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Biodegradation of Phenanthrene by Mixed Culture Consortia in Batch Bioreactor using Central Composite Face-Entered Design

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ABSTRACT: Biodegradation of Phenanthrene (PHE) was studied in aqueous culture to demonstrate the potential of the mixed culture in degrading 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 by the isolated mixed culture. A full factorial Central Composite Design of experiments was used to construct response surfaces with the removal, the extent of PHE degradation and the specific growth rate as responses. The initial Phenanthrene concentration (X1) and the reaction time (X2) were used as design factors. The result was shown that experimental data fitted with the 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 (Y1) was found to be 0.100 mg/mg (degraded PHE/initial PHE). The maximum extent of biodegradation relative to initial PHE concentration and biomass (Y2) was 0.171 mg/mg/mg (degraded PHE/initial PHE/biomass). This maximum biodegradation correspond to the factors combination of middle level of PHE content (X1= 19.06 mg/L) and the highest level of reaction time (X2 = 132.00 hours). The removal efficiency of PHE biodegradation was achieved 100%. 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.

Key words: Biodegradation, Phenanthrene, Mixed culture, Response surface methodology, Central composite design

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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 (Freeman and Cattell, 1990; Wild and Jones, 1995). Wastewater analyses reveal high PAHs concentrations from sources of industrial waste, domestic sewage, atmospheric rainfall, airborne pollutants and road surface run-off (Carolyn, *et al.*, 2003).

Phenanthrene, one of the most abundant PAHs in the environment (Cerniglia, 1993), is included in the U.S. EPA list of priority pollutants (Keith and Telliard, 1979; Supakaa, 2001). Biological treatment has been used to treat contaminated sites for many years. As with other treatment strategies, the effectiveness and cost of bio-treatment technologies are both site-specific and contaminant-specific. Because of the potential advantages offered by bioremediation, there remains a strong interest in the continued development of bio-treatment processes. 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 (Vidali, 2001). 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 (Lei, *et al.,* 2002; White, 1986; Pahlman and Pelkonen, 1987; Kramers and Van der Heijden, 1990). Bioremediation is a low cost and low disturbance solution for cleaning those sites and has already been tested for several PAHs (Kastner and Mahro, 1996).

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 (Rigasa, *et al.,* 2005). In addition, the design expert too 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 analyses 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 (Montgomery, 2001). 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 the effects of variables and overall experimental error with a minimum number of experiments Montgomery, 2001). Center points in CCD design are usually repeated 4-6 times to get a good estimate of experimental error (pure error). Five center points will be created by default for experimental design with two factors. Central composite designs generally require 5 levels for each factor: -Alpha, -1, 0, 1 and +Alpha. 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 (Rigasa, *et al.,* 2005). The objective of the present work is to investigate the Phenanthrene biodegradation in aqueous culture of a mixed strain 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 experiment (data not shown). The interaction between factors influencing dependent (response) parameters such as removal of PHE (Y1), extent of biodegradation relative to initial PHE concentration and biomass (mg/mg/mg) (Y2) and the specific growth rate (Y3) were studied.

MATERIALS & METHODS

Mixed strains of microorganisms were obtained from Pria 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.,* 2002. 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. 1.5 mL of seed culture was transferred to MSM media in three different flask 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}).

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 culture volume (25 mL). The extracts were 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 of length, 0.25 mm of inside diameter and 0.25 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 °C for 3 min and then the temperature was increased to 280 °C at a rate of 10 °C/min. The injector and detector temperatures were set at 290 °C. The data were obtained and the experiments were repeated in triplicates for accuracy and the means were employed. Standard deviations were low, most of the data in the range of 5 to 8 percent.

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 (X1) and the reaction time (X2). 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 (Y1), extent of biodegradation relative to initial PHE concentration and biomass (mg/mg/mg) (Y2) and the specific growth rate (Y3). After running these 13 trials, the corresponding quadratic models for the above response and parameters were computed.

RESULTS & DISCUSSIONS

Experimental data were found to be best fitted to polynomial model and regression coefficient was determined. Once the experiments are performed the coefficients of the polynomial model were calculated using the equation as follow (Ramesh Balusu, *et al.,* 2005).

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

Where, *i* and *j* are the linear and quadratic coefficients, respectively, *ß* 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 regression variable is dropped from a regression model in statistical modeling the adjusted R^2 which takes the number of regression variables into account is usually selected (Ahmadi, *et al.,* 2005). 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 (Hamsaveni, *et al.,* 2001).

