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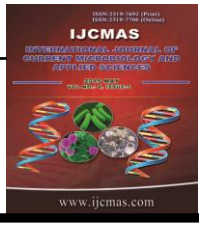

Original Research Article

Optimization of culture conditions for decolorization of Acid Red 10B by *Shewanella putrefaciens*

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Keywords

Decolorization, degradation, optimization, Plackett-Burman design, Response , Surface methodology, *Shewanella putrefaciens* sp.

A B S T R A C T

The aim of the present study was to investigate the potential of *Shewanella putrefaciens* to decolorize and degrade acid dye Red 10B. Percentage decolorization of Red 10B by bacteria was taken as the response to screen and optimize the media constituents using statistically valid response surface methodology (RSM) involving Plackett-Burman experimental design. The significance of the factors were analyzed in the form of analysis of variance which indicated that all the factors were found to be highly significant and were positively influencing the dye decolorization. Response surface methodology used in optimizing the important media constituents for maximizing the decolorization of Red 10B, gave optimum combinations of all factors which resulted in 98.92% decolorization with Yeast extract=0.019%, Sucrose=0.17%, Glucose=0.32%, Peptone=0.39%, $MgSO_4$=0.41%, K_2HPO_4=0.38%, NH_4Cl=0.09%, with pH 8, temperature 30°C and dye concentration 0.01%. The degradation of Red10B into different metabolites by the isolate was confirmed using Fourier transform infrared spectroscopy. These results suggest that the isolated bacterium was suitable for biological treatment of dye-containing wastewater.

Introduction

Synthetic dyes are widely used in several industries such as textile, paper, printing, cosmetics, pharmaceutical, color photography and petroleum (Marmion, 1991). It is estimated that over 10,000 different dyes and pigments are used industrially and over 7 x 10^5 tons of synthetic dyes are annually produced worldwide (Zollinger, 1987; Robinson *et al*., 2001; Ogugbue *et al*., 2011). In the textile industry, up to 200,000 tons of these dyes are lost in effluents every year during the dyeing and finishing operations, due to the inefficiency of the dyeing process (Ogugbue *et al*., 2011). Unfortunately, most of these dyes escape conventional wastewater treatment processes and persist in the environment as a result of their high stability to light, temperature, water, detergents, chemicals, soap and other parameters such

as bleach and perspiration (Couto *et al*., 2009). In addition, anti-microbial agents resistant to biological degradation are frequently used in the manufacture of textiles, particularly for natural fibers such as cotton (Couto *et al*., 2009; Neill et al., 1999). The synthetic origin and complex aromatic structure of these dyes make them more recalcitrant to biodegradation (Forgacs *et al*., 2004; Przystaś *et al*., 2011). However, environmental legislation obliges industries to eliminate colour from their dye-containing effluents, before letting into water bodies (Ogugbue *et al*., 2011; Neill *et al*., 1999).

The textile industry consumes substantial amount of water in its manufacturing processes used mainly in the dyeing and finishing operations of the plants. The wastewater from textile plants is classified as the most polluting among the industrial sectors, based on the volume generated as well as the effluent composition (Przystaś et al., 2011; Sen and Demirer, 2003). In addition, the increased demand for textile products and the proportional increase in their production, and the use of synthetic dyes have all contributed for the textile dye wastewater becoming one of the substantial sources of severe pollution problems in current times (Santos et al., 2007; Ogugbue et al., 2011).

Textile wastewaters are characterized by extreme fluctuations in parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, colour and salinity. The composition of the wastewater depends on different organic-based compounds, chemicals and dyes used in the dry and wet-processing steps (Santos *et al*., 2004; Talarposhti *et al*., 2001). Recalcitrant organics, colored toxicant, surfactant, chlorinated compounds and salts are the main pollutants of textile effluents (Mansour *et al*., 2012).

In addition, the effects caused by other pollutants in textile wastewater, and the presence of very small amounts of dyes (<1 mg/L for some dyes) in the water, which are nevertheless highly visible, seriously affect the aesthetic quality and transparency of water bodies such as lakes and rivers leading to damage to the aquatic environment (Ibrahim *et al*., 1996; Wijetunga *et al*., 2010).

