LOGICAL AND EXPERIMENTAL DESIGN FOR PHENOL DEGRADATION USING IMMOBILIZED *ACINETOBACTER SP.* CULTURE

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ABSTRACT **:** Phenol and phenolic compounds cause severe pollutant to the nature. Phenol degradation using microbes has been attracted the attention of many researchers. In this study Plackett-Burman design has been used to map and optimize the points affect in the degradation process. Nine variables include pH (X_1), ^oC (X_2), glucose (X_3), yeast extract (X_4), meat extract (X_5), NH_4NO_3 (X_6), K-salt (X_7), Mg-salt (X_8) and trace elements (X9) as a media constituents are optimized during the phenol degradation process using immobilized *Acinetobacter sp.* According to Plackett-Burman design experiments the maximum degradation rate was 31.25 mg/l/h. Logical and statistical analysis of the data lead to select pH, Temperature and Meat extract as the most important three factors affecting on the phenol degradation rate. These three variables have been used in Box-Behnken experimental design, which result in degradation rate equal to 35.7 mg/L/hr. Microsoft Excel 2002 solver tool was used to optimize the model created from Box-Behnken. The calculated degradation rate was 37.30 mg/l/hr while the experimental degradation rate was 38.45 mg/L/hr, which prove the model accuracy. The use of logical analyses of the data covers the limitation of the statistical methods in selecting the correct variables. We recommended using logical and statistical data analysis for optimization the phenol degradation rate and other related processes.

KEYWORDS: *Plackett-Burman, Box-Behnken, solver, phenol, degradation, optimization, Acinetobacter sp.*

1. INTRODUCTION

Phenol is a monoaromatic compound produced naturally or chemically [1]. Phenolic compounds found in polluted water resources, industrial effluents of many applications like winedistillery, olive oil extraction, green olive debittering, cork preparation, wood debarking, detoxification of coffee husk etc., and land filled run off wastes [2-7]. Many strategies have been developed for controlling phenol and preventing it from causing harmful effect on Nature [8-10]. Microbes are able to utilize phenolic compounds as a carbon source [11]. Experimental design is a versatile tool for optimization different parameters and conditions [11, 12]. Plackett-Burman [11] and Box-Behnken [13] design

were used in optimization of the environmental and nutritional condition of the phenol degradation process and the results analyzed statistically using different statistical methods. Logical interpretation of the statistical analysis as well as the crude data proved useful for selecting the most effective variables. The results of the optimization studies obtained from this study proved to be promising.

2. MATERIALS AND METHODS

2.1 Microorganism

Microbial strain used in this study was isolated from soil and characterized using standard criteria and identified as *Acinetobacter sp.*

2.2 Immobilization Technique

Two-gram agar and 0.75 g sodium alginate were dissolved in 80 mL water and stirred well before sterilization. After sterilization, 20 mL cell suspension was added to the mixture. The mixture (3 mg dry weight cells/ml gel) was aseptically extruded drop-wise through a needle into a cold solution of 2% $CaCl_2$. The beads were transferred to a sterile phosphate buffer solution (1.46% K_2HPO_4 and 0.22% KH_2PO_4) for 2 h. The beads were then washed and transferred to the Mineral medium containing 0.5 g/L phenol. Glucose, yeast extract and meat extract were prepared as stock solution and sterilized using bacterial membrane filter (0.22 μm) and added with the quantities as described in Table 1, 2 and 6.

2.3 Media Content (g/L)

NH_4NO_3, 1.5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1.0; KH_2PO_4 0.5; NaCl, 0.5; $CaCI_2$, 0.02; (0-4 ml), pH was adjusted as desired. After autoclaving at 12l^oC for 20 min, trace elements solution was then added [14].

2.4 Trace Element Solution (g/L)

$FeSO_4.7H_2O$, 5 g; H_3BO_4, 25 mg; $CuSO_4.5H_2O$, 5 mg; KI, 5 mg; $CoSO_4.7H_2O$, 0.3 g; $MnSO_4.4H_2O$, 3 g; $ZnSO_4.7H_2O$, 5 g; $NaMoO_4.7H_2O$, 12 mg. The solution was filter sterilized with a 0.22 μm membrane filter.

