International Journal of
Research in Chemistry and Environment
Available online at: www.ijrce.org
ISSN 2248-9649

Research Paper

Optimization of Fungal Decolorization of Isolan Grey using Plackett-Burman Experimental Design

Hema N. and *Suresha S.
Department of Environmental Science, Yuvaraja's College, Mysore-570005, Karnataka, INDIA

(Received 07th January 2015, Accepted 18th March 2015)

Abstract: *Penicillium oxalicum RF3 isolated from textile dye effluent was evaluated for its ability to decolorize the metal complex dye Isolan grey. Percentage decolorization of the dye by fungi 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 analysed in the form of ANOVA, in which Inoculum size showed significant effect on dye decolorization. Ammonium chloride showed suggestive significance indicating its importance in dye decolorization. Response surface methodology used in optimizing the important media constituents for enhancing the decolorization of Isolan grey, revealed optimum combinations of all factors which gave maximum percent dye decolorization of 50.75% with Inoculum size=29.28%, Fructose=0.67%, Urea=0.21%, Lactose=0.021%, K_2HPO_4=0.44%, NH_4Cl=0.54%, $MgSO_4$=0.60% with pH 7, temperature 25°C, dye concentration 0.01% and incubation duration=120 hours. The pareto chart plotted for all seven factors indicated that inoculum size, fructose, NH_4Cl and K_2HPO_4 positively influenced the dye decolorization process. The degradation of Isolan grey into different metabolites by the isolate was confirmed using Fourier transform infrared spectroscopy. These results suggest that the isolated fungus is suitable for the biological treatment of dye-containing wastewater.*

Keywords: Decolorization, degradation, optimization, *Penicillium oxalicum* sp, Plackett-Burman design, Response surface methodology

Introduction

The textile industry is one of the industries that generate a high volume of wastewater and creates potential for water pollution. Among the many chemicals in textile wastewater, dyes are considered as important pollutants [1]. The presence of even very low concentration of dyes in effluent is highly visible and degradation products of these textile dyes are often carcinogenic [2]. Currently, textile effluents are treated by physico-chemical methods that are often quite expensive. In addition, these methods do not generally degrade the pollutant, thereby causing an accumulation of the dye as sludge creating a disposal problem. Over the past decade, biological decolorization has been investigated as method to transform, degrade or mineralize azo dyes. Moreover such decolorization and degradation is an eco-friendly method and cost comparative alternative to chemical degradation process[3]. There are many variables or factors affecting enzyme production and decolorization that are expressed by different taxa of microorganisms and their culture conditions[4]. Response surface methodology is a very useful tool for this purpose, as it provides statistical models which helps in understanding the interactions among the parameters that have been optimized. The advantages of using RSM have been reported to include reduction in number of experimental trials needed to evaluate multiple parameters and the ability of the statistical tool to identity interactions. In addition to analyzing the effects of the independent variables, the experimental methodology also generates a mathematical model that describes the overall process [5, 6].Hence, the present study was aimed to examine the most influential variables for maximum decolorization

of Isolan grey through Plackett-Burman experimental design using *Penicillium oxalicum RF3*.

Material and Methods

Isolation and Screening of Dye Decolorizing Fungi

Dye decolorizing fungus was isolated from raw effluent collected from KSIC, textile factory in Mysore. 1ml of the effluent was transferred into 9 ml of distilled water in sterile test tubes. Serial dilution was done up to10^{-7} by thorough mixing. 0.1 ml of sample from each dilution was spread on potato dextrose agar plates containing chloramphenicol with the help of an L-rod. The petridishes were incubated at room temperature (28°C) for 5 days [7]. The predominant isolates were screened for decolorizing activity of the dye. Inoculum (10^{-5} spores/ml) of each isolate were added to 100 ml of Sabouraud dextrose broth supplemented with 0.01% dye and incubated at 25°C for 5 days and effective decolorization was observed visually. Those isolates showing decolorization of textile dye were selected for further studies. Out of the 5 fungal isolates screened, one was found to be a potential dye degrader. Further, identification of the dye degrading fungus was carried out at Chromous Biotech Pvt. Ltd, Bangalore, India by 16sRNA gene sequencing and construction of phylogenetic tree.

Preservation and maintenance

Pure fungal isolate was obtained on the PDA plates; this isolate was further sub-cultured on PDA slants and incubated at room temperature. After sufficient growth was obtained, the slants were stored in refrigerator and served as stock cultures. Subcultures were routinely made every 30-60 days [7].

Preparation of spore suspension

A mycelium disc of 1.2 cm diameter obtained from 5 days old culture plates of fungus were transferred to 25 ml PDA in 250 ml conical flask and incubated at 25°C for 5 days. At the end of incubation period, 30 ml of sterile water was added to each culture and the flasks were kept on a shaker for 1 hour. Then the contents of each conical flask were filtered through glass wool. The filtrate containing spore suspension was used for decolorization experiments [7].