During the dyeing process, it has been estimated that the losses of colorants to the environment can reach 10-50% (Forgacs *et al*., 2004; Przystaś *et al*., 2012; Mansour *et al*., 2012; Vaidya and Datye, 1982; Rajaguru *et al*., 1999). It is known that some dyes are highly toxic and mutagenic, and also decrease light penetration and photosynthetic activity, causing oxygen deficiency and limiting downstream beneficial uses such as recreation, drinking water and irrigation (Forgacs *et al*., 2004; Przystaś *et al*., 2012; Hubbe *et al*., 2012).

With respect to number and production volume, azo dyes are the largest group of colorants, constituting 60-70% of all organic dyes produced in the world (Bafana *et al*., 2011; Carliell *et al*., 1998). The usage of azo dyes in textile industries is due to their ease of application and cost effectiveness for synthesis as compared to natural dyes, and also their structural diversity, high molar extinction coefficient, and medium-to-high fastness properties in relation to light as well as to wetness (Bafana *et al*., 2011; Seesuriyachan *et al*., 2007). In light of these presumptive evidences, the present study was taken up to explore the potential of dye degrading bacteria in the textile effluent. The isolate, *Shewanella putrefaciens* was used to optimize the decolorization process under different culture conditions and optimal bioremediative capacity was studied.

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 10B was provided by Karnataka Silk Industries Corporation (Mysore, India). All other reagents were of analytical grade.

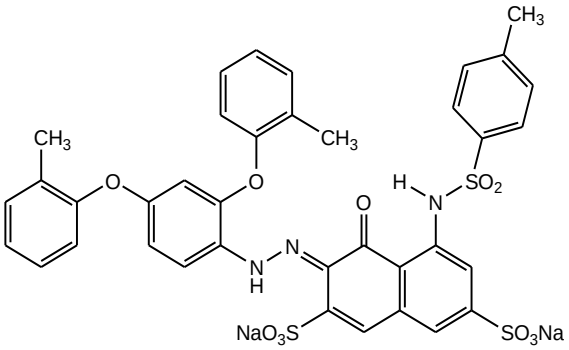


Fig.1 Structure of Red 10B

Bacterial strain and culture condition

The bacterial strain was isolated from the textile effluent obtained from KSIC, Mysore, Karnataka, India. Further, the identification of the bacterial isolate was carried out at the Department of Studies in Biotechnology, University of Mysore, Mysore and was identified to be *Shewanella putrefaciens.*

Isolation and screening of dye decolorizing bacteria

The effluent sample obtained from the textile industry 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 (Hayase *et al*., 2000; Kumar *et al*., 2005; Chen, 2002).

All the effluent isolated pure colonies were inoculated in 10ml Nutrient broth (inoculum). 2.5ml of inoculum was inoculated into 250 ml flasks containing 100ml 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; tab water up to 1 liter and 1ml of trace element solution per liter. The trace element solution had the following composition (g/l):$MgSO4.7H_2O$, 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) of yeast extract (Bayoumi *et al*., 2010). 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 10B decolorization by *Shewanella putrefaciens*. RSM was employed to identify the main effects of the

seven operational variables. The experimental range and levels of media constituents used in Plackett-Burman design is shown in Table 1.

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

Parameters	Range and levels	
	-1	+1
Yeast extract	0.1	0.5
Sucrose	0.1	0.5
Glucose	0.1	0.5
Peptone	0.1	0.5
$MgSO_4$	0.1	0.5
KH_2PO_4	0.5	1.0
NH_4Cl	10	20

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 bacterial strain was identified by analysis of 16SrRNA sequences. The genomic DNA was extracted from the isolate by using the DNeasy Plant Minikit (Qiagen, Germany). Two primers Forward = 5'-TGG TAGTCCACGCCCTAAC-3' and Reverse = 5' CTGGAAAGTTCC GTGGATGT-3' were applied for the amplification of the 16SrRNA 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 (Saitou and Nei, 1987) was employed to construct the phylogenetic tree using MEGA4 software (Tamura et al., 2007), and the maximum likelihood method was adopted for calculating the evolutionary distance.

Decolorization assay

To monitor the decolorization process, the degraded 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 10B was estimated by using UV-Visible Spectrophotometer 108 (Systronics) and the λ_{max} was found to be at 573nm. The response % decolorization was calculated using the following equation:

$$D = [A_o - A_1/A_o] \times 100$$

Where D is percent decolorization; A_o initial absorbance; A_1 final absorbance.

