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Molecular Characterization of Isolated Bacteria and Application of RSM for Decolorization of Acid Black 1

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Pure culture of Acid Black -1 dye degrading bacteria was isolated from azo dye contaminated soil in the industrial zone of Thirupur, Tamil Nadu, India. The bacterium was identified by 16S rRNA as *Pseudomonas aeruginosa*. The optimization of process conditions (pH, temperature, and carbon source) for dye degradation during incubation with *P.aeruginosa* was carried out using Response Surface Methodology (RSM) based on a central composite design (CCD). Maximum degradation was achieved at pH 6.5, temperature 35^{0}C with carbon source as 1.5 % after 48 hrs of incubation. This design of experiment methodology increased the dye degradation in many folds. The degraded dye sample was assessed by GC – MS, which indicated that the decolorization took place due to dye degradation.

Key words: 16S rRNA, *Pseudomonas aeruginosa,* RSM, Biodegradation, GC – MS analysis.

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceuticals, food, cosmetic and other industries[1]. Many azo dyes and their degradation intermediates are mutagenic and carcinogenic[2, 3] and contribute to the mutagenic activity of ground and surface water that are polluted by textile effluents[4,5]. Microbial decolorization and degradation is an environmentally friendly and cost-competitive alternative to chemical decomposition processes[6]. In the present study, we report the isolation, characterization of a highly efficient azo dye decolorizing bacterium and application of RSM in optimizing medium conditions for its best decolorization.

Response surface methodology (RSM) is widely applied in the optimization of various industrially important microbiological, biochemical and biotechnological products such as chemicals and enzyme. Based on the principles of design of experiment, the methodology encompasses the use of various types of experimental designs, generation of poly nominal equations, and mapping of the response over the experimental domain to determine the optimum product. The technique requires minimum experimentation and time, thus proving to be far more effective than the conventional methods of developing such

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products. The present work is aimed at statistically optimizing three variables of pH, temperature, and carbon source for enhanced degradation of azo dye. In the present study, we attempted to decolorize and degrade azo dye-Acid Black 1, by a novel bacterial consortium which has the potential to decolorize and degrade poly phenoloic compounds present in textile effluent. Acid Black 1 dye is extensively used in textile, printing and leather industries. As microbial consortium was used in the study there was every possibility of occurrence of its unusual activity with the process variables and thus finally influencing the response. Central composite design was employed for modeling of the media components. Various intermediates formed during dye decolorization have been analyzed and identified by GC-MS.

MATERIAL AND METHODS

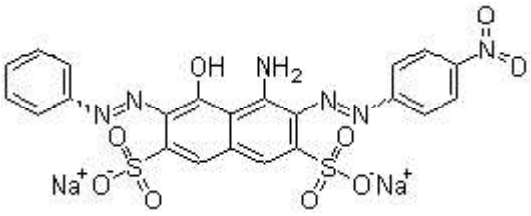
Selection of Dye

The dye Acid black 1 was procured from Sigma chemicals, Mumbai, India.

Name

Acid Black 1 Synonyms C.I. 20470; Amido Schwarz; Aniline Blue Black; Naphthol blue black; 4-Amino-5-hydroxy-3-(p-nitrophenylazo)-6-(phenylazo)-2,7-naphthalenedisulfonic acid disodium salt; Naphthalene black 10B; Amido black 10B.

Molecular structure



Mol. Formula $C_{22}H_{14}N_6Na_2O_9S_2$: Mol. Wt. 616.49

Measurement of dye concentration

The dye concentrations were measured in the sample with UV/VIS spectrophotometer (Hitachi 2000) at regular intervals during the decolorization process. The concentration of azo dye was detected spectrophotometrically by reading the culture at its specific λmax after centrifugation at 10,000 rpm for 10 minutes. The dye concentrations were determined from the absorbance (OD) of the culture at 490 nm.

Isolation and identification of the bacterial consortium

Soil samples were collected from the azo dye contaminated industrial zone of Thirupur, Tamil Nadu, India. One gram of soil sample was weighed and serially diluted up to 10^{-7}. Each dilution was plated on nutrient agar and potato dextrose medium with azo dye (0.25 %), incubated at 37^0C and observed for the formation of colonies. Then the single colonies were picked up randomly and plated on selective media for screening of particular species.

