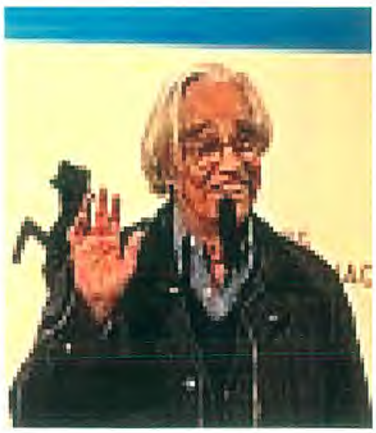


Robin L. Plackett (September 3 1920) was a statistician best known for his contribution to the history of statistics and to experimental design, most notably the Plackett-Burman design. Plackett–Burman designs are experimental designs presented in 1946 by Robin L. Plackett and J. P. Burman while working in the British Ministry of Supply. Their goal was to find experimental designs for investigating the dependence of some measured quantity on a number of independent variables (factors).

Chapter II

EXPERIMENTAL DESIGN TO OPTIMIZE THE DEGRADATION OF THE SYNTHETIC DYE ORANGE II BY AZOREDUCTASE FROM *PSEUDOMONAS OLEOVORANS PAMD_1*

ABSTRACT

Pseudomonas oleovorans PAMD_1 produced azoreductase as the more prominent enzyme that catalyze the reductive cleavage of the azo bridge (N=N) in azo dyes during the dye decolorization process. In order to optimize the catalytic action of azoreductase, statistically-based experimental designs were applied. Eleven components were screened for their significant effect on azoreductase production (% decolorization) using Plackett–Burman design. Dye, NADH, glucose and peptone were found to have highest positive influence on the azoreductase production. The concerted effect of these factors on azoreductase production was studied using Central Composite Design of Response surface methodology. This method showed that the medium containing dye (200 mgl^{-1}), NADH (1.14 mM), glucose (2.07 gl^{-1}) and peptone (6.44 gl^{-1}) to be optimum for the decolorization of Orange II up to 85.18 %. The applied methodology was validated through adequacy and the accuracy of the model, and results showed that the predicted value agreed well with the experimental values.

2.1 INTRODUCTION

Azo dyes and pigments are extensively used for several industrial applications such as textiles, leather, paper, pharmaceuticals and food industries. Azo dyes are xenobiotic compounds characterized by the presence of one or more azo linkages (-N=N-) and aromatic rings (Dias *et al.,* 2003). Azo dyes are known to be very toxic and mutagenic to living organisms (Moller and Wallin, 2000; Mechichi *et al.,* 2006). Discharge of waste water from textile industries results in pollution of aquatic systems (Keharia and Madamwar, 2003). Hence treatment of such dye containing effluent is essential to prevent deterioration of ecosystem (Sarayu Mohana *et al.,* 2008). Microbial degradation and decolorization of dyes is an environment friendly and cost competitive alternative to chemical decomposition process (Swamy and Ramsay, 1999; He *et al.,* 2004; Rodriguez Couto *et al.,* 2006). Various microorganisms have been found to reduce azo bonds with specific azoreductase enzyme system leading to carcinogenic dye decolorization (Khehra *et al.,* 2005). Hence, the color removal of azo dyes is the prime solution for the elimination of xenobiotics from the environment. Apparently, there exists a need to develop novel enzymatic decolorization processes leading to more effective clean up of azo dyes (Padmavathy *et al.,* 2003).

The growth of microorganisms, and consequently production of azoreductase, is influenced by the medium composition and the physical environment (Monteiro and De Carvalho, 1998). Screening and selection of the optimum concentration of medium components are very important to determine the overall economic feasibility of the production process and for industrial application. The reach of optimized fermentation conditions, particularly associated to physical and chemical parameters, is of primary and

great importance for the development of any process, due to their impact upon its economics and practicability (Levin *et al.*, 2005).

Different statistical designs has been used for the medium optimization in the production of various enzymes including protease, xylanase, amylase, lipase, glucanase, and laccase production by microbial cultures (Chhaya and Gupte, 2010; Khouni *et al.*, 2010; Rajendran and Thangavelu, 2009; Quaratino *et al.*, 2009; Majumder *et al.*, 2009; Deepak *et al.*, 2008; Palvannan and Sathishkumar, 2010). The Plackett-Burman experimental design was used to evaluate the relative importance of various nutrients for azoreductase enzyme activity. This design assumes that there are no interactions between the variables. A linear approach is considered to be sufficient for screening (Levin *et al.*, 2005; Palvannan and Sathishkumar, 2010).

