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Optimization of Process parameters for Decolorization of Azo Dye Remazol Golden Yellow by *Bacillus firmus* using Biostatistical Designs

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ABSTRACT

A halo tolerant dye decolorizing bacterium *Bacillus firmus* (TSL9) was isolated from activated textile sludge and identified by 16S rRNA sequencing method. Due to the effect of casein enzymic hydrolysate, and yeast extract, a maximum of 97.23% remazol golden yellow decolorization was manifested by the strain in Luria Bertani medium. Bacterial dye decolorization was insignificant in the aqueous medium, when carbon and nitrogen sources were absent. Plackett-Burman experiments were carried out to screen the significance of factors like beef extract, size of the inoculum and pH on decolorization. Important factors were optimized at various levels by response surface methodology with central composite design. We concluded through model validation that the optimal values of the ingredients required to perform 78.33% decolorization are as follows: beef extract 0.48% (w/v), the size of the inoculum 7.71% (v/v) and pH 6.96. The obtained result specifies that the native bacterium had positive signs to perform effective dye decolorization under derived optimal conditions.

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INTRODUCTION

Textiles industries are known to be the largest water consumers and one of the imperative sources for the global economy and environmental pollution. Among different industrial sectors, they release a prodigious amount of waste water into the environment (Mondal et al., 2017). In textile industrial practices, the fabric dyeing process contributes to major environmental problems as it involves the utilization of numerous chemicals like lubricants, sequestering agents, dye stuff, soda ash, sodium chloride, acetic acid, soap, fixing and softener (Arslan-Alaton et al., 2008; Julkapli et al., 2014). Dyes are synthetic complex aromatic colored compounds that are more toxic in nature and difficult to degrade, more than 10-90% of dye does not bind with fiber are entered into the environment and sewage treatment plant (Bhattacharya et al., 2018). Consequently investigation on decolorization and degradation of dye molecules from waste water has increased worldwide in the last few decades (Jadhav et al., 2008; Morales-Alvarez et al., 2018).

Among the 12 classes of chromogenic groups of dyes released into the environment, 70% are azo dyes (Tony et al., 2009). Azo dyes, are the major colorants used in textile industries worldwide, and improper disposal of azo dyes cause serious threat to both environment and aquatic organism. The effluent from textile industries containing azo dye causes eutrophication, rapid depletion of dissolved oxygen level in surface water and ground water when released into the environment (Rawat et al., 2016; Solis et al., 2012). Physical and chemical technologies such as photolysis, flocculation, membrane filtration, ultrafiltration, advanced oxidation, electrophotocatalysis, and coagulation with alum, ferric chloride, magnesium, carbon, polymer, mineral sorbents or biosorbents have been studied for the treatment of azo dyes in waste water (Mojsov et al., 2016; Robinson et al., 2001). Although these techniques are effective in removing dyes from contaminated water, they are very expensive for pilot scale operation with a limited potential application leads to the production of the vast quantity of sludge and land pollution. Considering these limitations with regard to chemical and physical processes, the most versatile and widely used technology is biological method (Morales-Alvarez et al., 2018).

Biological decolorization of dyes occur in two ways; one is adsorption on the microbial biomass and another is biodegradation of dyes using bacteria, fungi, actinomycetes and yeasts in aerobic, anaerobic, anaerobic / aerobic (sequential) treatment processes (Bhattacharya et al., 2018; Liu et al., 2006). Biodegradation of dyes is currently viewed as an effective, specific, lower energy-demanding and environmentally benign method. Bacterial decolorization is nonspecific and faster compared to other microorganisms (Kalyani et al., 2009). The biological treatment of textile effluent also has numerous challenges, majorly the capacity of microorganisms to utilize dye as a substrate should be considered (Saratale et al., 2011). Predominantly, azo dyes are chemically stable due to binding of azo group with aromatic amines being difficult to biodegrade aerobically and cannot be utilized as a

carbon source by bacteria and it requires additional co-metabolite for the decolorization of dye. Azo dye decolorization is effective in an anaerobic/static condition compared to the aerobic shaking condition, while azoreducatase enzyme has higher activity in reductive cleavage of azo groups (Khehra et al., 2005; Mojsov et al., 2016).

