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Statistical optimisation for improvement of phenol degradation by *Rhodococcus* sp. NAM 81

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Abstract

Phenol is an important compound widely used in formulation of dyes, pesticides, in fabrication of plastics, as well as being generated in several industrial effluent other industries. In the present study, ability of phenol-degrading bacteria *Rhodococcus* sp. NAM 81 was characterised by optimising the important parameters that were able to support phenol degradation abilities using two-step statistical. Fractional factorial design (FFD) was applied to select significant variables (temperature, pH, phenol concentration, ammonium sulphate concentration, sodium chloride concentration, incubation time) that affects percentage of phenol degradation. pH (B), phenol concentration (C), sodium chloride (E) and incubation time (F) were observed to be significant ($p<0.05$), which was further optimised for phenol degradation and cell growth, using response surface methodology (RSM) based on central composite design (CCD). From the optimisation by CCD, pH 7.25, 0.74 g l^{-1} of phenol with 0.06 g l^{-1} of NaCl concentration in 23 hrs of incubation time is the best condition for phenol degradation. Meanwhile, *Rhodococcus* sp. NAM 81 grow best in pH 7.72, 0.85 g l^{-1} of phenol, 0.06 g l^{-1} of NaCl for 24 hrs incubation time were the best condition predicted to produce 0.867 g l^{-1} of *Rhodococcus* sp. NAM 81 cells. Verification of experiment indicated no significant different between the predicted and experimental data ($P>0.05$) for phenol degraded performance, as well as *Rhodococcus* growth. Under optimum conditions, the proposed method was successfully applied to improve 1.2 fold of phenol degradation and 1.3 for cell growth as compared to non-optimal cultivation.

Key words

Central composite design, Fractional factorial design, Phenol degradation, Response surface methodology, *Rhodococcus* sp.

Introduction

Phenol is an aromatic organic compound that is also known as carbolic acid and its derivatives are very commonly used or generated in several industries. It was normally found in effluent of gas and coke oven industries, polymeric resin production, petroleum refineries, varnish industries, textile industries because it acts as a key material for the formulation of dye, pharmaceutical drugs, detergents, explosive, polycarbonate and many more. This substance creates a worldwide environmental problem. It has been considered as a priority pollutant by the American Environmental Protection Agency (EPA) as it highly toxic (Rao *et al.,* 2009). On ingestion it is causes lethal to human. The industries

runoff waters will treat aquatic organism and simultaneously reduce the effectiveness of wastewater bio-treatment even at a low level of phenol (Arif *et al*., 2013; Zidkove *et al*., 2013).

Biodegradation is a promising method to treat phenol-containing wastewater, as it is inexpensive, environmental friendly and able to convert toxic substances into harmless products as compared to conventional methods that need high cost and may produce hazardous by-products that can affect the environment. Fortunately, several reports have proved that diverse microorganisms including yeast, bacteria, fungi and algae does have their own mechanism and condition to degrade phenol (Ahmad *et al.,* 2011; Paca *et al.,* 2007). Degradation of phenol is sensitive and normally affected by physical and cellular factors, which influence growth and enzymes involved (Khleifat, 2006). Degradation is normally affected by physical and cellular factors, which influence growth and enzymes involved. Among the crucial factors are type of medium, temperature, pH, salinity, oxygen level, compound structure and concentration, nitrogen source, and accessibility to inorganic nutrients (Suhaila *et al.,* 2013a; Khleifat, 2006; Shourian *et al*., 2009).

