Role of *Shewanella putrefaciens* in decolorization of Acid dye Red 3BN

N. Hema, S. Suresha

Department of Environmental Science, Yuvaraja's College (Autonomous), University of Mysore, Mysore, Karnataka, 570005, India, Email: es_hema@yahoo.com

ABSTRACT

Investigating the potential of the bacterium *Shewanella putrefaciens* to decolorize and degrade Acid dye Red 3BN was the main aim of the study. Percentage decolorization of Red 3BN by bacterium was taken as the response to screen and optimize the media constituents using statistically valid Plackett-Burman and response surface methodology (RSM) design of experiments, respectively. The significance of the factors was analyzed in the form of analysis of variance which indicated that, $FeSO_4$ was a significant factor influencing the process of dye decolorization. Response surface methodology used in optimizing the important media constituents for enhancing the decolorization of Red 3BN, revealed optimum combinations of all factors which gave maximum percent dye decolorization of 82.44% with KH_2PO_4=0.35%, yeast extract=0.35%, ferrous sulphate=0.56%, magnesium sulphate=0.34%, ammonium chloride =0.47%, sucrose= 0.87%, inoculum size=20.5%, with pH 8, temperature 30°C and dye concentration 0.01%. The pareto chart plotted for seven factors reveals that, ferrous sulphate, inoculum size and ammonium chloride play a more positive and significant role in dye decolorization. The degradation of Red 3BN by the isolate was confirmed using Fourier transform infrared spectroscopy. These results suggest that the isolated bacterium is suitable for the biological treatment of dye-containing wastewater.

Keywords: Decolorization, degradation, Red 3BN, response surface methodology, *Shewanella putrefaciens*, Plackett-Burman design

INTRODUCTION

Effluents containing textile dyes are usually discharged in large quantities worldwide into natural water bodies on a daily basis [1]. Over 10,000 dyes with an annual production over 7×10^5 metric tons worldwide are commercially available and 5-10% of the dyestuffs are lost in industrial effluents [2]. Once in the environment, they can show their toxic and genotoxic effects on organisms. There are some reports on toxicity and genotoxicity of textile dyes [3, 5]. Moreover, the discharge of those colored wastewaters into rivers and lakes leads to reduction of sunlight penetration in natural water bodies which in turn decreases both the photosynthetic activity and the dissolved oxygen concentration. This will create anaerobic conditions thereby killing aerobic marine organisms. The elimination of colored substances in wastewater is based mainly on physical and chemical methods [6]. All these techniques have certain disadvantages [7]. On the other hand, microbial degradation and decolorization of azo dyes is considered environment friendly and cost competitive compared to physico-chemical processes [8].

The success of biological process for color removal from a given effluent depends in part on the utilization of microorganisms that effectively decolorize synthetic dyes of different chemical

Research Article, Acta Biologica Indica 2014, 3(2):731-742

pISSN 2319-1244, eISSN 2279-0160

structures. Many bacteria, actinomycetes, yeast and mitosporic fungi are able to decolorize dyes, with color removal by these microorganisms being mainly attributed to adsorption of dyes [6, 9]. Color removal processes with active microorganisms have two different simultaneous steps: adsorption of dyes on the surface of the organisms and degradation of dyes by the enzymes produced by these organisms [10, 11, 12].

Most of the optimization studies during the development of a process, involve the variation of one factor at a time, keeping all other factors constant. These conventional methods are time-consuming and incapable of detecting the true optimum, especially the absence of interactions among factors [13] and in defining the effect of the independent variables, alone or in combination, on the processes [14, 15]. The experiments conducted using the factorial designs, enable all factors to vary simultaneously. This helps in quantifying the linear, square and interactive effects of the test variables. Another advantage is that, the experimental designs could be changed progressively until a fitted model is found to describe the studied phenomenon [16, 17].

Response surface methodology (RSM) is an efficient experimental strategy to determine optimal conditions for a multivariable system rather than optimization by the conventional method. Optimization of conditions for maximum removal of Red 3BN by statistical approach has been planned to determine the exact conditions for removal by *Shewanella putrefaciens* which would be useful for industrial applications. RSM approach is used to generate the best conditions for a system comprising of many variables to calculate the combined effect [13, 18].

Hence, the present study was aimed to examine the most influential variables for maximum decolorization of the Acid dye Red 3BN through Plackett-Burman experimental design using *Shewanella putrefaciens*. The degradation process was assessed by FT-IR spectroscopy.

MATERIALS AND METHODS

Chemicals

The chemicals used for the experiments were of highest available purity and were obtained from the Hi-Media Laboratories (Mumbai, India). The textile dye, Red 3BN was provided by KSIC (Mysore, India). All other reagents were of analytical grade.

Bacterial strain and culture condition

The bacterium was isolated from the textile effluent obtained from textile industry (Karnataka Silk Industries Corporation Ltd) situated in Mysore, Karnataka, India. Further identification of the isolate was carried out at the Department of Studies in Biotechnology, University of Mysore, Mysore.

Isolation and screening of dye decolorizing bacteria

The effluent sample obtained from KSIC, Mysore was serially diluted with 9ml of distilled water from 10^{-1} to 10^{-7} dilution. About 0.1 ml of the serially diluted sample of 10^{-5} and 10^{-7} dilution was poured onto the nutrient agar [(g/l) Peptone - 5g, Sodium chloride- 5g, Yeast extract - 3g, Agar-15g and pH -7] plates and spread evenly under the laminar air flow providing aseptic condition and incubated at 37°C for 24 hours. Individual colonies of the predominant types of microorganisms were purified by streaking (zigzag streaking) on the same medium. By Gram's staining, the purified isolates were examined microscopically to check their purity. Obtained pure cultures were maintained on nutrient agar at 4°C [19, 20, 21].