However, the effectiveness of biological treatment system is greatly influenced by various operational parameters such as level of salts, aeration, nutrients, initial concentration of dye, temperature, pH and the amount of inoculum. Operating parameters play a crucial role in the decolorization activity of microorganism (Mondal et al., 2017). Therefore, the effect of various parameters on the color removal process must be investigated and optimized to produce the maximum rate of dye decolorization. The conventional OVAT approach implemented to investigate the media parameters for an ameliorated dye decolorization was unsatisfactory as it was circuitous and needs numerous trials (Bhavsar et al., 2018). Recently bio-statistical tools including design of experiments (DOEs) with response surface methodology (RSM) overcome these classical method limitations and convenient tool to achieve an optimized condition for effective dye removal process. Furthermore, it helps to understand the statistical models, interaction with media parameters at varying levels in dye deolorization (Mohana et al., 2008; Pillai, 2017). RSM is an effective optimization tool; it concurrently predicts the factors and their interactions in few experimental trials. Karthikeyan et al. (2010) reported that optimization of culture conditions for dye decolorization by *Aspergillus niger* HM11 using response surface methodology. Similarly, response surface methodology was studied in the optimization of azo dye amido black 10B decolorization by *Kocuria kristinae* RC3 (Uppala et al., 2018)

Recent studies have reported that *Bacillus firmus* that was isolated from local sewage and textile waste water had up to 97-98% dye decolorization capacity (Arora et al., 2007; Ogugbue et al., 2011). Therefore, Remazol Golden yellow (RNL), an azo dye, which is widely used colorant in textile dyeing and printing industries of study area was used as model dye substance, to evaluate the decolorization efficiency of native bacterium. The present study was aimed to investigate the ability of *Bacillus firmus* (TSL9) strain to decolorize remazol golden yellow azo dye and also to optimize the conditions of various parameters in order to achieve maximum dye decolorization by using statistical tools such as Plackett-Burman and central composite design.

MATERIALS AND METHODS

Isolation and Screening of Dye Decolorizing Bacteria

Activated sludge was collected from a textile industry in Tiruppur, India and processed for isolation of native decolorizing bacterial strains. Pour plate method was employed on nutrient agar containing (g l^{-1}): 5g of peptone, 5g of sodium chloride, 3g of yeast extract, 3g of beef extract and 20g of agar. Consequently inoculated plates were incubated at

35 and 45 °C for 24-48 hours incubation intended for the isolation of mesophilic and thermotolerant bacteria respectively. Morphologically distinct colonies were isolated, and experimented for azo dye decolorization ability on Luria Bertani agar plates containing (g l^{-1}): 10g of casein enzymic hydrolysate, 5g of yeast extract, 10g of sodium chloride and 15g of agar amended with various concentrations (50, 100, 150, 200 and 250 mg l^{-1}) of remazol golden yellow dye (RNL). Spot inoculated plates were subsequently incubated at 37 °C for 4 days. The bacterial isolates showing clear zone in all the concentrations of dye were preferred as potential strain and used for further studies. The strain *Bacillus firmus* (TSL9) was found to be the most potential strain since it showed significant dye decolorizing efficiency in the agar plate.

Identification of Dye Decolorizing Bacteria

The selected decolorizing bacterium was identified by 16S rRNA sequencing method. Extraction of genomic DNA, PCR amplification and 16S rRNA sequencing were carried out in Xcelris Labs Ltd, Ahmedabad, India. The 16S rRNA consensus sequence analysis and alignment of closely related species were executed in BLASTN (www.ncbi.nlm.nih.gov/BLAST) and multiple alignment program (Clustal W) respectively. A Phylogenetic tree was constructed in MEGA version 5.0 via Neighbor Joining (NJ) method. The nucleotide sequences were deposited in NCBI GenBank with an accession number: JX316004.

Effect of Co-substrates on Azo Dye Decolorization

The presence and absence of nourishments on azo dye decolorization with bacterial strain TSL9 was studied in diverse broth media. About 100 ml of Luria Bertani broth containing (g l^{-1}): 10g of casein enzymic hydrolysate, 5g of yeast extract, 10g of sodium chloride**;** Yeast Extract broth containing (g l^{-1}): 5g of yeast extract, 5g of sodium chloride**;** and Bushnell Hass broth containing (g l^{-1}): 0.2g of magnesium sulphate, 1g of di-potassium hydrogen phosphate, 0.02g of calcium chloride, 0.05g of ferric chloride and 1g of ammonium nitrate was prepared and amended with 100 mg.l^{-1} concentration of remazol golden yellow dye. About 1% aliquot of 18 h bacterial culture (TSL9) was inoculated in these flasks and incubated at 37 °C for 3 days under a static condition. Samples were withdrawn aseptically at periodic intervals and centrifuged at 5000 rpm for 20 minutes. Absorbance (OD) of the cell free supernatant was recorded using UV spectrophotometer (Model: Cyberlab UV-100 USA) at 412 nm for remazol golden yellow dye. Decolorization efficiency was experimented in triplicates with abiotic control and the decolorization percentage (D %) was determined by the Equation (1), where A1 and A2 are the initial and final absorbance value.

