Central Composite Face-Entered Design (CCFD) for the Biodegradation of Phenanthrene by Mixed Culture Consortia in Batch Bioreactor

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Abstract

Biodegradation of Phenenthrene (PHE) was studied in aqueous phase to demonstrate the potential of the mixed culture in degradation of high concentration of PHE. The experiments were conducted to monitor biodegradation of phenanthrene for duration of 6 days. Biodegradation of PHE was successfully achieved in low and middle concentration using specific isolated mixed culture. The PHE biodegradation was carried out in batch bioreactor with response surface methodology (RSM) based on central composite face entered design (CCFD). A full factorial Central Composite Design of experiments was used to construct response surfaces with the removal of PHE degradation and the specific growth rate as responses. The initial phenanthrene concentration (X_1) and the reaction time (X_2) were used as design factors. The experimental results were shown that experimental data fitted with the proposed polynomial model. Analysis of variance showed a high coefficient of determination value in the range of 0.936–0.999. The maximum biodegradation of PHE in terms of the removal of PHE (Y_1) was found to be 0.98 mg/mg (degraded PHE/initial PHE). The maximum extent of biodegradation relative to initial PHE concentration and biomass (Y_2) was 0.08 mg/mg/mg (degraded PHE/initial PHE/biomass). This maximum biodegradation correspond to the factors combination of middle level of PHE content (X_1= 43.01 mg/L) and the highest level of reaction time (X_2 = 103.53 hours). The optimum specific growth rate (Y_3) was found to be 0.0081 h^{-1}. A 98% removal efficiency of PHE biodegradation was achieved. Polynomial model was found useful to predict PHE degradation under the experimental studied. It was observed that optimum biodegradation of PHE can be successfully predicted by RSM.

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Keywords: *Biodegradation; phenanthrene; mixed culture; response surface methodology; central composite design*

1. Introduction

Toxic organic compounds (xenobiotics) cause serious environmental and health risks. Polycyclic aromatic hydrocarbons (PAHs) are the xenobiotics compounds that are products of incomplete combustion of organic matter. Currently, the vast majority of environmental PAH concentrations are generated from industrial activities such as gasification/liquification of fossil fuels, coke and coal-tar production, wood preservation and treatment processes, fuel and asphalt production [1-3]. Wastewater analysis reveals that high PAHs concentrations are originated from sources of industrial wastes, domestic sewage, atmospheric rainfall, airborne pollutants and road surface run-off [3]. Phenanthrene, one of the most abundant PAHs in the environment [4], is included in the U.S. EPA list of priority pollutants [5-6]. Biological treatment has been used to treat contaminated sites for many years. Bioremediation techniques are typically more economical than traditional methods such as incineration and some pollutants can be treated on site, thus reducing exposure risks for clean-up personnel or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation the public considers it more acceptable than other technologies [7]. In recent years, a biodegradation study on polycyclic aromatic hydrocarbons (PAHs) has received great attention because PAHs are ubiquitous environmental pollutants and some are toxic, mutagenic or carcinogenic and resistant to biodegradation [8-12].

Statistical design of experiments is a time and money saving method by decreasing significantly the number of trials needed to study a multi-variable phenomenon. This is very useful when screening probable factors for cases involving second-order models [13]. In addition, the design expert is used to compute the multiple interactions between the main factors.

Response surface method (RSM) is a collection of mathematical and statistical techniques that could generate three dimensional plots and display statistical analysis on how the responses are influenced by the process variables. RSM also applied the optimum operating conditions for the system and to identify the region which satisfies the operating specifications [14]. The most popular RSM design is the central composite design (CCD) for analysis of experimental data. The CCD is applied to estimate the coefficients of a particular model equation. The CCD method is efficient and flexible, providing sufficient information on effects of variables and overall experimental error with a minimum number of experiments [14]. Center points in CCD design are usually repeated 4-6 times to get a good estimate of experimental error (pure error). Five center points are created by default experimental design with two factors. Central composite designs generally require 5 levels for each factor: Alpha, with negative, zero and positive values (-1, 0, 1). In this study, Alpha value is taken as one resulting in 3 levels, Lowest (-1), middle (0), highest (+1) which is more specifically known as central composite face entered design (CCFD). Full factorial design for two variables study requires 13 experimental trials. RSM has successfully been applied to study and optimize the biodegradation of toxic compound (PAHs, PCBs and Pesticides) such as lindane [13].

