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STATISTICAL OPTIMIZATION OF SYNTHETIC AZO DYE (ORANGE II) DEGRADATION BY AZOREDUCTASE FROM *Pseudomonas oleovorans* PAMD_1

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□ Pseudomonas oleovorans *PAMD_1 produced an intracellular azoreductase as the more prominent enzyme that reduces the azo bridge during the azo dye decolorization process. In order to optimize the expression of azoreductase, statistically based experiments were applied. Eleven significant factors were screened on decolorization activity using Plackett–Burman design. Dye, NADH, glucose, and peptone were identified as having highest positive influence on the decolorization activity. Central composite design of response surface methodology was employed for the concerted effect of these four factors on decolorization activity. This method showed that the optimum medium containing dye (200 mg L^{-1}), NADH (1.14 m*M*), glucose (2.07 g L^{-1}), and peptone (6.44 g L^{-1}) for the decolorization of Orange II up to 87% in 48 hr. The applied methodology was validated through the adequacy and accuracy of the overall experiments, and the results proved that the applied methods were most effective. Further, the enzyme was purified ninefold with 16% yield by anion-exchange chromatography and a specific activity of 26 U mg^{-1}. The purified enzyme with a molecular mass of 29,000 Da gave a single band on sodium dodecyl sulfate (SDS) gel, and the degradation products sulfanilic acid and 1-amino-2-napthol of Orange II by azoreductase were analyzed by using an ultraviolet–visible (UV-Vis) spectrophotometer and hish-performance liquid chromatography (HPLC).*

Keywords azoreductase, decolorization, orange II, *Pseudomonas oleovorans*, RSM

INTRODUCTION

Azo dyes are widely used in several industries like textiles, leather, paper, paint, cosmetics, and food. The xenobiotic nature of the azo dyes is characterized by the existence of -N=N- (azo) groups and aromatic rings.[1] They pose toxicity (genotoxicity, mutagenicity, carcinogenicity,

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etc.) to living organisms.[2] Discharge of wastewater mainly from the textile industries adversely affects water resources and environmental integrity. Therefore, effluent containing azo dyes needs effective treatment before discharging, to prevent deterioration of ecological integrity.[3] Using microorganisms for the decolorization of dyes is an ecofriendly and cost-competitive decomposition process. Reduction of azo bonds with specific azoreductase by various microorganisms leading to carcinogenic dye decolorization has been identified.[4] Hence, the color removal of dyes is the prime solution for the reduction of toxicity from the environment. Therefore, it needs to develop a potential microbial enzymatic decolorization processes for the effective removal of synthetic dyes.[5]

Optimizing the concentration of medium components is very important to determine the overall economic feasibility of the production process in industries.[6] Different statistical designs have been used for the medium optimization in the production of various enzymes, including protease, xylanase, amylase, lipase, glucanase, and laccase, by microorganisms.[7–13] The Plackett–Burman statistical model was employed to analyze the correlation of different nutritional factors for azoreductase enzyme activity.[14]

The optimum level interactions between the positive variables and their response were observed through response surface methodology (RSM).[15] It is a right approach for finding the optimal conditions and the better statistical interpretation of a multifactorial system.[16,17]

The aims of the present study were (i) to optimize the decolorization of Orange II by azoreductase produced by *Pseudomonas oleovorans* PAMD_1, (ii) to purify the azoreductase, and (iii) to analyze the decolorized product of the azo dye Orange II. To best of our knowledge, this is the first report of purification of an azoreductase from *P. oleovorans*. This study was focused into the enzymatic degradation of azo dyes in bioremediation.

MATERIALS AND METHODS

Chemicals and Dye

NADH, Flavin Mono Nucleotide (FMN), yeast extract, and Orange II were purchased from Himedia, India, Na_2HPO_4, NaH_2PO_4, and ammonium sulphate were purchased from SRL, India. DEAE–cellulose was purchased from Sigma Aldrich. The molecular weight marker kit was obtained from Genei, Bangalore, India.

Microbial Strain and Culture Condition

The active microbial strain was isolated from the textile waste effluent-contaminated soil. Selective enrichment batch culture principle

was used to select the dye decolorizers. The most active microbial strain was picked based on its decolorizing ability to form a clear zone on dye agar medium and was identified through 16S rRNA gene sequencing as *P. oleovorans* PAMD_1.[18] The organism from the preserved stock cultures was used after preculturing in nutrient broth. For the degradation study, the mineral salt medium contained ($g\,L^{-1}$): NaCl 2.0, $MgSO_4 \cdot 7H_2O$ 0.42, KCl 0.29, K_2HPO_4 1.27, $NaNO_3 \cdot 2H_2O$ 0.42, KH_2PO_4 0.85, deionized water (pH 7.0) and EDTA 0.5 mL (0.5 m*M*). The medium was autoclaved for 20 min. One hundred milliliters of the production medium was taken in a 250-mL Erlenmeyer flask containing 0.5 g of Orange II inoculated with activated (24 hr age culture) 2% inoculum and incubated at 200 rpm in the incubator shaker for 24 hr at 35°C.