Degradation studies

Fourier transform infrared spectroscopy was applied for analysis of Red 10B and its decolorization products. After complete decolorization, cells were removed through centrifugation (10,000 x g, 10 min).

The product metabolites produced after decolorization of dye mixture 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 (Moosvi *et al*., 2005; Jadhav *et al*., 2007). FTIR analysis was carried out using Perkin Elmer 783 Spectrophotometer and changes in % transmission 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 isolate used in this study was identified on the basis of 16SrRNA gene sequencing. The closest neighbor in GenBank database 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 Fig.2

Effects of the process parameters on decolorization of Red 10B by *S. putrefaciens*

The effect of process parameters (independent variables) such as yeast extract, sucrose, glucose, peptone, $MgSO_4$, KH_2PO_4 and NH_4Cl on decolorization and degradation of Red 10B 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 72% to 92%. Based on the results, the regression model equation for dye decolorization is presented below:

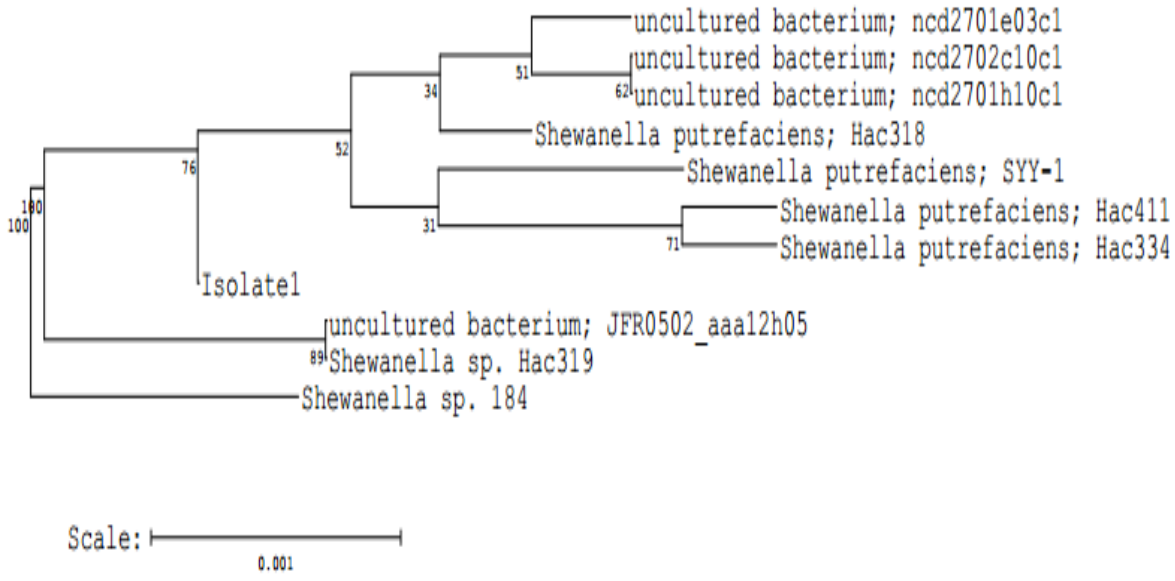


Fig.2 Phylogenetic tree of bacterial strain *S.putrefaciens* based on 16SrRNA gene sequence

% Decolorization = 92.67-18.75*A-5.00*B+1.25*C+37.50*D+6.00*E-23.33*F-13.75*G

The magnitude of effects indicates the level of significance of the variables on decolorization of the dye.

From the ANOVA table, it can be deduced that all the factors were highly significant in dye decolorization with *p*-value of 0.0001. The coefficient of variation (CV) was 15% indicating that the experiment performed was highly reliable. The model's goodness of fit was checked by the determination co-efficient (R^2 = 99.2%). There was very good correlation between the observed and the predicted responses. This implies that 99.2% of the variation for dye removal efficiency was explained by the independent variables and this also means that the model did not explain only about 0.8% variation.

It is quite evident from the table 4 that the predicted values of percent dye decolorization have matched exactly with the experimentally measured values (actual values). The graph shows that the values are positively correlating.

The Pareto chart illustrates the order of significance of the variables affecting the dye decolorization. Peptone is shown to positively influence dye decolorization. Yeast extract, NH_4Cl, Sucrose, KH_2PO_4 negatively influences the dye decolorization. Other factors have least or no effect which is below t-value limit.