$$D(\%) = \frac{A1 - A2}{A1} \times 100 \qquad [1]$$

Optimization of Dye Decoloriztion by Design of Experiments (DOEs)

Design of experiments (DOEs) is a statistical tool that exposes the individual and cumulative effects of factors that are involved in the dye decolorization by screening up to 'n-1' variables in just 'n' number of trails. Regression coefficients of response and fittest model prediction were analyzed with statistical software Minitab Version 15 (Tripathi & Srivastava, 2012).

Plackett-Burman Design for Screening of Significant Factors

The 2 k-factorial Plackett-Burman design of 12 trials were used to screen the significant factors of remazol golden yellow decolorization by *Bacillus firmus* (TSL9) and the effect of significant/ insignificant factors were studied. Screening of dye decolorization influencing factors and their actual values was shown in Table 1. Plackett-Burman design (Table 2) was generated on the basis of following first-order model equation.

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j \quad [2]$$

where, Y is decolorization, X_i is factor level, i is factor number, β_0 is model intercepts term, β_i *is* the linear effect, β_{ii} *is* squared effect, β_{ij} is interaction effect. Static decolorization experiments were performed in a basal broth medium, as per the statistical design with cell free control and the decolorization was assayed.

Table 1

Actual values of factors for Plackett-Burman design

Test variables	Lactose % (w/v)	Beef extract % (w/v)	pH	Temperature (°C)	Inoculums size % (v/v)	Dye concentration (mg/l)	Incubation period (hours)
Low level (-)	0.1	0.1	5	30	5	100	24
High level (+)	1.0	1.0	9	45	10	300	72

Table 2

Plackett-Burman design for screening of significant factors

Run order	LA % (w/v)	BE % (w/v)	pH	Temp (°C)	IS % (v/v)	DC (mg/l)	IP (hours)	DV-1	DV-2
1	1.0	0.1	9	30	5	100	72	1	1
2	1.0	1.0	5	45	5	100	24	1	1
3	0.1	1.0	9	30	10	100	24	-1	1
4	1.0	0.1	9	45	5	300	24	-1	-1

Table 2 *(Continued)*

Run order	LA % (w/v)	BE % (w/v)	pH	Temp (°C)	IS % (v/v)	DC (mg/l)	IP (hours)	DV-1	DV-2
5	1.0	1.0	5	45	10	100	72	-1	-1
6	1.0	1.0	9	30	10	300	24	1	-1
7	0.1	1.0	9	45	5	300	72	-1	1
8	0.1	0.1	9	45	10	100	72	1	-1
9	0.1	0.1	5	45	10	300	24	1	1
10	1.0	0.1	5	30	10	300	72	-1	1
11	0.1	1.0	5	30	5	300	72	1	-1
12	0.1	0.1	5	30	5	100	24	-1	-1

Where, LA - Lactose; BE - Beef Extract, Temp - Temperature, IS - Inoculum size, DC – Dye Concentration, IP - Incubation Period, DV1 & DV2 - Dummy variable, +1 denoted for high concentration; -1 denoted for low concentration

Significant Factors Optimization using Response Surface Methodology (RSM)

Optimization of the significant factors that resulted in the Plackett-Burman design was used for the maximum decolorization of remazol golden yellow by TSL9 by using response surface methodology (RSM). A central composite design (CCD) was applied to optimize the screened significant factors for effective dye removal. Significant factors, beef extract, inoculums size, and pH, were studied at five coded levels, -α, -1, 0, +1 and +α (Table 3). The actual values of these factors were calculated by following equation (Paul et al., 1992). Where $\alpha = 2^{n/3}$; here "n" was the number of factors and "0" was the central point.

$$\text{Coded value} = \frac{\text{Actual value} - (\text{high level} + \text{low level})/2}{(\text{High level} - \text{low level})/2} \quad [3]$$

The full factorial experimental runs were performed in triplicates as per the central composite design shown in Table 4, other than significant factors (sodium chloride 0.50% (w/v), dye concentration 200 mg/l, lactose 0.55% (w/v), temperature 37 °C and incubation period (48 hours) were kept constant. The correlation of significant factors with decolorization in central composite design can be described by following polynomial model shown in the equation.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 \quad [4]$$

Where Y is the response, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficient of significant factors, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X_1, X_2 , X_3, X_4 are the coded factors. The most fitting model for an effective response was found by determination of coefficient R^2 (Zhao et al., 2010).