Response surface methodology (RSM) provides information about the optimum level of each variable along with its interactions with other variables and their effects on particular response (Park *et al.*, 2002). It is also suitable for describing a near optimum region and exact conditions in a multifactorial system (Cavalitto and Mignone, 2007). This multivariate approach also improves statistical interpretation and relative significance of factors in the presence of complex interactions (Padma Iyer *et al.*, 2008).

In this chapter, we attempted to optimize decolorization of azo dye Orange II, by azoreductase produced by the isolated strain *Pseudomonas oleovorans PAMD_1*. Plackett-burman design (Plackett and Burman, 1946) followed by RSM was employed to optimize the culture conditions for the optimal catalytic activity of azoreductase from *Pseudomonas oleovorans PAMD_1*.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and dye

NADH, 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.

2.2.2 Microbial strain

The most active isolated microbial strain *P. oleovorans PAMD_1* (please refer first chapter) was used for the production of azoreductase. The growth condition parameters are as in the previous chapter.

2.2.3 Optimization of culture condition for decolorization

For any microbial decolorization process, media components such as carbon source, nitrogen source, dye concentration, redox mediators, pH and temperature are the most important parameters which affect the process (Wong and Yuen, 1996; Nachiyar and Rajkumar, 2003; Chen *et al.,* 2003; Khehra *et al.,* 2005). Hence it was necessary to investigate the influence of these variables on the production of azoreductase (decolorization process)*.

The optimization of azoreductase production was carried out in two steps, the first step involved the screening of significant variables and the second step involved the optimization of significant variables concentration for the maximum production of enzyme.

Note: * *Production of azoreductase denotes azoreductase activity represented interms of percentage of Orange II decolorization.*

2.2.3.1 Plackett-Burman design

For the present study the selected 11 variables were glucose, starch and sucrose (carbon source), peptone, yeast extract and ammonium sulphate (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. Table 1 shows the factors under investigation as well as levels of each factor used in the experimental design.

Trials were performed in triplicates and the average of decolorization observation results were treated as the response (Table 2). The highest positive variables (dye, NADH, glucose and peptone) influencing enzyme production (azoreductase mediated decolorization) on each category were selected from Pareto chart analysis. The levels of the significant variables were further optimized by Central composite design (CCD) of Response surface methodology (RSM).

2.2.3.2 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), as the percentage dye decolorization was the dependent response variable. Each of the independent variables was studied at five different levels (Table 3), with a total of 30 experiments. Percentage dye decolorization corresponding to combined effects of four components was studied with respect to, dye concentration (50 – 250 mgl^{-1}), NADH (0.4 – 2.0 mM), glucose (1 – 3 gl^{-1}) and peptone (2 – 10 gl^{-1}). The plans of CCD in coded levels of the four independent variables are shown in table -4. For statistical calculation independent variables were coded as,

$$X_i = \frac{Z_i - Z_0}{\Delta Z_i} \quad (1)$$

Where, X_i is the coded value of the variable, Z_i is the actual value (uncoded) of i^{th} independent variable, Z_0 is the midpoint value of the i^{th} independent variable (Sarayu Mohana *et al.*, 2008; Elibol, 2004), ΔZ_i is the step change value in Z_i and i= 1,2,3,4.

Dye decolorization (response), was explained as a second order response surface model in four independent variables.

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (2)$$

Where, $\hat{Y}$ is the predicted response (percentage decolorization); β_0 is a constant coefficient; 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 (Sarayau Mohana *et al.*, 2008).

2.2.3.3 Software and data analysis

The results of the experimental design were analyzed and interpreted using Design Expert 7.0 (Stat Ease, USA). Quadratic and interaction effects of the independent variables were evaluated for RSM. The Fisher's F-test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The statistical significance of regression coefficients were evaluated using Students t-test. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on azoreductase production*. The optimum values of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour plots (Myers and Montgomery, 2002). Experimental validation of the predicted model was also performed.

2.2.4 Analysis of decolorization

The percentage decolorization of the substrate Orange II was determined at their respective maximum absorption wavelength of 482 nm using a UV-Vis spectrophotometer (Shimadzu UV-2450). The efficiency of color removal was expressed as the percentage ratio of the decolorized dye concentration to that of initial one based on the following equation (Chen *et al.*, 2003).