In the perturbation graph, the reference point is set at the middle of the design space (the coded zero level of each factor). The deviation of factors B, D and E are equal from the reference point and its produces relatively equal effect on dye decolorization. The deviation of factor C from 0 to +1.5 has relatively greater effect than deviation towards -1.5 coded units, indicating that this factor would greatly enhance the dye decolorization. Whereas in case of factor A, F and G the deviation from 0 to -1.5 coded units has greater effect than towards +1.5 coded units indicating that, these factors would negatively influence the dye decolorization. So, it can be inferred that higher concentration of factor C and lower concentration of factors A, F and G would greatly enhance the dye decolorization.

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

	Experimental Variables							
Run no.	**Yeast extract (%)**	**Sucrose (%)**	**Glucose (%)**	**Peptone (%)**	**$MgSO_4$ (%)**	**KH_2PO_4 (%)**	**NH_4Cl (%)**	**Decolorization (%)**
1	0.5	1.0	0.5	0.15	0.5	0.35	0.1	78.0
2	0.5	1.0	0.1	0.35	0.25	0.35	0.5	78.0
3	0.5	0.1	0.5	0.15	0.25	0.50	0.5	72.0
4	0.1	1.0	0.1	0.15	0.5	0.50	0.5	76.0
5	0.5	0.1	0.1	0.35	0.5	0.50	0.1	86.0
6	**0.1**	**0.1**	**0.5**	**0.35**	**0.5**	**0.35**	**0.5**	**92.0**
7	0.1	1.0	0.5	0.35	0.25	0.50	0.1	88.0
8	0.1	0.1	0.1	0.15	0.25	0.35	0.1	88.0

Table.3 Analysis of variance for Red 10B decolorization

Parameter	Medium components	Degree of Freedom	Sum of Square	Mean Square	F value	P value
Model	-	7	507.66	382.29	Infty	<.0001**
A	Yeast extract	1	150.0	150.0	Infty	<.0001**
B	Sucrose	1	54.0	54.0	Infty	<.0001**
C	Glucose	1	0.66	0.66	Infty	<.0001**
D	Peptone	1	150.0	150.0	Infty	<.0001**
E	$MgSO_4$	1	6.00	6.00	Infty	<.0001**
F	KH_2PO_4	1	32.66	32.66	Infty	<.0001**
G	NH_4Cl	1	80.66	80.66	Infty	<.0001**

CV = 15.0, R2 = 99.2%

Table.4 Comparison of actual and predicted responses for dye removal

Exp. run	Experimental variables							Decolorization efficiency (%)	
	A	B	C	D	E	F	G	Actual value	Predicted value
1	0.5	1.0	0.5	0.15	0.5	0.35	0.1	78.0	78.0
2	0.5	1.0	0.1	0.35	0.25	0.35	0.5	78.0	78.0
3	0.5	0.1	0.5	0.15	0.25	0.50	0.5	72.0	72.01
4	0.1	1.0	0.1	0.15	0.50	0.50	0.5	76.0	76.01
5	0.5	0.1	0.1	0.35	0.50	0.50	0.1	86.0	86.01
6	0.1	0.1	0.5	0.35	0.50	0.35	0.5	92.0	92.0
7	0.1	1.0	0.5	0.35	0.25	0.50	0.1	88.0	88.01
8	0.1	0.1	0.1	0.15	0.25	0.35	0.1	88.0	88.0

Fig.3 Comparison of the experimental results of decolorization efficiency with those calculated via PB design resulted equation