Hence, in the present investigation, an attempt was made to optimise essential variables that could improve biodegradability of phenol. The common process for optimising a multivariable system normally applies "one-variable at a time" approach (Ray *et al.,* 2009). However, this technique is time consuming, and the materials used increases the cost of the experiment. Besides, this technique unable to estimate and detect interactions between independent variables and might overlook the optimal settings of factors as only one factor tested in a batch instead of all simultaneously (Bezerra *et al.,* 2008). With the development of knowledge in statistic and information technology, various statistical software for experimental design have been developed as a tool of optimisation to simplify the process and to view the effects and relation of the variables and economic especially for bioreactor design. Fractional factorial design along with response surface method is common by chosen method. Fractional factorial design is normally applied at first stage for preliminary screening of many factors in order to eliminate insignificant factors for targeted response that will be studied in further optimisation process. These factors can be used in RSM as an experimental design tool to determine optimal condition and to observe mutual interactions of variables by building models, which have been widely used for optimisation in the recent years (Adepoju *et al.,* 2014; Sivasubramanian and Namasivayam, 2014). It is a statistical technique for designing experiment, evaluating the effects of several factors for optimum conditions and building models. RSM minimises the number of experiments, which led to reduces expenses, as it has been reported to be capable of recognising accurate optimum condition of a particular parameter and define interaction between variables (Farliahati *et al.,* 2010; Zhou *et al.,* 2011). Among the experimental design offered are Central Composite Design (CCD), Box-Behnken, 3-level factorial, hybrid and one factor. In biological research, CCD is commonly employed, as it is well suited for fitting a quadratic surface, which is usually efficient for optimisation process with an acceptable number of runs (Candioti *et al.,* 2006; Hank *et al.,* 2010).

In the present study paper, statistical optimisation was applied to investigate significant variables between the temperature, pH, phenol, $(NH_4)SO_4$ concentration, NaCl concentration and incubation time to optimise growth and phenol degradation capability of *Rhodococcus* sp. NAM 81. The process began with two-level factorial technique using FFD, followed by optimisation of selected variables by RSM using practical CCD.

Materials and Methods

Microorganism and inoculum preparation : *Rhodococcus* sp. NAM 81 used for the experiment this study, was maintained in UNISEL Culture Collection Unit, Institute of Bio-IT Selangor. Bacteria from stock culture, maintained in glycerol, was transferred into nutrient broth (Merck, Germany) and incubated at 30 °C in an incubator shaker (Jeio Tech SI-600R, Korea) agitated at 160 rpm for 24 hrs. The cultures were used as standard inoculums throughout study.

Cultivation and phenol biodegradation experiments : Biodegradation of phenol was done in minimal salt media (MSM) with following composition in g l^{-1} : pH 7.5; $MgSO_4$ (0.1), K_2HPO_4 (0.4), KH_2PO_4 (0.2), NaCl (0.1), $MnSO_4$ (0.01), $FeSO_4H_2O$ (0.01), $Na_2MoO_4.2H_2O$ (0.01), $(NH_4)_2SO_4$ (0.4) and phenol (0.5). Different concentration of phenol, ammonium sulphate and NaCl were varied in each stage as shown in Table 1 and 2. Initial pH was 7.5, which was adjusted prior to autoclaving using NaOH (30%). After that, phenol was added to the cooled medium. Phenol solution was sterilised using cellulose membrane (0.2 µm). 10% inoculum was inoculated in 250 ml flask that contained 100 ml medium to initiate phenol degradation. The flasks were incubated in an incubator shaker (Jeio Tech SI-600R, Korea) at 30, 37 and 45 °C agitated at 160 rpm. During cultivation, 10 ml of culture samples was withdrawn at time interval for analysis.

Analytical procedures : MSM liquid cultures were withdrawn to monitor phenol degradation ability, and the residual phenol from the spent medium was determined using 4-aminoantipyrine method. Absorbance was measured at 500 nm using BioMate 3 UV-Vis Spectrophotometer, Thermoscientific, USA and subsequent concentrations were

Table 1: Experimental range and levels of the independent variables used in FFD in terms of actual and coded units

Independent variables	Symbols	Coded level	
		Low level (-1)	High level (+1)
Temperature (°C)	A	30	40
pH (pH)	B	6.5	8
Phenol concentration ($g\,l^{-1}$)	C	0.5	1.0
Ammonium sulphate ((NH_4SO_4)) ($g\,l^{-1}$)	D	0.1	0.5
Salinity (NaCl) ($g\,l^{-1}$)	E	0.05	0.25
Incubation time (hr)	F	8	24