Table 1. Arrangement of the CCFD for the two independent variables and their coded, experimental and predicted values for removal of PHE (Y1), extent of degradation relative to initial PHE concentration and biomass (Y2) and the specific growth rate (Y3)

Run	Variables/Coded		Actual	Predicted	Actual	Predicted
	X1	X2	Y1		Y2	
1	17(-1)	12(-1)	0.052	0.130	0.018	0.015
2	94(+1)	12(-1)	0.11	-0.051	0.022	0.019
3	17(-1)	132(+1)	1.00	0.999	0.170	0.180
4	94(+1)	72(0)	0.830	0.810	0.020	0.022
5	17(-1)	72(0)	0.830	0.760	0.047	0.062
6	94(+1)	12(-1)	0.410	0.590	0.012	0.027
7	55.5(0)	132(+1)	0.076	0.160	0.011	0.017
8	55.5(0)	72(0)	0.990	1.020	0.100	0.099
9	55.5(0)	72(0)	0.830	0.800	0.040	0.045
10	55.5(0)	72(0)	0.890	0.0800	0.070	0.045
11	55.5(0)	72(0)	0.840	0.0800	0.050	0.045
12	55.5(0)	72(0)	0.700	0.0800	0.060	0.045
13	55.5(0)	72(0)	0.830	0.800	0.035	0.045

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

Variables	Y1	Y2
R^2	0.936	0.937
R^2 adjusted	0.905	0.892
Prob.>F	<0.0001 (Significant)	0.0005 (Significant)
Lack of fit (LOF)	0.1108	0.4279
*S.D	0.11	0.015
**PRESS	0.34	0.006
Adequate precision	15.823	15.899

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

In the present study, the adjusted R^2 ranged from 0.936 (Y1) to 0.999 (Y3). The regression coefficient of determination (R^2) of the model in this study indicated that the polynomial model adequately represent the relationship between the variables study.

In order to gain a better understanding of the results, the predicted values for removal of PHE (Y1), extent of biodegradation relative to initial PHE and biomass (Y2) and the specific growth rate (Y3) 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. About 13 trails were generated for the 2- factorial CCFD.

Statistical parameters for removal of PHE (Y1) 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 different 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 was in the 0.936 indicating that Y1 response is significantly fitted by polynomial model (Table 2).

Based on the statistical analysis, LOF was not significant and regression was significant for the polynomial model (Table 2). The *p*-values of regression for the resulted experiment were 0.0001. 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 (Design Expert Version 6.0.7). Also, the predicted values obtained from model fitting technique were seen to be sufficiently correlated to the observed values in Table 2. Therefore, the polynomial model (built with codified factors) was selected to describe the response surface of PHE biodegradation within this region:

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

In the polynomial models obtained equation 2, positive signs for coefficient β_2 (+ 0.43) for removal of PHE (Y1) indicated that the biodegradation increase with increased level of factor reaction time (X2). Moreover, a negative sign for regression coefficient of β_1 (- 0.088) for Y1 show removal of PHE decreases with increase in the initial PHE concentration value (X1).

The Fig. 1a shows three dimensional plots of Y1 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 h of reaction time (X2) when the highest initial concentration of PHE was used (i.e. 94 mg/L). This Fig. (1a) also shows that the removal of PHE increase with increased reaction time (X2). Therefore, the removal of PHE is likely to increase with increased reaction time (X2) and decrease with increased initial concentration of PHE. Initial PHE concentration should be made to the lowest possible level because this compound is toxic for mixed culture in high concentration and exhibited inhibitory effect.

The extent of biodegradation relative to initial PHE and biomass (Y2) based on experimental design is shown in Table 1. A polynomial regression model was made by using coded values from the estimation of data:

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

Based on this statistical analysis, LOF was not significant and regression was significant for the polynomial model for equation 3. The *p*-values of the LOF and regression for the polynomial model were 0.4279 and 0.0005, respectively.

Table 3. The calculated and measured values of dependent response

Response	Criteria	Predicted values (DOE)	Actual values (exp.)	Error*	S.D
Y1	Maximum	0.100 (mg/mg)	0.987 (mg/mg)	+ 0.007	± 0.005
Y2	Maximum	0.171 (mg/mg/m)	0.125 (mg/mg/mg)	- 0.019	± 0.016

*Error: (*Yi*) exp " (*Yi*) DOE.
The experimental data were 43 mg/l for initial PHE concentration (X1) and 104 hours for reaction time (X2).