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

2.3. RESULTS AND DISCUSSION

2.3.1 Plackett–Burman design

Statistical methods have proved to be a powerful tool for the medium optimization. Table I shows the elucidation of medium components affecting azoreductase decolorization, as examined by Placket-Burman design experiments. The data indicated that there was a wide variation of azoreductase activity* in the 12 runs. This variation reflected the importance of medium optimization to attain higher activity.

Fig 1 (Pareto chart) shows, the positive and negative influence of the screened variables on the azoreductase activity*. The presence of high level of dye concentration, NADH, glucose, peptone and yeast extract in the growth medium affects decolorization positively. The factors which showed highest positive influence in the pareto chart analysis were included in the RSM optimization.

Several studies have previously demonstrated the ability of bacterial dye decolorization (El-Sersy, 2001; Hu, 1994). Glucose has been identified as the best carbon source supporting maximum dye decolorization in certain earlier reports (Radha, 2005). Peptone and Yeast extract has been the most commonly used nitrogen source for dye decolorization processes with different organisms like *Pseudomonas luteola* (Hu, 1998), *Klebsiella pnuemoniae* (Wong and Yuen, 1996), *Bacillus* and *Clostridium sp.* (Knapp and Newby, 1995). Bacterial culture generally exhibit maximum decolorization at a neutral pH value and the rate of color removal tends to decrease rapidly at highly acidic or alkaline pH conditions (Pearce *et al.*, 2003; Adedaya *et al.*, 2004; Bhatt *et al.*, 2005).

Substantial differences in Orange II decolorization appeared as a result of changing the variables concentration as done by Plackett-Burman experiment. This is one of the advantages of applying multifactorial experiments that consider the interaction of independent variables and provide a basis for model to search for the non linear nature of the response in short term experiment (Ravikumar *et al.*, 2005).

2.3.2 Production optimization by Response surface methodology*

The highest positive influence factors in each category were selected for CCD-RSM experiments. The levels of each factor are given in Table 3. The results of 30 run CCD-RSM using four variables (dye, NADH, glucose, peptone) chosen for optimization of bacterial dye decolorization process are shown in Table 4. It shows the percentage decolorization of orange II dye corresponding to the combined effect of four components in their specified ranges. Decolorization varied with the conditions tested in the range of 42 - 87 %. Decolorization values above 85 % were observed when high concentration of

dye and peptone and low concentration of NADH and glucose were used. The experimental results suggest that these variables strongly affect the decolorization process. The experimental results were evaluated and confirmed by the following polynominal equation:

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

The above equation can be converted in to the uncoded unit where,

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

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

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

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

From the signs and importance of regression coefficient for the four variables, the decolorization process can be well interpreted. Concentration of the dye has linear effect on the response, maximum decolorization achieved at high dye concentration. It was reported that dye concentration can influence the efficiency of decolorization (Pearce *et al.*, 2003). Increasing the concentration of glucose shows increasing decolorization but higher concentrations were found to be inhibitory effect on decolorization in previous studies (Knapp and Newby, 1995). NADH had synergistic effect on dye decolorization. The increasing concentration has minimal significance on the enzyme activity. Hence, a proper choice of level combination of dye and glucose is desirable for maximizing

decolorization. However, the nutrient requirement for optimum decolorization depends on the nature of the microbial species used (Sarayu Mohana *et al.*, 2008).

The statistical analysis of equation (1) was checked by Fisher's 'F' test. Analysis of variance (ANOVA) for percentage decolorization shows that fitted response surface model is highly significant with F-test with a very low probability value (Prob>F=0.0006) as show in Table 5. The Fisher's 'F' test value (6.18) also implies a high significance for the regression model. Each of the observed values (Y_j) was compared with the predicted value ($\hat{Y}_j$) calculated from the model. All of these considerations indicate a good adequacy of the regression model. Table 6 shows the fitness of the model as checked by the R^2 (determination coefficient, 0.8523) and adjusted R^2 (0.7144), which indicate a high correlation between the observed and the predicted values. Therefore this significant model provides an explanation of the relationship between the independent variables and the response.

The response surface 3D plots were constructed by plotting the central values of the variables that influence the azoreductase activity* are given in figure 2a-f. The figure reveals the behavior of % decolorization with respect to the interaction between variables. Decolorization values above 85 % were obtained when dye, NADH, glucose and peptone concentration were at the coded value of 1.0, -0.15, 0.14 and 0.22 respectively.