Table 2 : Experimental range and levels of the independent variables used in RSM in terms of actual and coded units

Independent variables	Symbols	Coded level	
		Low level (-1)	High level (+1)
pH	B	6.5	8
Phenol concentration ($g\,l^{-1}$)	C	0.5	1.0
Salinity (NaCl) ($g\,l^{-1}$)	E	0.05	0.25
Incubation time (hr)	F	8	24

determined using a calibration curve. Bacterial growth was determined using predetermined correlation between 660 nm optical density using BioMate 3 UV-Vis Spectrophotometer (Thermoscientific, USA), dry cell weight from filtration and oven dried method. Standard curve for determination of cell concentration in the sample was plotted.

Experimental design and data analysis for optimisation of phenol degradation : Firstly, FFD as a screening design was carried out to determine out of several experimental variables and their interactions which contributed to significant effects. Then, a multivariate statistic technique, RSM based on CCD was used to optimise the level of effective parameters for improving the efficiencies of phenol degradation using Design Expert Software (version 6.0.8-Stat-Ease, Inc. Minneapolis, USA).

Screening of significant physico-chemical determinants by FFD: Half FFD consisting of set of 16 experiments were carried out to determine effective phenol degradation under different combination of six parameters (temperature (A), pH (B), concentration of phenol (C), ammonium sulphate (D) and sodium chloride (E), as well as incubation time (F)), in order to screen significant factors. A complete matrix for screening was designed using standard half fractional factorial design (Table 1).

Optimisation of key determinants by RSM : Significant factors were further optimised using RSM with four parameters. Important four key determinants (pH (B), phenol concentration (C), sodium chloride concentration (E) and time (F) were further optimised in order to improve phenol degradation, as well as cell growth activities (Table 2). The pH range was between 6.5 and 8, 0.5 gl^{-1} to 1.0 gl^{-1} for phenol concentration, 0.05 gl^{-1} to 0.25 gl^{-1} for salt concentration. Minimum and maximum incubation time was set for 8 hrs and 25 hrs, respectively. The response surface method, involving a central composite design, was adopted in the investigation to define relationship between the responses and the variables by a group of statistical estimation. A set of 30 experiments including of six centre points, were carried out using central composite design with four selected significance factors. Total number of design point (N) determined by equation ($N=2^f+2f+C_p$), where k is number of factors in experiments and C_p is number of centre point. Data were analysed using Design Expert Software (version 6.0.8-Stat-Ease, Inc. Minneapolis, USA) for regression analysis of experimental data and to plot response surface in order to obtain optimisation of significant parameters.

Statistical analysis and modelling : Analysis of variance (ANOVA) was applied to determine the relationship between the process variables and the responses. The experimental results of RSM were fitted by R^2 coefficient and the statistical significant of model was verified by F-test. Combination of factors represents interaction between individual (Azaman *et al.*, 2010). The chosen variables can be related to the response by quadratic model as shown in the equation:

$$Y=b_o+\sum b_1X_1+\sum b_2X_2+\sum b_3X_3+\sum b_4X_4+\sum b_{11}X_2+\sum b_{22}X_2+\sum b_{33}X_2+\sum b_{44}X_2\ldots.X_m$$

Where Y is predicted response for phenol degradation, b_o, b_1, b_2, b_3, b_4, b_{11}, b_{22}, b_{33}, b_{44}, b_{12}, b_{13}, b_{14}, b_{23}, and b_{24} are the constant regression coefficient of the model. Where b_o is the intercept term, b_1, b_2, b_3 and b_4 is the linear coefficient, b_{11}, b_{22}, b_{33} and b_{44} is the interaction coefficient (squared coefficient), and X_m are the independent variables, where in this study independent

variables were coded as B, C, E and F.

