoculum size and time at constant values.

decolorization was found at 485 mg/L dye concentration and fermentation time of 26 h. Further increase in dye concentration, decreases the percentage dye decolorization. Figure 1c exhibits the interactive effect of inoculum size and dye concentration on percentage dye decolorization. We can deduce from the plot that the increase in inoculum size up to 5.5 %(v/v) increases the dye decolorization, but started decreasing upon further

augmentation while the dye decolorization increased as the dye concentration is decreased. Figure 1d exhibits the interactive effect of decolorization time and temperature on dye decolorization. We can infer from the plot that the rise in temperature above 38°C was detrimental to the dye decolorization, while the dye decolorization increased as the decolorization time increases. Figure 1e displays the interactive effect of Inoculum size and temperature. We can deduce from the plot that the rise in temperature above 38°C was detrimental to the percentage dye decolorization, while the inoculum size above 5.5% decreases the dye decolorization. Figure 1f displays the interactive effect of initial dye concentration and temperature on dye decolorization. Dye decolori-zation increased as the temperature was increased from 30 to 38°C, but further increase causes decrease in dye decolorization, whereas decrease in dye concentration favours dye decolorization. Maximum percentage decolorization was obtained at dye conc. of 530 mg/L and temperature of 38°C. Figure 1g depicts the interaction of decolorization time and pH. Maximum decolorization was obtained at 24 h of decolorization time and pH 6.9. Further increase or decrease in pH and decolorization time causes decrease in the dye decolorization. Figure 1h depicts the interaction of inoculum size and pH on dye decolorization. Maximum decolorization was obtained at pH 6.9 and at inoculum size 5.5 % (v/v). Figure 1i depicts interaction of Dye concentration and pH on dye decolorization. It was found that maximum percentage dye decolorization was obtained at pH 6.9 and at dye concentration of 524 mg/L. Further increase or decrease in both pH and dye concentration causes decrease in percentage dye decolorization. Figure 1j depicts interaction of temperature and pH on percentage dye decolorization. It was found out that maximum percentage dye decolorization occurred at temperature 38°C and at pH 6.9. As the temperature and pH values are increased from 30°C and 5.0, respectively; the percentage dye decolorization increases up to 38°C and pH 6.9, respectively. Further increase in temperature and pH values leads to decrease in percentage dye decolorization. The optimal combination of the process parameters for Orange G decolorization as obtained from the surface plots are as follows: p, 6.9; temperature, 37.0°C; dye concentration, 517 mg/L, inoculum size (v/v), 5.5% and time, 23.7 h. At these optimum levels of process parameters, Orange G decolorization by *B. megaterium* ITBHU01 of 94.48% was optimized.

Validation of the optimized decolorization parameters

Experiments were done in triplicate using the optimized condition to verify the modeling results. It was found that for dye concentration of 520 mg/L, pH 6.9 and temperature 37°C, percentage decolorization of 95% was obtained in 24 h. Majority of azo dye reducing bacteria reported (Chang and Lin, 2007; Suzuki et al., 2001) so far were able to reduce the dye at near neutral pH, showing similarity with the present report.

Identification of metabolic intermediates

Bacillus megaterium ITBHU01 successfully resulted in the decolorization of the dye, Orange G. The decolorization was confirmed by UV-VIS spectrum. The UV-VIS spectrum, as shown in Figure 2a, corresponds to initial and final samples of decolorization experiments. The absorbance values were analyzed from 300 to 800 nm. The initial dye solution, before decolorization showed high peak at the wavelength of 480 nm. The decolorized sample showed lowering of peak to a smaller absorbance value for dye concentration of 500 mg/L, which informs that the decolorization is due to removal or degradation of dye (Figure 2a).