The objective of the present work is to investigate the phenanthrene biodegradation in aqueous phase with mixed strains using the central composite face-centered design (CCFD). The main factors (variables) investigated were the initial PHE concentration and reaction time based on preliminary screening experiments (data not shown). The interaction between factors influencing dependent (response) parameters such as removal of PHE (Y_1), extent of biodegradation relative to initial PHE concentration and biomass (mg/mg/mg) (Y_2) and the specific growth rate (Y_3) were examined.

2. Material and Method

2.1. Microbial Strains and Degradation experiments

Mixed strains of microorganisms were obtained from Prai industrial zone, Butterworth, Malaysia. The mixed culture contains Gram positive and negative microorganisms. Nutrient broth was used to screen the strains. The propagation was carried out in basal media at pH 7.2 based on the method proposed by Lei *et al.* [8]. A stock solution of phenanthrene was made by dissolving PHE in ethanol solution (less than 1%) and then it was transferred to mineral salt media (MSM) for final concentration. Phenanthrene degradation experiments were conducted in 100-ml Erlenmeyer flasks containing 50 ml of MSM media. The acclimated seed culture was prepared and harvested at mid-exponential phase. A 1.5 ml of seed culture was transferred to MSM media in three different flasks containing 17, 55.5, 94 mg/l phenanthrene, respectively. The media were incubated at 25 ^{o}C with a rotary shaker at 150 rpm. Since the presence of phenanthrene solid affected the measurement of broth optical density, cultures were filtered through cotton wool to remove solid phenanthrene and then rinsed by magnesium sulfate to release microbe into the vessel. The optical densities of the filtrates containing suspended cells were measured by spectrophotometer (Cecil, 1010, England) at a wavelength of 600nm (OD_{600nm}).

2.2. Analysis of phenanthrene

The samples taken from culture broth were acidified to pH 2 with 2N sulfuric acid and were extracted three times, with ethyl acetate using half of culture volume (25ml). The extracted organic phase was pooled, dried with anhydrous Na_2SO_4 and the solvent was removed by gentle nitrogen current. Phenanthrene was quantified by GC (Perkin Elmer Clarus 500, USA) equipped with a flame ionization detector (FID) using a PTE-5 capillary column, 30 m length, 0.25 mm of inside diameter and 0.025 mm of coated film thickness (Supelco, USA). Squalene is used as an internal standard. Helium was used as carrier gas for the FID. The PHE was determined on temperature programming. The oven temperature was initially set at 50^{o}C for 3 min and then the temperature was increased to 280^{o}C at a rate of 10 ^{o}C min^{-1}. The injector and detector temperatures both were set at 290^{o}C.

2.3. Statistical design of experiments

The statistical analyses were performed using Design-Expert (DOE) software (version 6.0.7). The response surface method (RSM) of statistical analysis system and design expert was used to statically analyze the experimental data. A central composite face entered design (CCFD) was applied with two design factors, which are the initial phenanthrene concentration (X_1) and the reaction time (X_2). The factor levels are such that the upper level corresponds to +1, the lower level to -1 and the middle level to zero. Table 1 shows the 3^2 full factorial designs based on CCFD with both coded and actual values are

presented. The response selected were the removal of PHE (Y_1), extent of biodegradation relative to initial PHE concentration and biomass (mg/mg/mg) (Y_2) and the specific growth rate (Y_3). After running these data with 13 trials, the corresponding quadratic models for the above response and parameters were computed.