All the effluent isolated pure colonies were inoculated in 10ml Nutrient broth (inoculum). Inoculum of 2.5 ml was inoculated into 250 ml flasks containing 100 ml mineral salts medium and dye (0.01%). Mineral salts Basal medium had the following composition (g/l): Na_2HPO_4, 2.13; KH_2PO_4, 1.3; NH_4Cl, 0.5; $MgSO_4$, 0.2; 1litre of tap water with 1ml of trace element solution per liter. The trace element solution had the following composition (g/L): $MgSO_4.7H_2O$, 7.12; $ZnSO_2.7H_2O$, 0.044; $MnSO_4.4H_2O$, 0.081; $CuSO_4.5H_2O$, 0.0782; $Na_2MoO_4.2H_2O$, 0.025; $FeSO_4.7H_2O$, 0.498; Boric acid 0.1+0.27ml of H_2SO_4. The final pH was adjusted to 7.0. The mineral salts medium was supplemented with 1(g/L) yeast extract [22]. Each flask with 2.5 ml of inoculum was incubated on a rotary incubator at 150 rpm for six days at room temperature to check the dye degrading ability.

Optimization of culture media for dye decolorization by response surface methodology

To approach a near optimal response region of the medium composition, a fractional factorial Plackett-Burman design was applied. A total of eight experimental runs, including two center-point replicates were used to screen the media constituents for their effects on Red 3BN dye decolorization by *Shewanella putrefaciens*. Based on the previous reports [23], RSM was employed to identify the interactions between seven operational variables. The experimental range and levels of media constituents used in Plackett-Burman design is shown in Table 1. Plackett-Burman design was employed to study the interactions of different variables and various concentration of factors were chosen as the critical variables, and dye decolorization experiments were carried out according to the arrangement presented in Table 2.

Molecular characterization of the bacterial strain

After purification by successive single colony isolation on an agar plate, the bacterium was identified by analysis of 16S rRNA sequences. The genomic DNA was extracted from the isolate by using the DNeasy Plant Minikit (Qiagen, Germany) as per manufacturer's instruction. Two primers Forward = 5'-TGGTAGTCCACGCCCTAAC-3' and Reverse = 5' CTGGAAAGTTCCGTGGATGT-3' were applied for the amplification of the 16S rRNA gene. Polymerase chain reaction (PCR) was performed as follows: pre-denaturation at 94°C for 5 min, 94°C for 30 s, 60°C for 30 s and 72°C for 45 s, 72°C for 2 min and hold at 4°C. Steps 2, 3 and 4 were repeated for 35 cycles. Nucleotide sequencing for these samples was performed using ABI 3500XL Genetic Analyzer at the Department of Studies in Biotechnology, University of Mysore, Mysore. The nucleotide sequences of the isolate obtained were compared to the sequences available in the public database using BLAST software (www.ncbi.nlm.nih.gov). Neighbor-joining method [24] was employed to construct the phylogenetic tree using MEGA4 software [25], and the maximum likelihood method was adopted for calculating the evolutionary distance.

Decolorization assay

To monitor the decolorization process, the degraded dye samples were taken from the experimental system and centrifuged at 6000 rpm for 20 min to remove the bacterial cells. The absorbance of the supernatant was measured at the maximum wavelength of the dye. The maximum absorbance of Red 3BN was estimated by using UV-Visible Spectrophotometer 108 (Systronics) and the λ_{max} was found to be at 495nm. The response percent decolorization was calculated using the equation 1.

$$D = \left(\frac{C_0 - C_t}{C_0}\right) \times 100 \quad (1)$$

where D=percent dye decolorization, C_o=initial dye concentration (mg/L) at time t=0 and C_t=dye concentration (mg/L) at time t (h).

Degradation studies

Fourier transform infrared spectroscopy analysis was carried for Red 3BN dye and its decolorization products. After complete decolorization, cells were removed through centrifugation at 10,000 rpm for 10 mins. The products produced after decolorization of dye were extracted by using equal volume of ethyl acetate and dried by anhydrous Na_2SO_4 in a rotary evaporator.This sample was then subjected to FT-IR analysis to confirm degradation of the dye [26-27] using Perkin Elmer 783 Spectrophotometer and changes in percent absorbance at different wavelengths were observed. The FT-IR analysis was done in the mid-infrared region of 400-4000 cm^{-1}.

RESULTS AND DISCUSSION

Molecular Identity of the Potential Strain

The bacterial isolate was identified on the basis of 16S rRNA gene sequencing. The closest neighbor in GenBank database to the one used in study was found to *be S. putrefaciens* with the homology of 99.0%. The sequence was submitted to GenBank with an accession number of JN555612. The phylogenetic relationship of the isolate is shown in Figure 1.

Effects of the process parameters on decolorization of Red 3BN by *S. putrefaciens*

The effect of process parameters (independent variables) such as, KH_2PO_4, yeast extract, $FeSO_4$, $MgSO_4$, NH_4Cl, sucrose and inoculum size on decolorization and degradation of Red 3BN by *Shewanella putrefaciens* was studied. Experiments were performed as per the combinations of factors shown in Table 2. The corresponding response for dye decolorization varied from 50.0 to 72.2%.