The degradation of azo dye, Orange G, by *B. megaterium* ITBHU01 was further supported by thin layer chromatography (TLC). The spots observed with the initial dye solution varied a lot from the spot observed with the supernatant obtained after decolorization (Figure 2b). The original dye was quite different from the supernatant obtained after dye decolorization, which was suggested by different values of retention factors obtained in the TLC experiment. This difference confirms that decolorization was due to breakdown of dyes into unknown intermediate products. The most important step in bacterial degradation of dye is reductive cleavage of N=N (azo) bond leading to formation of colorless aromatic amines. These amines are converted to simpler forms, subjected to oxidation (Lourenco et al., 2000). FT-IR analysis was done to characterize the breakdown products generated. The FT-IR analysis of the dye Orange G and sample obtained after decolorization showed many peaks (Figure 3). The FT-IR spectra of Orange G control dye display peaks at 3535.9, 1634, 1494, 1198.2 and 1035.4. The peak characteristic of azo bond at 1494 of Orange G was not found in the decolorized sample, indicating degradation of Orange G to aromatic amines as metabolites. Peaks at 3341 and 3161 cm^{-1} shows the presence of amine, whereas 3482 cm^{-1} indicates phenolic group. Degradation of aromatic amines obtained after break down of Orange G, leads to formation of aldehyde as an intermediate, which was confirmed by the spot test using 2, 4-dinitro phenyl hydrazine reagent which indicates color test due to presence of aldehyde. Similar results were obtained by Kolekar et al. (2008).

ESI-MS analysis of the supernatant obtained after decolorization confirms degradation to intermediate compounds (Figure 4). During the degradation, there is asymmetric cleavage of azo bonds in Orange G resulting in formation of Phenyl hydrazine, while the naphthalene part of the dye was degraded giving rise to smaller compounds. Further biodegradation of naphthalene part with opening of ring, giving rise to the formation of aldehyde

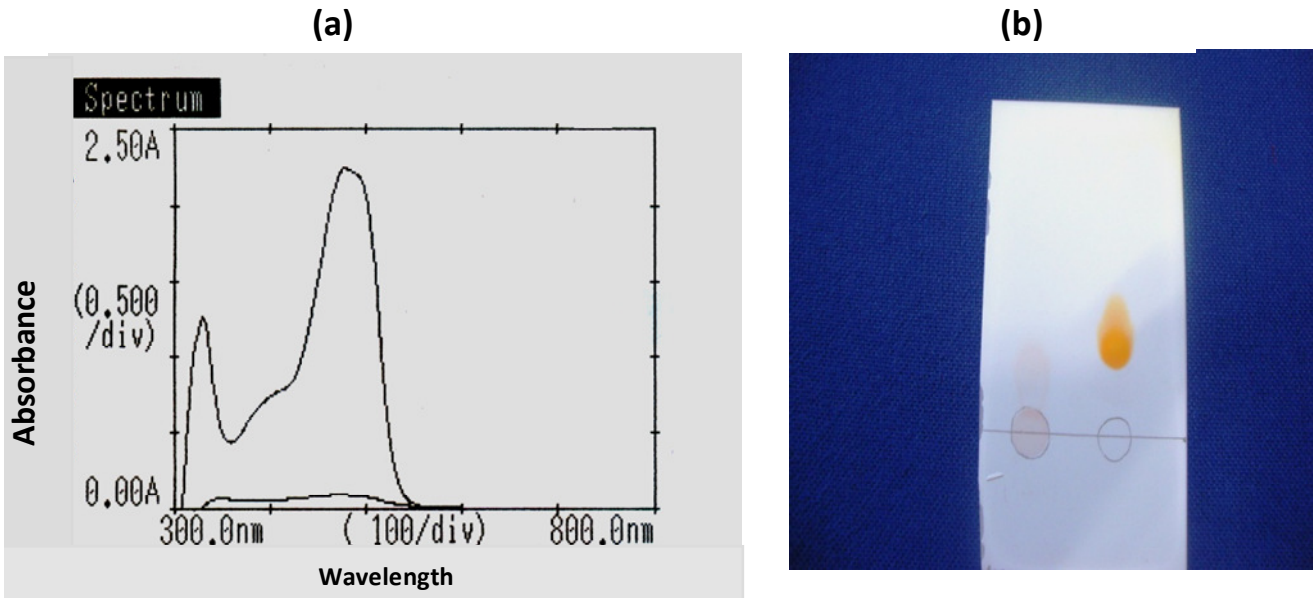


Figure 2. (a) UV-VIS spectrum of Orange G degradation by *B. megaterium* ITBHU01. Lowering of peak clearly indicates decolorization due to removal of Orange G. (b) TLC experiments showing different Rf values of Orange G solution and decolorized sample, indicating its degradation to intermediate products. All the experiments are performed in static condition with the optimized process parameter values from RSM.