With the support of response optimizer, the optimized values of significant factors were analyzed in statistical software Minitab Version 15. Optimal level of significant factors for maximum decolorization of remazol golden yellow by *Bacillus firmus* (TSL9) was verified with validation experiments by comparing the experimental and predicted values of decolorization percentage.

Table 3

Actual values of the significant factors for CCD

Variables	Unit	Five levels of variables				
		-α (-1.68179)	-1	0	1	+α (+1.68179)
Beef extract	% (w/v)	-0.20681	0.1	0.55	1	1.306807
Inoculums size	% (v/v)	3.295518	5	7.5	10	11.70448
pH	-	3.636414	5	7	9	10.36359

Table 4

Central composite design for optimization of decolorization with significant factors

Run Order	Pt Type	Blocks	Beef extract % (w/v)	Inoculums size % (v/v)	Ph
1	1	1	0.1	5	5
2	1	1	1	5	5
3	1	1	0.1	10	5
4	1	1	1	10	5
5	1	1	0.1	5	9
6	1	1	1	5	9
7	1	1	0.1	10	9
8	1	1	1	10	9
9	-1	1	-0.20	7.5	7
10	-1	1	1.31	7.5	7
11	-1	1	0.55	3.29	7
12	-1	1	0.55	11.71	7
13	-1	1	0.55	7.5	3.63
14	-1	1	0.55	7.5	10.36
15	0	1	0.55	7.5	7
16	0	1	0.55	7.5	7
17	0	1	0.55	7.5	7
18	0	1	0.55	7.5	7
19	0	1	0.55	7.5	7
20	0	1	0.55	7.5	7

RESULTS AND DISCUSSION

Screening of Dye Decolorizing Bacteria

About 20 bacterial strains were isolated from textile activated sludge and screened for ability of textile dye decolorization. These bacterial strains showed a wide variation of clear zones at different concentrations (50-250 mg/l) of remazol golden yellow dye amended in agar plates. Among the bacterial strains tested, TSL9 was found to be the most effective dye-decolorizing bacteria when subjected to further investigations. The efficiency of textile dye decolorization depends on the metabolic activity of microorganisms and their flexibility with the recalcitrant nature of dye (Khan et al., 2012). The bacterium TSL9 decolorized a mono azo dye, remazol golden yellow completely in all the concentrations that were incorporated.

Identification of Dye Decolorizing Bacteria

The dye decolorizing bacterial strain was identified by 16S rRNA sequencing method. Analysis of evolutionary history revealed that the sequence of TSL9 was closely associated with the diverse spectrum of the genus *Bacillus* depicted in the phylogenetic tree (Figure 1). The dye decolorizing bacterium was confirmed as *Bacillus firmus* (TSL9) and their sequence was submitted in the genbank database with the accession number of JX316004. In earlier studies, several *Bacillus* sp. have been reported for decolorization of textile dyes. Liao et al. (2013) reported that *Bacillus cereus* HJ-1 strain from azo dye contaminated river sediment is capable of decolorizing reactive black B (RBB).

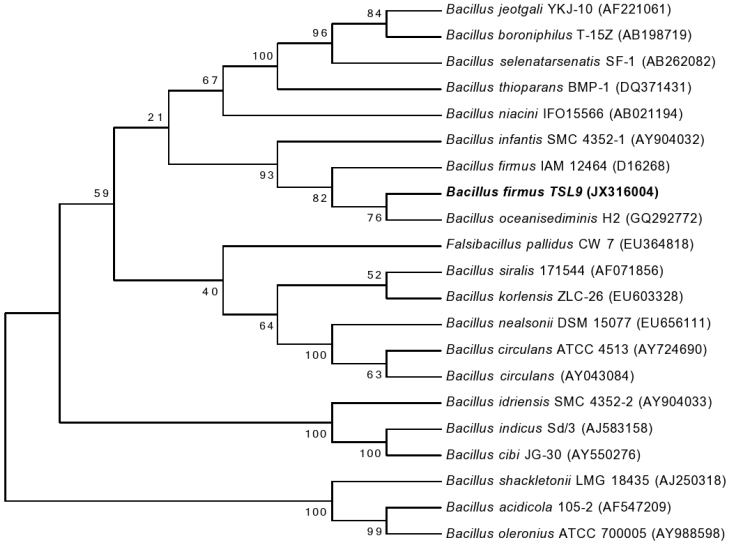


Figure 1. Phylogenetic tree of *Bacillus firmus* (TSL9) showing relationship between selected bacterial strains. Percentage numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 replicates. Brackets represent the sequence accession numbers