2.5 Fermentation Procedures

Phenol degradation was conducted using 250 mL Erlenmeyer-flask containing 100 mL media and 20 mL gel (immobilized cells), at a shaking rate of 180 rpm. The media composition, pH, and temperature were changed when running both of Plackett-Burman and Box-Behnken design as described in Table 2 and 6.

2.6 Phenol Assay

The method described by Korenman *et al.* (1998) was used to determine the phenol degradation rate as mg/L/hr [15].

2.7 Experimental Designs

A. Media Constituents Effect

Two experiments with or without the media constituents as described above, in presence of 0.5 g/L phenol, were conducted. One experiment was conducted using all the above described media constituents while the other contain none of them. The phenol degradation rate was determined as mg/L/hr.

B. Plackett-Burman

Plackett-Burman design was used for screening the effect of nine variables include pH (X_1), temperature (X_2), glucose (X_3), yeast extract (X_4), meat extract (X_5), NH_4NO_3 (X_6), K-salt (X_7), Mg-salt (X_8) and trace elements (X_9). The experimental design was created following the design described by Plackett and Burman [12] where sixteen experiments were created to analyze the effect of the different variables on the phenol degradation rate as summarized in Table 1.

C. Multiple Regression Analysis of the Plackett-Burman Experiments

The results of the Plackett-Burman experiments were analyzed by multiple regression analysis using Microsoft Excel 2002 as described in details by Abdel-Fattah and Olama [16]. From the statistical analysis of the data as in Table 2, which has been tabulated in Table 3, the variables whose confidence levels ≥ than 90% were considered to be significant. Variables with confidence level between 90% and 70% were considered as being effective [17].

Table 1: Phenol degradation rate with or without media constituents

Expt. No.		pH	°C	Glucose g/L	Yeast extract g/L	Meat extract g/L	NH_4NO_3 g/L	K salt g/L	Mg salt g/l	Trace element ml/100 ml	Phenol degradation rate mg/L/hr
+	1	+1 (8)	+1 (40	+1 (15)	+1 (4)	+1 (4)	+1 (1.5)	+1 (1.5)	+1 (0.5	+1 (4)	0
-	18	-1 (4)	-1 (20	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	13.6

Table 2: Plackett-Burman experiments.

Expt. No.		pH	°C	Glucose g/L	Yeast extract g/L	Meat extract g/L	NH_4NO_3 g/L	K salt g/L	Mg salt g/l	Trace element ml/100 ml	Phenol degradation rate mg/L/hr
Plackett Burman Experiments	1	+1 (8)	-1 (20)	+1 (15)	+1 (4)	-1 (0)	+1 (1.5)	+1 (1.5)	+1 (0.5)	-1 (0)	0
	2	+1 (8)	+1 (40)	-1 (0)	+1 (4)	+1 (4)	-1 (0)	+1 (1.5)	+1 (0.5)	+1 (4)	31.25
	3	-1 (4)	+1 (40)	+1 (15)	-1 (0)	+1 (4)	+1 (1.5)	-1 (0)	+1 (0.5)	+1 (4)	0
	4	-1 (4)	-1 (20)	+1 (15)	+1 (4)	-1 (0)	+1 (1.5)	+1 (1.5)	-1 (0)	+1 (4)	0
	5	-1 (4)	-1 (20)	-1 (0)	+1 (4)	+1 (4)	-1 (0)	+1 (1.5)	+1 (0.5)	-1 (0)	8.62
	6	-1 (4)	-1 (20)	-1 (0)	-1 (0)	+1 (4)	+1 (1.5)	-1 (0)	+1 (0.5)	+1 (4)	17.86
	7	+1 (8)	-1 (20)	-1 (0)	-1 (0)	-1 (0)	+1 (1.5)	+1 (1.5)	-1 (0)	+1 (4)	15.63
	8	-1 (4)	+1 (40)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	+1 (1.5)	+1 (0.5)	-1 (0)	17.86
	9	-1 (4)	-1 (20)	+1 (15)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	+1 (0.5)	+1 (4)	0
	10	+1 (8)	-1 (20)	-1 (0)	+1 (4)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	+1 (4)	22.73
	11	+1 (8)	+1 (40)	-1 (0)	-1 (0)	+1 (4)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	31.25
	12	+1 (8)	+1 (40)	+1 (15)	-1 (0)	-1 (0)	+1 (1.5)	-1 (0)	-1 (0)	-1 (0)	0
	13	-1 (4)	+1 (40)	+1 (15)	+1 (4)	-1 (0)	-1 (0)	+1 (1.5)	-1 (0)	-1 (0)	0
	14	+1 (8)	-1 (20)	+1 (15)	+1 (4)	+1 (4)	-1 (0)	-1 (0)	+1 (0.5)	-1 (0)	0
	15	+1 (8)	+1 (40)	-1 (0)	+1 (4)	+1 (4)	+1 (1.5)	-1 (0)	-1 (0)	+1 (4)	27.78
	16	-1 (4)	+1 (40)	+1 (15)	-1 (0)	+1 (4)	+1 (1.5)	+1 (1.5)	-1 (0)	-1 (0)	0

