OPTIMIZATION OF BIODEGRADATION OF ANTHRACENE BY CORYNEBACTERIUM SP AND PSEUDOMONAS PUTIDA

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Abstract

The carcinogenic potential of industrial effluent discharged of anthracene on the ambient environment withcontamination to soils and aquifers is not only environmental concern but difficulty in implementation of biodegradation of anthracene due to inability to optimizing the process parameters. The optimization of process parameters for biodegradation of anthracene with the activity of Corynebacterium sp and Pseudomonas putida using response surface methodology was aimed to be investigated. A 2^3 of full factorial central composite design was employed for developed quadratic polynomial model to achieve optimal performance of the process. Statistically,high correlation coefficient (R^2) for quadratic polynomial model for biodegradation of anthracene by activity of Corynebacterium sp 0.9659 ($p<0.05$)and Pseudomonas putida 0.9939 ($p<0.05$)were obtained. The optimum conditions for anthracene degradation by Corynebacterium sp were 69.99g/L, 83.20hours and 3.4735vvm with about 98.48 percent of anthracene degraded while Pseudomonas putida were 69.989g/L, 83.58hours and 3.5vvm with 89.51percent of anthracene degraded.

Key words: Anthracene, biodegradation, Corynebacterium sp, Pseudomonas putida, Optimization

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INTRODUCTION

The drastic oil bunkering, oil spilling and industrial effluent discharges which affect the survival of organisms was attributed to the toxicity and concentration of oil in the environment (Kumar et al, 2010). Polycyclic aromatic Hydrocarbon (PAH) was identified as secondary pollution generated by the oil bunkering and spilling, and anthropogenic discharged into the environment which induced health problems due to its toxicity, teratogenic, mutagenic and harmful effects to living organisms (Mrozik et al, 2003; Kumar et al, 2010; Faust, 1991; Rumaet al, 2007; Samanta et al, 2002). Anthracene is a hydrophobic, persistent and tri-cyclic hydrocarbon that found in high concentrations in polycyclic aromatics hydrocarbon – contaminated sediments, surface soils, water and waste sites which are toxic to aquatic organism (Azeez et al, 2012; Rumaet al, 2007). PAHs enter into the human body through inhalation, ingestion of food and water that are contaminated with PAHs (Azeez et al, 2012). Humans could be exposed to PAHs, if the skin touches PAH contaminated soil or products like heavy oils, coal tar, roofing tar or creosote (Abd-Elsalam et al, 2009).

The physical and chemical methods for remediation of anthracene have not been economically implemented due to expensive nature of the process and management of the generated waste. Varieties of living organisms have been used to utilize anthracene as the sole source of carbon and energy (Abd-Elsalam et al, 2009; Kumar et al, 2010; Van Herwijnen et al, 2003; Alcalde et al, 2002; Azeez et al, 2012; Samanta et al, 2002; Korade and Fulekar, 2008). Degradation of anthracene into non-toxic substance using biological agents had been considered a better clean up technique due to its ecofriendly but have not being implemented due to unknown optimal conditions of degradation of anthracene and microbes (Kastner et al, 1999; Eibes et al, 2005).

Many researchers have work on the degradation of anthracene, metabolism and its kinetics with the activity of some microbes such as *Escherichia coli, Soil bacterium, Alcaligenes sp, Thiobactersubterraneus, Pseudomonas* sp, *Pseudomonas putida, Corynebacterium sp, Sphingomonas sp,Sphingopyxis sp, Nocardia sp, Beijerinckia sp, Rhodococcus sp* and *Mycobacterium sp* (Kumar et al, 2010; Mrozik et al, 2003; Shokrollahzadel et al, 2012; El-Gendy et al, 2010; Van Herwijnen et al, 2003; Moody et al, 2001; Rodrigo et al, 2005; Surani et al, 2011). Kinetics of bioremediation of anthracene have been studied **(**Prasanna, 2008**).** More so, Azeez et al (2014) and Owabor et al (2010) have studied the diffusion and kinetics of degradation on anthracene with the activity of *Corynebacterium sp* and *Pseudomonas putida* in a saturated porous medium without signified the optimal conditions for the degradation. Kostenko et al, (2014) reported the significant effect of *Pseudomonas putida* in the separation of solids, water and oil from oil sands tailing without optimizing the conditions of separation.