Crude Enzyme Preparation

The grown cells were harvested from the culture medium by centrifugation for 10 min at 10,000 × g and the pellet was suspended in 100 m*M* phosphate buffer (pH 7.0). Cells were disrupted at 4°C by 6 × probe sonication for 30 s at 70% amplitude.[19] The cell debris was removed by centrifugation for 20 min at 12,000 × g, and the supernatant was used for further experiments. The remaining cell lysate was stored at 4°C.

Azoreductase Assay

The azoreductase activity was determined based on the Zimmermann's procedure[20] with minor modifications. A typical assay mixture with a total volume of 3 mL was carried out in cuvettes having 1 cm path length: 100 m*M* phosphate buffer (pH 7.0), 20 μ*M* Orange II, and 0.5 mL of enzyme solution. The reaction was initiated by the addition of 0.5 m*M* NADH to the air-saturated and 35°C preincubated solutions after 4 min. The decreasing color intensity was monitored at 482 nm.

The slope of the initial linear decrease of absorption was used to calculate the azoreductase activity based on the molar absorption coefficient of Orange II ($\varepsilon = 18.2\,mM^{-1}\,cm^{-1}$). One unit (U) of azoreductase was defined as the reduction of 1 nmol dye per minute.

Analysis of Decolorization

The percentage decolorization of the substrate Orange II was determined at its maximum absorption wavelength of 482 nm using an ultraviolet–visible (UV-Vis) spectrophotometer (Shimadzu UV-2450). The

percentage decolorization was calculated based on the following equation[21]:

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance value} - \text{Final absorbance value}}{\text{Initial absorbance value}} \times 100$$

Optimization of Culture Condition for Decolorization

Composition of the growth media is the essential parameter for any microbial decolorization process. Components like carbon, nitrogen source, redox mediators, dye concentration, and other environmental factors affect the decolorization process.[22] The optimization of decolorization activity of the azoreductase was carried out initially by screening of the significant variables through a Plackett–Burman experiment followed by optimizing the significant variables concentration through RSM.

Plackett–Burman Design

In this study, for the screening of significant variables, the selected 11 factors were glucose, starch and sucrose (carbon source), peptone, yeast extract and ammonium sulfate (nitrogen source), dye, NADH, riboflavin (inducers), pH, and incubation period. These variables were evaluated by 12 runs and the levels of each variable were determined. Factors used for the initial screening and the levels for the experimental design are shown in Table 1.

Trials were performed in triplicates and the average of decolorization observation results were treated as the response. The highest positive variables (dye, NADH, glucose, and peptone) influencing enzyme activity on

TABLE 1 Plackett–Burman Design for Screening Variables for Decolorization Activity

Factors	Concentration	Code	Low Level (−1)	High Level (+1)	Effect
Glucose	$g\,L^{-1}$	X_1	0.5	1.5	Positive
Starch	$g\,L^{-1}$	X_2	0.5	1.5	Negative
Sucrose	$g\,L^{-1}$	X_3	0.5	1.5	Positive
Peptone	$g\,L^{-1}$	X_4	3.0	5.0	Positive
Yeast extract	$g\,L^{-1}$	X_5	3.0	5.0	Positive
Ammonium sulphate	$g\,L^{-1}$	X_6	3.0	5.0	Negative
Riboflavin	m*M*	X_7	0.5	1.5	Positive
NADH	m*M*	X_8	0.5	1.5	Positive
Temperature	°C	X_9	29	45	Negative
pH	Value	X_{10}	6.0	8.0	Negative
Dye	mg	X_{11}	50	150	Positive

TABLE 2 Plackett–Burman Design Variables with Decolorization Activity in Terms of Percentage Decolorization

	Variable Levels (coded)											
Experiment	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Decolorization (%)*
1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	82.93
2	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	32.92
3	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	65.85
4	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	73.17
5	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	26.83
6	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	32.92
7	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	43.90
8	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	93.90
9	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	31.71
10	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	47.56
11	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	79.26
12	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	78.04

*Average of triplicate.

each category were selected from a Pareto chart analysis. The levels of the significant variables were further optimized by central composite design (CCD) of response surface methodology.

Response Surface Methodology

The four positive variables from the Pareto chart analysis (PB design) were chosen as independent variables, denoted as A (dye), B (NADH), C (glucose), and D (peptone), and the dependent response is the percentage dye decolorization. Concerted effects of all the four independent variables on the percentage dye decolorization were studied with respect to dye concentration (50–250 mg L^{-1}), NADH (0.4–2.0 mM), glucose (1–3 g L^{-1}), and peptone (2–10 g L^{-1}) using CCD with a total of 30 runs at five different levels (shown later, in Table 3). Table 4 (shown later) shows the levels of the four independent variables in CCD experiment. For statistical calculation independent variables were coded as

$$X_i = \frac{Z_i - Z_0}{\Delta Z_i} \tag{1}$$

The coded, uncoded, and midpoint value of the *i*th independent variable were represented as X_i, Z_i, and Z_0, respectively[3,23]; ΔZ_i is the step change value in Z_i and $i = 1, 2, 3, 4$. Second-order multiple regression procedure for the four independent variables on the dye decolorization