D. Generating 1st Order Model

The model created from the analysis of Plackett-Burman experimental design [12] using multiple regression analysis is based on the 1st order-model:

$$Y = \beta_0 + \sum \beta_i X_i$$

Where Y is the predicted response, β_0 model constant, β_i variables linear coefficient.

E. Box-Behnken

Response surface using Box-Behnken experimental design, modeling and analysis were carried out using the Microsoft Excel 2000 and Essential Exp., Version 2.205 software. Box-Behnken experimental design was used to optimize three variables represented at three levels (high, medium and low) which were denoted by +1, 0 and -1 respectively. The variables were pH (X_1) 8, 4 and 6, Meat extract (X_2) 4, 2 and 3 g/L and Temperature (X_3) 40, 20 and 30^{o}C. Fifteen experiments were conducted as in Table 6.

F. Multiple Regression Analysis of Box-Behnken Experiments

The phenol degradation results obtained from the different Box-Behanken experiments were analyzed by multiple regression analysis using Microsoft Excel 2000 and Essential Exp., version 2.205 software.

G. 2nd Order Model

The created model was applied using the coefficient result of each variable. For the three variables this equation was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

X_1, X_2 and X_3 represent independent variables, β_1, β_2 , and β_3 are liner coefficients, β_{12} , β_{13} and β_{23} are cross product coefficients and β_{11} , β_{22} and β_{33} are the quadratic coefficients.

The various response surface and counter plots for each two variables and their related response (the phenol degradation rate) were performed using Microsoft Excel 2000 and Essential Exp., Version 2.205 software.

H. Microsoft Excel Solver Optimization

The value of each pH (X_1) , Meat extract (X_2) and Temperature (X_3) was further optimized to calculate the best Y (degradation rate) value using Microsoft Excel 2002 solver. The experiments for calculated optimum pH (X_1), Meat extract (X_2) and Temperature (X_3) were then conducted.

I. Determination of the 2nd Order Model Accuracy

To prove the accuracy of the model, the % accuracy was calculated from the following formula:

$$Accuracy\ of\ the\ model = [Y_{Experiment} / Y_{Calculated}] \times 100$$

3. RESULTS

3.1 Phenol Degradation Rate With or Without Media Constituents

The preliminary experiments for phenol degradation in the presence or absence of the media constituents indicated that the microbe could degrade phenol in absence of all of the media constituents. The presence of the media constituents appears to hinder the phenol degradation process while the absence of them give degradation rate of 13.6 mg/L/hr.

3.2 Statistical Analysis of Plackett-Burman Experiments

The experimental design using Plackett-Burman method was produced using +1 and -1 value for each variable as in Table 2 where 16 experiments have been conducted and the results are summarized as phenol degradation rate (mg/L/hr) as in Table 2. The variations in the results prove the importance of using experimental design in the media optimization. For each variable, the responses mean of the experiments with high value [+1] have been calculated using the following formula $(\sum+1)/n_{(+1)}$, while the responses mean of the experiments with the low value [-1] have been calculated using the following formula $(\sum-1)/ n_{(-1)}$. The main effect of the responses for each variable has been calculated from the following formula: Main effect = $\sum(+1)/n_{(+1)}-\sum(-1)/n_{(-1)}$. The different main effect of the different variables were calculated and summarized in Fig. 1 and Table 3.