The results of dye decolorization were analyzed in the form of ANOVA, which is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources variation for the purpose of testing the null hypotheses on the parameters of the model [15]. The mean sum of squares (MS) of the model term is obtained from the ratio of sum of squares (SS) and degrees of freedom (df). The Fisher's F value is calculated by dividing the MS owing to the model by the MS owing to error.

From the Table 3, it is clear that factor C ($FeSO_4$) is significant with the F-value = 4.48 and the P-value =0.0787. Factors E (NH_4Cl) and G (inoculum size) show similar trend with lower p-value of 0.2873 and 0.1586 respectively. Therefore, from the screening process, the $FeSO_4$ was found to have maximum effect on percent dye decolorization. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the experiment is indicated by high value of CV. In the present case, a low CV (5.03) denoted that the experiments performed were highly reliable. The model's goodness of fit was checked by the determination co-efficient (R^2=63.65%). Similar observations were reported by an earlier study [28]. With CV value, R^2 value, P-value=0.039 and F-value=8.07, the model indicates that, it is precise and significant. Using response surface regression (RSREG) analysis in response surface methodology, optimized media compositions as well as culture conditions were obtained. The estimated optimal response for Red 3BN dye decolorization was 82.44% when KH_2PO_4=0.35%,

yeast extract=0.35%, ferrous sulphate=0.56%, magnesium sulphate=0.34%, ammonium chloride =0.47%, sucrose= 0.87%, inoculum size=20.5%, with pH 8, temperature 30°C and dye concentration 0.01%. The pareto chart in Figure 2 illustrates the order of significance of the variables affecting dye decolorization. The chart, plotted for all seven factors reveals that ferrous sulphate, inoculum size and ammonium chloride play a more positive and significant role in dye decolorization. Other factors have least or no effect as the values are below t-value limit.

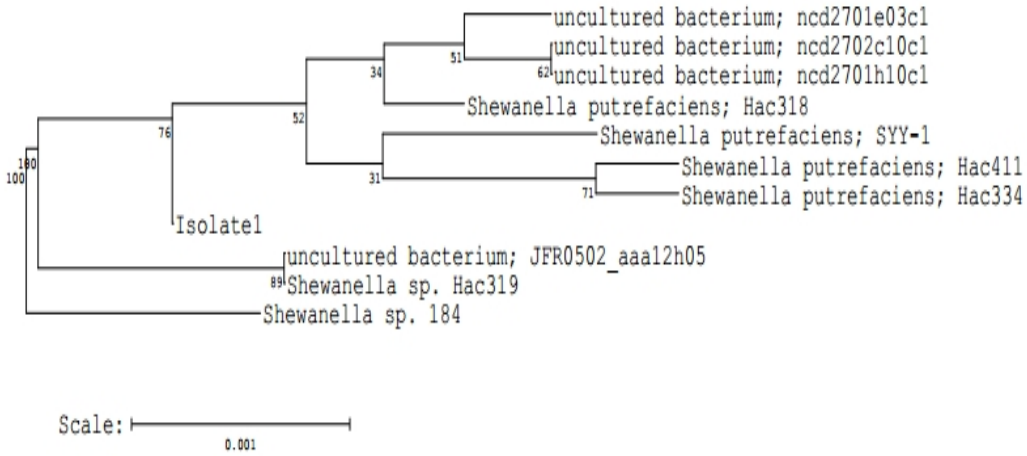


Figure 1. Phylogenetic tree of bacterial strain *S. putrefaciens* based on 16S rRNA gene sequence.