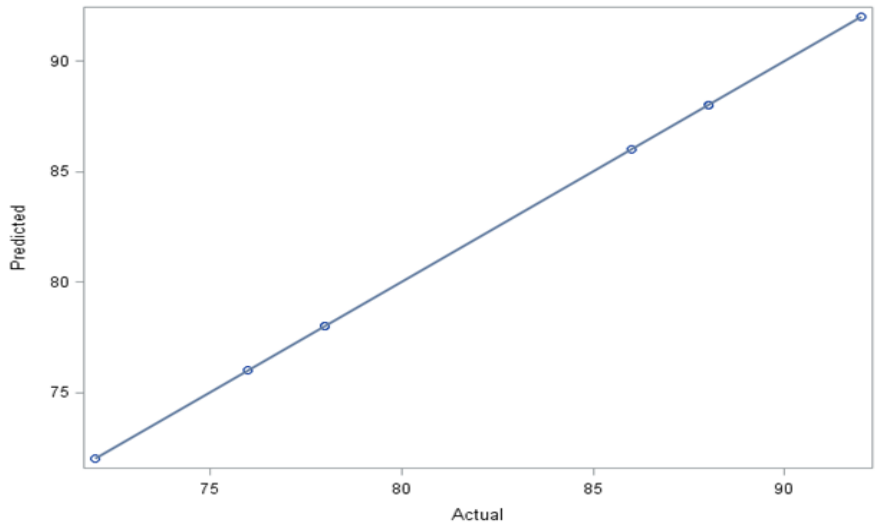


Fig.4 Pareto chart for Plackett-Burman design for 7 factors on Red 10B decolorization

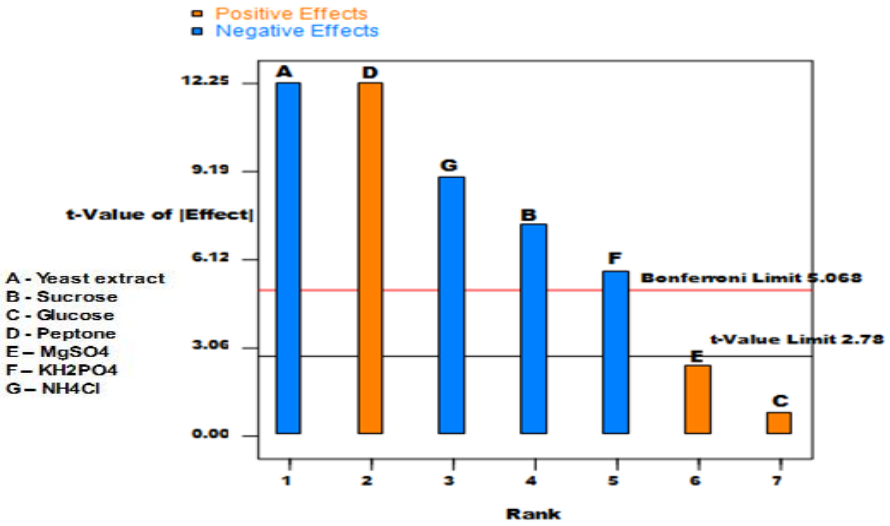
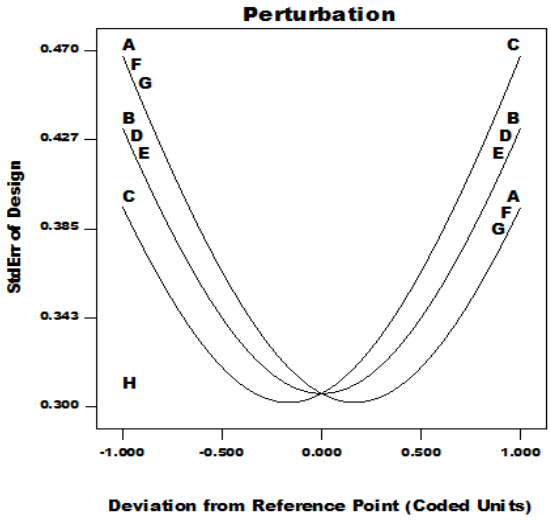


Fig.5 Perturbation graph showing the optimum values of the medium component



Optimization of the media constituents using RSM

To investigate the interactive effect of two factors on the decolorization of dye, response surface plots were drawn. Some typical 3D response surface plots to illustrate these salient findings on the interaction between the media constituents on Red 10B decolorization are depicted in Fig 5 .

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 (Yeast extract=0.019%, Sucrose=0.17%, Glucose=0.32%, Peptone=0.39%, $MgSO_4$=0.41%, KH_2PO_4=0.38%, NH_4Cl=0.09% with inoculum size=20%, pH 8, temperature 30°C and dye concentration 0.01%).