TABLE 3 Experimental Range and Levels of Independent Variables Selected for RSM

Variable	Code	Range and Levels					Change Value (ΔZ_i)
		Very Low (−2)	Low (−1)	Mid (0)	High (+1)	Very High (+2)	
Dye ($mg\,L^{-1}$)	A	50	100	150	200	250	50
NADH (m*M*)	B	0.4	0.8	1.2	1.6	2.0	0.4
Glucose ($g\,L^{-1}$)	C	1.0	1.5	2.0	2.5	3.0	0.5
Peptone ($g\,L^{-1}$)	D	2.0	4.0	6.0	8.0	10.0	2.0

(response) was measured by using the following equation:

$$\hat{\mathbf{Y}} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \qquad (2)$$

TABLE 4 Full Factorial CCD Matrix and Their Observed Responses for Decolorization Activity Using RSM

Run Order	Dye, $mg\,L^{-1}$	NADH, m*M*	Glucose, $g\,L^{-1}$	Peptone, $g\,L^{-1}$	Decolorization (%)*
1	−1	−1	−1	−1	65.0
2	1	−1	−1	−1	76.0
3	−1	1	−1	−1	71.0
4	1	1	−1	−1	60.0
5	−1	−1	1	1	53.0
6	1	−1	1	−1	74.0
7	−1	1	−1	1	52.0
8	1	1	1	−1	64.0
9	−1	−1	−1	1	58.0
10	1	−1	−1	1	87.0
11	−1	1	−1	1	69.0
12	1	1	−1	1	81.0
13	−1	−1	1	1	62.0
14	1	−1	1	1	76.0
15	−1	1	1	1	66.0
16	1	1	1	1	70.0
17	−2	0	0	0	42.0
18	2	0	0	0	68.0
19	0	−2	0	0	79.0
20	0	2	0	0	75.0
21	0	0	−2	0	79.0
22	0	0	2	0	73.0
23	0	0	0	−2	72.0
24	0	0	0	2	79.0
25	0	0	0	0	80.0
26	0	0	0	0	78.0
27	0	0	0	0	80.0
28	0	0	0	0	80.0
29	0	0	0	0	79.0
30	0	0	0	0	78.0

*Average of triplicate.

where $\widehat{Y}$ is the predicted percentage decolorization; β_0 is a intercept; β_i, linear terms coefficients; β_{ii}, quadratic effect of X_i; and β_{ij}, interaction effect between X_i and X_j on dye decolorization.[3]

Statistical Analysis

Design-Expert 7.0 (Stat Ease, USA) was used to analyze the results and interpretation. The statistical significance of the experimental design was evaluated through Fisher's *F*-test for analysis of variance (ANOVA). Student's *t*-test was used to evaluate the significance of regression coefficient. The interaction among the four independent variables on dye decolorization was illustrated by three-dimensional surface plots. By solving the regression equation, the optimum concentration of the selected independent variables was calculated.[24] The model was experimentally validated by testing the concerted effect of the optimized parameters.

Purification of Azoreductase

Purification of azoreductase was performed using a procedure similar, with minor modification, to that described previously by Valli Nachiyar.[25] The bacterial cells grown in the optimized culture medium were centrifuged for 10 min at 10,000 × g. The cell pellet was dissolved in 100 m*M* phosphate buffer (pH 7.0). Cells were disrupted at 4°C by 6 × probe sonication for 30 s at 70% amplitude. Cell debris was removed by centrifugation at 12,000 × g for 20 min and the supernatant was collected. The collected supernatant was then fractionated with $(NH_4)_2SO_4$(ammonium sulfate) up to 80% saturation, then centrifuged at 10,000 × g for 30 min at 4°C. After standing in the ammonium sulfate solution for 5 hr at 0°C, the precipitate was collected by centrifugation at 10,000 × g for 30 min and resuspended with 100 m*M* of phosphate buffer (pH 7.0) and dialyzed exhaustively against the same buffer for 12 hr.

The dialyzed enzyme sample was then applied to anion-exchange chromotography (DEAE- cellulose column of 45 cm × 2 cm) at a flow rate of 60 mL hr^{-1}, then equilibrated and washed with 100 m*M* phosphate buffer (pH 7.0). After that the enzyme-loaded column was washed with 500 mL of the buffer to remove loosely and unbound sample components. Elution was carried out with a linear gradient of 0 to 1 *M* NaCl (total volume of 200 mL) in the same buffer. Two-milliliter fractions were collected, and those that showed enzyme activity were pooled, dialyzed against the same buffer, and preserved.

Molecular Weight Determination

The SDS-PAGE analysis of purified azoreductase was performed as described previously by Laemmli.[26] After electrophoresis, the protein

bands were stained with Coomassie brilliant blue R-250 at 0.025% (w/v) in methanol/acetic acid/water (4:1:5) for molecular weight determination.

Analytical Methods

UV-Vis Analysis

The degradation pattern of Orange II azo dye was analyzed by a UV-Vis spectrophotometer (Shimadzu UV-2450). The spectra of dye and azoreductase-treated solutions were observed in the range of 200 to 600 nm.