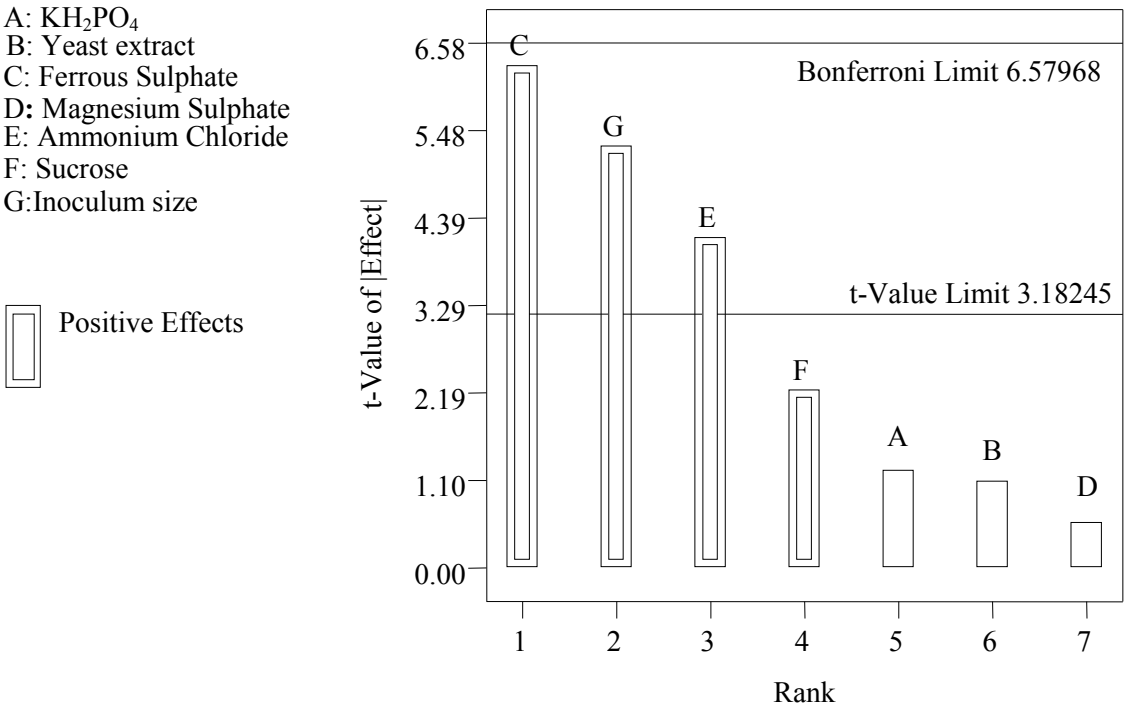


Figure 2. Pareto chart for Plackett-Burman design for 7 factors on Red 3BN decolorization by *S. putrefaciens*

Table 1. Experimental range and levels of media constituents used in Plackett-Burman design.

Parameters	Range and levels	
	-1	+1
KH_2PO_4	0.1	0.5
Yeast extract	0.1	0.5
$FeSO_4$	0.1	0.5
$MgSO_4$	0.1	0.5
NH_4Cl	0.1	0.5
Sucrose	0.5	1.0
Inoculum size	10	20

Table 2. Plackett-Burman design for screening the media constituents.

Run no.	Experimental Variables							
	KH_2PO_4 (%)	Yeast extract (%)	$FeSO_4$ (%)	$MgSO_4$ (%)	NH_4Cl (%)	Sucrose (%)	Inoculum Size (%)	Decolorization (%)
1	0.5	0.5	0.5	0.1	0.5	0.5	10	62.5
2	0.5	0.5	0.1	0.5	0.1	0.5	20	50.0
3	0.5	0.1	0.5	0.1	0.1	1.0	20	68.75
4	0.1	0.5	0.1	0.1	0.5	1.0	20	62.5
5	0.5	0.1	0.1	0.5	0.5	1.0	10	50.0
6	0.1	0.1	0.5	0.5	0.5	0.5	20	72.2
7	0.1	0.5	0.5	0.5	0.1	1.0	10	55.5
8	0.1	0.1	0.1	0.1	0.1	0.5	10	27.7

Table 3. Analysis of variance for Red 3BN decolorization.

Parameter	Degree of freedom	Sum of Square	Mean Square	F-value	P-value	R Square	CV	Significance
A	1	22.27	22.27	0.10	0.76	0.01	26.81	NS
B	1	17.55	17.55	0.08	0.79	0.01	26.86	NS
C	1	590.82	590.82	4.48	0.07	0.42	20.45	S
D	1	4.88	4.88	0.02	0.88	0.003	26.98	NS
E	1	255.94	255.94	1.36	0.28	0.18	24.40	NS
F	1	74.11	74.11	0.34	0.58	0.05	26.30	NS
G	1	416.88	416.88	2.59	0.15	0.30	22.59	NS

S-Significant, NS-Not significant

Optimization of the media constituents using RSM

To investigate the interactive effect of two factors on the decolorization of dye, response surface methodology was used and surface plots were drawn. Response surface plots of two factors at a time, maintaining other factors at fixed levels, are more helpful in understanding both the main and the interactive effects of the two factors. Similar studies have been carried out by earlier investigators [29]. Some typical response surface plots to illustrate these salient findings on the

interaction between the media constituents on Red 3BN decolorization in the present study are depicted in Figure 3 and Figure 4. The quadratic polynomial equation was used to facilitate plotting of response surfaces. The equation can generally be represented as:

