



## RESEARCH ARTICLE

### OPTIMIZATION OF BIODEGRADATION PROCESS OF RIVER WATER CONTAINING PHENOL USING RESPONSE SURFACE METHODOLOGY BY *PSUEDOMONAS PUTIDA* (*P. PUTIDA*)

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#### ABSTRACT

The biodegradation process was investigated using an isolated single bacterial culture of *P. putida*. The *P. putida* was able to reduced 75-80% pollution load within 48 hrs under aerobic condition as term of Chemical Oxygen Demand (COD) in River Water containing Phenol (RWP). Obtained data showed that *P. putida* was potent bacterial culture for RWP treatment. The influence of three process parameters such as temperature (20-50°C), pH (4-9) and agitation speed (30-150 rpm) influenced the rate of biodegradation extent. Central composite design (CCD), an experimental design for response surface methodology (RSM), was used to create a set of 36 experimental runs needed for optimizing operating conditions. The optimal degradation conditions were determined by response surface methodology based on three-variable central composite design to obtain maximum reduction and to determine the significance and interaction effect of the variables on degradation. The optimal conditions of response were found to be 35 °C, pH 8.05 and 120 rpm agitation speed, respectively, giving an experimental degradation value of 80.07%. Very high regression coefficient between the variables and the response ( $R^2 = 0.6447$ ) indicated a good evaluation of experimental data by polynomial regression model.

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## INTRODUCTION

River water containing phenol (RWP) is considered as an emerging environmental problem due to their toxicity and chemical persistence in the environment. They can remain in the environment for a long time and their residence time is considered as dangerous at both low and high concentrations. It has been estimated that up to half of the wastewater produced worldwide is released without any treatment and the components are difficult to destroy or decompose by common treatment in conventional wastewater treatment plants and discharged in to the river streams (Baughman and Weber 1994; O'Neill *et al.*, 2000). Hence, the treatment strategy for such type of wastewater is under considerable pressure to efficiently and effectively treat the effluents discharged to the environment to comply with environmental legislation restricting the discharge of wastewater and to prevent deterioration of ecosystems. However, these techniques have shown limited success for the treatment of wastewater due to the nature and composition of effluents. Therefore, other technologies have been explored with the aim to further reduce

the concentration of phenolic contaminants in wastewaters. These technologies include membrane separation, advanced oxidation technology, and electrochemical techniques such as electro-flotation and electro-coagulation. Electro-coagulation has shown the potential to largely overcome the drawbacks of conventional processing techniques. Until now, the effects of environmental factors on microbial degradation of complex wastewater are usually examined with the conventional single-factor optimization (Parshetti *et al.*, 2006; Khataee *et al.*, 2009) in which experiments were conducted by varying systematically the studied parameter while keeping other parameters constant (Sedighi *et al.* 2009). This is usually repeated for all the parameters influencing degradation thus, resulting in an unreliable number of experiments. In addition, the combined effect of the effective influence parameters cannot be determined using this exhaustive procedure. Hence, a novel experimental design method such as the response surface methodology (RSM) which can estimate quadratic effects and linear interaction of the factors and predict a model for the response with a minimum number of experiments could be a useful tool for optimization of effective parameters of water containing phenol degradation. Here, we report the isolation and identification of a phenol degrading bacterial culture. *P. putida*, capable of degradation of generated high pollution load

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in a aerobic process. There are almost no reports on the biodegradation of phenol by *P. putida* and hence, it was significant to develop this new microbial procees in bioremediation. The effect of process parameters for degradation was determined by an optimized condition using the Response surface methodology (RSM) based on central composite design (CCD).

**MATERIALS AND METHODS**

**Sample collection area and its preservation**

Water samples of river Gola were collected in Tarson bottles previously rinsed with non-ionic detergent, rinsed with tap water and later leached with 10% nitric acid for 24 hours and finally rinsed with deionised water prior to use. This process has importance because certain metal ions like chromium, cadmium, lead are subject to loss or adsorption or ion exchange with walls of container. Before sample collection these bottles were rinsed with the sample. All the samples after maintaining in cold chain were brought to the laboratory to store in sampling box at 4°C.