According to the main effect analysis, pH, temperature, yeast extract, meat extract and trace elements give positive effect on the phenol degradation rate while glucose, NH_4NO_3, K-salt, Mg-salt give negative effect. The nine variables were analyzed using linear multiple regression analysis method and the % coefficient level were calculated from the *P* value and summarized in Table 4. The analysis of variance using ANOVA test was generated and summarized in Table 5 which gives $P = 0.0040$. This indicates that there is a statistically significant relationship between the variables at 99% confidence level.

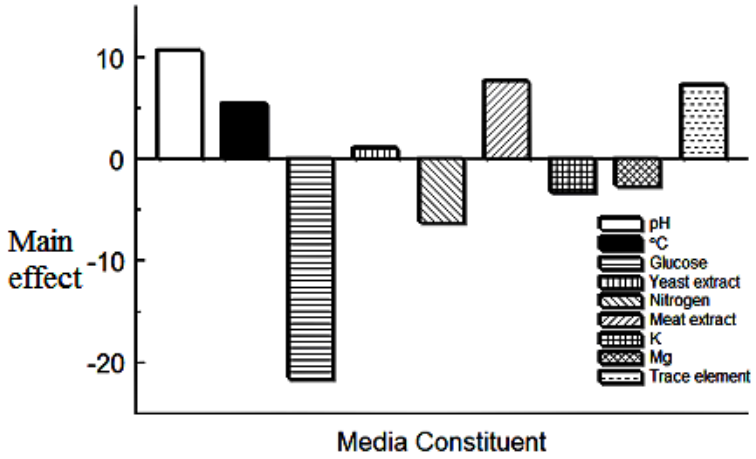

Fig. 1: Effect of environmental and media composition on phenol degradation rate using immobilized *Acinetobacter sp.* culture.

Table 3: Main effect.

Code	Variable	Values		Unit	Main effect $\sum(+1)/n_{(+1)}-\sum(-1)/n_{(-1)}$
		$(\sum+1)/ n_{(+1)}$	$(\sum-1)/ n_{(-1)}$		
A	pH (4/8)	16.08	5.5424		10.5375
B	Temperature (20/40)	13.5175	8.105	^oC	5.4125
C	Glucose (0/15)	0	21.6225	g/l	-21.6225
D	Yeast Extract (0/4)	11.2975	10.325	g/l	0.9725
E	Meat Extract (0/4)	14.595	7.0275	g/l	7.5675
F	NH_4NO_3 (0/1.5)	7.65875	13.96375	g/l	-6.305
G	K-Salt (0/0.5)	9.17	12.4525	g/l	-3.2825
H	Mg-Salt (0/0.5)	9.44875	12.17	g/l	-2.725
I	Trace element (0/4)	14.4062	7.216	mL/100 mL	7.19

Table 4: Linear multiple regression analysis of Plackett-Burman experiments.

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	10.8112	1.16063	9.31496	0.0001	99.99
Temperature	2.98734	1.41294	2.11427	0.0789	92.11
Glucose	-8.77488	1.54846	-5.66683	0.0013	99.87
k-salt	-0.0576222	1.54846	-0.0372125	0.9715	2.85
Meat extract	0.969583	1.51081	0.641766	0.5447	45.53
Mg-salt	-0.563177	1.41294	-0.398585	0.7040	29.6
NH_4NO_3	-1.71674	1.52635	-1.12473	0.3037	69.63
pH	2.89627	1.46897	1.97163	0.0961	90.39
Trace element	2.5629	1.46897	1.74468	0.1317	86.83
Yeast extract	0.094239	1.52635	0.0617413	0.9528	4.72

Table 5: ANOVA test of Plackett-Burman experiments.

Analysis of Variance					
Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	2198.62	9	244.291	11.33	0.0040
Residual	129.319	6	21.5531		
Total (Corr.)	2327.94	15			

R-squared = 94.4449 percent
R-squared (adjusted for d.f.) = 86.1123 percent
Standard Error of Est. = 4.64253
Mean absolute error = 2.36908

The *R*-squared statistic indicates that the model as fitted explains 94.4449% of the variability in the degradation rate. The adjusted *R*-squared statistic is 86.1123%. The standard error of the estimate shows the standard deviation of the residuals to be 4.64253. The mean absolute error of 2.36908 is the average value of the residuals.