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

b_0 = Intercept

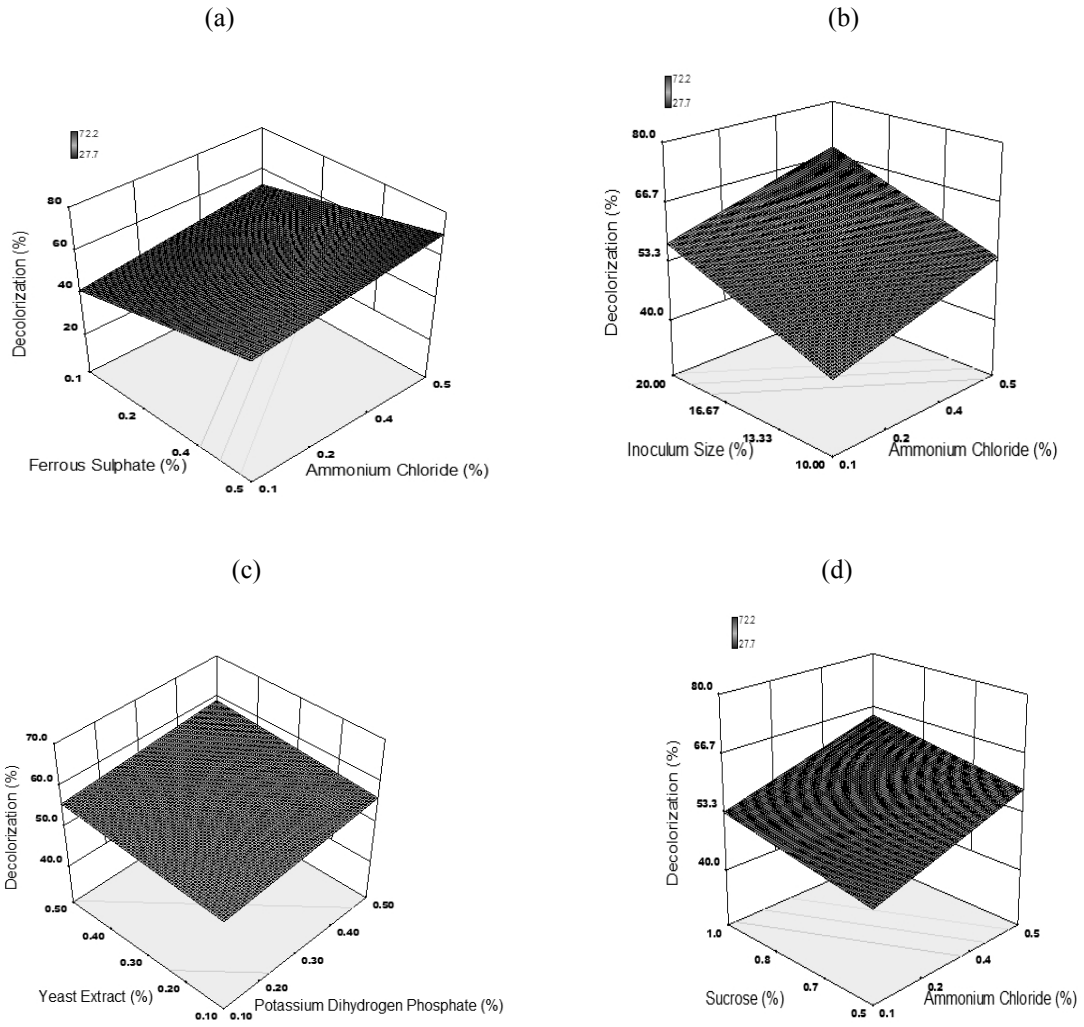


Figure 3. Response surface plot of decolorization showing the interactive effect of (a) $FeSO_4$ and NH_4Cl, (b) inoculum size and NH_4Cl, (c) yeast extract and KH_2PO_4, (d) sucrose and NH_4Cl.

Two parameters were plotted at a time on the x_1 and x_2 axes respectively, with the other remaining parameters set at their optimized value (KH_2PO_4=0.35%, yeast extract=0.35%, $FeSO_4$=0.56%, $MgSO_4$ =0.34%, NH_4Cl=0.47%, sucrose= 0.87%, inoculum size=20.5%, with pH 8, temperature 30°C and dye concentration 0.01%). Effect of carbon and nitrogen sources on degradation efficiencies of microorganisms have been studied by few researchers. Earlier works

have reported that there is an increased efficiency of bacterial cultures with addition of carbon and nitrogen sources to the degradation medium [30-31]. Many different co-substrates have been found to suit as electron donors like glucose and yeast extract [32-33].