**Culture media, strain isolation and characterization**

Nutrient Agar Media and Tris Minimal Salt Media were used for the isolation. Further, the shake flask studies were conducted using river wastewater supplemented with minimal mineral media. The soil samples for the isolation of the microorganisms were collected in the sterilized zip lock polybags to prevent environmental contamination. The samples were immediately transported to the lab and maintained below 4°C, till their utilization. For isolation of microbes serial dilution method was adopted; 1 g of contaminated soil sample was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of nutrient broth medium and spread in Nutrient Agar Media at 37°C in 24 hrs. isolates were identified by biochemical characterization and bacterial growth kinetics. The organism was used for subsequent treatment and optimization studies.

**Treatment of river water by *Psuedomonas putida* (*P. putida*)**

The treatment capability of *P. putida* was determined using river water (100 ml/l) in minimal salt media. Bacterial culture was inoculated in pre-autoclaved minimal salt media (10<sup>7</sup> cfu/ml approximate cell density, pH-7.2) and incubated 35°C for 48 hours under shaking condition. Using the above performance parameters aerobic bacteria was encourage to decrease the high pollution load in 48 hours incubation time and determine the equilibrium time required for maximum treatment of RWP. Control consists of river water and minimal salt media without bacterial culture. Further experiment were performed to determine the effect of incubation temperature, pH and agitation speed condition on river water treatment by varying the incubation temperature (20–45°C), medium pH (5–9) and shaking condition (50–150 rpm), while keeping other conditions constant. The pH of the MSM solution was adjusted using 0.1 M HCl or 0.1 M NaOH. All the experiments were performed in triplicates.

**Optimization of treatment conditions by response surface methodology**

RSM is a collection of mathematical and numerical techniques that are useful for modelling and analysis of the processes

having numerous variables influencing the response and the objective is to optimize process settings in an efficient use of the resources (Sharma et al., 2009). It can be used for predicting the functional relationship between a set of experimental design variables and a response variable. The RSM approach was applied to determine the optimal levels of three input variables, namely temperature (factor A), pH (factor B), incubation time (factor C) and agitation speed (factor D) and to identify the relationship between the response functions and process variables. Design Expert 8.0.2 software was used to analyze the obtained results. The values used were based on results of preliminary experiments carried out to determine range of values of parameters for effective treatment. For four variables (n = 3) and five levels [low (-) and high (+)], the total number of experiments was 36 determined by the expression: 2n (2<sup>3</sup> = 8 factorial points in triplicates) + 2n (2×3 = 6 axial points) + 6 (center points: six replications) as given in Table 1.

**Table 1. Observed and predicted values of RWP treatment by *P. putida* using of RSM**

Run	temperature	pH	Agitation speed	% response
1	32.5	7.5	90	69.88
2	32.5	7.5	222.8	60.08
3	45	10	30	76.09
4	45	10	150	45.05
5	20	10	150	32.02
6	45	10	150	34.06
7	45	10	150	34.03
8	20	10	30	15.05
9	32.5	7.5	90	68.32
10	45	5	30	45.76
11	20	10	150	17.03
12	20	5	150	17.04
13	20	5	30	16.23
14	20	5	30	16.5
15	32.5	13.0	90	11.03
16	4.8	7.5	90	12.03
17	32.5	7.5	90	80.02
18	45	5	150	55.97
19	45	10	30	63.07
20	45	5	30	55.06
21	20	5	30	66.04
22	32.5	7.5	90	82.09
23	45	10	30	70.03
24	20	5	150	55.06
25	45	5	150	33.04
26	20	10	30	35.05
27	20	10	150	50.09
28	45	5	150	44.04
29	20	10	30	44.07
30	60.2	7.5	90	11.03
31	32.5	7.5	90	88.04
32	32.5	1.97	90	10.04
33	45	5	30	56.02
34	32.5	7.5	-42.8	78.05
35	32.5	7.5	90	89
36	20	5	150	67.08