Decolorization assay

Decolorization assay was carried out in triplicates, by taking 100 ml of SD broth with 0.01% dye, pH 7 and 10% inoculum, in 250 ml conical flask and were subjected to the experimental setup as shown in Table 2. 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 cells. The absorbance of supernatant was measured at maximum wavelength of the dye. The maximum absorbance of Isolan grey was estimated by using UV-Visible Spectrophotometer 108 (Systronics) and the λ_{max} was found to be 587 nm. The response percent decolorization was calculated using the following formula:

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

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

Molecular characterization of the fungal strain

The isolated fungal strain was identified using 16SrRNA gene sequencing. The extraction of genomic DNA was done using the fungal genomic DNA isolation kit (RKT13). The forward and reverse primers were applied for the amplification of the 16SrRNA gene. The ~500 bp ITS region was amplified using high–fidelity PCR polymerase. Polymerase chain reaction was performed as follows: Initial denaturation: 94°C for 5 min, denaturation: 94°C for 30 sec, annealing: 55°C for 30 sec, extension: 72°C for 1min, final extension: 72°C for 5 min, $MgCl_2$: 1.5mM final conc.-35 cycles. Nucleotide sequencing for this sample was performed using ABI 3500XL Genetic Analyzer at Chromous Biotech Pvt. Ltd, Bangalore, India. 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). Neighbour-joining method [8] was employed to construct the phylogenetic tree using MEGA4 software [9] and the maximum likelihood method was adopted for calculating the evolutionary distance.

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 (coded as -1 for low level and +1 for the high level) were used to screen the media constituents for their effects on Isolan Grey by *Penicillium oxalicum*. 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
Inoculum size	10	20
Fructose	0.1	0.5
Urea	0.25	0.75
Lactose	0.1	1.0
K_2HPO_4	0.25	0.50
NH_4Cl	0.1	0.5
$MgSO_4$	0.50	0.75

The Plackett-Burman design under RSM was employed in order to get the combination of value that optimizes the response within the region of 3D spaces. RSM was carried out using the statistical software, SAS Version 9.2.

Degradation Studies

The metabolites produced after decolorization of dye mixture were extracted by using equal volume of ethyl acetate. The extracted product metabolites were completely dried and then dissolved in a small volume of HPLC grade methanol. This sample was then subjected to FTIR analysis to confirm degradation [10, 11]. FTIR analysis was carried out using Perkin Elmer 783 Spectrophotometer and changes in % transmissions at different wavelengths were observed. The FTIR analysis was done in the mid-infrared region of 400-4000 cm^{-1}.

Results & Discussion

Molecular Identity of the Potential Strain

The isolate used in this study was identified on the basis of 16SrRNA gene sequencing. The closest neighbour in GenBank database was found to *be Penicillium oxalicum RF3* with the homology of 99.0%. The sequence was submitted to GenBank with an accession number GQ856686.1. The phylogenetic relationship of the isolate is shown in Figure 1.