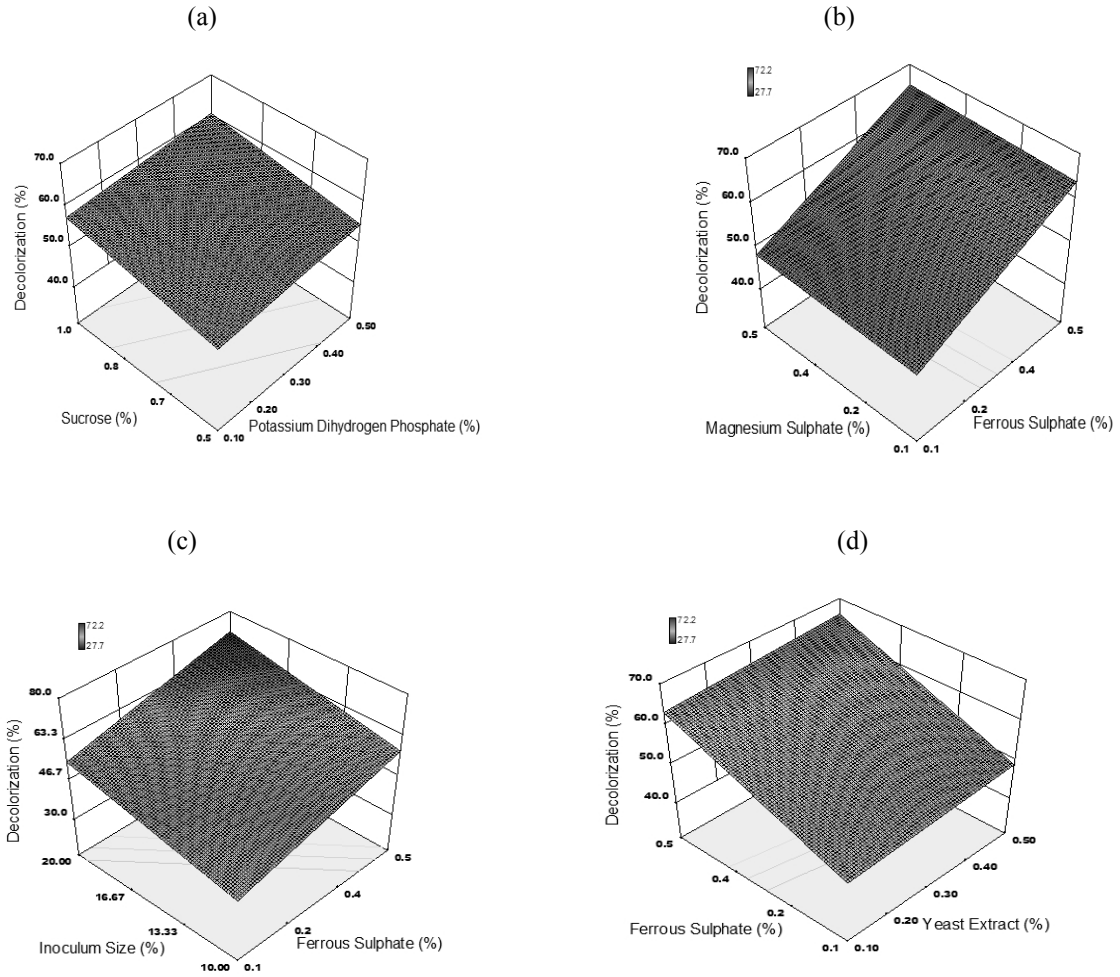


Figure 4. Response surface plot of decolorization showing the interactive effect of (a) sucrose and KH_2PO_4, (b) $MgSO_4$ and $FeSO_4$ (c) inoculum size and $FeSO_4$ (d) $FeSO_4$ and yeast extract.

The genus *Shewanella* is a Gram-negative, rod-shaped, oxidase-positive, aquatic, facultative anaerobic bacterium with genomic DNA containing 38-54 mol% G+C. Members of *the Shewanella* genus are mostly isolated from seas, but a few have been isolated from industrial wastewater, sediment and spoiled foods. [34-36]. Based on the genetic and phenotypic analyses, more than 40 species are currently listed in the genus *Shewanella* [37]. Many studies have shown that *Shewanella* has the ability to use various terminal electron acceptors in the absence of oxygen, allowing them to survive in extreme environments, such as low temperatures, various salt concentrations and barometric stress [38-39].

Many *Shewanella* species have been reported to be capable of decolorizing textile dyes. A study reported that *S. oneidensis* was capable of decolorizing 95% of Reactive Black 5 from the medium

containing 100µm after 12 h [40]. *S. decolorationis* decolorized Victoria Blue-B to the extent of 94.83% with minimum dye concentration of 50 mg/L in 42 h [41]. Congo Red was decolorized to 96.2% by *S. xiamenensis* isolated from sediments [42]. *S. putrefaciens* AS96 was isolated from activated sludge which reported its efficient decolorization capacity under saline conditions [38]. *S. decolorationis* was suggested to be capable of obtaining energy to support growth during dissimilatory azo dye reduction [43]. Another study, observed biomass increase of *S. putrefaciens* AS96 during dye decolorization under saline conditions of NaCl (5-30 g/L) which was helpful for cell growth. Earlier study reported that complete or partial decolorization of azo dye mixture by *S. putrefaciens* AS96 and the other five bacterial strains isolated from activated sludge [38]. Work on *S. oneidensis* MR-1 revealed that it could not completely decolorize 200 mg/L of acid yellow 199 under microaerophilic and non-saline conditions, and there was negative correlation between dye removal and initial dye concentration [44].

Figure 4a shows the interactive effect of ferrous sulphate and ammonium chloride on Red 3BN decolorization. With increase in $FeSO_4$ from 0.1-0.5%, there is an increase in percent decolorization till 50%. Similarly, an increase in ammonium chloride from 0.1-0.5%, leads to a rise in percent decolorization. The interaction between inoculum size and ammonium chloride shows a different trend as shown in Figure 4b. With an increase in inoculum size, there is a increase in the percent decolorization, whereas an increase in ammonium chloride concentration beyond 0.1% bears a negative impact. Maximum decolorization is seen at 20% inoculum size and 0.1 % ammonium chloride. Figure 4c shows that with increase in yeast extract, there is an increase in the percent decolorization but increase in potassium dihydrogen phosphate concentration beyond 0.1% has negative impact. Maximum decolorization is seen at 0.5% yeast Extract and 0.1 % KH_2PO_4. With increase in sucrose levels from 0.5-1.0%, the level of percent decolorization rises to 53%, but with increase in ammonium chloride from 0.1-0.5%, there is negative impact as shown in Figure 4d.

Figure 5a shows that with increase in sucrose from 0.1-0.5%, there is increase in percent decolorization. Similarly an increase in potassium dihyrogen phosphate, leads to an increase in percent decolorization till 65%. An increase in $MgSO_4$ from 0.1-0.5%, there is an increase in percent decolorization as represented in figure 5b. Similarly, with increase in $FeSO_4$ from 0.1-0.5%, there is substantial increase in percent decolorization. Maximum decolorization is observed at 0.5% $MgSO_4$ and 0.5% $FeSO_4$. From Figure 5c it was inferred that, with increase in inoculum size from 10 to 20%, there was a rise in percent decolorization upto 75%. With increase in ferrous sulphate from 0.1 to 0.5 percent, there was increase in percent decolorization from 30-70%. Figure 5d shows that with increase in $FeSO_4$ from 0.1-0.5%, there is increase in percent decolorization till 70%. Similarly, an increase in yeast extract has also resulted in an increase in percent decolorization.