Though, *Corynebacterium sp* and *Pseudomonas putida* have been identified as promising degraders of Anthracene (Azeez et al, 2012; Kumar et al, 2010). The use of *Corynebacterium sp* and *Pseudomonas putida* have not been optimally practice due to inability to quantify the optimal conditions of degradation of anthracene with specific microbes, thereby resulted in the waste of time, concentration and available oxygen used. The aim of the present research was to evaluate the optimal conditions such as degradation time, concentration and aeration for biodegradation of the anthracene.

MATERIALS AND METHOD

Anthracene, dichloromethane and hexane (Analytical grade Chemicals) were purchased from Patanne Chemicals, a renowned laboratory chemicals and equipment dealer in Benin City.

Preparation of Mineral Salt Medium and Isolation of Microbes

The microorganisms *Corynebacterium sp* and *Pseudomonas putida* for the experiment were isolated from the subsurface soil of about 0-15cm depth obtained from an uncultivated land in the Nigerian Institute for oil palm research (NIFOR), Benin City in Nigeria. The subsurface soil used for isolation of microbes has been described by (Azeez et al, 2010). The soil was sieved using 2mm mesh screen for uniform particle size and stored in sterilized polyethylene bag at room temperature covered with aluminium foil for further use. Mineral salt medium (MSM) was used to avoid drastic fluctuation of pH which may be detrimental to the viability of the microbes in the batch medium and it was carbon free before anthracene was added after autoclaved at 121^0C for 15 minutes. The MSM was prepared with Analytical grade chemicals composition: KH_2PO_4 (9.0g/l), K_2HPO_4 (1.5g/l), NH_4Cl (1.5g/l), $CaCl_2$ (20mg/l), and $MgSO_4$ (0.2g/l). The pH of the medium was standardized to 7.2 using 0.1N NaOH. The MSM was sterilized in an autoclaved at 121^0C for 15 minutes and then stored in a secured corner in the laboratory until the experiment was set up.

0.5 g of soil samples were added into 100 ml MSM. The medium containing the soil and 0.1% w/v anthracene was incubated at 28 ± 2^0C on a rotary incubator shaker at 150 revolutions per minute for 24 h. The pure culture of colonies of *Corynebacterium sp* and *Pseudomonas putida* were maintained on nutrient agar plates for 72hours at 28 ± 2^0C temperature for production of the microbes' enmasse mainly for reduction of the lag phase and suitability of the inoculums in anthracene contaminated environment before the biodegradation.

Biodegradation Analysis of Anthracene

The quantity of anthracene as presented in the Table 1 was dissolved in 10% dichloromethane solution and make up to 1 liter by water. The solvent was volatized from anthracene solution under fume-hood.

250 ml of each of the anthracene solution measured into bioreactor vessel and 15 ml of inoculums was transferred from each agar plate of *Corynebacterium sp* and *Pseudomonas putida* into anthracene contaminated water and incubated at 28±2^{0}C on a rotary incubator shaker at 150 revolutions per minute and supernatant was withdrawn for analysis at designed biodegradation time (3.546, 24, 54, 84 and 104.45 hour), centrifuged, decanted and cells of *Corynebacterium sp* and *Pseudomonas putida settled down at the bottom of the centrifuged tube were scooped and dried in an oven at* 60^{0}C for 8 hours.