Effect of Co-Substrates in Azo Dye Decolorization

Bacillus firmus (TSL9) was subjected to further experimentation by investigating the ability of dye removal in aqueous medium with/without carbon and nitrogen sources. The bacterial culture in Luria Bertani broth showed 69.78% remazol golden yellow removal after 24 hours, and 82.55% followed by 48 hours of incubation. After 72 hours, the rate of decolorization reached 97.23%. Carbon and nitrogen sources have different impacts on dye removal using micro-organisms; these elements may either be utilized for growth or may acted as an electron donor for dye reduction (Gonzalez-Gutierrez & Escamilla-Silva, 2009). In this study, the bacterial strain TSL9 accomplished maximal decolorization within a short period of incubation in Luria Bertani broth. This was mainly due to the amendment of casein enzymic hydrolyzate as a vitamin source for growth and metabolic activities. In contrast, yeast extract broth culture reached 27.9% dye removal with 24 hours, 62.03 and 73.81% decolorization were observed after 48 and 72 hours incubation respectively. Beyond 72 hours of incubation, dye removal efficiency did not increase. Addition of organic nitrogen sources, yeast extract and peptone in the medium enhanced the removal of dye as they act as NADH electron donor (Chang et al., 2000). In the case of Bushnell Haas broth, the strain TSL9 did not decolorize the dye significantly even at extended incubation period of 72 hours. When the carbon and nitrogen sources were absent in the basal broth medium, the bacterial cells failed to multiply. This can be consequently correlated to the absence of azo dye-catabolizing enzyme activity in the broth. These oxidoreductase enzymes such as azoreductase, laccase, and peroxidase are responsible for azo dye decolorization process (Mahmood et al., 2016). Due to deficiency of carbon and nitrogen compounds in dye structure, microbial cells require added nutritional factors to mediate the decolorization (Pillai, 2017; Sani & Banerjee 1999).

Optimization of Dye Decoloriztion by Plackett-Burman Design

Plackett-Burman design (PBD) results are shown in Table 5. There was a wide variation in remazol golden yellow decolorization using *Bacillus firmus* (TSL9), it ranged from 2.07 to 52.26%. The statistical analyses of estimated effects and regression coefficients indicate that a higher concentration of beef extract, incubation period, pH and temperature had a positive influence on decolorization. The reduction of the azo bond increased with higher levels of pH and led to the formation of basic aromatic amine metabolites (Willmott 1997). It was observed that maximum dye removal was obtained under optimal temperature, as it was favorable for azo reductase enzyme production (Chang et al., 2001). Consistently, extended incubation (70 hours) with pure culture of *Pseudomonas* sp. favored Reactive Blue 13 decolorization (Lin et al., 2010). On the contrary, lactose and size of the inoculums revealed the negative influence in lower concentration as shown Table 6. Bacterial cells did not prefer lactose as they assimilated dye as a carbon source (Bhavsar et al., 2018; Saratale

et al., 2009) A vast volume of microbial cells is desirable for decolorization, owing to the toxic nature of dye molecule (Tan et al., 2013).

The most fitting model was confirmed by the determination of correlation coefficient (R^2) (R^2 = 0.8975) nearer to 1 for the regression equation (5), which means that the model can express up to 89.75% variation in the experiment. The value of the adjusted determination coefficient (adj R^2 = 71.81) corresponds to high significance of the model. Analysis of variance of the model is represented in Table 7. The F value of dye decolorization is 4.42. It was demonstrated that the model term was significant, the value of probability (P) > F is less than 0.085. The factors whose P-value were less than 0.10, indicated that the factors were highly significant (Khelifi et al., 2012).