Validation of the experimental model

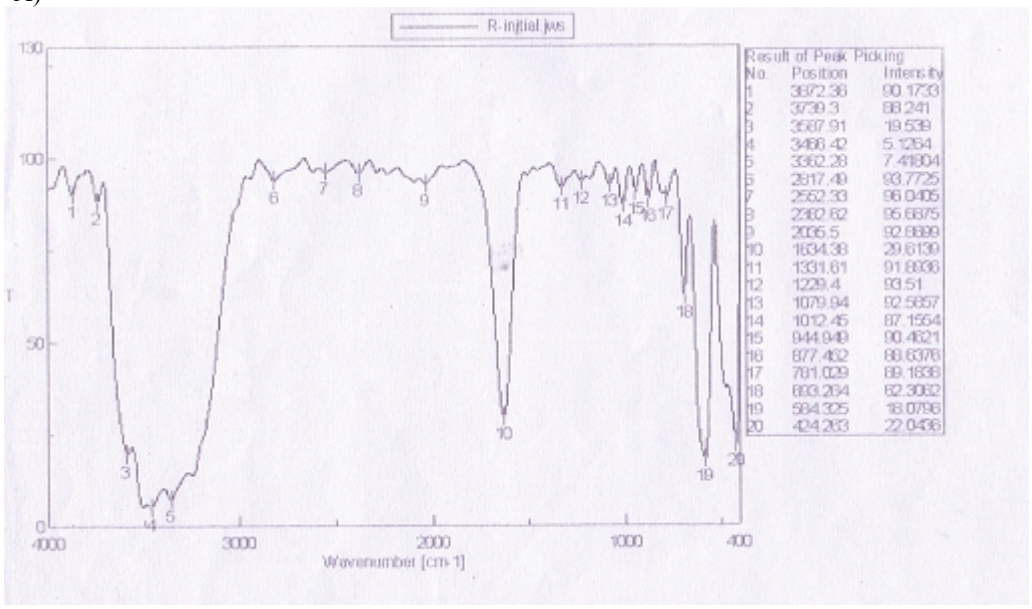
To validate the optimum combination of the process variables, confirmatory experiments were carried out. The selected combinations of the seven variables resulted in 69% decolorization. Hence, maximum decolorization of the dye can be obtained by using the following factors: KH_2PO_4=0.35%, yeast extract=0.35%, $FeSO_4$=0.56%, $MgSO_4$=0.34%, NH_4Cl=0.47%, sucrose= 0.87%, inoculum size=20.5%, with pH 8, temperature 30°C and dye concentration 0.01%.

Degradation product analysis

FTIR spectral comparison between the dye and its product formed after decolorization by bacterial strain confirmed biodegradation of the dye. The FTIR spectrum of the degraded dye showed significant changes in position of peaks when compared with the original dye spectrum. The FTIR spectrum obtained from Red 3BN dye before degradation showed C=0 stretching at 1650 cm^{-1}

showing the presence of carbonyl group. Peak at 1700 cm^{-1} showed the presence of C=N stretching. The other predominant peaks observed were, amide stretching at 1690 cm^{-1} and NH stretching band at 3530 cm^{-1}. The degraded compound showed the disappearance of N-H at 3550 cm^{-1} indicating the degradation of the dye. The by product showed the presence of the following peaks at C=0 at 1650 cm^{-1}, NH at 3550 cm^{-1} and NH_2 at 3680 cm^{-1}. Similar results were reported by earlier studies [45, 46].

A)



B)

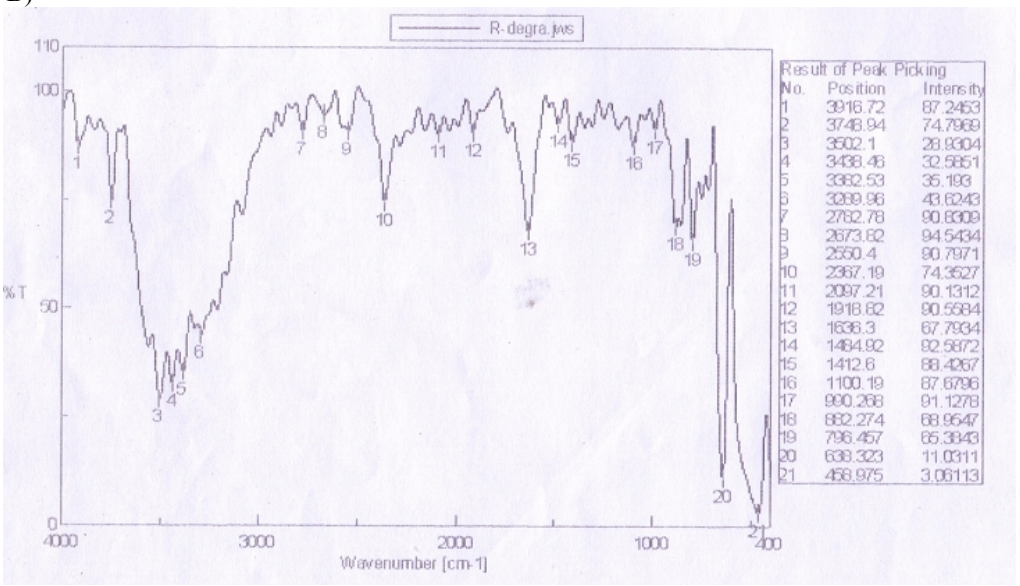


Figure 5. A – FTIR spectrum of the original dye; B – FTIR spectrum of the dye degradation product.

CONCLUSION

In this study, a bacterial isolate, *Shewanella putrefaciens* was used to treat the textile dye Red 3BN. RSM was applied to optimize the process parameters. From the results it was found that a maximum of 82.44% decolorization occurs at the optimized condition. The ability of the strain to tolerate, decolorize and degrade acid dye, gives it an advantage for treatment of textile industry wastewater. However, potential of the culture needs to be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate bioreactors.