RWP treatment was selected as the response for the combination of the independent variables. Randomised experimental runs were performed to reduce to the barest minimum the effects of unexpected variability in the observed responses (Montgomery 2004). Design Expert 8.0.7.1 software was used to analyze the obtained results. For statistical analysis, a quadratic polynomial equation by central composite design was developed to predict the response as a function of independent variables and their interaction. In general, the response for the quadratic polynomials is described below:

$$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{12}x_1x_2 \dots\dots\dots (1)$$

where Y is the response (COD reduction);  $\beta_0$  is the intercept coefficient,  $\beta_1$  is the linear terms,  $\beta_{ii}$  is the squared terms and

$\beta_{ij}$  is the interaction terms, and  $x_i$  and  $x_j$  are the uncoded independent variables (Ghadge and Raheman 2006). Data from the central composite experimental design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models.

The F test was then used to evaluate the significance of the model equation and model terms. The statistical significance of the model was evaluated using the analysis of variance (ANOVA) while, the optimal values were obtained by solving the regression equation and analyzing the response surface plot.

**Analytical method for Chemical Oxygen Demand Determination**

Original and treated samples were measured by monitoring as per APHA guidelines for waste water. COD vials and caps were rinsed with 20% H<sub>2</sub>SO<sub>4</sub> to prevent contamination. 2.5 ml of the sample was added to the COD vial and for blank 2.5 ml distilled water was added to the COD vial and then 1.5 ml of digestion solution was added to each COD vials. 3.5 ml sulphuric acid reagent was added in the same manner. At this time caution should be taken because COD vials are hot now. COD vials was then placed in COD digester and heated at 150°C for 2 hrs. After digestion the vials was allowed to cool at the room temperature in test tube rack. After cooling, the content of the vials was transferred to conical flask. 1 to 2 drops ferroin indicator was then added in conical flask containing sample. The solution was titrated against the Mohr’s salt and the titer volume required for the color change from blue-green to reddish brown was noted. The procedure was repeated for blank titration.

**Calculation**

$$\text{COD (mg/L)} = [(B-A) \times N \times 8000] / V \quad \dots\dots\dots (2)$$

Where: B = volume of titrant used against blank, A = volume of titrant used against sample  
 N = normality of FAS, V= volume of the sample

**RESULT AND DISCUSSION**

**Reduction Capabilities and growth kinetic study of Bacterial Strains for River water containing phenol**

The six bacterial cultures showing maximum growth capability in river water was selected.

Time course study was done for the isolated strains among them, *Psuedomonas putida* (*P. putida*) showed the maximum degradation capability. Further, to determine the growth and multiplication of bacterial cells, the highest cell numbers were observed after 24h of incubation and this corresponds to the late log phase/early stationary phase of *Psuedomonas putida* growth cycle.

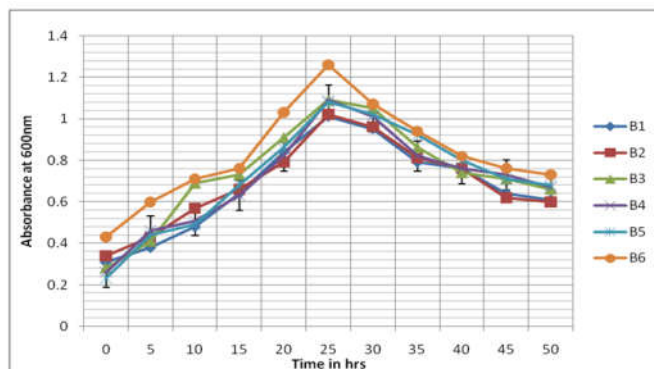


Fig. 1. Time interval study of growth kinetics of different isolated bacterial culture in Minimal salt media (MSM)