Table 5

Plackett-Burman design of experimental and predicted decolorization (%) results

Run order	LA % (w/v)	BE % (w/v)	pH	Temp (°C)	IS % (v/v)	DC (mg/l)	IP (hours)	Percentage decolorization	
								Experimental	Predicted
1	1.0	0.1	9	30	5	100	72	17.28	16.6
2	1.0	1.0	5	45	5	100	24	14.90	17.14
3	0.1	1.0	9	30	10	100	24	16.00	15.87
4	1.0	0.1	9	45	5	300	24	20.63	23.66
5	1.0	1.0	5	45	10	100	72	10.12	7.87
6	1.0	1.0	9	30	10	300	24	20.47	20.6
7	0.1	1.0	9	45	5	300	72	52.26	44.33
8	0.1	0.1	9	45	10	100	72	4.24	9.66
9	0.1	0.1	5	45	10	300	24	2.27	1.75
10	1.0	0.1	5	30	10	300	72	2.07	0.58
11	0.1	1.0	5	30	5	300	72	20.24	29.35
12	0.1	0.1	5	30	5	100	24	8.99	3.95

Where, LA - Lactose; BE - Beef Extract, Temp - Temperature, IS - Inoculum size, DC – Dye Concentration, IP - Incubation Period

Table 6

Statistical analysis of Plackett-Burman design

S. No	Variables	Effect	Coef	SE Coef	T	P
1	Constant		15.852	2.064	7.68	0.002*
2	Lactose	-3.270	-1.635	2.064	-0.79	0.473
3	Beef extract	13.353	6.677	2.064	3.23	0.032*
4	pH	11.873	5.937	2.064	2.88	0.045*

Table 6 *(Continued)*

S. No	Variables	Effect	Coef	SE Coef	T	P
5	Temperature	3.107	1.553	2.064	0.75	0.494
6	Inoculum size	-13.310	-6.655	2.064	-3.22	0.031*
7	Dye concentration	8.000	4.000	2.064	1.94	0.125
8	Incubation time	4.043	2.022	2.064	0.98	0.383
	R-Sq = 89.75% R-Sq (adj) =71.81%					

*Significant ($p<0.05$)

Table 7

ANOVA for Plackett Burman design

S. No	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Main effects	7	1754.20	1754.20	1754.20	4.42	0.085
2	Lactose	1	28.61	28.61	28.61	0.50	0.517
3	Beef extract	1	513.65	513.65	513.65	9.06	0.040*
4	pH	1	435.49	435.49	435.49	7.68	0.050*
5	Temperature	1	31.27	31.27	31.27	0.55	0.499
6	Inoculum size	1	521.80	521.80	521.80	9.20	0.039*
7	Dye concentration	1	179.49	179.49	179.49	3.16	0.150
8	Incubation period	1	43.89	43.89	43.89	0.77	0.429
9	Residual error	4	226.87	226.87	56.72	-	-
	Total	11	1981.07				

*Significant ($p<0.05$)

$$Y= 15.852 - 1.635 \times \text{lactose} + 6.677 \times \text{beef extract} + 5.937 \times \text{pH} + 1.553 \times \text{temperature} - 6.655 \times \text{inoculum size} + 4.000 \times \text{dye concentration} + 2.022 \times \text{incubation period} \quad [5]$$

Standardized effects in a Pareto chart demonstrated the significant factors as shown in Figure 2. According to their statistical significance ($p=0.10$), beef extract, inoculum size and pH were found to be most important variables that influence dye decolorization by *Bacillus firmus* (TSL9).

Central Composite Design

The significant factors (beef extract, inoculums size and pH) and their mutual interactions were studied using response surface methodology (RSM), for maximum removal of remazol golden yellow dye using *Bacillus firmus* (TSL9). According to a central composite design (CCD), the decolorization output varied from 3.98 to 84.55% as shown in Table 8.