3. Result and discussion

3.1. Data analysis

Experimental data were found to be best fitted to polynomial model and regression coefficient was determined. Once the experiments were performed the coefficients of the polynomial model were calculated based on following equation [15]:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i . X_i + \sum_{i=1}^{k} \beta_{ii} . X_i^{\,2} + \sum_{i_{i\leq j}}^{k} \sum_{j}^{k} \beta_{ij} . X_i . X_j + e \qquad (1)$$

Where, i and j are the linear and quadratic coefficients, respectively, β is the regression coefficient, k is the number of factors studied and optimized in the experiment and e is the random error. Statistical parameters obtained from the analysis of variance (ANOVA) for the quadratic models of the PHE biodegradation are given in Table 2. Since R^2 always decreases when a regressor variable is dropped from a regression model in statistical modeling the adjusted R^2 which takes the number of regressor variables into account is usually selected from literature [16]. The R^2 coefficient gives the proportion of the total variation in the response variable explained or accounted for by the predictors (X's) included in the model [17]. In the present study, the adjusted R^2 ranged from 0.936 (Y_1) to 0.999 (Y_3). The regression coefficient of determination (R^2) of the model in this study indicated that the polynomial model adequately represent the relationship between the studied variables.

In order to gain a better understanding of the experimental results, the predicted values for removal of PHE (Y_1), extent of biodegradation relative to initial PHE and biomass (Y_2) and the specific growth rate (Y_3) in the present study are given in Table 1 (the regression coefficients of the polynomial models). A detailed discussion on Table 1 is presented in the following sections. 13 trails were generated for the 2- factorial CCFD.

3.2. Removal of PHE (Y1) and extent of biodegradation relative to initial PHE and biomass (Y2)

Statistical parameters for Removal of PHE (Y_1) and extent of biodegradation relative to initial PHE and biomass (Y_2) obtained from the analysis of variance (ANOVA) for the quadratic models of the PHE biodegradation are given in Table 2. The significant effects (factors and interactions) were indicated by p-values less than 0.05, since these are significantly differences from zero at the 95% confidence level. The adequacy of the model, the lack-of fit (LOF) test and the adequacy of precision were presented for suggested quadratic model. The regression coefficient were in the range of 0.936 – 0.937 indicating that Y_1 and Y_2 responses are significantly fitted by polynomial model (Table 2).

Based on the statistical analysis, LOF was not significant and regression was significant for the quadratic model (Table 2). The p-values of regression for the resulted experiment were 0.0001 and 0.0005, respectively. The model summary statistics values shows the standard

deviation value, the R-squared value, adjusted R-squared value and the predicted residual error sum of squares value (PRESS) statistic for complete model, at which low standard deviation, R-squared near 1 and relatively low PRESS are desirable [18]. Also, the predicted values obtained from model fitting technique were seen to be sufficiently correlated to the observed values in Table 2.

Table 1: *Arrangement of the CCFD for the two independent variables and their coded, experimental and predicted values for removal of PHE (Y_1), extent of degradation relative to initial PHE concentration and biomass (Y_2) and the specific growth rate (Y_3)*

Run	Variables/Coded		Actual	Predicted	Actual	Predicted	Actual	Predicted
	X_1	X_2	Y_1		Y_2		Y_3	
1	17(-1)	12(-1)	0.052	0.130	0.018	0.015	0.036	0.036
2	94(+1)	12(-1)	0.11	-0.051	0.022	0.019	0.017	0.017
3	17(-1)	132(+1)	1.00	0.999	0.170	0.180	0.005	0.006
4	94(+1)	72(0)	0.830	0.810	0.020	0.022	0.007	0.007
5	17(-1)	72(0)	0.830	0.760	0.047	0.062	0.013	0.012
6	94(+1)	12(-1)	0.410	0.590	0.012	0.027	0.012	0.012
7	55.5(0)	132(+1)	0.076	0.160	0.011	0.017	0.030	0.030
8	55.5(0)	72(0)	0.990	1.020	0.100	0.099	0.007	0.006
9	55.5(0)	72(0)	0.830	0.800	0.040	0.045	0.013	0.014
10	55.5(0)	72(0)	0.890	0.0800	0.070	0.045	0.014	0.014
11	$_{55}$.5(0)	72(0)	0.840	0.0800	0.050	0.045	0.014	0.014
12	55.5(0)	72(0)	0.700	0.0800	0.060	0.045	0.013	0.014
13	55.5(0)	72(0)	0.830	0.800	0.035	0.045	0.013	0.014