The multiple linear regression model of Plackett-Burman screening method describes the relationship between the phenol degradation rate and 9 independent variables as indicated in Table 3 and 4. The equation of the fitted model is:

$$Y_{Degradation\ rate} = 10.8112 - 8.77488*Glucose - 0.0576222*K\text{-}Salt + 0.969583*Meat\ extract - 0.563177*Mg\text{-}Salt - 1.71674*NH_4NO_3 + 2.89627*pH + 2.98734*Temperature + 2.5629*Trace\ elements + 0.094239*Yeast\ extract$$

From the statistical analysis, Glucose, Temperature and pH significantly effect the phenol degradation rate while they have confidence level > 90%. The trace elements are considered as being effective [17], whereas NH_4NO_3 which give 69.63 % confidence level might be affected by the presence of other media constituents containing nitrogen like meat extract.

3.3 Statistical Analysis of Box-Behnken Experiments

Based on the *t*-statistic and *P* values as well as the % confidence level obtained from Plackett-Burman experiments (Table 3), Temperature and pH have positive effect and being statistically significant. They were selected for further optimization.

Glucose which has a negative effect and statistically being significant in phenol degradation has been omitted from the media where it gives the optimum degradation rate when it equals to 0 mg/L/hr. NH_4NO_3 is omitted too while it has % confidence level less than 70%, even it is so close to 70% (the minimum accepted limit) [17]. Trace elements were not used based on its performance in experiment 2 and 11. To get the optimum conditions in Box-Behnken, variables were used with the same amounts which give maximum phenol degradation rates according to Plackett-Burman experiment no. 11 (Table 2). Based on experiment no. 11 the media constituents which have 0 amount were omitted. These constituents represent glucose, yeast extract, NH_4NO_3, K-salt, Mg-salt and trace elements (Table 2). Meat extract, which contains different nutrients could substitute these constituents as shown in experiment no. 11 (Table 2). pH (X_1) was used in three level 8, 6, 4; Meat extract (X_2) 4, 2, 0 g/L and Temperature (X_3) 40, 30, 20 ^oC. The Box-Behnken was created using 15 experiments and the phenol degradation rate was determined and summarized in Table 6.

Table 6: Box-Behnken experiments.

Experiment No.	pH X_1	Meat Extract X_2	^oC X_3	Phenol degradation rates mg/L/hr
1	6 (0)	0 (-1)	40 (1)	25
2	6 (0)	2 (0)	30 (0)	35.7
3	6 (0)	4 (1)	40 (1)	22.73
4	4 (-1)	0 (-1)	30 (0)	15.63
5	6 (0)	2 (0)	30 (0)	35.7
6	6 (0)	0 (-1)	20 (-1)	25
7	4 (-1)	2 (0)	40 (1)	25
8	4 (-1)	2 (0)	20 (-1)	17.86
9	8 (1)	2 (0)	40 (1)	22.81
10	4 (-1)	4 (1)	30 (0)	17.86
11	8 (1)	4 (1)	30 (0)	27.78
12	8 (1)	2 (0)	20 (-1)	31.25
13	6 (0)	2 (0)	30 (0)	35.7
14	6 (0)	4 (1)	20 (-1)	25
15	8 (1)	0 (-1)	30 (0)	35.7

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where X_1=pH , X_2=Meat extract and X_3= ^oC

Table 7: Linear multiple regression analysis of Box-Behnken experiments.

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	35.7	1.74571	20.4501	0.0000	
X1	5.14875	1.06903	4.8163	0.0048	99.52
X1X1	-5.83	1.57356	-3.70497	0.0139	98.61
X1X2	-2.5375	1.51183	-1.67843	0.1541	84.60
X1X3	-3.895	1.51183	-2.57635	0.0497	95.03
X2	-0.995	1.06903	-0.930754	0.3947	60.56
X2X2	-5.6275	1.57356	-3.57628	0.0159	98.40
X2X3	-0.5675	1.51183	-0.375373	0.7228	27.71
X3	-0.44625	1.06903	-0.417436	0.6937	30.61
X3X3	-5.64	1.57356	-3.58422	0.0158	98.42

Table 8: ANOVA test of Box-Behnken experiments.