HPLC Analysis of Metabolites

Ten milliliters of the decolorized supernatant was used to analyze the product produced by the action of azoreductase on Orange II. The reverse-phase C-18 column (4.6 mm × 25 cm) with a 5-μm particle size was used. The sample injected volume was 20 μL at a flow rate of 1.0 mL min^{-1} with an aqueous solvent system consisting 100 m*M* phosphate buffer (pH 7.0) over a period of 30 min. A linear gradient (20% methanol increased to 100%) was then maintained for 10 min with the same flow rate. The standards of Orange II and sulfanilic acid (SA) were injected for comparison.

RESULTS AND DISCUSSION

Plackett–Burman Design

Table 2 shows the elucidation of medium components affecting azoreductase decolorization activity, as examined by Plackett–Burman design experiments. The experimental response shows the diverse decolorization activity among the 12 runs. This discrepancy shows the importance of medium optimization to obtain maximal activity.

The positive influence and negative influence of the selected variables on the decolorization activity are shown in Figure 1 (Pareto chart). The presence of a high level of dye concentration, NADH, glucose, peptone, and yeast extract in the growth medium affects decolorization positively. From the Pareto chart, the factors that showed greatest positive effect were selected for the RSM optimization.

Several studies have previously demonstrated the ability of bacterial dye decolorization.[27] Among the three carbon sources glucose has been identified as good for supporting maximum dye decolorization in the previous literature.[28] For the dye decolorization process with different organisms like *Pseudomonas luteola*,[29] *Klebsiella pnuemoniae*,[30] *Bacillus*, and *Clostridium*

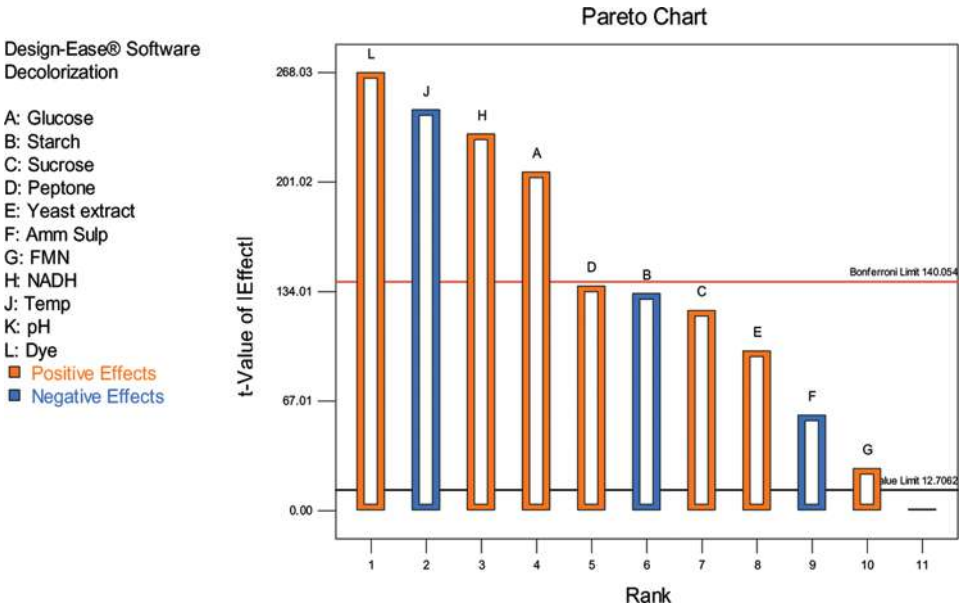


FIGURE 1 Pareto chart of 11-factor effects on decolorization activity (color figure available online).

sp.,[31] peptone and yeast extract has been the widely used nitrogen source. Optimal decolorization by the bacterial culture exhibits generally at a neutral pH, but it is greatly reduced at high acidic and alkaline conditions.[32,33]

Changing the variable concentration shows the considerable differences in Orange II decolorization as done by the Plackett–Burman experiment. The advantage of using a multifactorial experiment is an understanding of the interactions of the selected independent variables and the nonlinear response of the experimental model.[34]

Decolorization Optimization by RSM

The highest positive influence factors from the Plackett–Burman experiment were chosen for CCD-RSM experiments. Table 3 shows the different levels of each variable used for RSM. The results of four variables (dye, NADH, glucose, peptone) chosen for the dye decolorization optimization process for 30-run CCD-RSM are shown in Table 4. The collective effect of four positive factors on the percentage decolorization of Orange II ranges from 42 to 87%. At high concentrations of dye and peptone, low concentrations of NADH and glucose show more than 85% dye decolorization. Hence, this result proves the concentration of these variables has a strong influence on the dye decolorization process. From the

following polynominal equation the experimental results were evaluated and subsequently converted to uncoded units:

$$\hat{Y} = 85 + 5.29A + 0.46B - 0.54C + 2.38D - 2.81AB + 1.44AC + 1.19AD - 0.94BC + 2.31BD + 1.31CD - 5.91A^2 - 4.28B^2 - 3.52C^2 - 5.91D^2 \quad (3)$$

$$A = \frac{Z_1 - 150}{50} \quad (4)$$

$$B = \frac{Z_2 - 1.2}{0.4} \quad (5)$$

$$C = \frac{Z_3 - 2.0}{0.5} \quad (6)$$

$$D = \frac{Z_4 - 6.0}{2.0} \quad (7)$$

The decolorization process can be interpreted from the regression coefficients of the four variables. The results prove dye concentration has a significant effect on dye decolorization process. A similar result was reported earlier on decolorization efficiency.[32] Increasing the concentration of glucose shows increasing decolorization, but at higher concentration it has inhibitory effect, shown in previous studies.[31] The results also depict that NADH had synergistic effect with dye on decolorization process. Therefore, an appropriate combination of these variables is important for the optimal dye decolorization process.