Table 2: *Summery of analysis of variance (ANOVA) for the polynomial model*

Variables	Y_1	Y_2	Y_3
R^2	0.936	0.937	0.999
R^2 adjusted	0.905	0.892	0.997
Prob.>F	<0.0001 (Significant)	0.0005 (Significant)	<0.0001 (Significant)
Lack of fit(LOF)	0.1108	0.4279	0.1245
*Std.Dev.	0.11	0.015	0.005
**PRESS	0.34	0.006	0.006
Adequate precision	15.823	15.899	83.63

* Standard deviation, ** Predicted Residual Error Sum of Squares

Therefore, the polynomial model (built with codified factors) was selected to describe the response surface of PHE biodegradation within this region:

$$Y_1 = 0.8 - 0.088X_1 + 0.43X_2 - 0.12X_1^2 - 0.20X_2^2 \quad (2)$$

$$Y_2 = 0.045 - 0.018X_1 + 0.041X_2 + 0.013X_2^2 - 0.039X_1X_2 - 0.02X_1X_2^2 \quad (3)$$

In the polynomial models obtained equation 2 and 3, positive signs for coefficient β_2 (+ 0.43) for removal of PHE (Y_1) and (β_2 = + 0.041) for extent of biodegradation relative to initial PHE and biomass (Y_2) indicated that the biodegradation increase with increased level of factor reaction time (X_2). Moreover, a negative sign for regression coefficient of β_1 (- 0.088) for Y_1 and β_1 = - 0.018 for Y_2 show removal of PHE decreases with increase in the initial PHE concentration value (X_1).

The Figure 1a shows three dimensional plots of Y_1 for removal of PHE. The maximum achievable removal of PHE was almost 100% when the PHE concentration lower than 55.5 mg/l was used. The rapid reduction of PHE in the medium might be due to assimilation of PHE in the cell. The removal of PHE was minimal after 132 hours of reaction time (X_2) when the highest initial concentration of PHE was used (i.e. 94 mg/l). This Figure (1a) also shows that the removal of PHE increase with increased reaction time (X_2). Therefore, the removal of PHE is likely to increase with increased reaction time(X_2) and decrease with increased initial concentration of PHE. Initial PHE concentration should be made to the lowest possible level because this compound at high concentration is toxic for mixed culture and exhibited inhibitory effect.

Figure 1b shows the extent of biodegradation relative to initial PHE and biomass (Y_2). It can be seen that the optimum conditions for extent of biodegradation relative to initial PHE and biomass (Y_2) differs from removal of PHE (Y_1). This is because Y_2 is defined by taking into account the amount of biomass produced in the system, at which the biomass undergoes different growth phase throughout the reaction time (X_2), consequently making Y_2 values inconsistent (since the biomass is the denominator in Y_2).

Thus, a lower initial PHE concentration (X_1) and a higher reaction time (X_2) are needed for the maximum relative biodegradation Y_2. Figure 3b also shows that an increase in initial PHE concentration (X_1) decreases the Y_2 because at high concentration this compound is toxic for microorganisms. The maximum biodegradation of PHE, expressed as the extent of biodegradation relative to initial PHE concentration and biomass (Y_2) was found equal to 0.17 mg/mg/mg (degraded PHE/initial PHE/biomass) when the lowest initial PHE concentration (X_1) was used (i.e.17 mg/L) after 132 hours reaction time (X_2).