Bacterial identification service by genotyping using 16S rRNA

The bacterial consortium was identified based on the morphological biochemical as well by 16S rRNA based molecular approach. Selected microorganisms were picked up by the formation of clearing zone around the colonies and characterized them up to genus level by biochemical traits[7]. Differentiation of the bacterial isolates present in the consortium was done by amplified rDNA restriction analysis of the 16S rRNA genes. Upon obtaining the sequences from the NCBI, phylogenic tree was constructed by the neighbor joining method using MEGA package. The sequence has been deposited in the gene bank.

EXPERIMENTAL

Central composite design (CCD) for optimization of the culture condition for dye decolorization and RSM methodology

For any microbial decolorization process, media components such as carbon source, pH, temperature and nitrogen source, are the most important parameters which affect the process[8, 9, 10, 11]. Hence it is necessary to investigate the influence of these variables on the decolorization process. Hence the present study had a fewer components from the list mentioned such as pH, temperature and carbon source.

Due decolorization studies were carried out in the nutrient broth medium containing glucose (1 – 2%), pH adjusted to 6.5 to 7.0 and temperature 32 – 38 ^{0}C with 0.25 mg/L Acid Black 1, inoculated with 15% (v/v) of bacterial culture under agitation (120rpm). Samples were withdrawn and

analyzed for growth and decolorization.

Central composite design (CCD) with three factors at three levels was employed to investigate the first and higher order main effects of each factor and interactions amongst them. The design involved 20 factorial with $\alpha \pm 1$. The three coded levels investigated in the current study are -1, 0, +1. The statistical software package Design Expert® version 8.0 was used to generate polynomials and the contour plots.

Table 1. Full factorial central composite design for decolorization of Acid Black 1

Std	Run	pH (A)	Temperature (^{0}C) (B)	C source (C)	% decolorization
7	1	-1	1	1	75
10	2	1	0	0	79
1	3	-1	-1	-1	56
16	4	0	0	0	89
4	5	1	1	-1	73
20	6	0	0	0	87
8	7	1	1	1	66
2	8	1	-1	-1	63
18	9	0	0	0	85
14	10	0	0	1	70
19	11	0	0	0	89
13	12	0	0	-1	78
3	13	-1	1	-1	69
17	14	0	0	0	90
12	15	0	1	0	80
11	16	0	-1	0	79
5	17	-1	-1	1	71
6	18	1	-1	1	68
15	19	0	0	0	86
9	20	-1	0	0	78

All experiments were carried out in triplicate and the average value was given in the result.

Soft ware and data analysis

The results of the experimental design were analyzed and interpreted using Design Expert® version 8 statistical software.

Analysis of decolorization

Decolorization was determined by measuring the absorbance of the culture supernatants at the absorbance maxima of the dye under study.

Detection of the metabolites by Gas chromatography-mass spectrometry (GC-MS)

Cultures were gravity filtered with P8 filter paper. The filtrate from each flask (around 120 ml) was extracted with methylene chloride three times. The combined organic layers, approximately 30 ml, were concentrated to about 1 ml under reduced pressure after drying with sodium sulfate. The ionization voltage was 70 V. The initial column temperature was held at 40 °C for 4 min, then increased linearly to 270°C at 10 °C/min, and held for 4 min at 270°C. The temperature of the injection port was 275 °C and the GC/MS interface was maintained at 300°C. Helium was used as carrier gas with a flow rate of 1.0 ml/min. Injection was split less to increase sensitivity. Identification of degradation products was made by comparison of retention time and fragmentation pattern with known reference compounds.

RESULTS AND DISCUSSION

Isolation, identification of bacterial consortium and effect of culture conditions on dye decolorization Varied numbers of colonies were observed at various dilutions after overnight

incubation. A total number of six different bacterial strains were isolated in which four of them was gram negative bacteria. The subsequent morphological and biochemical characterization of the cultures showed the presence of *Pseudomonas* species. PCR amplified profile of 16S rRNA was done to identify the isolated bacteria.