The method described by (Azeez et al, 2012) was employed using UV visible spectrophotometer to measure absorbance of the anthracene in aliquot. The absorbance of the anthracene was recorded at a wavelength of 267 nm in the UV region after isolation of the microbes by centrifuge 10 ml aliquots of rotating at 10,000 revolutions per minute for 20 minutes and allowed to settle for 30minutes to get a clear supernatant. 5 ml of the clear supernatant was extracted with 5ml of hexane for 10 minutes in a separating funnel. The top solution in a separating funnel at the end of the extraction was a solution of the anthracene in hexane and poured into the corvettes of the spectrophotometer and absorbance readings at a wavelengths of 267 nm was recorded. The procedure was repeated in designed biodegradation time immediately after inoculation with *Corynebacterium sp and Pseudomonas putida* for a period of 104.45 hours of incubation and solutions of anthracene with hexane were prepared to give a concentration of 0.3mg/ml. The absorbance of the solutions was read at the appropriate wavelengths 267nm for the anthracene solution. The standard model obtained by Azeez et al (2012) was used for the conversion of anthracene absorbance to mg/L. The percentage of biodegradation of anthracene was evaluated as follows:

$$\%\, Degraded\ (Y_i) = \frac{C_0 - C_i}{C_0}\ x\ 100\% \qquad (1)$$

Where Y_i is the concentration of anthracene utilized or degraded, C_0 and C_i is the initial concentration of anthracene and concentration of anthracene at any time after inoculums respectively measured in mg/L

Experimental design and Statistical Optimization

A 2^3 full factorial Central Composite Design (CCD) with response surface methodology (RSM) of Design – Expert software version 6.0.8 (2002 East Hennepin ave., Suite 480 Minneapolis, MN 55413, stat Ease, Inc.) was used. Eight hundred milliliters of the autoclaved MSM and 0.4 liters of anthracene solution with variable concentration and inoculum of 200 mL were introduced aseptically to make up 0.5 liters of the working volume. Three factors were considered to perform response surface methodology (RSM) with CCD at variable concentration of anthracene (X_1), fermentation time (X_2) and aeration (X_3). The bioreactor was operated for variable concentration (9.66, 25, 47.5, 70, 85.34 g/L), aeration (2.159, 2.5,

3.0, 3.5 and 3.84 vvm) and biodegradation time (3.546, 24, 54, 84 and 104.45 hours) at a temperature of 28±2^{0}C as presented in the Table 1. Aliquot was withdrawn for analysis based on designed factors of bioreactor for degradation of anthracene as presented in the Table 2 for coded and experimental variables. The range of these values was considered since it characterized the optimum range for the microbes and the expected range in which the process could be operated.

Table1: Levels of different process variables for biodegradation

	Ranges and Levels				
Process Variables	-1.682	-1	0	1	+1.682
Concentration of Anthracene (X_1) (g/L)	9.660	25	47.5	70	85.34
biodegradation time (X_2) (hr)	3.546	24	54	84	104.45
Aeration (X_3) (vvm)	2.159	2.5	3.0	3.5	3.841

All the experiments were done in triplicates and the average of anthracene degraded by *Corynebacterium sp* (Y_1) and *Pseudomonas putida* (Y_2) obtained were taken as the response function (Y) of the factors. The Second degree polynomials equation (2) which contains factors with interaction terms were used to calculate the predicted response:

$$Y_i = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

Where Y_i is the response of anthracene degraded by *Corynecbacterium sp* and *Pseudomonas putida* as dependent variables; n is the number of independent variables (factors), X_i (i=1, 2, 3…) and X_j(j = 1, 2, 3…) are the concentration of anthracene degraded, degradation time and aeration respectively; ε is the random error; β_0 is offset term, and β_i, β_{ij} and β_{ii} are the coefficients of linear, interaction and quadratic term respectively.