Analysis of Variance					
Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	621.23	9	69.03	7.550	0.01924
Residual	45.71	5	9.143		
Total (Corr.)	666.95	14			

R-squared = 93.1433 percent
R-squared (adjusted for d.f.) = 80.8011 percent
Standard Error of Est. = 7.56375
Mean absolute error = 3.42139

The analysis of variance used in Box-Behnken experiment (Table 6) was created using ANOVA test as in Table 8 which gives $P = 0.01924$. Since the P-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the variables at the 95% confidence level. The R-squared statistic suggests that the model as fitted explains 93.146% of the variability in the degradation rate. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 80.8087%. The standard error of the estimate shows the standard deviation of the residuals to be 3.02366. The mean absolute error of 1.36667 is the average value of the residuals.

The multiple linear regression model of the Box-Behnken analysis experiments describe the relationship between the degradation rate and 3 independent variables as in Table 6 and 7. The equation of the fitted model is:

$Y_{Degradation\ rate}$ = *35.7 - 5.83* pH* pH - 2.5375* pH *Meat extract - 3.895* pH *Temperature - 5.6275* Meat extract* Meat extract – 0.5675* Meat extract * Temperature - 5.64* Temperature * Temperature - 0.995*Meat extract+ 5.14875*pH - 0.44625*Temperature*

From the statistical analysis, it can be concluded that pH is the most significant variable affecting the phenol degradation rate. The best level of the three variable as obtained from the maximum point of polynomial model were estimated using the solver function of Microsoft Excel 2000 tool and found to be pH =7.12, Meat extract = 1.6 g/L, and Temperature= 27.77^{o}C, with a prediction calculated degradation rate equal to 37.30 mg/L/hr. The various response surface and counter plots for each two variables and the response (the phenol degradation rate) were drawn using Excel 2000 and Essential Exp. Version 2.205 software as shown in Fig. 2 to 7.

The data obtained from the response surface, counter plots and Table 7, show that pH and ^{o}C are the most significant variables affecting on the phenol degradation rate. The interaction between pH and ^{o}C is significant [16] with confidence level % equal to 95.03, and between pH and meat extract is effective [17] with confidence level % equal to 84.60. On the other hand the interaction between meat extract and temperature is insignificant with confidence level % equal to 27.71. This also clearly proves that pH is the most effective variable.. Meat extract has a limit in improving the degradation rate but plays an essential role in stabilizing the media composition. The role of meat extract must be subjected to further investigation concerning its composition.

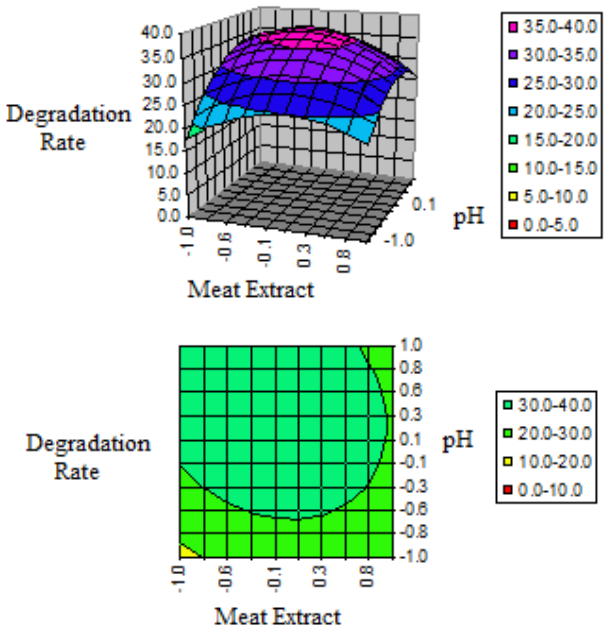

Fig. 2 and 3: The response surfaces plot and corresponding contour plot showing the effects of Meat extract (above) and pH (below) on phenol degradation rate.

The shape of response surface and counter plots (Fig. 2 and 3) show high interaction between pH and meat extract. The results obtained from Fig. 4 and 5 concerning the interaction between pH and temperature show high interaction. In case of Fig. 6 and 7 the interaction between meat extract and temperature gives only small interaction. The results obtained from Fig. 2 to 7 agree with that in Table 7.