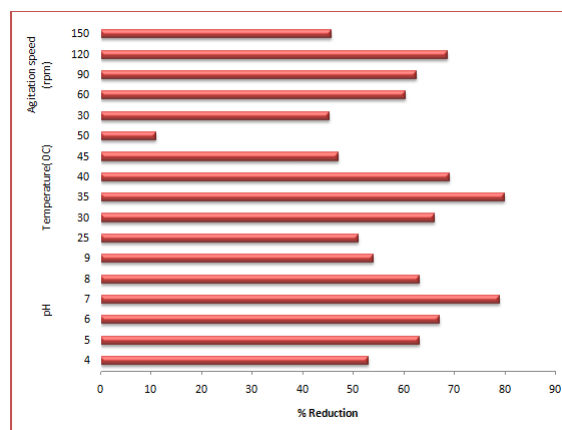
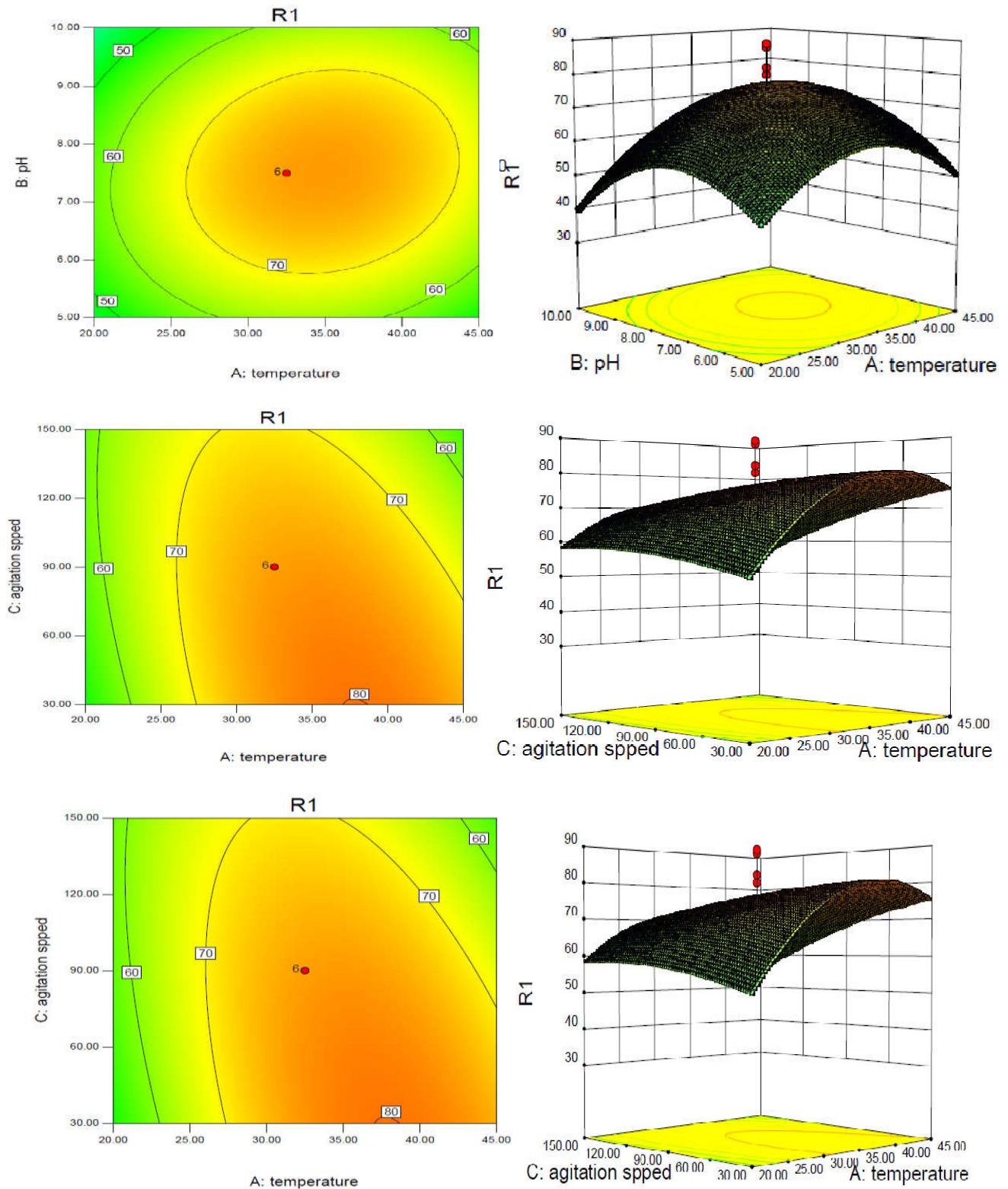