REFERENCES

[1] Meehan C, Banat IM, McMullan G, et al. Environ Int. 2000, 26:75 -9.
[2] Wong Y, Yu J. Wat Res. 1999, 33:12-20.
[3] Walthall WK, Stark JD. Environ Pollut. 1999, 104:207-15.
[4] Al-Sabti K. Ecotox Environ Safe. 2000, 47:149-55.
[5] Hu TL, Wu SC. Biores Technol. 2001, 77:93 -5.
[6] Banat IM, Nigam P, Singh D, et al. Biores Technol. 1996, 58:217-27.
[7] Zhang F, Yu J. Bioproc Eng. 2000, 23:295-301.
[8] Bumpus JA, Brock BJ. World J Microbiol Biotechno. 1998, 54:1143-1150.
[9] Zhou W, Zimmermann W. FEMS Microbiol. Lett. 1993, 107:157-162.
[10] Cripps C, Bumpus JA, Aust ST. Appl Environ Microbiol. 1990, 56: 1114-1118.
[11] Khalaf MA, Bioresour Technol. 2008, 99:6631-6634.
[12] Tatarko M, Bumpus JA. *Water Res*. 1998, 32:1713-1717.
[13] Liu BL, Tzeng YM. Bioprocess Eng. 1998, 18: 413-418.
[14] Anjum MF, Tasadduq I, Al-Sultan K. Eur J Oper Res. 1997, 101: 65-73.
[15] Montgomery DC, Design and analysis of experiments. Wiley, 3rd Edition, New York, 1991.
[16] Box GE, Draper NR. Empirical Model Building and Response Surfaces, Wiley, New York, 1987.
[17] Mason RI, Gunst RF, Hess JL. Statistical Design and Analysis of Experiments, Wiley, New York, 1989.
[18] Myers RH, Montgomery DC. Response surface methodology: Process and product optimization using designed experiments. John Wiley & Sons Inc, New York, 1995.
[19] Hayase N, Kouno K, Ushio K. Jour. of Biosci. Bioengin. 2000, 90: 570-573.
[20] Kumar K, Devi SS, Krishnamurthi K, et al. Bioresource Techno. 2005, 1-7.
[21] Chen B, Process Biochem. 2002, 38: 437-446.
[22] Bayoumi RA, Musa SM, Bahobil AS, et al. J. Appl. Environ. Biol. Sci. 2010, 1:1-25.
[23] Sathian S, Rajasimman M, Karthikeyan C, Biotech. 2013, 3:71-79.
[24] Saitou N, Nei M. Mol Biol Evo.1987, l 4:406-425.
[25] Tamura K, Dudley J, Nei M, et al. Molecular Biology and Evolution. 2007, 24:1596-1599.
[26] Moosvi S, Keharia H, Madamwar D. World J. Microbiol. Biotechnol. 2005, 21: 667-672.
[27] Jadhav JP, Parshetti GK, Kalme SD, et al. Chemosphere. 2007, 68:394-400.
[28] Usha MS, Sasirekha B, Bela RB, et al. J. Chem. Pharm. Res. 2011, 3:450-457.
[29] Karthikeyan K., Nanthakumar K., Shanthi K, et al. Iranian Journal of Microbiology. 2010, 2: 213-222.
[30] Nosheen S, Nawaz R, Arshad M, et al. Int. J. Agr. Boi. 2010, 12: 426-430
[31] Wang X, Cheng X, Sun D, et al. J. Envtl. Sci. 2008, 20: 1218-1225.
[32] Carliell CM, Barclay SJ, Naidoo N, et al. Water SA. 1995, 21:61-69.
[33] Nigam P, Bana IM , Singh D, et al. Proc Biochem.1996, 31: 435-442.
[34] Pearce CI, Christie R, Boothman C, et al. Biotechnol Bioeng. 2006, 95: 692-703.
[35] Xu M, Guo J, Sun G, Appl Microbiol Biotechnol. 2007, 76: 719-726.
[36] Cai PJ, Xiao X, He YR, et al. Appl Microbiol Biotechnol. 2011, 93: 1769-1776.
[37] Fredrickson JK, Romine MF, Beliaev AS, et al. Nat.Rev Microbiol. 2008, 6: 592-603.
[38] Khalid A, Arshad M, Crowley DE. Appl Microbiol Biotechnol. 2008, 79:1053-1059.
[39] Mugerfeld I, Law BA, Wickham GS, et al. Appl Microbiol Biotechnol. 2009, 82: 1131-1141.
[40] Wu J, Kim KS, Sung NC, et al. J.Gen.appl.Microbiol. 2009, 55: 51-55
[41] Babu SS, Mohandass C, Raj ASV, et al. Water, Air, Soil Pollution. 2013, 224:1500.

[42] Ng IN, Chen T, Lin R, et al. Applied Microbiol Biotechnol. 2013, 253:013-5151z.
[43] Hong Y, Xu M, Guo J, et al. Applied Microbiol Biotechnol. 2007, 73:64 -72.
[44] Yang YY, Du LN, Wang G, et al. Water Sci Technol. 2011, 63: 956-963.
[45] Jadhav JP, Phugare SS, Dhanve RS, et al. Biodegradation. 2010, 21:453-463.
[46] Parshetti GK, Parshetti SG, Telke AA, et al. J. Environ. Sci. 2011, 23:1384-1393.