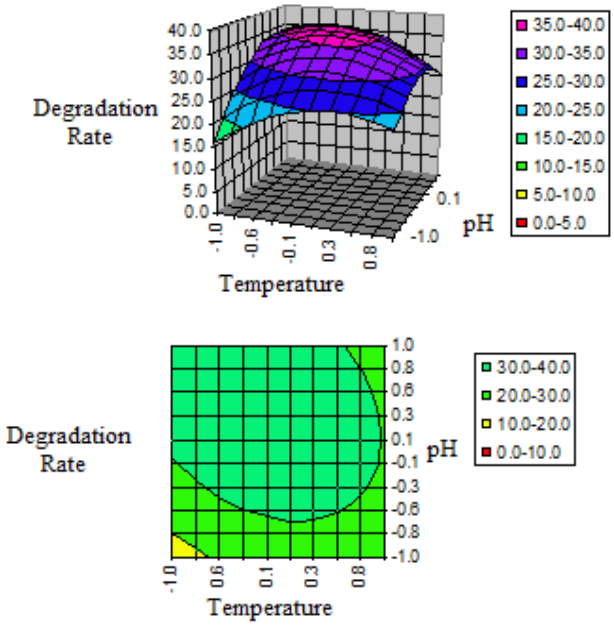

Fig. 4 and 5: The response surfaces plot and corresponding contour plot showing the effects of Temperature (above) and pH (below) on phenol degradation rate.

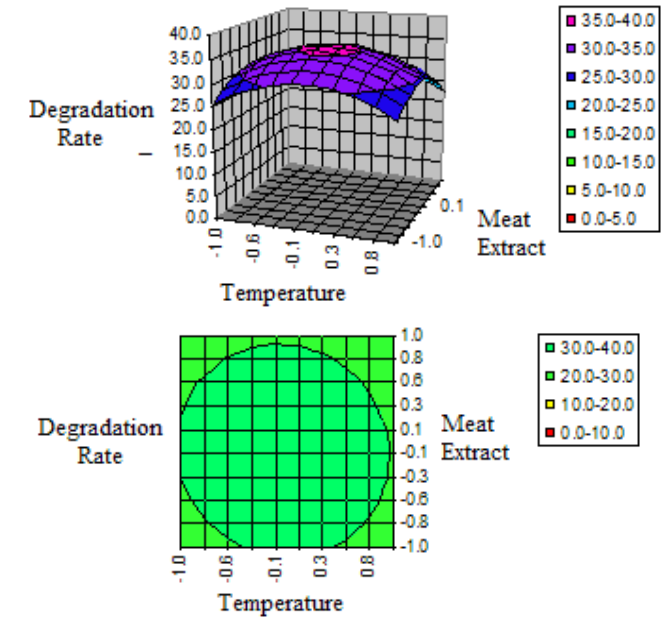

Fig. 6 and 7: The response surfaces plot and corresponding contour plot showing the effects of Meat extract (above) and Temperature (below) on the phenol degradation rate.

3.4 Confirming Accuracy of Model

The *Y* value which has been optimized from the model was calculated using Microsoft Excel found to be 37.30 at pH=7.12, meat extract=1.6 and temperature=27.77 ^{o}C. The *in vivo* experiment shows that *Y* value of 38.45 mg/L/hr. The calculated model accuracy was 103.08 %.

4. DISCUSSION

Degradation of chemical compounds is an important microbial criterion. Phenol, which is a toxic natural or synthetic compound, can be degraded to non-toxic by different microbes [1-11]. Phenol can be degraded by aerobic and anaerobic bacteria [18-20]. Phenol can be a growth inhibitor to microorganisms at elevated concentration [21]. This criteria lead to a conclusion that each strain, which is able to degrade phenol should be treated, analyzed and optimized as a single case.