Statistical analysis of Eq. (1) was checked by Fisher's *F* test. Table 5 shows the ANOVA result for percentage decolorization and depicts the selected RSM model is highly significant. The low probability (prob >0.0006) and Fisher's *F* test value (6.18) also imply the significance of the selected model. From the determination coefficient ($R^2 = 0.8523$) and adjusted R^2(0.7144), fitness of the model was verified (Table 6). It shows a good correlation between experimental and predicted values. Therefore, the interaction between the variables and the response was well interpreted by this significant model.

Response surface three-dimensional (3D) plots of the influencing variables that influence the dye decolorization activity are given in Figure 2, a–f. The interaction between the variables on dye decolorization has been observed form the 3D plots (Figure 2, a–f). Maximal decolorization (above 85%) was achieved at the coded value of 1.0, −0.15, 0.14, and 0.22 for the variables dye, NADH, glucose, and peptone concentrations respectively.

TABLE 5 Significance of Regression Coefficients, Response 1 % Decolorization, ANOVA for Response Surface Quadratic Model, and Analysis of Variance Table [Partial sum of squares—Type III]

Source	Sum of Squares	df	Mean Square	*F*- Value	*p*- Value	Prob > *F*
Model	3114.55	14	222.47	6.18	0.0006	Sig.
A-A	672.04	1	672.04	18.68	0.0006	
B-B	5.04	1	5.04	0.14	0.7134	
C-C	7.04	1	7.04	0.20	0.6645	
D-D	135.38	1	135.38	3.76	0.0715	
AB	126.56	1	126.56	3.52	0.0803	
AC	33.06	1	33.06	0.92	0.3530	
AD	22.56	1	22.56	0.63	0.4408	
BC	14.06	1	14.06	0.39	0.5413	
BD	85.56	1	85.56	2.38	0.1439	
CD	27.56	1	27.56	0.77	0.3953	
A^2	956.81	1	956.81	26.59	0.0001	
B^2	502.74	1	502.74	13.97	0.0020	
C^2	342.03	1	342.03	9.51	0.0076	
D^2	956.81	1	956.81	26.59	0.0001	
Residual	539.75	15	35.98			
Lack of fit	539.75	10	53.97			
Pure error	0.000	5	0.000			
Cor total	3654.3029					

Note. The model *F*-value of 6.18 implies the model is significant. There is only a 0.06% chance that a "model *F*-value" this large could occur due to noise. Values of "Prob>*F*" less than 0.0500 indicate model terms are significant. In this case A, A^2, B^2, C^2, and D^2 are significant model terms.

With respect to the individual variables, Eq. (3) was maximized by the maximum and minimum principle of differential calculus. The partial, (8)–(11), and second-order, (12)–(15), differential equations are:

$$\frac{\partial \widehat{Y}}{\partial A} = -11.82A + 2.81B + 1.44C + 1.19D + 5.29 \tag{8}$$

$$\frac{\partial \widehat{Y}}{\partial B} = -2.81A - 8.56B - 0.94C + 2.31D + 0.46 \tag{9}$$

TABLE 6 ANOVA Results for the Quadratic Equation

Std. dev.	6.00	*R*-squared	0.8523
Mean	69.30	Adj *R*-squared	0.7144
C.V.%	8.66	Pred *R*-squared	0.1492
PRESS	3108.96	Adeq precision	8.065

Note. The "pred *R*-squared" of 0.1492 is not as close to the "adj *R*-squared" of 0.7144 as one might normally expect. "Adeq precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 8.065 indicates an adequate signal. This model can be used to navigate the design space.