Maximum and minimum principle of differential calculus was used to maximize the equation 3 with respect to individual tested variables. The partial differential equations obtained are:

$$\frac{\partial \hat{Y}}{\partial A} = -11.82A + 2.81B + 1.44C + 1.19D + 5.29 \quad (8)$$

$$\frac{\partial \hat{Y}}{\partial B} = -2.81A - 8.56B - 0.94C + 2.31D + 0.46 \quad (9)$$

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

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

The second order differential equations are:

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

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

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

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

The negative values of second order partial differential equations (equation: 12-15) indicate the absence of local maximum and applicability of maximization (Arulkumar *et al.*, 2010). The equations 8 to 11 are equated to zero and solved for A, B, C and D, which give the maximum value of $\hat{Y}$.

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

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

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

A = 1.0, B = -0.15, C = 0.14 and D = 0.22 values were obtained from the above equations (16-18). These values correspond to the uncoded values of Z_1= 200 mgl^{-1}, Z_2= 1.14 mM, Z_3= 2.07 gl^{-1} and Z_3= 6.44 gl^{-1}. At these optimum values, the maximum predicted azoreductase activity in terms of percentage decolorization of Orange II removal was 85.18 %. The simulated data was validated by carrying out the actual experiments with predicted variables. The results obtained for decolorization of orange II dye decolorization was 82.3 %. The good correlation between these two results verifies the validity of the response model and the existence of an optimal point. The optimization of dye decolorization by RSM showed an increase of approximately 20 % over the unoptimized condition.

The developed model showed a better decolorization percentage when compared to pure cultures of *S. paucimobilis* [(97.19 %), Lamia Ayed *et al.,* 2010] and bacterial consortium [(90 %), Sarayu Mohana *et al.,* 2008] for 100 ppm obtained through RSM. The decolorization efficiency of *Pseudomonas oleovorans PAMD_1* produced azoreductase indicates its potential application for decolorizing textile dyeing effluents containing azo dyes.

2.4 CONCLUSION

Pseudomonas oleovorans PAMD_1 produced azoreductase as the potential azo reductive enzyme during dye decolorization. Therefore, utilization of azoreductase for decolorization of azo dye (Orange II) seems to be a practical approach. The present study shows that RSM was an appropriate method to optimize the best culture conditions for obtaining maximum decolorization of the dye. By applying this experiment, we could analyze the process variable completely and achieve decolorization above 85 %. Moreover it indicates its potential application for decolorizing textile dyeing effluents.

Table 1: Plackett-Burman design for screening variables for azoreductase activity

Factors	Concentration	Code	Low level (-1)	High level (+1)	Effect
Glucose	gl^{-1}	X_1	0.5	1.5	Positive
Starch	gl^{-1}	X_2	0.5	1.5	Negative
Sucrose	gl^{-1}	X_3	0.5	1.5	Positive
Peptone	gl^{-1}	X_4	3.0	5.0	Positive
Yeast extract	gl^{-1}	X_5	3.0	5.0	Positive
Ammonium sulphate	gl^{-1}	X_6	3.0	5.0	Negative
Riboflavin	mM	X_7	0.5	1.5	Positive
NADH	mM	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 azoreductase activity in terms of percentage decolorization

Expt.	Variable levels (Coded)											% Decolorization*
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
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

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 (mgl^{-1})	A	50	100	150	200	250	50
NADH (mM)	B	0.4	0.8	1.2	1.6	2.0	0.4
Glucose (gl^{-1})	C	1.0	1.5	2.0	2.5	3.0	0.5
Peptone (gl^{-1})	D	2.0	4.0	6.0	8.0	10.0	2.0

Table 4: Full factorial CCD matrix and their observed responses for azoreductase activity using RSM

Run order	Dye mgl^{-1}	NADH mM	Glucose gl^{-1}	Peptone gl^{-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

Table 5: Significance of regression coefficients

Response 1 % Decolorization

ANOVA for Response Surface Quadratic Model

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	3654.30	29				

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, D^2 are significant model terms.

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

The "Pred R-Squared" of 0.1492 is not as close the the "Adj R-Squared" of 0.7144 as one might normally expect.

"AdeqPrecision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.065 indicates an adequate signal. This model can be used to navigate the design space.