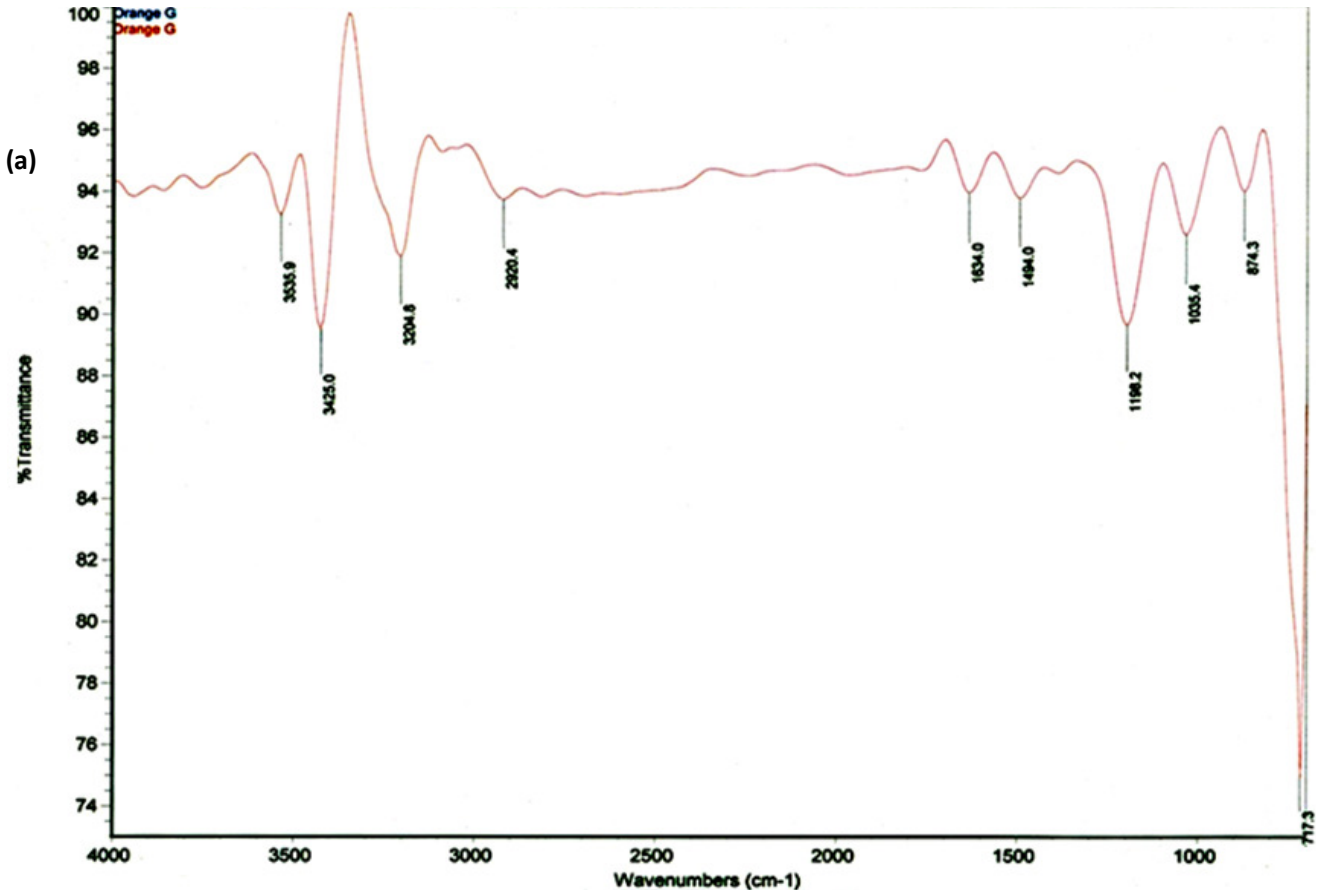


Figure 3. FT-IR spectra of (a) Orange G, control dye, and (b) degraded sample after treatment with *B. megaterium* ITBHU01 with optimized process parameters obtained from RSM.