Fig.6 Three dimensional response surface plot showing the interactive effect of (a) glucose and yeast extract (b) peptone and glucose (c) sucrose and glucose (d) peptone and sucrose (e) MgSO4 and peptone (f) KH2PO4 and peptone (g) KH2PO4 and glucose (h) KH2PO4 and sucrose

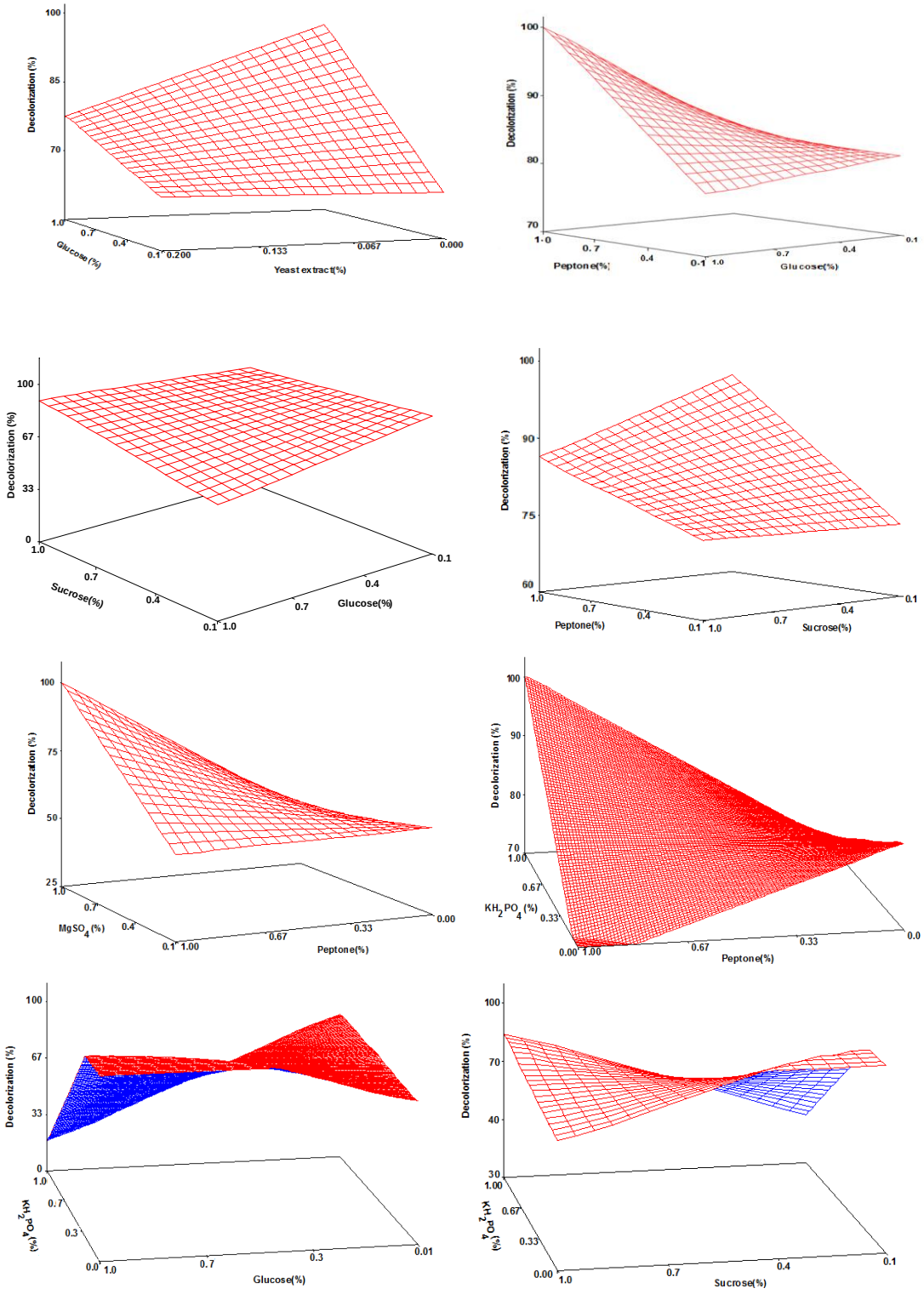
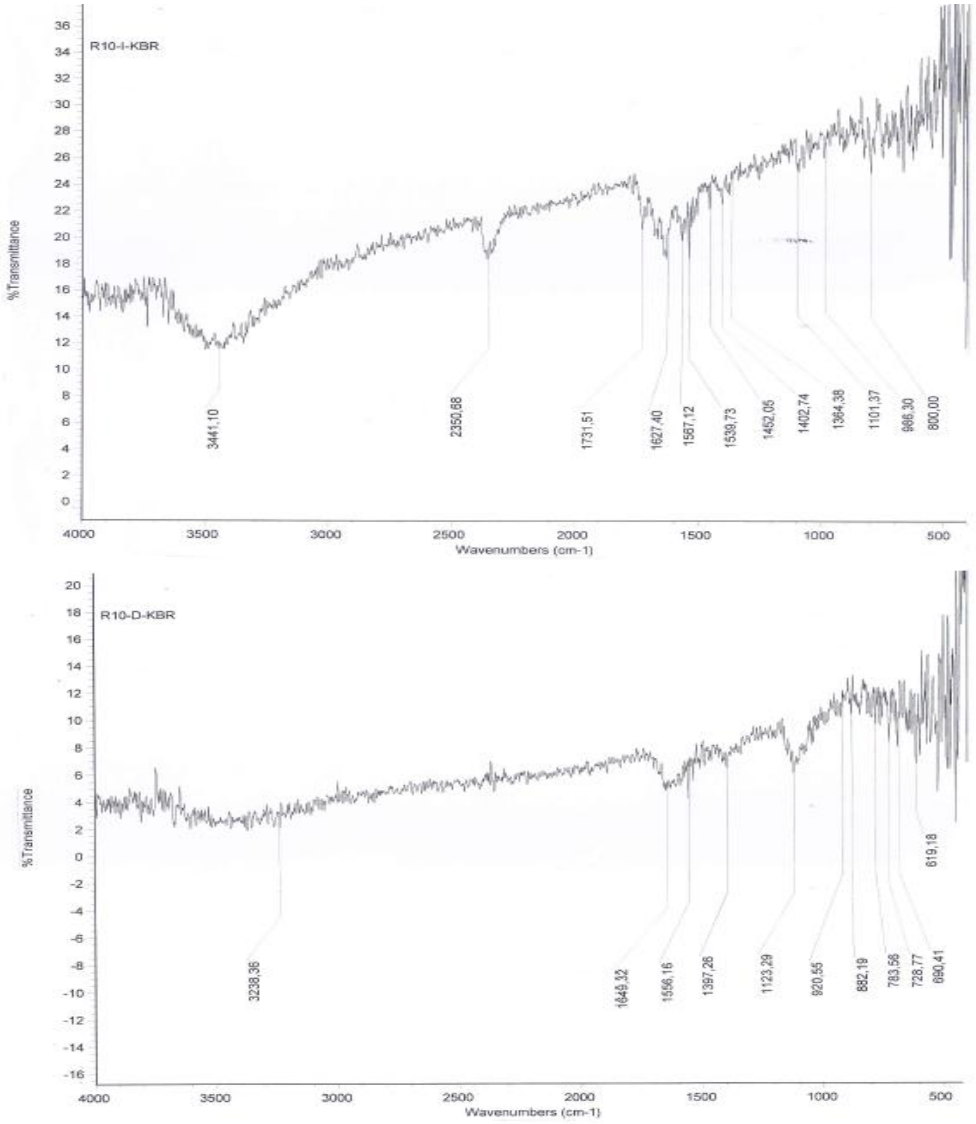