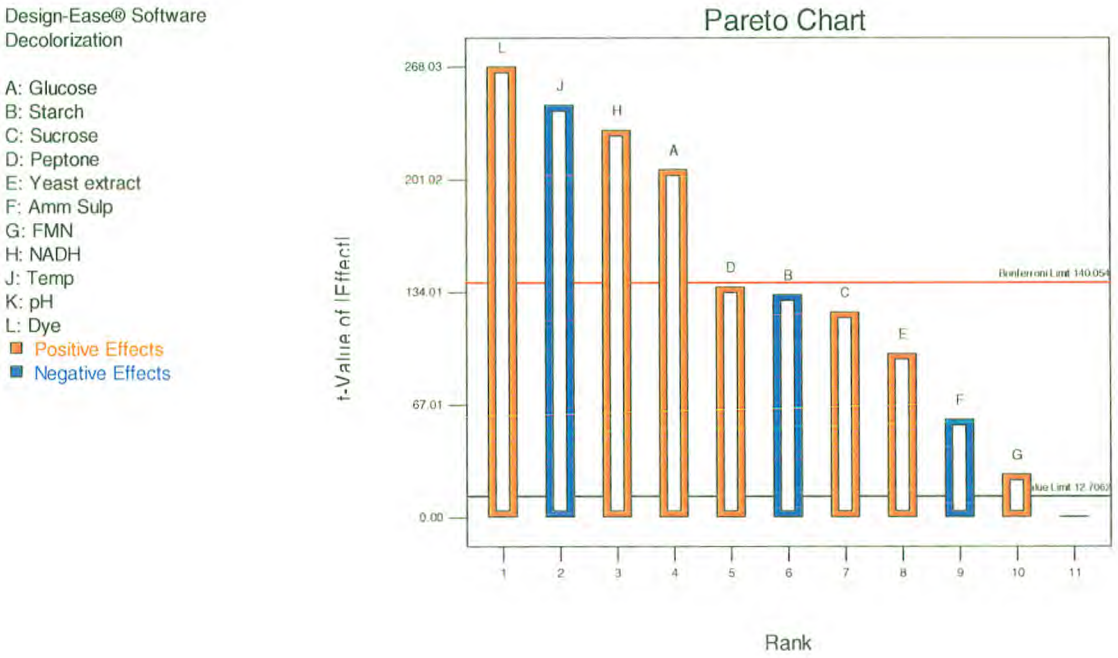
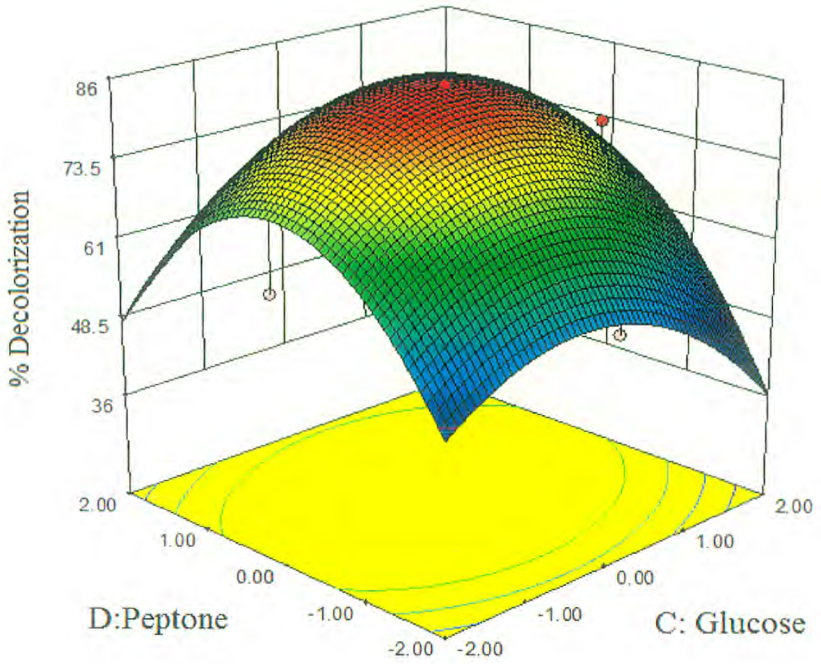
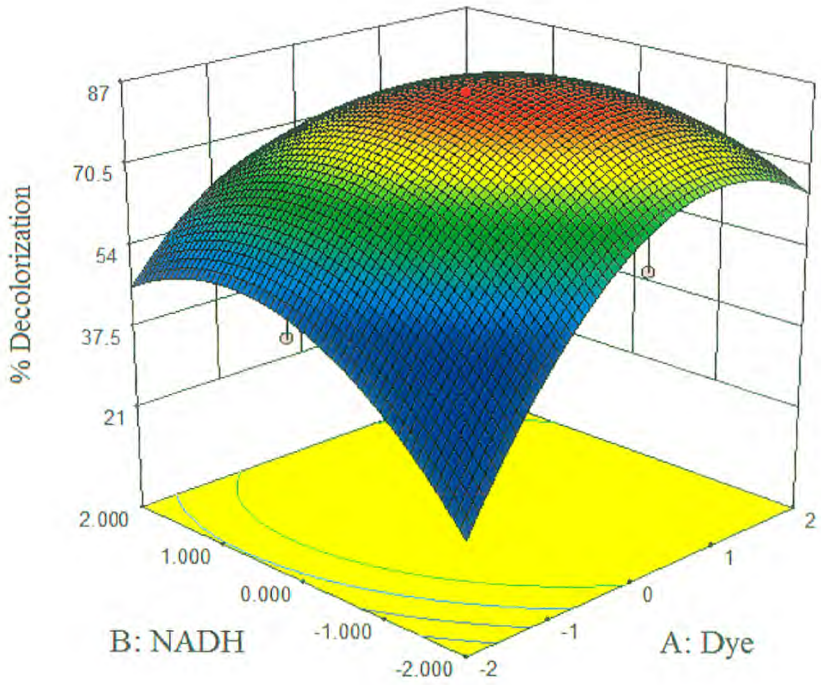