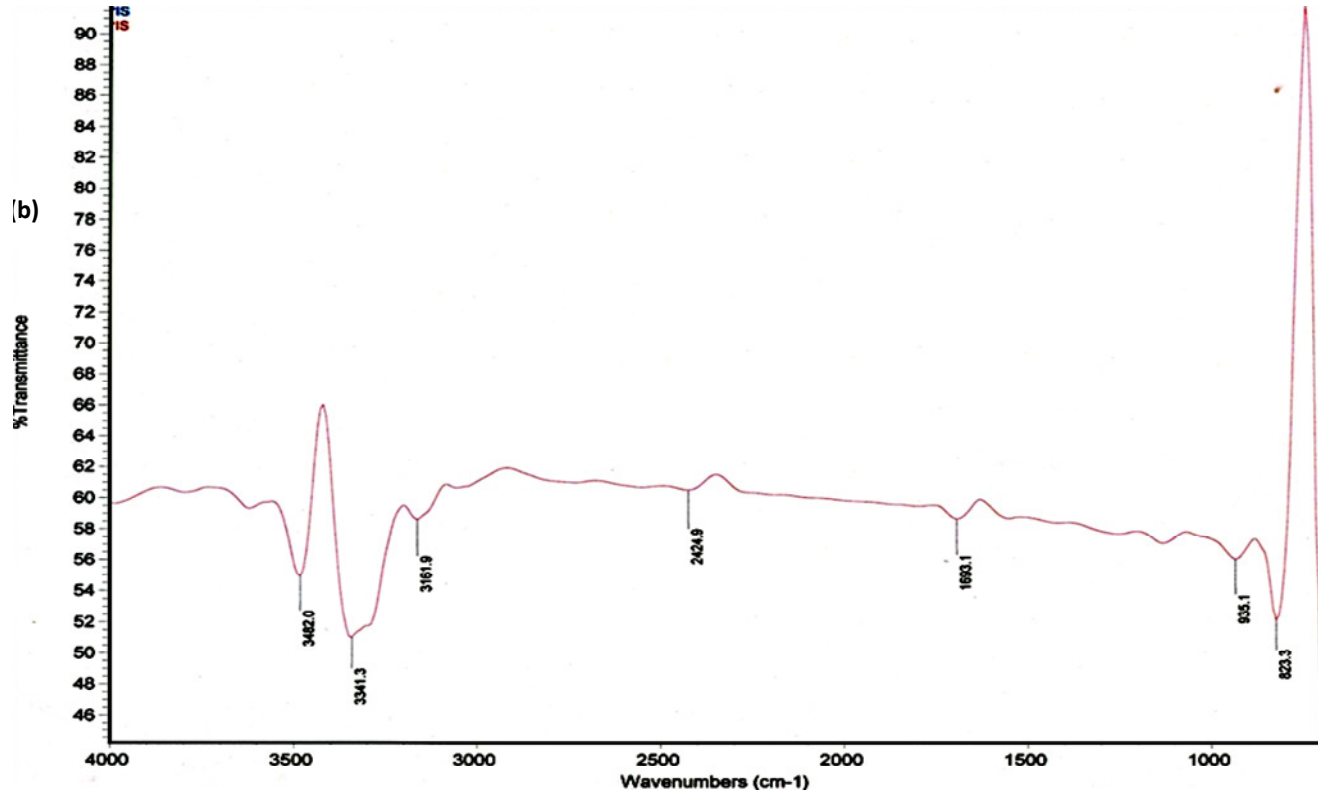


Figure 3. Contd

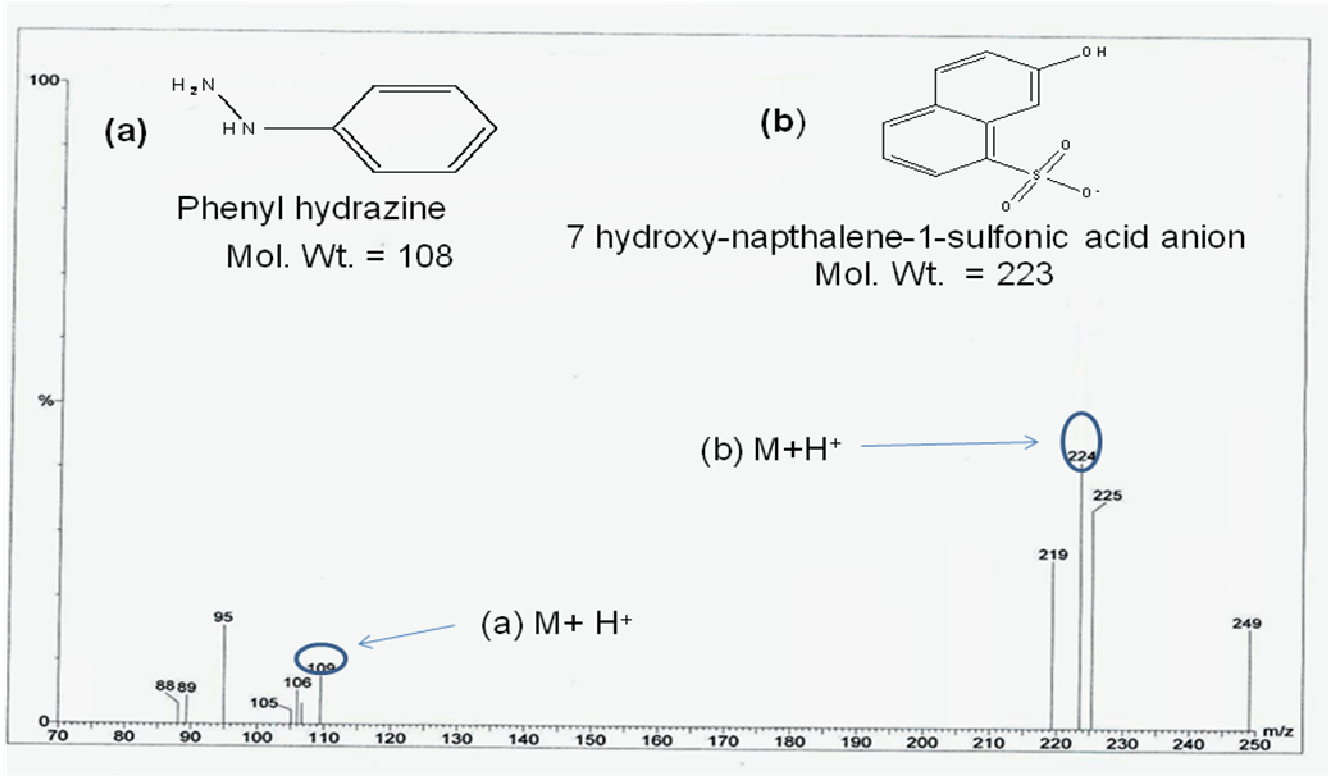


Figure 4. ESI-MS spectra of degradation of Orange G by *B. megaterium* ITBHU01 revealing the degradation mechanism.

as one of the intermediate is confirmed from the IR data.

Thus, it is clear from the analytical methods used that the azo dye, Orange G, is degraded to intermediate compounds as a result of cleavage of azo bond (N=N). The color produced by Orange G was only due to the presence of azo (N=N) group. The intermediates, obtained, phenyl hydrazine, naphthalene derivatives and aldehyde are devoid of any chromophores like azo group (N=N) and hence are colorless and thus are responsible for the decolorization caused by *B. megaterium* ITBHU01.

Conclusions

The present study confirms the ability of newly isolated bacterial culture *B. megaterium* ITBHU01 to decolorize the textile dye Orange G with decolorization efficiency of 95%, thus suggesting its application for decolorization of dye bearing industrial wastewaters. The anaerobic decolorization of Orange G dye occurs as a result of reduction of N=N- bond accompanied by the formation of aromatic amines. The ability of the novel strain needs to be tested in continuous reactor containing real dye bearing wastewater. There