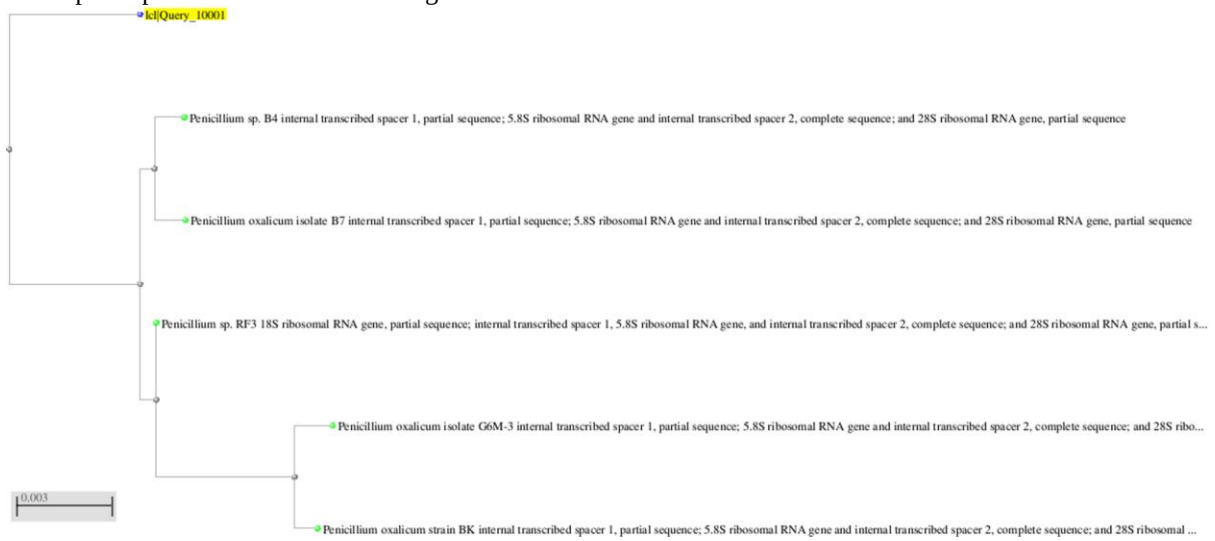


Figure 1: Phylogenetic tree of the fungal strain *P. oxalicum* based on 16SrRNA gene sequence

Effects of process parameters on decolorization of Isolan Grey by *Penicillium oxalicum RF3*

The effect of process parameters (independent variables) such as inoculums size, fructose, urea, lactose, potassium dihydrogen phosphate, ammonium chloride and magnesium sulphate on decolorization and degradation of Isolan Grey by *P.oxalicum RF3* was studied. Experiments were performed as per combinations of factors shown in Table 2. The corresponding response for dye decolorization varied from 2.70 to 27.02%. Based on the results, the regression model equation for dye decolorization is presented below:

% Decolorization = (-1.5) +1.15*A+18.58*B-9.46*C-5.26*D+8.1*E+11.83*F-2.7*G-2.36*AB +1.86*AC+1.05*AD+0.54*AE-3.72*AF-1.62*AG+6.7*BC-11.25*BD+94.6*BE-1.456*BF +90.6*BG-51.04*CD-75.68*CE-20.25*CF-11.88*CG6.04*DE+3.75*DF-4.04*DG+94.6*EF+3.68*EG+94.6 *FG

The results of dye decolorization were analysed 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 [12]. 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 [12]. The ANOVA table indicates that factor A-Inoculum size was significant with the F-value=7.59 and the *p*-value =0.04. Factor F-Ammonium Chloride showed suggestive significance with *p*-value=0.05. Therefore, from the screening process, Inoculum size was found to have maximum effect on 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 usually indicated by high value of CV[13].

Table 2: Plackett-Burman design matrix for screening the media constituents

Run	Inoculum size (%)	Fructose (%)	Urea (%)	Lactose (%)	K_2HPO_4 (%)	NH_4Cl (%)	$MgSO_4$ (%)	% Decolorization
1	**20**	**0.5**	**0.75**	**0.1**	**0.50**	**0.1**	**0.50**	**27.02**
2	20	0.5	0.25	1.0	0.25	0.1	0.75	24.32
3	20	0.1	0.75	0.1	0.25	0.5	0.75	21.62
4	10	0.5	0.25	0.1	0.50	0.5	0.75	24.32
5	20	0.1	0.25	1.0	0.50	0.5	0.50	24.32
6	10	0.1	0.75	1.0	0.50	0.1	0.75	2.70
7	10	0.5	0.75	1.0	0.25	0.5	0.50	13.51
8	10	0.1	0.25	0.1	0.25	0.1	0.50	10.81

Table 3: Analysis of variance for Isolan grey decolorization

Source	Degree of freedom	Sum of squares	Mean square	F- value	*p* -value
Model	7	517.57	382.2	33.32	0.03*
A	1	263.81	263.81	7.59	0.04*
B	1	110.40	110.40	0.62	0.58
C	1	44.74	44.74	1.09	0.41
D	1	44.74	44.74	0.30	0.75
E	1	8.20	8.20	0.67	0.56
F	1	44.74	44.74	6.26	0.05^+
G	1	0.91	0.91	0.07	0.92

* - p= 0.01-0.05 - significant
$^+$ - p=0.05-0.1- suggestive significance CV=15.2%, R^2=98.1%

In the present case, a low CV value (15.2%) denoted that the experiments performed were highly reliable. The model's goodness of fit was checked by the determination co-efficient (R^2 = 0.98). In this study, the value of R^2 was closer to 1, which denoted good correlation between the observed and the predicted responses. This implies that 98.1% of the variation for dye removal efficiency was explained by the independent variables and this means that the model did not explain only about 1.9% variation. With CV value, R^2 value, p-value=0.0317 and F-value=33.3, the model indicated that, it is precise and significant. Similar observations were reported by an earlier study [13].

Using RSREG procedure in response surface methodology, optimized media compositions as well as culture conditions were obtained. The estimated optimal response for Isolan Grey 2S SBL decolorization was Inoculum size=29.28%, Fructose=0.67%, Urea=0.21%, Lactose=0.021%, K_2HPO_4=0.44%, NH_4Cl=0.54%, $MgSO_4$=0.60% with pH 7, temperature 25°C, dye concentration 0.01% and incubation duration=120 hours.