PCR amplification profile of 16S rRNA from bacterial isolates

>Isolate 1_1466 bp

CGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCT
GGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT
CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGT
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCAT
GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTT
ACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAACTACTGATCTTAGAGTACT
GNAGAGGGGGGTAGAATTCCTTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC
TGATACTGACACTGAAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAG
CCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAG
AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCC
GCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATC
GTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCT
AGTCTAACCGCAAGGGGACGGTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTA

Phylogenetic analysis

The sequences of these 16S rRNA genes were compared with the sequences available from GenBank using the BLASTN program[12] and were aligned using CLUSTAL Software[13]. Distances were calculated according to Kimura's two-parameter correction[14]. Moreover Phylogenetic tree was constructed using the neighbor-joining method[15]. Bootstrap analysis was done based on 1000 replications for which The MEGA3 package was used for all analyses (Fig. 1).

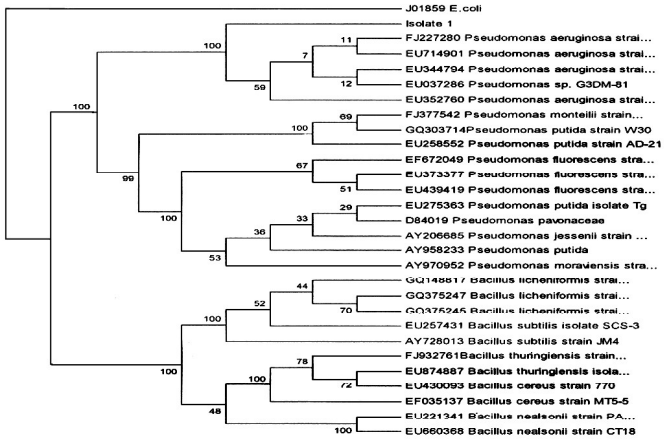


Fig. 1. Phylogenetic tree analysis based on 16S rRNA gene sequence comparisons over 1466 bases showing the comparisons with of *Pseudomonas, Bacillus* and *E.Coli* was used as out group. The sequence has been retrieved from NCBI database and the tree has been drawn using neighbor joinining method in MEGA software. The bar represents distance values calculated in MEGA

Central composite design for optimization of the culture conditions for dye decolorization

Microorganisms are capable of utilizing a variety of complex chemicals including dyes as their sole source of carbon but only few researchers have been successful in isolating such culture[16]. The bacterial consortium however, was not able to decolorize the dye Acid Black 1 using it as the sole source of carbon and energy. While performing media optimization studies for obtaining maximum decolorization of aerobically treated textile effluent, we observed that amongst various carbon sources tested, glucose was the best carbon source supporting maximum decolorization. Bacterial culture generally exhibit maximum decolorization at a neutral pH value or a slightly alkaline pH value and the rate of colour removal tends to decrease rapidly at highly acidic or alkaline pH conditions [17,18]. The temperature required to produce the maximum rate of colour removal tends to correspond with the optimum cell culture growth temperature of 35-45^{0}C [17].

On the basis of the reports published earlier, preliminary studies with several variables (Table 2) were standardized by one variable at a time method. The dye concentration used was 2.5 mg/L throughout this study and the dye decolorization was determined as the response. This study revealed several of the parameters to be important and influential on dye decolorization. The study had provided us with the optimum level for each of the parameter for dye decolorization which was given in the following table.

Table 2. Parameters examined for conventional one variable at a time optimization of degradation of Acid Black 1

Parameter	Range	Optimum
Different pH	3.5 to 11.5	6.5
Different Temperature	4 to 60^0 C	35^{0}C
Effect of Salinity	0 to 10(%)	Growth up to 0.5%
Carbon source (1.0% (w/v)	Glucose,Sucrose, Fructose,Maltose,Lactose.	Glucose
Nitrogen source(1.0% (w/v).	Peptone, Beef extract, yeast extract, tryptone	Yeast extract
Metal salts (2.0 mM)	Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, Ca EDTA and Mg^{2+}	The growth was highest in the presence of Zn^{2+},
Incubation time	(12 to 84 h)	48 hrs
Agitation	(0 to 150)	120 rpm
Inoculum size	1-15%	More than 5%

The dye decolorization observed with all the optimized culture conditions was 79 % with 24 hrs and further incubation did not enhance the decolorization.