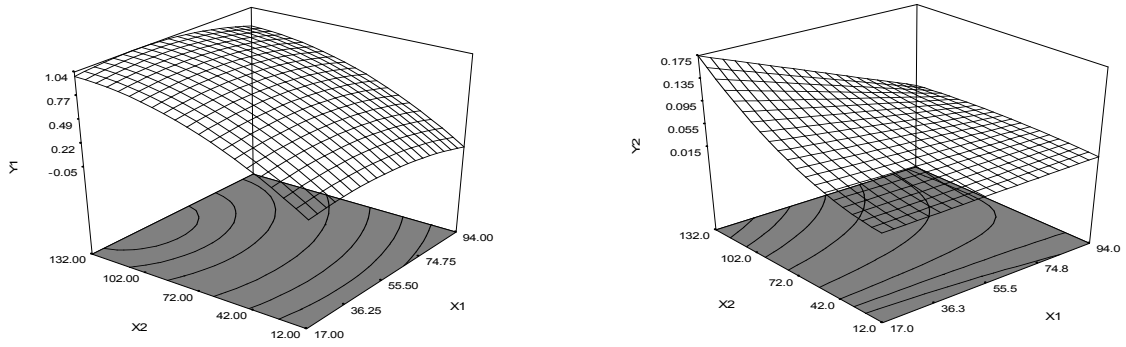


Figure 1: *Three-dimensional plots of the polynomial model within a full factorial central-composition design: (a) Removal of PHE (mg/mg) (Y_1) (b) Extent of biodegradation relative to initial PHE mass and biomass (mg/mg/mg) (Y_2).*

3.3. Specific growth rate

The response of specific growth rate (Y_3) based on experimental design is shown in Table 1. A polynomial regression model was made by using coded values from the estimation of data:

$$Y3 = 0.014 - 0.0002X_1 + 0.012X_2 - 0.0014X_1^2 - 0.004X_2^2 + 0.005X_1X_2 + 0.0014X_1^2X_2 - 0.004X_1X_2^2 \quad (4)$$

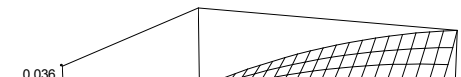
The corresponding analysis of ANOVA is presented in Table 2. Based on above statistical analysis, LOF was not significant and regression was significant for the polynomial model for equation 4. The *p*-values of the LOF and regression for the polynomial model were 0.1245 and <0.0001, respectively. This is associated with a regression coefficient of 0.999 indicating the experimental results were best fitted by a quadratic model. The adequate precision ratio was 85.63 indicates an adequate signal. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable [19]. The standard deviation was 0.005 and PRESS was 0.006 which are found to be satisfing with suggested polynomial model. The predicted and actual values are shown in Table 1. The highest specific growth rate (0.0362 h^{-1}) was observed at experimental run number 1, where the factors of initial PHE concentration (X_1) and reaction time (X_2) were used at lower level of 17 mg/l and 12 h, respectively, but at higher reaction time (X_2) and higher initial PHE concentration (X_1) this response decreased.

Figure 2 shows the three-dimensional plot of Y_3. At high concentration of PHE the specific growth rate (μ) was smaller as compared to low concentration of PHE due to the fact that a high PHE concentration is normally associated with toxicity effect. The response curvature shows a maximum region towards minimum value of 17 mg/l (X_1) and minimum value of 12 hours (X_2). It is observed that Y_3 was more susceptible to the change in X_1 at both low PHE concentrations (i.e. $17 < X_1 < 36$ mg/l) than that in high PHE concentration (i.e. $85 < X_1 < 94$ mg/l) and high reaction time (X_2 = 102 -132 hours).

Thus, a maximum value for Y_3 response is desirable at minimal reaction time and minimal initial PHE concentration because of the toxicity effect exhibited by the PHE compound when it is present at high initial concentration which is inhibitory to mixed culture. A minimum reaction time is desired as the mixed culture showed a relatively short lag phase and resulted in a much sooner log phase. Therefore, a relatively low initial PHE concentration (X_1) which is less than 55.5 mg/l and reaction time (X_2) that is less than 72 hours were observed to be favorable for higher specific growth rate.