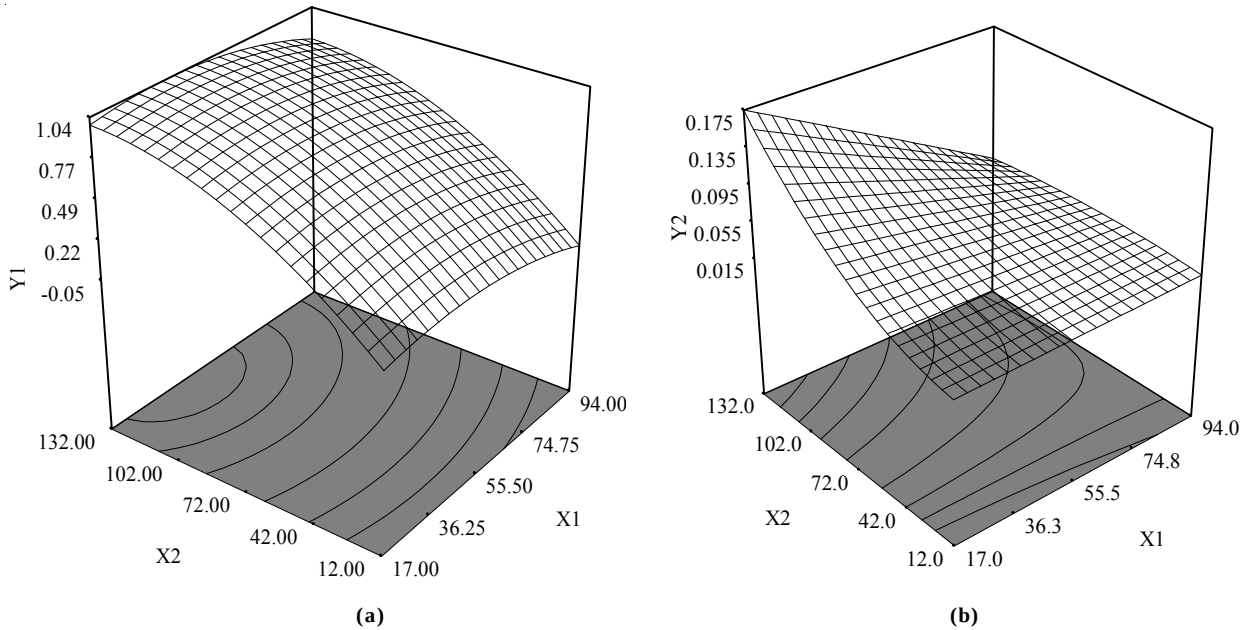

Fig. 1. Three-dimensional plots of the polynomial model within a full factorial central-composition design: (a) Removal of PHE (mg/mg) (Y1) (b) Extent of biodegradation relative to initial PHE mass and biomass (mg/mg/mg) (Y2)

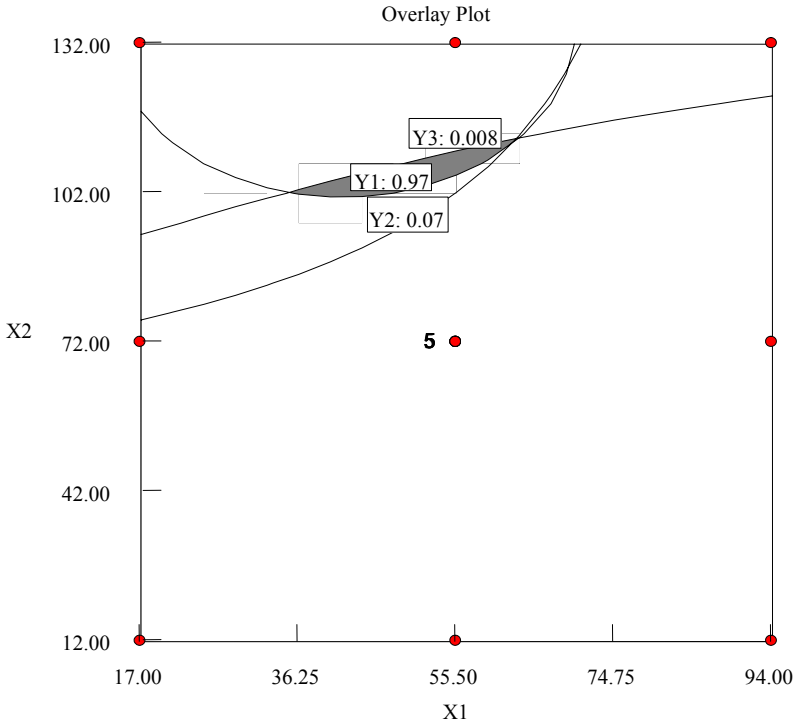

Fig. 2 . Overlay plot for the factors initial concentration (X1) and reaction time (X2) in the optimum region

This is associated with a regression coefficient of 0.937 indicating the experimental result is best fitted by a polynomial model.