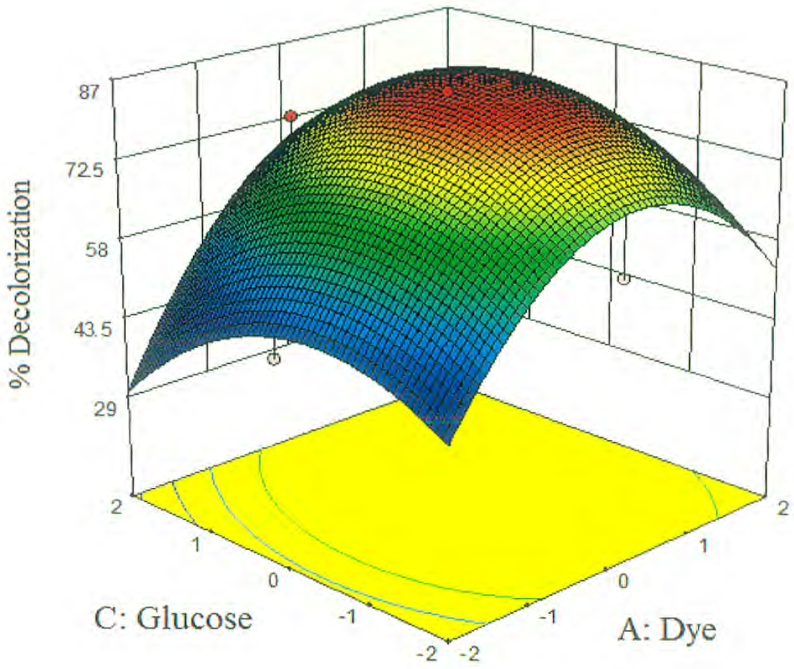
Figure 1: Pareto chart of eleven-factor effects on azoreductase activity


% Decolorization
86
73.5
61
48.5
36
2.00
1.00
0.00
-1.00
-2.00
D:Peptone
2.00
1.00
0.00
-1.00
-2.00
C: Glucose