3.4. Overlay plot Analysis

Since the optimum condition of one response differs to the other, therefore it is crucial to optimize the design criteria favorable for responses desired. By overlaying critical response contours on a contour plot we can visually search for the best combination design parameters. The overplay plot was generated by superimposing the contours for the various response surfaces such as Y_1, Y_2 and Y_3. The shaded portion of the overlay plot defined the permissible values of the dependent variables as shown in Figure 3. The area that satisfies the constraints is grey coloured. The design criteria are at Y_1 to be more than 0.973, Y_2 more than 0.069 and Y_3 more than 0.008. A verification of the suggested design factors for optimal responses was justified by conducting experiment with initial PHE concentration (X_1) and reaction time(X_2) factors selected from the overlay region. Table 1



compares the experimental results with predicted values from the regression model of DOE. The standard deviation was between 0.0004-0.016 which is low (Table 3). These experimental results are in close agreement with the model prediction.

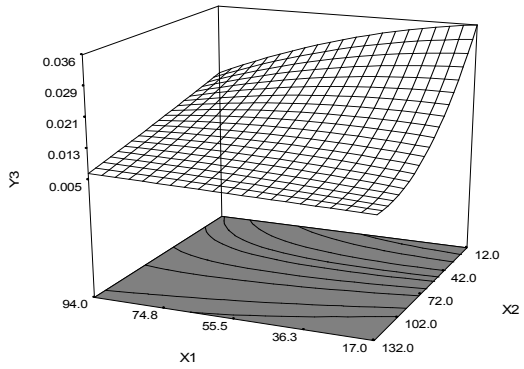


Figure 2: *Three-dimensional plot of the polynomial model for two variables of X_1 and X_2 on specific growth rate (Y_3)*

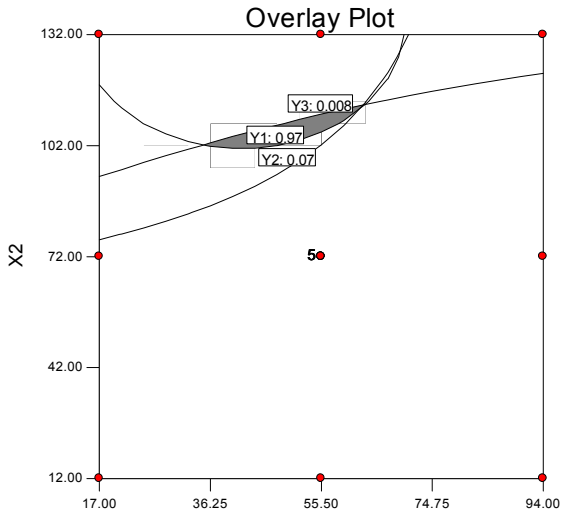


Figure 3: *Overlay plot for the factors initial concentration (X_1) and reaction time (X_2) in the optimum region*

Table 3. *The calculated and measured values of dependent response*

Response	Criteria	Predicted values (DOE)	Actual values (exp.)	Error*	Std.Dev.
Y1	Maximum	0.98 (mg/mg)	0.987 (mg/mg)	+ 0.007	± 0.005
Y2	Maximum	0.08 (mg/mg/m)	0.061 (mg/mg/mg)	- 0.019	± 0.016
Y3	Maximum	0.0081 (h^{-1})	0.0086 (h^{-1})	+ 0.0005	± 0.0004

*Error: (*Yi*) exp − (*Yi*) DOE.

The experimental data were 43 mg/l for initial PHE concentration (X_1) and 104 hours for reaction time (X_2).

4. Conclusion

Biodegradation of PHE was successfully achieved in low and middle concentration by the isolated mixed culture. The PHE biodegradation was carried out in batch bioreactor using response surface methodology (RSM) based on central composite face entered design (CCFD). The central composite design has been found to be a useful response surface methodology as suitable tools for analyzing bioremediation studies in systems containing toxic pollutant which is inhibitory to the growth of microorganisms. The comparison between predicted and experimental values was in good agreement, implying that empirical

models derived from RSM could adequately describe the relationship between the factors and response in the biodegradation of phenanthrene. These models can then be used to predict PHE degradation performance under experimental studied.

6. References

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