Thus, the second-order polynomial equation can be represented as follows :

$$Y = b_o + \sum b_1 B + \sum b_2 C + \sum b_3 E + \sum b_4 F + \sum b_{11} B + \sum b_{22} C + \sum b_{33} E + \sum b_{44} F \ldots . X_m$$

Validation of experimental model : The statistical model was validated for all variables within the design to study the capability for phenol degradation and growth of cells.

Results and Discussion

Prior to optimisation, screening of significant parameters which influence phenol degradation was carried out of using 2^{6-2} fractional factorial designs. The design was employed to screen numerous parameters that effect phenol degradation. The number of experiment in fractional factorial design was based on 2^{a-b}+C, where a is number of parameters/variables, b is whole number that indicated how fractionated the experimental design was, if b is zero, that indicates the experimental design is a full factorial design and C is the number of replicates in the experiment (Asadollahzadeh and Tavakoli, 2014).

Table 3 shows total number of 16 experimental designs with each row of table consisting of six independent parameters and phenol degradation as response. From this experiment, highest phenol degradation of 90% was obtained from experiment design in run 1. Analysis of variance result, showed that temperature (A) and concentration of ammonium sulphate (D) did not produce significant effect on phenol degradation by *Rhodococcus* sp. NAM 81 ($p>0.05$) with 0.2450 and 0.1154, respectively (Table 4). Temperature (^{0}C) might not be a critical parameter for this organism as it could tolerate high temperature because it was isolated from palm oil mill effluent. Nevertheless, *p*-values of the remaining four parameters; pH (B), phenol concentration (C), sodium chloride (E) and incubation time (F) were observed to be significant ($p<0.05$), thus explaining that all four parameters had a positive role in phenol degradation. The availability of phenol and nitrogen source in cultivation medium influences secretion of enzyme for biodegradation of aromatic compounds, especially phenol hydroxylase enzyme (Veenagayathri and Vasudevan, 2011; Norazah *et al.,* 2015). The model F-value of 0.0379 gave an indication that the model was significant for this study. Determination coefficient (R^2) of the model was 0.9941, which implied that sample variation of 99.41% for phenol degradation was attributed to the studied variables. Meanwhile, the adjusted R^2, which was more suitable for multiple regressions, was calculated to be 0.9559 that indicates a good agreement between the experimental and predicted values of phenol degradation. The percentage of phenol degradation obtained from 2^{6-2} FFD experiments showed variation, which indicates necessity for further optimisation. Significant variables were selected for further optimization using RSM based on CCD.

Results obtained from the central composite design experiments were fitted into a second order polynomial equation to explain the dependence of phenol degradation. Application of RSM represented the effect of independent factors, and their interaction and response was predicted. The model F-value implied that phenol degradation and growth of *Rhodococcus* sp. NAM 81 did have a good fit with the model. As depicted in Table 5 and 6, *p*-value of the model for both responses were statistically significant with $p < 0.0001$. Reddy *et al.* (2008) reported that significant model l was good fit and acceptable. Meanwhile as for lack of fit, the data obtained in the present study showed that the value was not

Table 3 : Half FFD matrix for 6 independent variables for phenol degradation by *Rhodococcus* sp. strain NAM 81

Run	A	B	C	D	E	F	Response (%)
1	30	8	1	0.5	0.05	24	89
2	30	8	0.5	0.5	0.25	8	22
3	30	6.5	1	0.5	0.25	8	10
4	40	6.5	0.5	0.1	0.25	8	10
5	30	6.5	0.5	0.5	0.05	24	35
6	30	8	0.5	0.1	0.25	24	72
7	40	8	1	0.1	0.25	8	23
8	40	6.5	1	0.5	0.05	8	28
9	40	6.5	1	0.1	0.05	24	75
10	40	6.5	0.5	0.5	0.25	24	35
11	40	8	0.5	0.1	0.05	24	60
12	30	6.5	1	0.1	0.25	24	49
13	30	6.5	0.5	0.1	0.05	8	23
14	40	8	0.5	0.5	0.05	8	22
15	40	8	1	0.5	0.25	24	67
16	30	8	1	0.1	0.05	8	52

significant for both responses and gave an indication of acceptable fitness of selected model (Aghaie *et al.,* 2009). Lack-of-fit values for phenol degradation and cell growth activities were obtained at 0.0698 and 0.6764, respectively. As for an adequate precision measures, the signal to noise ratio showed higher than four and this value was preferable (Mohamad *et al.,* 2011). Therefore, 31.0 and 20.5, for both responses showed an adequate signal that signified that the model was illegible to envisage the response. The percentage of coefficient of variation (CV%) is considered as an evaluation of residual variation of the data comparative to the mean. A model is considered reproducible with CV if it is not