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 factor C, F and G 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 factors A and B 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 deducted that higher concentration of factor C, F and G and lower concentration of factors A and B would greatly enhance the dye decolorization.

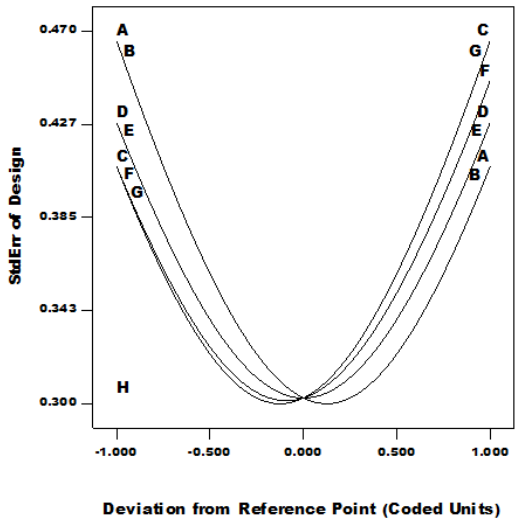


Figure 2: Perturbation graph showing the optimum values of the medium composition

The pareto chart illustrates the order of significance of the variables affecting dye decolorization. The chart, plotted for all seven factors reveals that inoculum size and fructose positively influences the dye decolorization. Other factors have least or no effect which is below t-value limit.

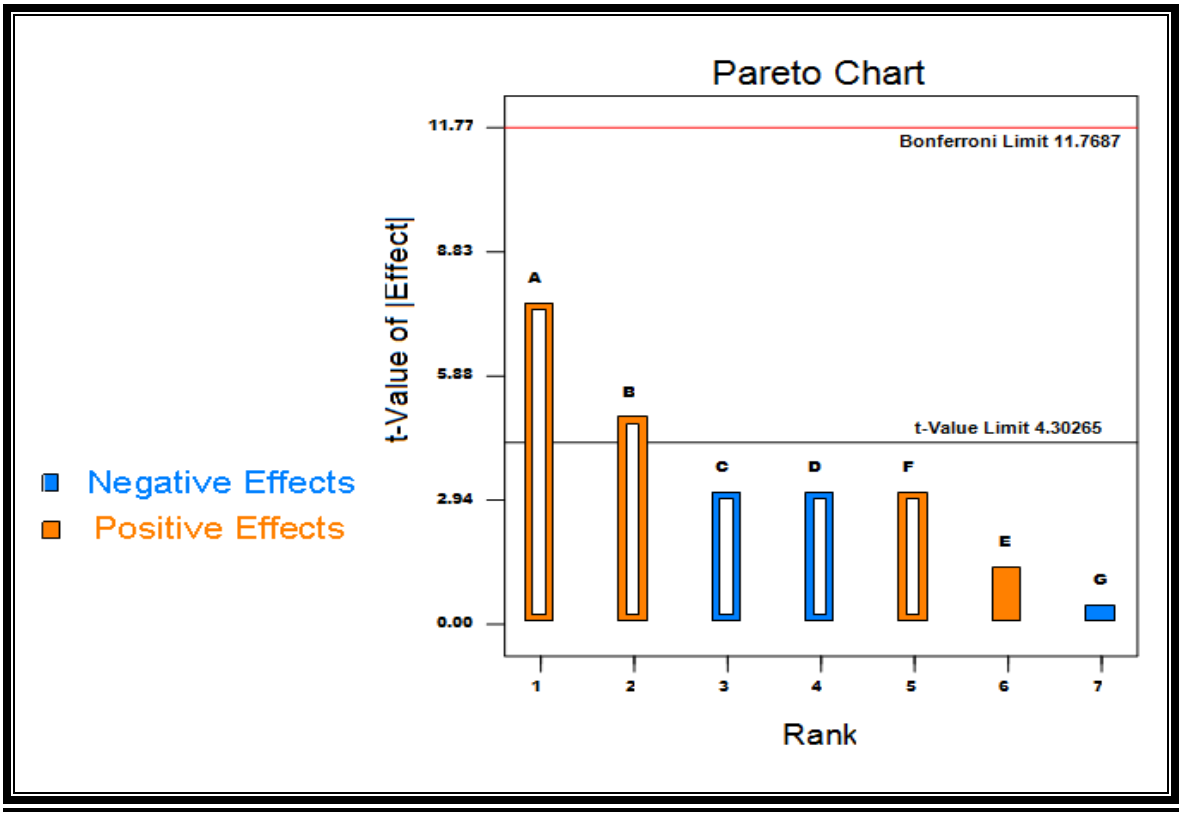


Figure 3: Pareto chart of 7 factor effects on Isolan Grey decolorization by *Penicillium oxalicum*