is no information available in open literature concerning optimization of process parameters by applying statistical software for decolorization of Orange G by *B. megaterium* ITBHU01. Validity of the response model was verified by agreement of the predicted and experimental values as by keeping pH, 6.9 and temperature, 37.0°C, decolorization of 95% was obtained for Orange G concentration of 520 mg/L in 24 h. The results of this study could be used to design a suitable process to get higher percentage decolorization of Orange G.

REFERENCES

Aksu Z, Kilic NK, Ertugrul S, Donmez G (2007). Inhibitory effects of chromium (VI) and Remazol Black B on chromium (VI) and dyestuff removals by Trametes versicolor. Enz. Microb. Technol. 40: 1167-1174.

Chang JS, Lin CY (2001). Decolorization kinetics of a Recombinant E.coli strain harboring azo dye decolorizing determinants from Rhodococcus sp. Biotechnol. Lett. 23: 631-636.

Choudhari S, Singhal R (2007). Media optimization for the production of b-carotene by Blakeslea trispora: A statistical approach. Bioresour. Technol. 99: 722-730.

Dos Santos AB, Cervantes FJ, Van Lier JB (2007). Review Paper on Current Technologies for Decolourisation of Textile Wastewaters: Perspectives for Anaerobic Biotechnology. Bioresour. Technol. 98: 2369-2385.