Table 4 : Analysis of variance (ANOVA) for fitted second-order polynomial model as per half FFD

Independent variables	Sum of squares	*F* value	*p* value
Model	9221.25	26.03	0.0376 Significant
A	72.25	2.65	0.2450
B	1296	47.56	0.0204
C	841	30.86	0.0309
D	196	7.198	0.1154
E	600.25	22.03	0.0425
F	5329	195.56	0.0051
AB	576	21.14	0.0442
AC	16	0.598	0.5236
AD	36	1.32	0.3693
AE	0.25	0.00917	0.9324
AF	16	0.59	0.5236
BD	110.25	4.05	0.1820
BF	132.25	4.85	0.1585
Residual	54.5		
Cor total	9275.75		

Table 5 : Analysis of variance (ANOVA) for phenol degradation

Independent variables	Sum of squares	*df*	Mean square	*F* value	Prob>F (*p* value)
Model	25139.69	14	1795.69	69.13	<0.0001
B	218.40	1	218.41	8.41	0.0110
C	5.80	1	5.80	0.22	0.6433
E	691.22	1	691.23	26.61	0.0001
F	19244.00	1	19244.01	740.83	<0.0001
B^2	1228.21	1	1228.21	47.28	<0.0001
C^2	2387.20	1	2387.20	91.89	<0.0001
E^2	6.407619048	1	6.407619048	0.25	0.6266
F^2	1719.047619	1	1719.047619	66.18	<0.0001
BC	107.1225	1	107.1225	4.12	0.0604
BD	71.40	1	71.4025	2.75	0.1181
BE	51.12	1	51.1225	1.97	0.1810
CD	2.40	1	2.4025	0.09	0.7652
BD	80.10	1	80.1025	3.08	0.0995
DE	197.40	1	197.4025	7.599	0.0147
Residual	389.64	15	25.9762		
Lack of fit	346.31	10	34.6309	3.995	0.0698
Pure error	43.335	5	8.667		
Cor Total	25529.33	29			
Std dev.	5.09				
Mean	60.78				
C.V	8.38				
Press	2057.14				
R-squared	0.9847				
Adj R-squared	0.9705				
Pred R-squared	0.9194				
Adeq Precision	31.43				

Table 6 : Analysis of variance (ANOVA) for cell growth

Independent variables	Sum of squares	*df*	Mean square	*F* value	Prob>F (*p* value)
Model	1.672	14	0.119	30.6094	<0.0001
B	0.027	1	0.027	6.9019	0.0190
C	0.00006017	1	0.00006017	0.0154	0.9028
E	0.07889	1	0.0789	20.216	0.0004
F	1.23488	1	1.2349	316.442	<0.0001
B^2	0.136262	1	0.1362	34.9177	<0.0001
C^2	0.08789	1	0.0879	22.5234	0.0003
E^2	0.00139	1	0.0014	0.3585	0.5583
F^2	0.094617	1	0.0946	24.2461	0.0002
BC	0.01525	1	0.0153	3.9084	0.0667
BD	0.023716	1	0.0237	6.0773	0.0262
BE	0.01199025	1	0.0120	3.0725	0.1000
CD	0.0049	1	0.0049	1.2556	0.2801
BD	0.00070225	1	0.0007	0.1799	0.6774
DE	0.0036	1	0.0036	0.9225	0.3520
Residual	0.058535792	15	0.0039		
Lack of fit	0.035036583	10	0.0035	0.7455	0.6769
Pure error	0.0235	5	0.0047		
Cor Total	1.7308	29			
Std dev.	0.0625				
Mean	0.48757				
C.V	12.8123				
Press	0.2356				
R-squared	0.9662				
Adj R-squared	0.9346				
Pred R-squared	0.8639				
Adeq Precision	20.54				