Figure 2. Effect of process parameters (pH, incubation temperature and agitation speed) on percentage reduction of RWP by *P. putida* under 48 h incubation time

It has been observed that maximum degradation capability occurs in the initial stage of the stationary phase which corresponds to the maximum numbers of the viable cells of bacteria in growth media. Fig.1 showed the adaptation period of 15 hours for the bacterial strain *Psuedomonas putida* with the wastewater supplemented media, followed by a rapid

Table 2. ANOVA analyses for the response surface quadratic model

Source	Sum of Squares	Df	Mean Square	F Value	p-value	Prob > F
Model	15731.38	9	1747.931	8.05652	< 0.0001	Significant
A-temperature	945.334	1	945.334	4.357209	0.0468	
B-pH	2.96395	1	2.96395	0.013661	0.9079	
C-agitation speed	386.1014	1	386.1014	1.779609	0.1938	
AB	247.5553	1	247.5553	1.141025	0.2953	
AC	1137.402	1	1137.402	5.242484	0.0304	
BC	483.3038	1	483.3038	2.227631	0.1476	
A^2	7419.884	1	7419.884	34.19954	< 0.0001	
B^2	7633.066	1	7633.066	35.18213	< 0.0001	
C^2	227.7704	1	227.7704	1.049834	0.3150	
Residual	5640.924	26	216.9586			
Lack of Fit	761.3377	5	152.2675	0.655305	0.6609	not significant
Pure Error	4879.586	21	232.3612			
Cor Total	21372.31	35				



**Fig 3. a** Contour plot and 3-D surface showing degerdation extent as a function of temperature and pH (Actual factor B: pH = 8.02). **b** Contour plot and 3-D surface showing degerdation extent as a function of temperature and agitation speed (Actual factor C: agitation speed = 120 rpm). **c** Contour plot and 3-D surface showing degerdation of RWP extent as a function of temperature and agitation speed (Actual factor A: temperature = 35.05)

growth period of 15-22 hours and consequently showed the decline phase after 35 hours of culture growth. The results showed a typical bacterial growth curve corresponding to the physiology of the bacterial growth kinetics. The *Pseudomonas putida* along with the other strains procured from MTCC was further subjected to the optimization studies under different nutritional parameters to observe the enhanced reduction capability of the strains.

#### Optimization of process parameter for Reduction of River water containing phenol by *Pseudomonas putida*

Data obtained on effect of three process parameters on degradation potential of *Pseudomonas putida* are as presented in Fig. 2

## Optimization of River Water containing Phenol treatment Process using Response Surface Methodology

A total of 36 experiments with different combinations of temperature (factor A, 20–45°C), pH (factor B, 7.5–10) and (factor C, 90–150 rpm) were performed within 48 h using the CCD method. RWP treatment was used as the response (R, %) and the results obtained from the experiments (observed and predicted) are summarized in Table. By applying multiple regression analysis, the following second-order polynomial equation (in coded units) that could relate degradation of RWP to the parameters studied was obtained as Eq. (3).