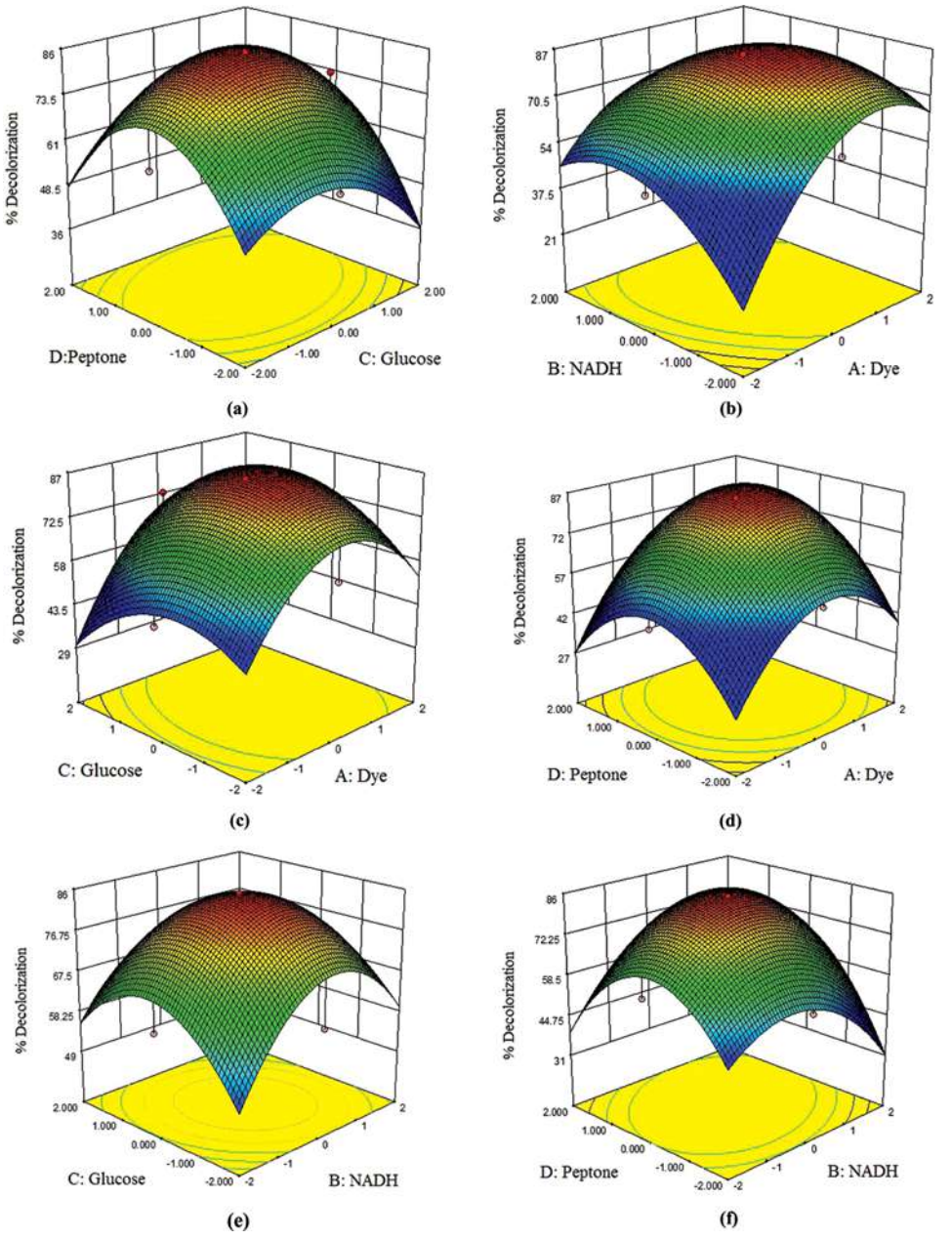


FIGURE 2 Three-dimensional (3D) response surface plot for decolorization activity by *Pseudomonas oleovorans* PAMD_1, showing the interaction between (a) glucose and peptone, (b) dye and NADH, (c) glucose and dye, (d) peptone and dye, (e) NADH and glucose, and (f) peptone and NADH (color figure available online).

$$\frac{\partial \hat{Y}}{\partial C} = 1.44A - 0.94B - 7.06C + 1.31D - 0.54 \quad (10)$$

$$\frac{\partial \hat{Y}}{\partial D} = 1.19A + 2.31B - 1.31C - 11.82D + 2.38 \quad (11)$$

$$\frac{\partial^2 \hat{Y}}{\partial A} = -11.82 \quad (12)$$

$$\frac{\partial^2 \hat{Y}}{\partial B} = -8.56 \quad (13)$$

$$\frac{\partial^2 \hat{Y}}{\partial C} = -7.06 \quad (14)$$

$$\frac{\partial^2 \hat{Y}}{\partial D} = -11.82 \quad (15)$$

The negative values from the Eqs. (12) to (15) indicate the lack of local maximum and applicability of maximization.[35] By equating Eqs. (8) to (11) to zero and solving for the variables A, B, C, and D, the optimum value of $\hat{Y}$ can be calculated:

$$-11.82A + 2.81B - 1.44C - 1.19D + 5.29 = 0 \quad (16)$$

$$-2.81A - 8.56B - 0.94C + 2.31D + 0.46 = 0 \quad (17)$$

$$1.44A - 0.94B - 7.06C + 1.31D - 0.54 = 0 \quad (18)$$

By solving Eqs. (16) to (18) the values of variables A, B, C, and D are 1.0, −0.15, 0.14, and 0.22, respectively. The corresponding uncoded values of Z_1, Z_2, Z_3, and Z_4 are 200 mg L^{-1}, 1.14 m*M*, 2.07 g L^{-1}, and 6.44 g L^{-1}, respectively. The result obtained for decolorization of orange II dye was about 87%. Experimental and predicted values shows good correlation with these optimum values and verify the validity of the response model. The optimization of dye decolorization by RSM showed an increase of approximately 10% over the unoptimized condition.