Droste RL (2004).Theory and Practice of water and wastewater treatment. John Wiley and Sons, Inc; Singapore. Droste.

Fu Y, Viraraghavan T (2001). Fungal decolorization of dye wastewaters: a review. Bioresour. Technol. 79: 251-262.

Fu Wen, Zhang Xiaoyong, Zhou Jinyan, Zhong Juan, Tan Hong (2009). Jiean-peptide production by immobilized cell fermentation of *Bacillus subtilis*. Chinese J. Appl. Env. Bio. 15(2): 230-234.

Ghodake GS, Telke AA, Jadhav JP, Govindwar SP. (2009) Potential of *Brassica juncea* in order to treat textile effluent contaminated sites. Int. J. Phytorem. 11: 297-312.

Hasan SH, Srivastava P, Talat M (2009). Biosorption of Pb(II)from water using biomass of Aeromonas hydrophila: central composite design for optimization of process variables. J. Hazard. Mater. 168: 1155-1162.

Jadhav JP, Parshetti GK, Kalme SD, Govindwar SP (2007). Decolourization of Azo Dye Methyl Red by Saccharomyces cerevisiae MTCC463. Chemosphere, 68: 394-400.

Kalil SJ, Maugeri F, Rodrigues MI (2000). Response surface analysis and simulation as a tool for bioprocess design and optimization. Proc. Biochem. 35: 539-550.

Kolekar YM, Pawar SP, Gawai KR, Lokhande PD, Shouche YS, Kodam KM (2008). Decolorization and degradation of Disperse Blue 79 and Acid Orange 10, by *Bacillus fusiformis* KMK5 isolated from the textile dye contaminated soil.Biores.Technol. 99: 8999-9003.

Lourenco ND, Novais JM, Pinheiro HM (2000). Reactive textile dye colour removal in a sequencing batch reactor. Water Sci. Technol.; 42(5-6): 321-328.

Maier J, Kandelbauer A, Erlacher A, Cavaco Paulo A, Gubits GM (2004). A new alkali – thermostable azoreductase from *bacillus sp.* Strain SF. Appl. Env. Microbiol. 70: 837-844.

Olukanni OD, Osuntoki AA, Gbenle GO (2006). Textile effluent biodegradation potentials of textile effluent-adapted and non-adapted bacteria. Afr. J. Biotechnol. 5: 1980-1984.

Paul GC, Kent CA, Thomas CR (1992). Quantitative characterization of vacuolization in Penicillium chrysogenum using automatic image analysis. Trans1 ChemE. 70: 13-20.

Rai H, Bhattacharya M, Singh J, Bansal TK, Vats P, Banerjee UC (2005). Removal of Dyes from the Effluent of Textile and Dyestuff Manufacturing Industry: A Review of Emerging Techniques with Reference to Biological Treatment. Crit. Rev. Environ. Sci. Technol. 35: 219-238.

Saratale RG, Saratale GD, Kalyani DC, Chang JS, Govindwar SP (2009). Enhanced Decolorization and Biodegradation of Textile Azo Dye Scarlet R by Using Developed Microbial Consortium-GR. Bioresour. Technol. 100: 2493-2500.

Shih I, Kuo C, Hsieh F, Kao, Sueysheng, Hsieh C (2008). Use of surface response methodology to optimize culture conditions for iturin A production by *Bacillus subtilis* in solid-state fermentation. J. Chinese Inst. Chem. Engg. 39(6): 635-643.

Suzuki T, Timofei S, Kurunczi L, Dietze U, Schuurmann (2001).Correlation of aerobic biodegradability of Sulfonated azo dyes with the chemical structure. Chemosphere, 45: 1-9.

Telke A, Kalyani D, Jadhav J, Govindwar S (2008). Kinetics and Mechanism of Reactive Red 141 Degradation by a Bacterial Isolate Rhizobium radiobacter MTCC 8161. Acta Chim. Slov. 55: 320-329.

Wang HJ, Su Q, Zheng XW, Tian Y, Xiong XJ, Zheng TL (2009). Bacterial Decolorization and Degradation of the Reactive Dye Reactive Red 180 by Citrobacter sp. CK3. Int. Biodeter. Biodeg. 63: 395-399.

Wesenberg D, Kyriakides I, Agathos SN (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Advances, 22: 161-187.