greater than 10% (Bisht *et al.,* 2013). Higher CV value indicated that reliability of experiment was low but here, low coefficient variation (CV=8.38 for percentage of phenol degradation and 12.81 for cell growth) showed the reliability of the experiments conducted. This finding was parallel with the optimisation of phenol degradation by *Rhodococcus* UKMP-5M with 32.55 and 18.68 as adequate precision and 4.7655 and 12.3828 of CV value for time required to degrade 1g l^{-1} phenol and cell growth (Suhaila *et al.,* 2013b). Minimum predicted residual sum of squares (Press) indicated fitness of the model to each data point in the design and in the present study values of press were 20 and 0.2356 for the respective responses.

The coefficient of determination (R^2 and adjusted R^2) expressed the quality of fit of polynomial model equation. R^2 of 0.9847 signified that model could explain 98.47% of variation in response to phenol degradation and 0.9667 or 96.7% for cell growth. The value measures the strength of linear relationship between experimental and the predicted values (Rajendran and Thangavelu, 2008). Adjusted R^2 evaluated the percentage of variation explained only by independent variables that actually affected the dependent variable. In the present study, the value was 0.9705 for phenol degradation and 0.9346 for cell growth that showed that regression model provides an excellent explanation on relationship between response and independent factors (variables). The significance of each coefficient was observed, based on *p* value of the individual term (Table 6). The result of phenol degradation showed that seven models terms were found to be significant; the linear terms were B, E and F, B^2, C^2 and F^2 as quadratic terms followed by interaction effects of DE with *p* value was less than 0.05. Simultaneously, the significant model terms for cell growth almost resembled the model term for phenol degradation, with B, E and F as linear terms and B^2, C^2 and F^2 as quadratic terms, but the interaction effect of BD gave significant effect to the model of cell growth. The effect order of linear terms on phenol degradation were as follows: incubation time (740), concentration of NaCl (F=26), pH (F=8) and phenol concentration (F=0.223), while for cell growth, orders were incubation time (F=316), NaCl concentration (F=20), pH (F=6.9), and phenol concentration (F=0.01). Multiple regression analysis simplified the quadratic model for phenol degradation (Y_1) in coded factor as in equation 1 and cell growth (Y_2) in equation 2.

$$Y_1 = 79.55 + 3.0167*B + 0.4917*C - 5.3667*E + 28.3167*F - 6.6917*B^2 + 9.3292*C^2 + 0.4833*E^2 - 7.9167*F^2 + 2.5875*B*C + 2.1125*B*E + 1.7875*B*F + 0.3875*C*E - 2.2375*C*F - 3.5125*E*F \quad (1)$$

Table 7 : The predicted and experimen value of phenol degradation and cell growth of *Rhodococcus* NAM 81 in the matrix of central composite design