The developed model showed a better decolorization percentage when compared to pure cultures of *S. paucimobilis* (97.19%)[36] and bacterial consortium (90%)[3] for 100 ppm obtained through RSM. The decolorization efficiency of *Pseudomonas oleovorans* PAMD_1-produced azoreductase shows its potential application in the bioremediation for untreated textile effluents containing azo dyes.

Purification of Azoreductase

Table 7 summarizes the results of intracellular azoreductase purification by different steps described in the Materials and Methods section. Specific activity of the final purified enzyme was 26 U mg^{-1}. From the crude material about ninefold purification was achieved with 16% recovery. As a result of reduction in other proteins and low-molecular weight-substances from the extract, the specific activity of the enzyme is greatly increased.[37] Yan[38] reported higher azoreductase concentration achieved from the *Escherichia coli* crude extract, about 5.8% recovery with 9.3-fold purification. However, the activity comparison with the previous literature is difficult because of different kinds of dyes with various concentrations have been used for the decolorization studies.

Molecular Weight Determination

Figure 3 shows the appearance of a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purification fraction, and the molecular mass was estimated to be 29,000 Da. The single band appearance on native PAGE also proves the purified enzyme is in monomeric form (data now shown). This value of purified azoreductase was greatly supported by previous literatures form various microorganisms and also all are monomers.[11,25,39]

Spectrophotometer Analysis of Metabolites

Spectrophotometric analysis was carried out for Orange II and its degradation products by considering their λmax values. The λmax values for the Orange II and its metabolites were taken from the existing literatures.[40,41] Figure 4a shows the sole peak of absorbance at 482 nm for the dye solution, characterized by an azo bond (-N = N-), and the absorption at 482 nm is due to the color of Orange II solution. The peak at 482 nm

TABLE 7 Purification Profile of Azoreductase from *Pseudomonas oleovorans* PAMD_1

Purification Steps	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U mg^{-1})	Fold Purification	Yield (%)
Crude enzyme	500	987.5	2884	2.92	1	100
Ultrafiltration	50	262.5	2142	8.16	2.79	74.27
Ammonium sulfate precipitation (80%)	20	209	2060	9.85	3.37	71.43
DEAE-cellulose	8	18	468	26.00	9.00	16.23

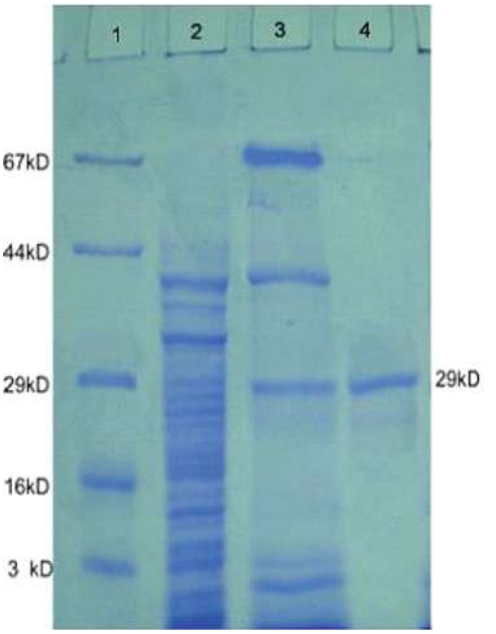


FIGURE 3 Molecular mass determination of purified azoreductase of *Pseudomonas oleovorans* PAMD_1 on SDS-PAGE. Lane 1, protein marker; lane 2, crude filtrate; lane 3, ammonium sulfate precipitation; lane 4, azoreductase purified by ion-exchange chromatography. Size markers are insulin (3 kD), lysozyme (16 kD), carbonic anhydrase (29 kD), ovalbumin (44 kD), and bovine albumin (67 kD) (color figure available online).

completely disappears, and no other apparent peaks were detected after the azoreductase treatment in the visible region (Figure 4b), which indicates the color removal due to the complete breakdown in the chromophore group.[42]