The adequate precision ratio was 15.899 indicates an adequate signal. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable (Parajo, *et al.,* 1992). The standard deviation was 0.015 and PRESS was 0.005 which are found to be satisfing with suggested polynomial model. The corresponding analysis of ANOVA is presented in Table 2.

Fig. 1b shows the extent of biodegradation relative to initial PHE and biomass (Y2). It can be seen that the optimum conditions for extent of biodegradation relative to initial PHE and biomass (Y2) differs from removal of PHE (Y1). This is because Y2 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 (X2), consequently making Y2 values inconsistent (since the biomass is the denominator in Y2).

Thus, a lower initial PHE concentration (X1) and a higher reaction time (X2) are needed for the maximum relative biodegradation Y2. Figure 2 also shows that an increase in initial PHE concentration (X1) decreases the Y2 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 (Y2) was found equal to 0.17 mg/mg/mg (degraded PHE/initial PHE/biomass) when the lowest initial PHE concentration (X1) was used (i.e.17 mg/L) after 132 h reaction time (X2).The response curvature shows a maximum region towards minimum value of 17 mg/l (X1) and maximum value of 132 h (X2). It is observed that Y was more susceptible to the change in X1 at both low PHE concentrations (i.e. 17< X1<36 mg/L) than that in high PHE concentration (i.e. 85 <X1<94 mg/L) and high reaction time (X2 = 102 -132 h). The predicted and actual values are shown in Table 1. The highest extent of biodegradation relative to initial PHE concentration and biomass (Y2) (0.17 mg/mg/mg (degraded PHE/initial PHE/ biomass) was observed at run number 3, where the factors of initial PHE concentration (X1) was used the lowest initial concentration and reaction time (X2) was used at highest level, respectively. Therefore, a relatively low initial PHE concentration (X1) which is less than 55.5 mg/l and reaction time (X2) that is less than 72 hours were observed to be favorable for higher extent of biodegradation relative to initial PHE concentration and biomass (Y2).

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 Y1, Y2. The shaded portion of the overlay plot defined the permissible values of the dependent variables as shown in Fig. 2. The area that satisfies the constraints are grey colored. The design criteria are at Y1 to be more than 0.97 and Y2 more than 0.125. The selected mixed culture had a maximum biodegradation of PHE, as expressed the removal of PHE (Y1) was found to be 0.100 mg/mg (degraded PHE/initial PHE) and was 0.171 mg/ mg/mg (degraded PHE/initial PHE/biomass) in terms of the extent of biodegradation relative to initial PHE concentration and biomass (Y2). This was achieved at the middle level of PHE content (X1 = 19.06 mg/L) and the highest level of reaction time (X2 = 132.00 hours). The removal efficiency of PHE biodegradation was achieved 100%. A verification of the suggested design factors for optimal responses was justified by conducting experiment with initial PHE concentration (X1) and reaction time(X2) 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. These experimental results are in close agreement with the model prediction.

The present work is a statistical and optimization studies concerned with process parameters to define a suitable range for the best treatment and the bio-remediation of PAH in the treated industrial effluents. The F/M ratio was not considered as a variable in this process since the sludge was not considered as a critical process parameter in digestion of PAH. The media pH was initially adjusted the change of pH was observed during the course of biodegradation of PAH.

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.

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NOMENCLATURE

DOE: design of expert
RSM: response surface methodology
CCD: central composite design
CCFD: central composite face entered design
X1: initial PHE concentration (mg/l)
X2: reaction time (h)
Y1: removal of PHE (mg/mg)
Y2: extent of biodegradation relative to initial PHE concentration and biomass (mg/mg/mg)
Y3: specific growth rate (1/h)
R^2: coefficient of determination
ANOVA: analysis of variance Greek symbols
β_0: intercept coefficient of Eq. 1
β_i, β_j: linear coefficient of Eq. 1
βij: interaction coefficient of Eq. 1
β_{ii}, β_{jj}: quadratic coefficient of Eq. 1
k: the number of factors studied
e: random error Eq. 1
μ: specific growth rate (h^{-1})

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