$$R1 = +76.58 + 5.29 A - 0.30B - 3.38 C + 3.21 AB - 6.88 AC - 4.49 BC - 14.24 A^2 - 14.44 B^2 - 2.49 C^2 \dots\dots\dots(3)$$

From the Eq. (2) above, the second-order response functions are represented by: R, the response for RWP treatment; A, the coded value of variable incubation temperature; B, the coded value of variable pH and C, the coded value of variable agitation speed (rpm). Data obtained on predicted values and observed values show the empirical models actually fit the actual data with  $R^2$  of 0.9624. When  $R^2$  is closer to unity as obtained in this study, the empirical models fit the actual data better whereas, the relevance of the dependent variables in the model in explaining the behaviour of variations cannot be ascertained by a smaller value of  $R^2$  (Cao *et al.*, 2008). Table 2 shows the data obtained from the analysis of variance (ANOVA) for the response surface reduced quadratic model. The Model F-value of 8.06 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise Values of "Prob > F" less than 0.0500 indicate In Values greater than 0.1000 indicate the model terms are not significant this case A, AC,  $A^2$ ,  $B^2$  are significant model terms If model reduction may improve your model. there are many insignificant model terms (not counting those required to support hierarchy), model terms are significant The "Lack of Fit F-value" of 0.66 implies the Lack of Fit is not significant relative to the pure error. There is a 66.09% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit. The "Pred R-Squared" of 0.4767 is in reasonable agreement with the "Adj R-Squared" of 0.6447. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 10.494 indicates an adequate signal.

This model can be used to navigate the design space. The choices for level combinations of the three variables such as, temperature, pH and agitation speed were made into contour plots (Fig. 3 a–c) which indicated the percentages of reduction within 48 h of incubation. The behavior of percentage reduction with respect to changes in temperature and pH is shown in Fig. a. These two parameters showed positive influence on RWP treatment. The percentage reduction increased with increase in temperature and pH until a certain level where further increases in both parameters led to a decrease in percentage reduction. In Fig. b, the variation of percentage degradation as specified by the variables, pH and temperature shows that with the increase in pH, the percentage reduction decreased. The result shows that optimal percentage reduction of RWP was between 35 and 40°C and slightly above basicity of pH. Three dimensional and contour plots for interaction effect of reaction pH and agitation speed toward RWP reduction are shown in Fig. C. RWP reduction increased as the pH increased to its medium level (pH 8) and agitation

speed increased to its central level (100 rpm). Optimal reduction of RWP was obtained in culture broth of pH 8 and 100 rpm agitation speed. Generally, a stronger influence of pH and agitation speed occurred when both parameters were at their median level. The decreasing the reduction of RWP at higher agitation and pH were probably as a result of increasing toxicity of the RWP to the microbial cells inactivation at such high concentrations (Ogawa *et al.*, 1989). In each contour plot, the other one variable was held constant. Generally, increase in the three process parameters to a certain threshold value resulted in increase in percentage reduction of RWP by *Pseudomonas putida*. This is due to the positive quadratic model as shown in Eq. 2. It also indicates that the experimental value must consider the running effect of these significant factors at the stipulated levels to maximize reduction of RWP by *Pseudomonas putida* (Mohana *et al.*, 2008; Lau and Ismail 2010).

## Conclusion

In this research work, treatment of RWP by *Pseudomonas putida* was investigated by CCD using RSM by varying three control factor such as temperature, pH and agitation speed. The function of these factors in terms of optimization of process parameter and its treatment operation was well explained by the results obtained from RSM. By applying the RSM design to the optimization experiments, the process variables were well studied and reduction efficiency extent up to 88 % was achieved in a shorter time (24 h). The experimental and the predicted values were very similar which reflected the accuracy and the applicability of RSM. Efficient RWP treatment by *Pseudomonas putida* suggests its potential for real industrial applications in treatment of RWP.

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