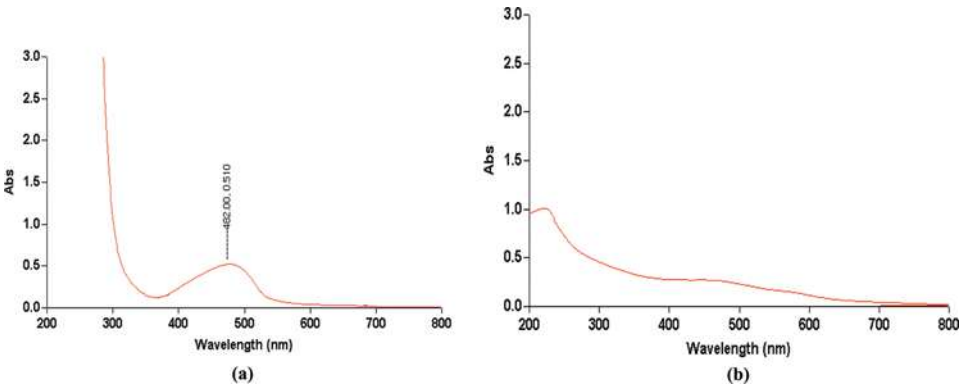
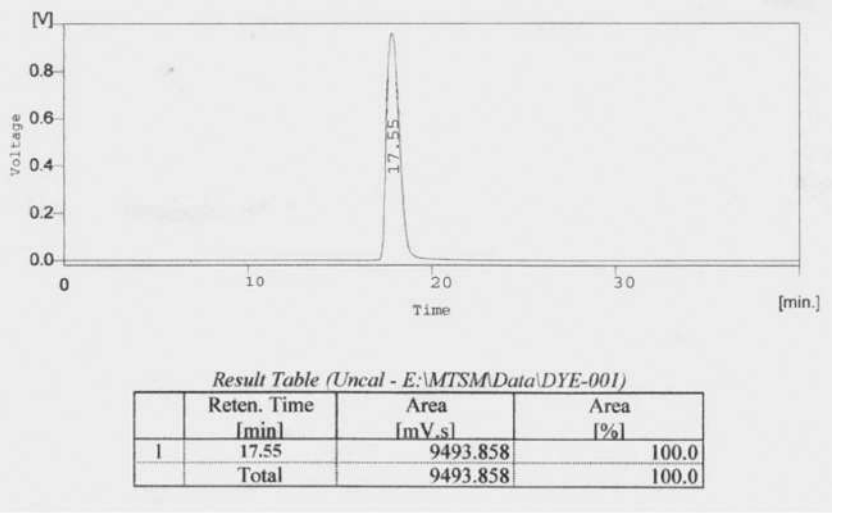


FIGURE 4 UV-vis absorption spectra of Orange II before (a) and after (b) enzymatic degradation by azoreductase from *Pseudomonas oleovorans* PAMD_1 after 48 hr (color figure available online).

HPLC Analysis of Metabolites

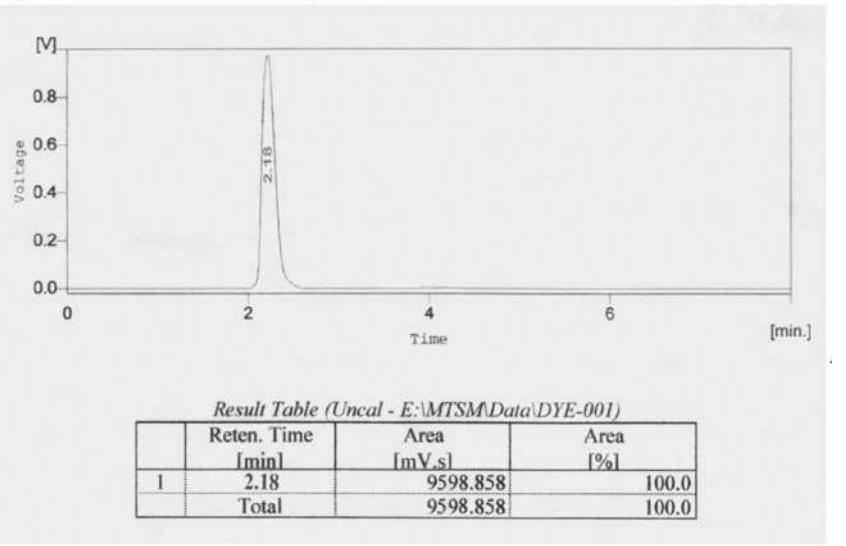
Figure 5a shows a single-peak high-performance liquid chromatogram (HPLC) of the assay mixture (without enzyme) corresponding to Orange II at a retention time of 17.55 min. The similar peak at the same retention



Result Table (Uncal - E:\MTSM\Data\DYE-001)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	17.55	9493.858	100.0
	Total	9493.858	100.0

(a)



Result Table (Uncal - E:\MTSM\Data\DYE-001)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.18	9598.858	100.0
	Total	9598.858	100.0

(b)

FIGURE 5 Chromatogram of the Orange II azo dye (a) without and (b) with azoreductase from *Pseudomonas oleovorans* PAMD_1.

time was obtained for the standard Orange II. Simultaneously, Figure 5b shows the HPLC chromatogram of the assay mixture (with purified enzyme), which depicts the disappearance of Orange II peak, and the appearance of a new peak with retention time 2.18 min. Metabolite of Orange II from the assay mixture was confirmed as sulfanilic acid (4-aminobenzenesulfonate) by comparing with retention time of the standard and previous degradation studies of Orange II dye. However, after Orange II degradation, aromatic amines such as sulfanilic acid (SA, 4-aminobenzenesulfonate) and 1-amino-2-naphthol (AN) were released. AN cannot be readily detected on HPLC due to its unstable nature.[43,44]

CONCLUSION

Pseudomonas oleovorans PAMD_1 produced azoreductase as the potential azo reductive enzyme during dye decolorization. Therefore, utilization of azoreductase for azo dye decolorization will be a practical approach. Optimization of the culture conditions for achieving maximum decolorization by using the RSM statistical tool was a suitable technical approach. In addition, the well-defined purification procedure with high yield and high specific activity can be used as a potential tool for bioremediation processes on untreated textile dyeing effluent.

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