These results led us to plan and carry out a systematic study of dye decolorization process and hence we proceeded with central composite design and response surface methodology. The main objective of RSM is to determine the optimum operational conditions for the system or to determine a region that satisfies operating specifications[19].

Central composite design (CCD) and Response surface methodology (RSM)

The results of 20- run CCD in three variables; pH, temperature and glucose concentration for optimization of bacterial dye decolorization process are shown in table 1. It shows %dye decolorization as the dependent response corresponding to combined effect of three independent factors in their specified ranges.

Decolorization varied markedly with the conditions tested, in the ranges of 56-90%. Lowest decolorization was observed when glucose concentration, temperature and carbon source was minimum (run 3). Appreciable decolorization was observed when at least two parameters were in the range of 0 in terms of the coded value. Best decolorization was shown with all the three parameters in the range 0 in the coded values. At these levels decolorization exhibited was was above 85%. Further RSM steps, the statistical analysis of the CCD experimental results, were carried out using Design Expert®. The statistical analysis

employed Fisher's 'F' test and student's t-test. Analysis of variance (ANOVA) for percentage decolorization shows that fitted second order response surface model is highly significant with F-Test=11.70610735 (p=0.0003) as shown in Table 3. The student's t test was used to determine the significance of the regression coefficients of the variables.

Table 3. Experimental Range and Levels of independent Variables in terms of coded factors for decolorisation of Acid Black 1

Variable	Range and levels		
	-1	0	+1
pH	6.0	6.5	7.0
Temperature	32	35	38
Carbon source(%w/v)	1.0%	1.5%	2%

Table 4. ANOVA of % decolorization for Acid Black 1; effect of pH, Temperature and carbon source

Source	Sum of Squares	df	Mean Square	F Value	p-value	
Model	1575.415909	9	175.0462121	11.70610735	0.0003	significant
A-pH	0	1	0	0	1.0000	
B-Temperature	67.6	1	67.6	4.520708261	0.0594	
C-Carbon source	12.1	1	12.1	0.809180029	0.3895	
AB	10.125	1	10.125	0.677103123	0.4298	
AC	66.125	1	66.125	4.422068546	0.0618	
BC	55.125	1	55.125	3.686450338	0.0838	
A^2	73.84090909	1	73.84090909	4.938065203	0.0505	
B^2	48.09090909	1	48.09090909	3.216049852	0.1032	
C^2	257.7784091	1	257.7784091	17.23877194	0.0020	
Residual	149.5340909	10	14.95340909			
Lack of Fit	130.2007576	5	26.04015152	6.734521944	0.0282	significant
Pure Error	19.33333333	5	3.866666667			
Cor Total	1724.95	19				

Values of "Prob > F" less than 0.0500 indicate model terms are significant. The regression coefficients, t and p values for all the linear, quadratic and interaction effects of the variables are given in Table 4.

For a three factor system the model equation generated in terms of coded factors was

$$\begin{aligned} \%\ \text{decolorization} = {} & 86.07272727 \\ & 0 * A \\ & 2.6 * B \\ & 1.1 * C \\ & -1.125 * A * B \\ & -2.875 * A * C \\ & -2.625 * B * C \\ & -5.181818182 * A^2 \\ & -4.181818182 * B^2 \\ & -9.681818182 * C^2 \end{aligned}$$

The coefficient of determination R^2 for the above predicting equation was 0.913311058. Therefore this equation generated can be used for predicting response at any combination of three variables in experimental range. Percent decolorization at specified combination of three variables can be predicted by substituting corresponding coded values in above equation. Contour plots were prepared in Design Expert®. The contour plots showcase the behavior of response (% decolorization) with respect to

simultaneous change in two variables[20]. Lack of fit for the model was given as 0.0282 which indicated that the model was insignificant by 2.8% and hence it is highly reliable. Apart from the linear effect of the variables on the decolorization process, the second order RSM also gives an insight into their quadratic and interaction effects. The coefficient for the linear effect of glucose (p=0.3895), pH (p=1.0000), Temperature (p=0.0594) were highly significant.