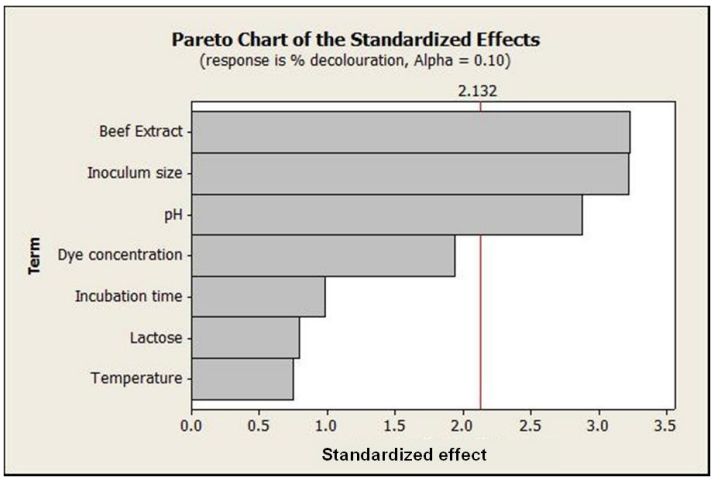


Figure 2. Effects of factors on dye decolorization in Pareto chart

Second order polynomial model equation (6) was fitted with the results of central composite design to reveal the dependence of the response. Smaller p-values with larger t-values related to coefficient value of factors were considered significant with a confidence level of 95% (Table 9). Correlation coefficient (R^2) was determined by regression analysis and it was found to be 98.68%, thus higher value of R^2 delivered presence of a good correlation between the factors and response (Zhao et al., 2010).

$$Y = 81.944 - 3.022 \times X_1 + 2.941 \times X_2 + 1.105 \times X_3 - 10.870 \times X_1^2 - 16.398 \times X_2^2 - 28.157 \times X_3^2 + 1.197 \times X_1 \times X_2 + 15.862 \times X_1 \times X_3 + 1.023 \times X_2 \times X_3 \quad [6]$$

Where Y is predicted response of dye decolorization (%), X_1, X_2 and X_3 were the coded values of significant factors.

In analysis of variance (Table 10) determined data, the F-value = 82.83 and probability value P =0.0 indicates the model is highly significant to predict the results. The linear (p=0.048), quadratic (p=0.000) and interaction (p=0.000) effect of the factors found in enhanced response/decolorization. Response counter plots with various levels of significant factors as shown in Figure 3, which signifies the relationship and interactive effect of two factors on dye decolorization, whereas a third factor value was maintained as middle level.

Table 8

Central composite design of experimental and predicted decolorization (%) results

Trails	Beef extract (X_1)	Inoculums size (X_2)	pH (X_3)	Percentage decolorization		Residual
				Experimental	Predicted	
1	0.1	5	5	36.64	43.57	-6.93
2	1	5	5	3.986	3.41	0.57
3	0.1	10	5	42.91	45.01	-2.10

Table 8 *(Continued)*

Trails	Beef extract (X_1)	Inoculums size (X_2)	pH (X_3)	Percentage decolorization		Residual
				Experimental	Predicted	
4	1	10	5	4.66	9.64	-4.98
5	0.1	5	9	12.71	12.01	0.69
6	1	5	9	33.12	35.30	-2.18
7	0.1	10	9	12.69	17.55	-4.86
8	1	10	9	48.27	45.62	2.64
9	-0.21	7.5	7	62.07	56.28	5.78
10	1.31	7.5	7	46.4	46.11	0.28
11	0.55	3.2	7	33.22	30.61	2.60
12	0.55	11.71	7	43.98	40.51	3.46
13	0.55	7.5	3.63	6.38	0.44	5.93
14	0.55	7.5	10.36	4.3	4.16	0.13
15	0.55	7.5	7	81.45	81.94	-0.49
16	0.55	7.5	7	84.55	81.94	2.60
17	0.55	7.5	7	78.16	81.94	-3.78
18	0.55	7.5	7	83.51	81.94	1.56
19	0.55	7.5	7	80.09	81.94	-1.85
20	0.55	7.5	7	82.86	81.94	0.91

Table 9

Estimated regression coefficients for Central composite design

S. No	Variables	Coef	SE Coef	T	P
1	Constant	81.944	1.953	41.954	0.000*
2	Beef extract	-3.022	1.296	-2.332	0.042*
3	Inoculum size	2.941	1.296	2.270	0.047*
4	pH	1.105	1.296	0.853	0.414
5	Beef extract*Beef extract	-10.870	1.262	-8.616	0.000*
6	Inoculum size*Inoculum size	-16.398	1.262	-12.998	0.000*
7	pH*pH	-28.157	1.262	-22.320	0.000*
8	Beef extract*Inoculum size	1.197	1.693	0.707	0.496
9	Beef extract*pH	15.862	1.693	9.368	0.000*
10	Inoculum size*pH	1.023	1.693	0.604	0.559
	R-Sq = 98.68% R-Sq (adj) = 97.49%				

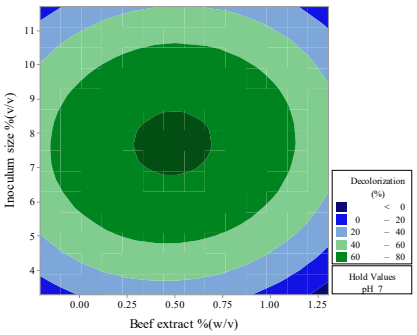
*Significant ($p<0.05$)