Validation of the experimental model

The maximum experimental response for Isolan Grey removal was 45.45% whereas the predicted response was 50.75%. To validate the optimum combination of the process variables, confirmatory experiments were carried out. The selected combinations of the three variables resulted in 89.55% decolorization. Hence, maximum decolorization of the dye can be obtained by using the following factors: (Incubation duration=120 hours, inoculum size=29.28%, Fructose=0.67%, Urea=0.21%, Lactose=0.021%, K_2HPO_4=0.44%, NH_4Cl=0.54% and $MgSO_4$=0.60% with pH 7, temperature 25°C and dye concentration 0.01%).

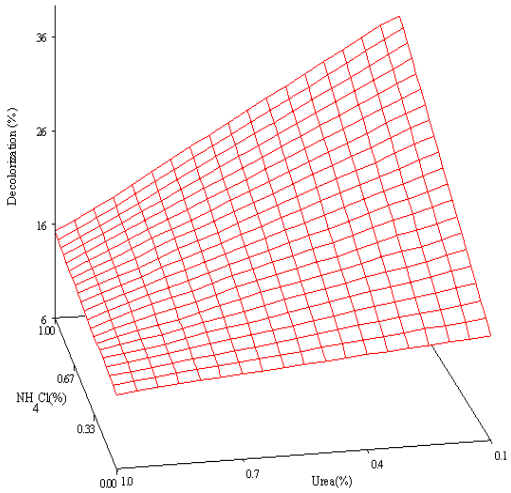
Optimization of the media constituents using RSM

To investigate the interactive effect of two factors on the decolorization of the dye, response surface methodology was used and surface plots were drawn. Response surface plots of two factors at a time, maintaining all other factors at fixed levels, are more helpful in understanding both the main and interactive effects of two factors [12]. Some typical response surface plots to illustrate these salient findings on the interaction between the media constituents on Isolan Grey 2S SBL decolorization in the present study are depicted in Fig 3. The quadratic polynomial equation was used to facilitate plotting of response surfaces. The equation can be represented as

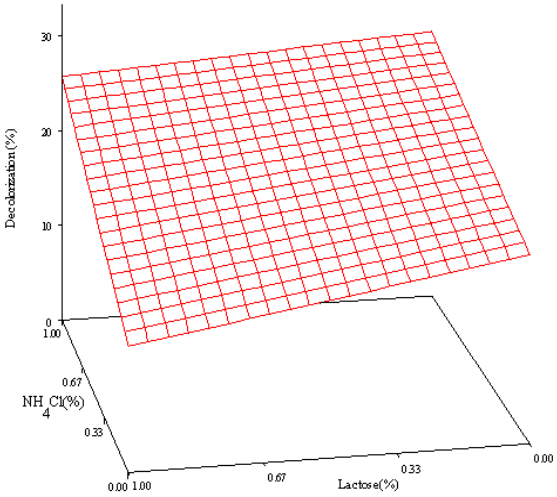
$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

b_0 = Intercept

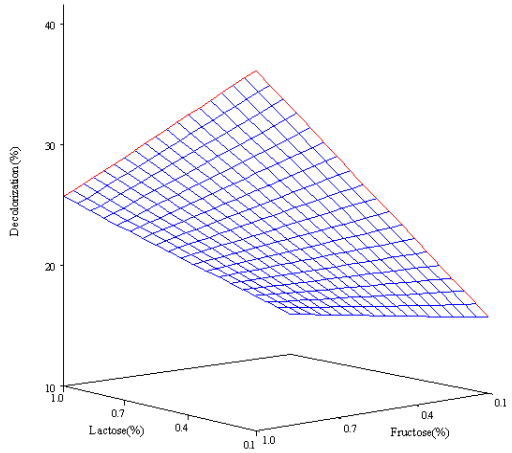
Two parameters were plotted at any one time on the x_1 and x_2 axes respectively, with the other remaining parameters set at their optimized values: pH 7, temperature 25°C, dye concentration 0.01%, incubation duration=120 hours, inoculum size=29.28%, Fructose=0.67%, Urea=0.21%, Lactose=0.021%, K_2HPO_4=0.44%, NH_4Cl=0.54% and $MgSO_4$=0.60%.