Table 5. Analysis of variance (ANOVA) table for Response Surface Methodology

Std. Dev.	3.866963808	R-Squared	0.913311058
Mean	76.55	Adj R-Squared	0.83529101
C.V. %	5.051552982	Pred R-Squared	0.498467994
PRESS	865.1176345	Adeq Precision	10.74126814

The isolated bacteria performed maximum decolorization at the glucose level of 1.5 %, at pH level of 6.5 and at 35^{0}C.

The choices for level combinations of the three variables; pH, temperature and glucose level can be made easily from contour plots. These recommendations are valid for future trials because they are based on well fitted response surface model (R^2=0.913311058).

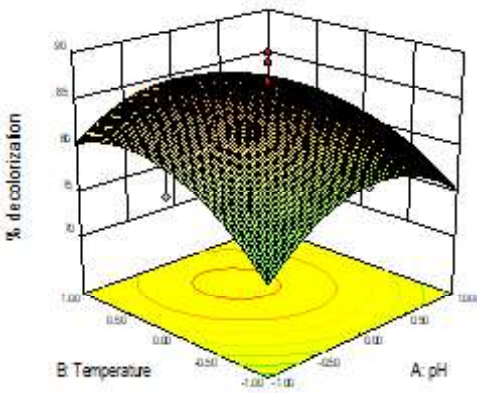


Fig. 2. Contour plot showing effect of pH and Temperature on % decolorization of Acid Black 1 dye by *P.aeruginosa*

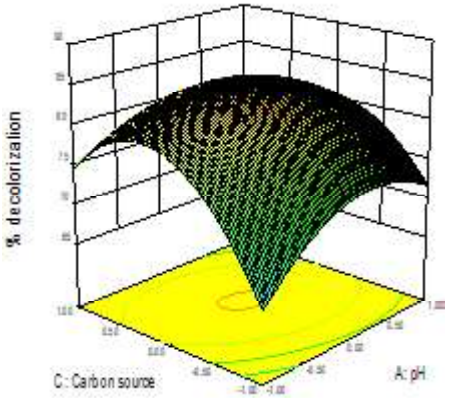


Fig. 3. Contour plot showing effect of pH and Carbon source concentration on % % decolorization of Acid Black 1 dye by *P.aeruginosa*

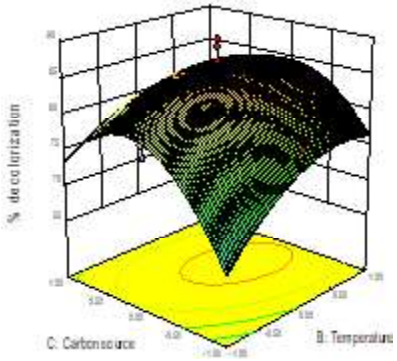


Fig. 4. Contour plot showing effect of Temperature and Carbon source concentration on % % decolorization of Acid Black 1 dye by *P.aeruginosa*

Spectrum of Acid Black 1 decolorization

Mass spec analysis showed that the initial dye solution had a peak of molecular weight (616) and after degradation it showed molecular weights of degraded products. By careful and peak to peak comparison of MS spectra of azo dye substrate, before and after degradation revealed that the molecular weight of the main dye product is lowered remarkably which gives information on the extent of degradation of the dye, caused by the enzyme. The M+1 peak in the first spectra, (taken before degradation) is at 5H. Also it is noted that the dye is a disodium salt. Hence the molecular weight of the dye substrate is

Molecular weight = {(M + 1) peak + 2 (weight of sodium)} – 1

= {571 + 2 (23)} – 1

= (571 + 46) – 1

= 617 – 1= 616.0

CONCLUSION

An attempt was made to isolate bacteria with best dye degrading efficiency from the bacterial consortium obtained from soil sample of dye contaminated area. The isolated bacterium was characterized at molecular level by 16S rRNA technique as *Pseudomonas aeruginosa*. Response surface methodology was applied to determine optimum conditions such as pH, temperature and carbon source for dye decolorization. The results of the study showed the values as 6.5, 35 ^{0}C and 1.5% respectively as the efficient conditions of decolorization. This study shows that response surface methodology was an appropriate method to optimize the best culture conditions for obtaining maximum decolorization of dye. The work could be further extended to express the gene responsible for dye decolorization in to appropriate host which could be used as a cheaper and easier source of azoreductase enzyme for azo dye decolorization.

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