Optimization of the media constituents as well as the other environmental variables like pH, temperature, etc., using well established experimental design can map the conditions affecting on the different processes concerning media optimization [21-25]. While biological system is complex and can be affected by unknown factors, using Plackett-Burman analysis could detect the weak and the strong points in the process under investigation [12]. Microbes have unique criterion and they are usually prefer and utilize simple carbon source such as glucose as opposed to complex carbon sources. In this study the presence of glucose in the media omit any chance that the bacterial strain start to degrade phenol as shown in Table 1 and experiments no. 3, 4, 9, 12, 13, 14 and 16 as (Table 2). The results from Table 1 lead the use of 0 amount as a low level [-1] to investigate which of the media constituents could be removed. The absence of glucose forces the microbe to degrade the phenol as shown in Table 2. Glucose as an example shows a significant effect on the degradation rate. Meanwhile, the main effect analysis as in Table 3 and Fig. 1 show that this to be a negative effect. Using glucose in 0 g/L gave the best result as indicated in Table 2. For these reasons, even glucose has the highest % confidence level, it is not considered. The main effect as shown in Table 3 and Fig. 1 gave clear view about each variable's mode of action. Main effect classified the variables to positive and negative, which support the analysis of variables and highlight in which direction the optimization should be conducted.

This study does not follow the usual way of optimization. Using the usual way of optimization will lead to select glucose, temperature and pH for further optimization (e.g Box-Behnken method). Temperature and pH are significant variables with % confidence level equal to 92.11 and 90.39, respectively and have been selected for optimizations. Based on the results obtained from experiment no. 11 using Plackett-Burman design beside glucose; yeast extract, NH_4NO_3, K-salt, Mg-salt and trace elements have been omitted while they gave the best degradation rate when they were at 0 amount as in Table 2. In experiment no. 2 and 11, meat extract was in its high value [+1] (4 g/L). Comparing the media constituents in experiment no 2 and 11 lead to a conclusion that meat extract perfectly could substitute the other factors used in 0 amount. However, this fact was not detected by statistical analysis.

Box-Behnken experimental design was performed using 15 experiments (Table 6), and four experiments give phenol degradation rate of 35.7 mg/L/hr, which indicate a possibility for further optimization. Using the solver tool in Microsoft Excel 2002 to optimize the model created from Box-Behnken the calculated degradation rate was 37.30 mg/L/hr in the optimum calculating condition. To prove the efficiency of using the Microsoft Excel solver the *in vivo* experiments were conducted. The degradation rate for the *in vivo* was 38.45 mg/L/hr.

Using direct phenol degradation (without media) gives 13.6 mg/L/hr. By using Plackett-Burman the maximum degradation rate was 31.25 mg/L/hr which represent 2.3 time increase than that at Table 1. Using Box-Behnken design the maximum degradation rate was 35.7 which is 2.71 fold increase than that without media and 14.24% more than Plackett-Burman. Using Excel solver optimization gives 37.30 mg/l/hr degradation rate mathematically and 38.45 mg/L/hr degradation rate experimentally. The *in vivo* experiment shows 103.08% model accuracy which indicates the advantage of the experimental design for optimization. Applying the conditions which were optimized using Excel solver for *in vivo* experiment result in degradation rate equal 38.45 mg/L/hr which is 2.92 fold increase than that without media and which is 23.04% more than Plackett-Burman and 7.70% more than Box-Behnken.

Different strategies have been developed for controlling phenol and preventing it from causing harmful effect on Nature [8-10]. Using immobilized technique has also been described [25]. The main problems in different strategies are the phenol degradation optimization. Most of the studies describe analysis of one variable at a time, which usually did not give the maximum expected optimization and is time consuming. Media optimization using experimental design proved to be more efficient [26]. As what has been done in this study many authors described using different nutrients to improve the growth of microbial strains which will be finally improve phenol degradation rate under some controlling conditions. An example is the glucose has been used to overcome the inhibitory effect of phenol on microbial growth as described by Mamma *et al*. [27]. In this study. glucose completely inhibit the phenol degradation which prove our concept that each microbe should be treated as a special case.

Experimental design using Plackett-Burman, Box-Behnken and Excel solver lead to subsequent improvements in phenol degradation rate. Logical analysis of the data leads to a better understanding of the roles of variables and investigation as to which one should be used. Statistical analysis help a lot in understanding the behavior of each variable but variables which gave the same results should be subjected to logical analysis with the aim to obtain the correct choice.

We should highlight that the phenol used in this study did not represent reality while in fact phenol was usually present as a contaminant with other wastes like olive oil. This fact should be taken into consideration in any future studies aimed. While Plackett-Burman and Box-Behnken are well-established tools for experimental design, we recommend using our strategy for the best use of both of them.

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