Table 10

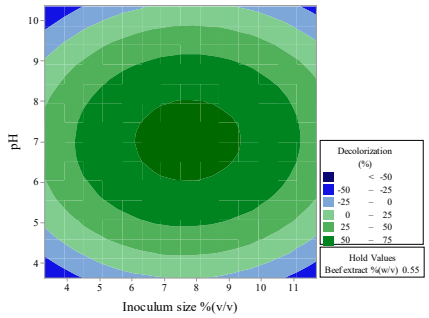
ANOVA for central composite design

S. No	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Regression	9	17097.8	17097.8	1899.75	82.83	0.000*
2	Linear	3	259.5	259.5	86.51	3.77	0.048*
3	Square	3	14805.6	14805.6	4935.21	215.19	0.000*
4	Interaction	3	2032.6	2032.6	677.53	29.54	0.000*
5	Residual error	10	229.3	229.3	22.93	-	-
6	Lack-of-fit	5	201.4	201.4	40.29	7.22	0.024*
7	Pure error	5	27.9	27.9	5.58	-	-
	Total	19	17327.1	-	-	-	-

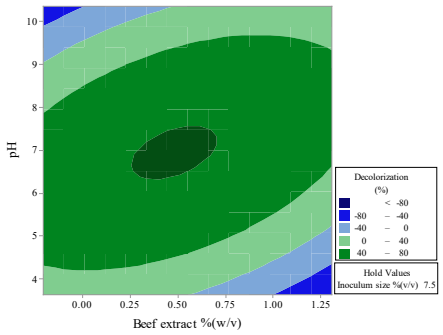
*Significant ($p<0.05$)



(a) Effect of beef extract and inoculum size



(b) Effect of pH and inoculum size



(c) Effect of pH and beef extract

Figure 3. Response contour plots between significant factors on decolorization (%)

The response optimizer tool was used to establish the most favorable point of significant factors for maximum dye removal rate, which was beef extract 0.48% (w/v), inoculum size 7.71% (v/v) and pH 6.96. Under the optimal level of significant factors, *Bacillus firmus* (TSL9) achieved 78.33% of decolorization, which agreed with the predicted decolorization (82.28%). The volume of inoculums provides sufficient biomass to perform decolorization of dye in an intermediate form. Similar to the present findings, beef extract 0.0025% (w/v) showed 97-98% decolorization of disperse dye by *Bacillus firmus* at a wide range of pH (6-9) (Arora et al., 2007). Ogugbue et al. (2011) reported the decolorizarion of Polar red B by *Bacillus firmus*, where maximum dye removal was obtained at pH 7-8. Bolstering the present investigation, Saraswathi et al. (2009) and Padhmavathy et al. (2003) accounted that *Bacillus firmus* acted as a potential candidate in the textile industry effluent treatment. Dafnopatidou and Lazaridis (2008) also stated that *Bacillus firmus* a halo tolerant strain had the ability to decolorize the dye at high salt concentration ranging from 0 to 60 mg/l.

CONCLUSION

The present investigation clearly demonstrated that the dye removal competence of *Bacillus firmus* (TSL9) can be related to the practical implementation constraints of textile industry. The dye decolorizing bacterial broth mediums require nutritional properties for their prospective decolorization and it was not able to decolorize remazol golden yellow dye in the absence of carbon and nitrogen sources. Application of statistical tool, Plackett-Burman design revealed the contribution of significant factors on dye removal using bacteria. Beef extract effectively utilized by the cells in the decolorization process than lactose as a carbon source in the medium. Thus, beef extract is suitable alternative organic nitrogen source which helps to avoid usage of inorganic nitrogen substances and generation of secondary pollutants usually occurred in the conventional effluent treatment at industrial level. Optimization of important factors for an effective RNL decolorization through Response surface methodology achieved a satisfactory response that supports in designing a cost effective process for dye decolorization. The optimized significant factors viz., beef extract, inoculum size and pH influenced the maximum dye removal efficiency in liquid broths, indicating the potential of native bacterium as an effective biodegradation tool for the decolorization of azo dyes from the effluents of the dye industries. Further scale-up studies are required on the decolorization of dye containing industrial effluents by isolated bacteria in order to assess the potential of *Bacillus firmus* as a prospective bioremediation agent.

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