Fig.7a– FTIR spectrum of the initial dye **b.** FTIR spectrum of the dye degradation product



Degradation product analysis

FTIR spectral comparison between the dye and its product formed after decolorization by bacterial strain confirmed biodegradation of the dye into different metabolites. The FTIR spectrum of extracted metabolites showed significant changes in position of peaks when compared with the initial dye spectrum. The FTIR spectrum obtained from Red 10B before degradation showed C=0 stretching at 1627 cm^{-1} showing the presence of carbonyl group. Peak at 3441.1cm^{-1} showed the presence of N-H indicating the presence of secondary amine. The degraded compound showed the disappearance of N-H indicating the degradation of the dye.

Validation of the experimental model

The maximum experimental response for Red 10B removal was 84% whereas predicted response was 98.92%. To validate the optimum combination of the process variables, confirmatory experiments were

carried out. The selected combinations of the seven variables resulted in 84.91% decolorization. Hence, maximum decolorization of the dye can be obtained by using the following factors: (Yeast extract=0.019%, Sucrose=0.17%, Glucose=0.32%, Peptone=0.39%, $MgSO_4$=0.41%, KH_2PO_4=0.38%, NH_4Cl=0.09% with Inoculum size=20%, pH 8, temperature 30°C and dye concentration 0.01%).

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