Decolorization (%)
36
26
16
6
NH4Cl(%)
1.00
0.67
0.33
0.00
1.0
0.7
0.4
0.1
Urea(%)

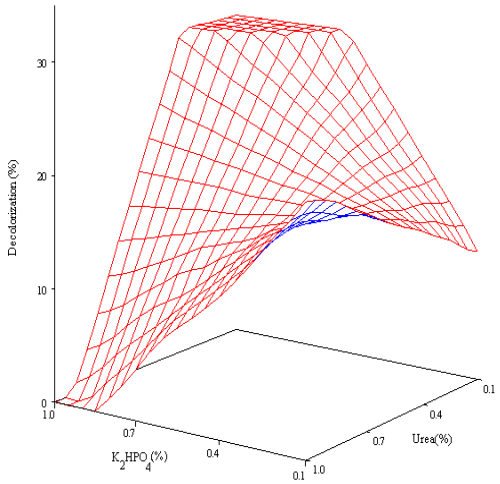
(A)


Decolorization (%)
30
20
10
0
NH4Cl(%)
1.00
0.67
0.33
0.00
0.00
0.67
0.33
0.00
Lactose(%)

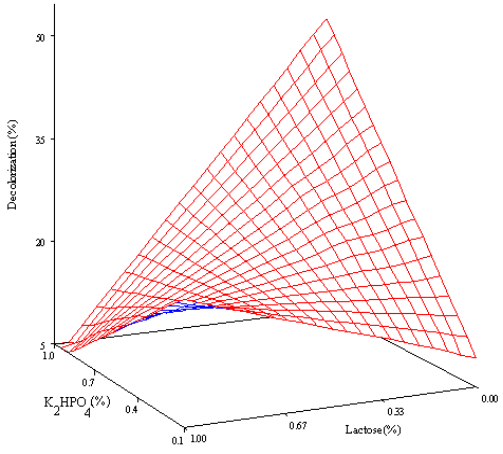
(D)


Decolorization (%)
40
30
20
10
Lactose(%)
1.0
0.7
0.4
0.1
Fructose(%)
1.0
0.7
0.4
0.1

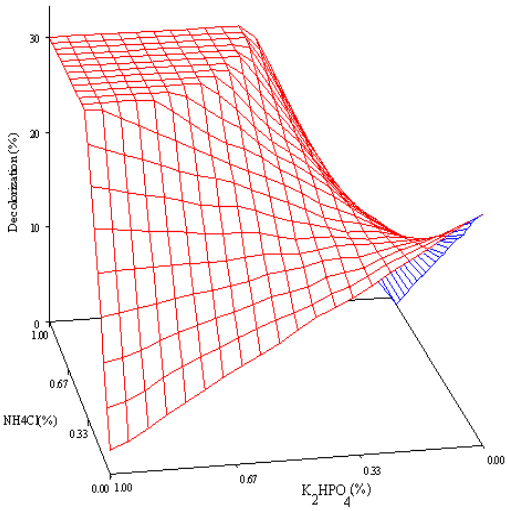
(B)


Decolorization (%)
30
20
10
0
K2HPO4(%)
1.0
0.7
0.4
0.1
Urea(%)
0.1
0.4
0.7
1.0

(E)


Decolorization (%)
50
35
20
5
K2HPO4(%)
1.0
0.7
0.4
0.1
Lactose(%)
1.00
0.67
0.33
0.00

(C)


Decolorization (%)
30
20
10
0
NH4Cl(%)
1.00
0.67
0.33
0.00
0.00
0.67
0.33
0.00
K2HPO4(%)

(F)