Run number	Factor 1 pH (B)	Factor 2 Phenol (C) concentration ($g l^{-1}$)	Factor 3 NaCl (E) concentration ($g l^{-1}$)	Factor 4 Incubation time (hour) (F)	Response 1 Percentage of phenol degradation (%)	Response 1 Percentage of phenol ($g l^{-1}$) degradation (%)	Response 2 Cell growth ($g l^{-1}$)	Response 2 Cell growth
1	6.5	0.5	0.05	8	28.4	30.76	0.255	0.308
2	8	0.5	0.05	8	20.9	23.82	0.188	0.182
3	6.5	1	0.05	8	25.6	30.27	0.259	0.291
4	8	1	0.05	8	31.3	33.68	0.271	0.288
5	6.5	0.5	0.25	8	16.5	22.05	0.146	0.181
6	8	0.5	0.25	8	26.3	23.56	0.222	0.209
7	6.5	1	0.25	8	19.9	23.11	0.075	0.095
8	8	1	0.25	8	33.2	34.97	0.224	0.246
9	6.5	0.5	0.05	24	95.1	95.32	0.731	0.750
10	8	0.5	0.05	24	98.1	95.53	0.776	0.733
11	6.5	1	0.05	24	82.5	85.88	0.717	0.707
12	8	1	0.05	24	100	96.44	0.808	0.814
13	6.5	0.5	0.25	24	74.3	72.56	0.604	0.564
14	8	0.5	0.25	24	83.9	81.22	0.692	0.701
15	6.5	1	0.25	24	65.6	64.67	0.403	0.450
16	8	1	0.25	24	85.4	83.68	0.787	0.711
17	5.75	0.75	0.15	16	53.8	46.75	0.351	0.282
18	8.75	0.75	0.15	16	54.4	58.82	0.364	0.416
19	7.25	0.25	0.15	16	40.6	41.25	0.405	0.407
20	7.25	1.25	0.15	16	46.5	43.22	0.421	0.401
21	7.25	0.75	-0.05	16	95.8	92.22	0.799	0.774
22	7.25	0.75	0.35	16	69.8	70.75	0.537	0.544
23	7.25	0.75	0.15	0	0	-8.75	0.013	-0.058
24	7.25	0.75	0.15	32	98.4	104.52	0.796	0.849
25	7.25	0.75	0.15	16	78.7	79.55	0.59	0.631
26	7.25	0.75	0.15	16	83.3	79.55	0.759	0.631
27	7.25	0.75	0.15	16	74.8	79.55	0.59	0.631
28	7.25	0.75	0.15	16	81.8	79.55	0.6591	0.631
29	7.25	0.75	0.15	16	80	79.55	0.592	0.631
30	7.25	0.75	0.15	16	78.7	79.55	0.593	0.631

$$Y_2 = 0.6305 + 0.0335*B - 0.001583*C - 0.0573*E + 0.2268*F - 0.0705*B^2 - 0.05661*C^2 + 0.007141667*E^2 - 0.05873*F^2 + 0.030875*B*C + 0.0385*B*E + 0.0274*B*F - 0.0175*C*E - 0.006625*C*F - 0.015*E*F \quad (2)$$

CCD based method of RSM observed minimum and maximum phenol degradation concentrations that were in run number 5 (16%) and run 12 (100%), respectively. The growth also had a minimum (0.1465 $g l^{-1}$) and maximum (0.808 $g l^{-1}$) in similar order (Table 7).

RSM tool have special features for point prediction and interpret graphically through contour plot and 3D response surface curve that could be used to determine the optimum value of essential factor combination for optimum phenol degradation. The results were interpreted based on 3D graphs obtained from the model. The optimization value was set on the range of parameters studied, meanwhile the response was set for maximum responses (100%).

Highest degradation rate was found at phenol concentration ranging from 0.63 to 0.75 $g l^{-1}$ and pH ranging from 7.25 to 7.63. *Rhodococcus* sp. strain NAM 81 was able to withstand phenol up to 1 $g l^{-1}$. The inhibitory effects became significant when phenol concentration increased beyond 1 $g l^{-1}$ in acidic condition, as *Rhodococcus* spp. preferred neutral condition (Wang *et al.,* 2008). Limited number of phenol-degrading bacteria were able to grow and degrade high concentration of phenol above 1.5 $g l^{-1}$ (Bajaj *et al.,* 2009), except after it were immobilised in matrixes as in *Rhodococcus opacus* 1G that was able to degrade 1.0 and 1.5 $g l^{-1}$ of phenol after 24 and 48 hrs (Shumkova *et al.,* 2009). It was noted that optimum phenol degradation was found at mid dose of (0.75 $g l^{-1}$) phenol and minimum salt concentration (0.05 $g l^{-1}$). The effect or interaction between phenol concentration and incubation time on phenol biodegradation by *Rhodococcus* sp. NAM 81, when pH was placed at the centre point (7.25) and 0.15$g l^{-1}$ of NaCl. 100% of phenol degradation could be achieved at 0.75 $g l^{-1}$ phenol in less than