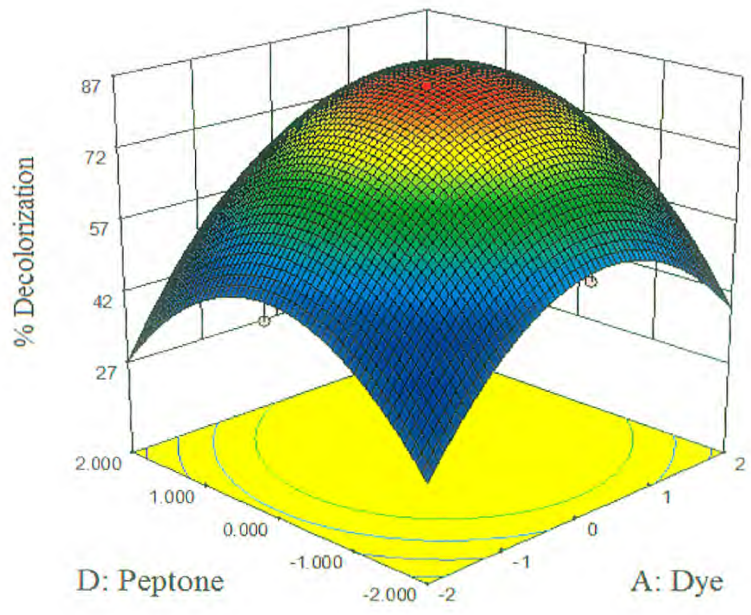
2 (a)


% Decolorization
87
70.5
54
37.5
21
2.000
1.000
0.000
-1.000
-2.000
B: NADH
2
1
0
-1
-2
A: Dye

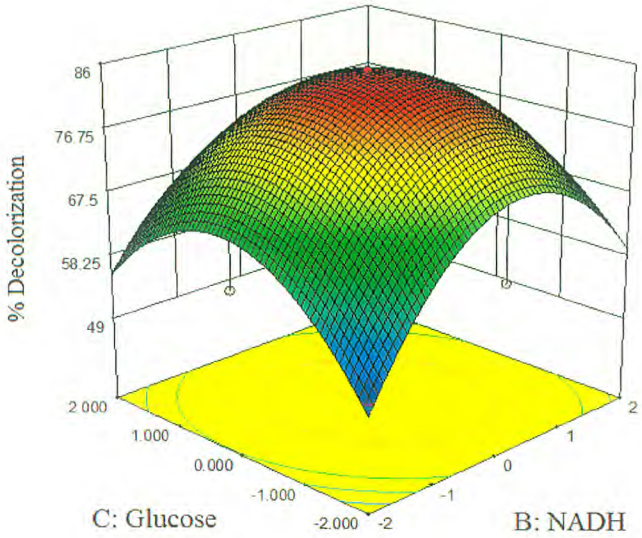
2 (b)


% Decolorization
87
72.5
58
43.5
29
C: Glucose
2
1
0
-1
-2
A: Dye
2
1
0
-1
-2

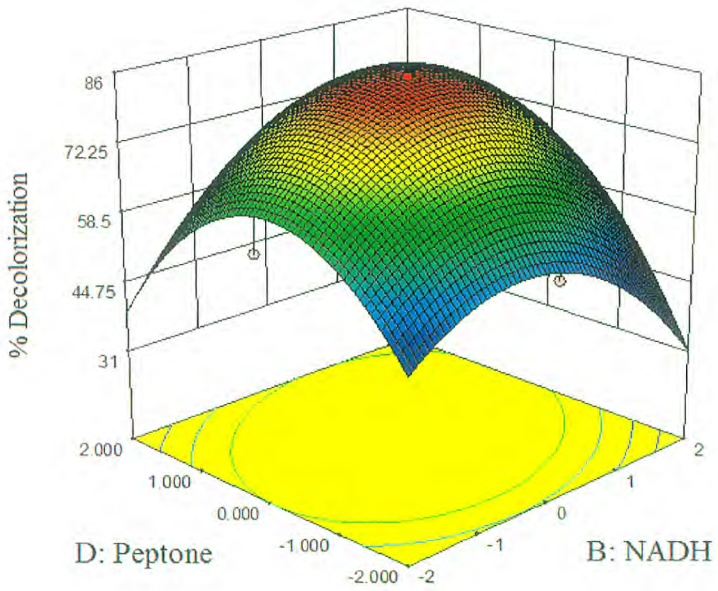
2 (c)


% Decolorization
87
72
57
42
27
D: Peptone
2.000
1.000
0.000
-1.000
-2.000
A: Dye
2
1
0
-1
-2

2 (d)



2 (e)



2 (f)

Figure 2: 3D response surface plot for azoreductase activity by *Pseudomonas oleovorans PAMD_1*, shows the interaction between (a) Glucose and peptone (b) Dye and NADH (c) Glucose and dye (d) Peptone and dye (e) NADH and glucose (f) Peptone and NADH.