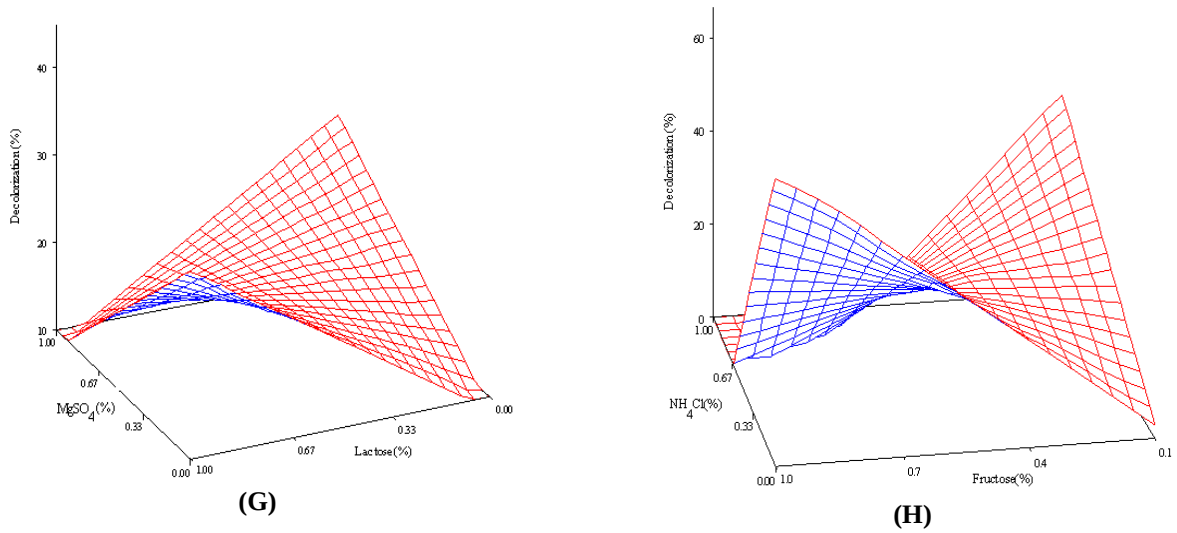
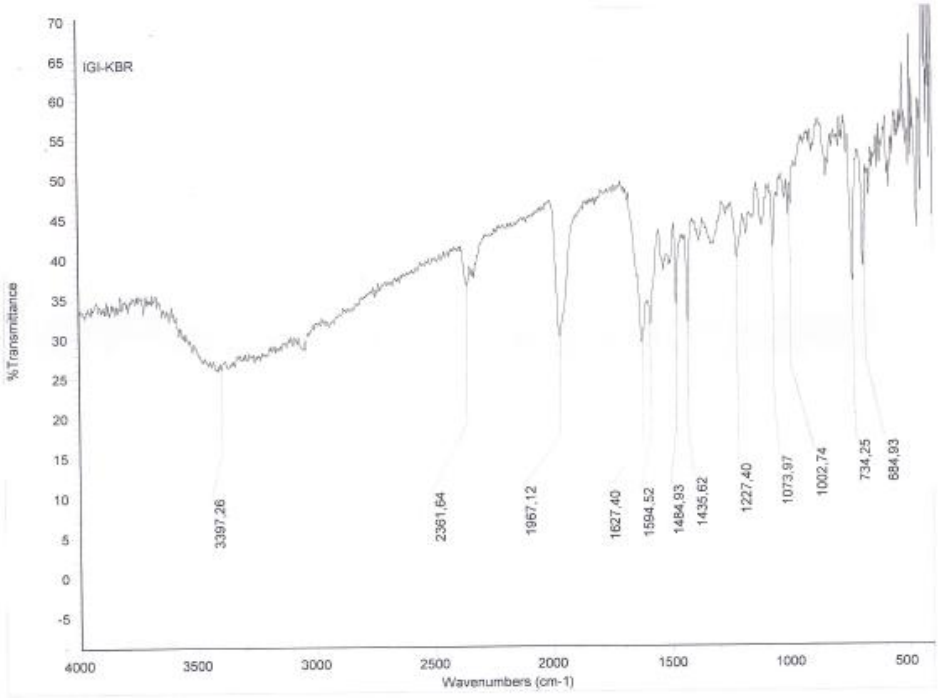


Figure 4: Three dimensional response surface plot for the effect of (a) NH_4Cl, Urea (b) Lactose, Fructose (c) K_2HPO_4, Lactose (d) NH_4Cl, Lactose (e) K_2HPO_4, Urea (f) NH_4Cl, K_2HPO_4 (g) $MgSO_4$ and Lactose (h) NH_4Cl and Fructose

Degradation product analysis

FTIR spectral comparison between the dye and its product formed after decolorization by the fungal 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 control dye spectrum as shown in Fig 4. In FTIR spectra, the original dye mixture showed N-H stretching at 3397 cm^{-1}. Peak at 1627 cm^{-1} indicated the presence of C=O. The other peaks observed were N=N stretching at 1484 cm^{-1} showing the presence of the azo bond and S=O stretching at 1227 cm^{-1}. The degraded compound showed the disappearance of N=N stretching indicating the cleavage of the azo bond. The other peak was at S=O stretching indicating the presence of sulphonyl group.