Table 8 : Validation of predicted and experimental cultivation variable

Cultivation variation	Predicted by RSM		Experimental result		Non-optimal cultivation
	Phenol degradation	Cell growth	Phenol degradation	Cell growth	Phenol degradation and cell growth
pH	7.25	7.72	7.25	7.72	7.5
Phenol concentration (g l^{-1})	0.74	0.85	0.74	0.85	0.75
NaCl (g l^{-1})	0.06	0.06	0.06	0.06	0.1
Incubation time (hour)	23	24	23	24	24
Percentage of degradation (%)	100	100	95.81	92.20	74.8
Cell growth (g l^{-1})	0.852	0.867	0.858	0.886	0.6311

24 hrs. Degradation rate increased with incubation time. Thus, addition of high phenol concentration in the media required more incubation time.

The effect or interaction between pH and phenol concentration on growth of *Rhodococcus* sp. strain NAM 81 at NaCl concentration and time was observed. Optimum cell growth was found at pH 7.25 and 0.75 g l^{-1} phenol concentration. The increasing or decreasing in the stated value resulted in reduction of cell growth. It was observed that as the initial concentration of phenol raised, growth also increased and started decreasing with increase in phenol concentration. This was attributed to the fact that cells were inhibited due to high phenol concentration. The preferred pH behavior was consistent with literature reported for biodegradation of phenol. Extremely low or high pH value of cell could be easily penetrated by acids or bases that tend to exist in undissociated form under this situation and the electrostatic force were unable to prevent them from entering cells (Robertson & Alexander, 1992; Balamurugan *et al.,* 2012). The growth of *Rhodococcus* sp. NAM 81 was maximum in medium phenol concentration (0.75 g l^{-1}) and low NaCl concentration. From the plot, *Rhodococcus* sp. NAM 81 could apparently grow at higher phenol concentration with minimum salt. High salt content caused a significant inhibitory effect on bacterial growth (Tabib *et al.,* 2012), but strain that seemed tolerant might be classified as facultative halophile. Prolonged incubation time might lead to increment of cell concentration.

Overall, from the optimization process using CCD, the best parameter for 100% phenol degradation was pH; 7.25, 0.74 g l^{-1} phenol with 0.06 g l^{-1} NaCl concentration after 23 hrs of incubation time. Meanwhile, pH 7.72, 0.85 g l^{-1} phenol, 0.06 g l^{-1} NaCl and 24 hrs incubation time were considered best for producing 0.8 g l^{-1} of *Rhodococcus* sp. NAM 81 cells.

Analysis of the results showed that experimental value for phenol degradation and cell growth at optimal condition was good, as the data predicted by model was not statistically significant different (p<0.05) (Table 8). Application of optimal conditions support that the strain was able to degrade 95.8% of 0.74 g l^{-1} phenol within 23 hrs with 0.8 g l^{-1} cells or 92.2% 0.85 g l^{-1} phenol within 24 hrs with 0.8 g l^{-1} cells. Improvement in percentage of phenol degradation and cell growth under optimal condition was about 1.2-fold and 1.3-fold as compared to non-optimal cultivation condition. The cultivation period for degradation of 0.5 and 1 g l^{-1} phenol by this strain was also tested using optimal parameters and it was discovered that it was able to degrade within 16 and 35 hrs of incubation time. The potential of *Rhodococcus* strain is comparable with other strain like *Rhodococcus* UKMP-5M that was able to degrade 1 g l^{-1} phenol in 27.6 hrs, unlike 222.22 hrs by *Rhodococcus erythropolis* and 283.7 hrs by *Rhodococcus* strain N014-1 (Suhaila *et al.,* 2013b; Goswami *et al.,* 2005; Margesin*et al.,* 2005). Therefore, with application of optimal condition in a control environment, this strain has good prospective as phenol degrader in bioremediation and biotransformation process.

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