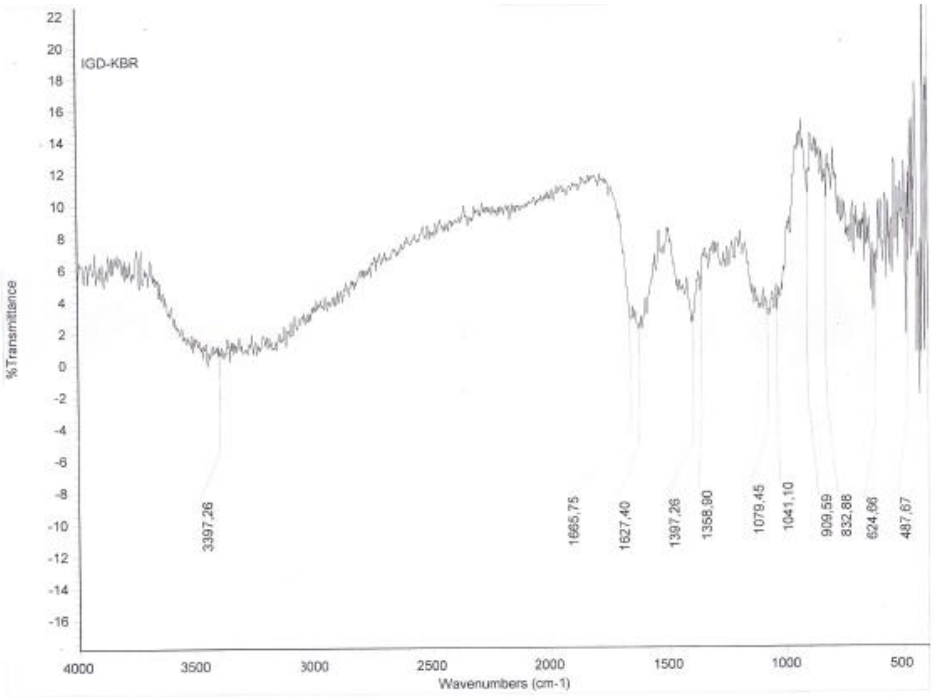


Figure 5: IGI - FTIR spectrum of the initial dye IGD - FTIR spectrum of the dye degradation product

Conclusion

The present study demonstrates the ability of *P. oxalicum RF3* isolated from the textile industry effluent to decolorize the dye. With further experimentation, involving other carbon and nitrogen sources and suitable culture conditions, it would be possible to enhance the percent decolorization to a greater extent. The potential of this fungus can be exploited for the removal of residual dyes from the wastewater streams for environmental cleanup and restoration of ecosystem.

Acknowledgement

The authors thank Dr. Suresh Dabade, Assistant Dyeing Master, Karnataka Silk Industries Corporation, Mysore for having provided the dye sample. The authors are grateful to Ms. Ramya Rao, Senior Research Fellow, National Institute of Animal Nutrition & Physiology, Bangalore for her assistance in statistical analysis. Authors acknowledge Chromous Biotech Pvt. Ltd, Bangalore for sequencing the fungal culture.

References

1. Huantian C., Ian R., Optimization conditions for microbial decolorization of textile waste water starch as carbon source, *AATCC Rev*., **1**, (37–40) (**2001)**

2. Kim S., Park C., Kim T.H., Lee J and Kim S.W., COD reduction and decolorisation of textile effluents using a combined process, *J. Biosci. Bioeng.,* **95**, 102-105 **(2003)**

3. Verma P., and Madamwar D., *World Journal of Microbiology and Biotechnology*, **19,** 615-618 (**2003)**

4. Wesenberg D., Kyriakides I., Agathos S.N., White-rot fungi and their enzymes for the treatment of industrial dye effluents, *Biotechnology Advances*, **22**, 161-187 **(2003)**

5. Montgomery D. C., *Design and analysis of experiments*, John Wiley & son, 427-472 **(2001)**

6. Nurdiyana H., Siti Mazlina M.K., optimization of protein extraction from fish waste using response Methodology, *Journal of applied sciences*, **9(17),** 3121-3125 **(2009)**

7. Ponraj M., Jamunarani P., Zambare V., Isolation and optimization of culture conditions for decolorization of True Blue using dye decolorizing fungi, *Asian. J. Exp. Biol. Sci.,* **2(2)**, 270-277 **(2011)**

8. Saitou N. and Nei M., The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol Biol Evo***, l (4)**, 406–425 (**1987)**

9. Tamura K., Dudley J., Nei M. and Kumar S., MEGA4: Molecular Evolutionary Genetics Analysis

(MEGA) software version 4.0., *Molecular Biology and Evolution*, **24**, 1596-1599 **(2007)**

10. Moosvi S., Keharia H., Madamwar D., Decolourization of textile dye Reactive Violet 5 by a newly isolated bacterial consortium RVM 11.1., *World J. Microbiol. Biotechnol*, **21**, 667-672 **(2005)**

11. Jadhav J.P., Phugare S.S., Dhanve R.S., Jadhav S.B., Rapid biodegradation and decolorization of Direct Orange 39 (Orange TGLL) by an isolated bacterium *Pseudomonas aeruginosa* strain BCH, *Biodegradation*, **21**, 453-463 **(2010)**

12. Montgomery D.C., *Design and analysis of experiments*, Wiley, 3rd Edition, New York, **(1991)**

13. Usha M.S., Sasirekha B., Bela R.B., Devi S., Kamalini C., Manasa G.A., and Neha P.M., Optimization of Reactive Black 5 dye and Reactive Red 120 dye degradation. *J. Chem. Pharm. Res*., **3(6)**, 450-457 **(2011)**