Table 2: CCD Matrix of Coded and Experimental Variables for Biodegradation of Anthracene

	Coded value			Actual Value		
Run	Concentration of Anthracene (X_1) (mg/L)	Degradation time (X_2) (hr)	aeration (X_3) (vvm)	Concentration of Anthracene (X_1) (mg/L)	Degradation time (X_2) (hr)	aeration (X_3) (vvm)
1	-1	-1	-1	25	24	2.5
2	1	-1	1	70	24	3.5
3	0	0	0	47.5	54	3
4	0	1.682	0	47.5	104.45	3
5	0	0	0	47.5	54	3
6	1	1	1	70	84	3.5
7	-1.682	0	0	9.660	54	3
8	0	0	1.682	47.5	54	3.841
9	-1	1	-1	25	84	2.5
10	0	0	-1.682	47.5	54	2.159

11	1	1	-1	70	84	2.5
12	0	0	0	47.5	54	3
13	0	0	0	47.5	54	3
14	-1	1	1	25	84	3.5
15	0	0	0	47.5	54	3
16	0	-1.682	0	47.5	3.546	3
17	1	-1	-1	70	24	2.5
18	1.682	0	0	85.340	54	3
19	-1	-1	1	25	24	3.5
20	0	0	0	47.5	54	3

Numerical analysis of the model was performed with statistical optimization using analysis of variance (ANOVA). The quadratic models were represented as contour plots (3D) and response surface curves were generated for variables.

RESULTS AND DISCUSSION

Fitting of data and Validation of the Models

Anthracene degradation by *Corynebacterium sp* (Y_1) and *Pseudomonas putida*(Y_2) were correlated with the three factors (concentration of anthracene, degradation time and aeration), using the second-order polynomial, as represented by Eq. (1). From the experimental data, quadratic regression models were obtained and reduced to Equation (3) and (4):

$$Y_1 = 199.10 - 0.8398X_1 + 0.7255X_2 - 123.13X_3 + 0.0141X_1^2 - 0.0037X_2^2 + 23.035X_3^2 + 0.0028X_1X_2 - 0.2004X_1X_3 + 0.1308X_2X_3 \quad (3)$$

$$Y_2 = 17.365 - 0.0951X_1 + 1.6188X_2 - 11.067X_3 + 0.00157X_1^2 - 0.00705X_2^2 + 2.2842X_3^2 + 0.0002X_1X_2 - 0.0220X_1X_3 - 0.0005X_2X_3 \quad (4)$$

It is usually necessary to check the fitted model to ensure it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with investigation and optimization of the fitted response surface is likely to give poor or misleading results. The predicted values generated from diagnostics case of statistics were compared with the experimental values for the activity of *Corynebacterium sp* and *Pseudomonas putida* as shown in the Figure 1 and Figure 2 respectively. The coefficient of determination (R^2) was evaluated from Figure 1 and 2 based on equation 5. The high coefficient of the determination (R^2) for both activities of *Corynebacterium sp* (0.9659) and *Pseudomonas putida* (0.9939) satisfied the fitness of the experimental data as also presented in the Table 3.

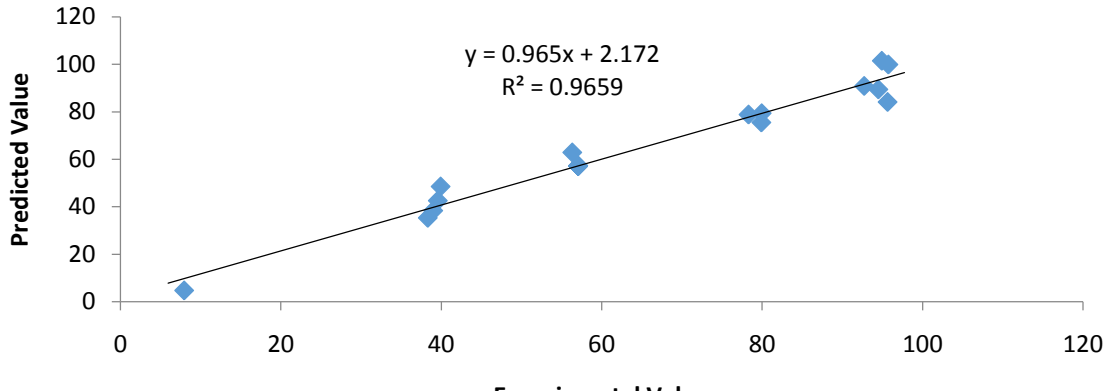

Figure 1: Predicted Value against experimental Value for Anthracene degraded by *Corynebacterium spp*

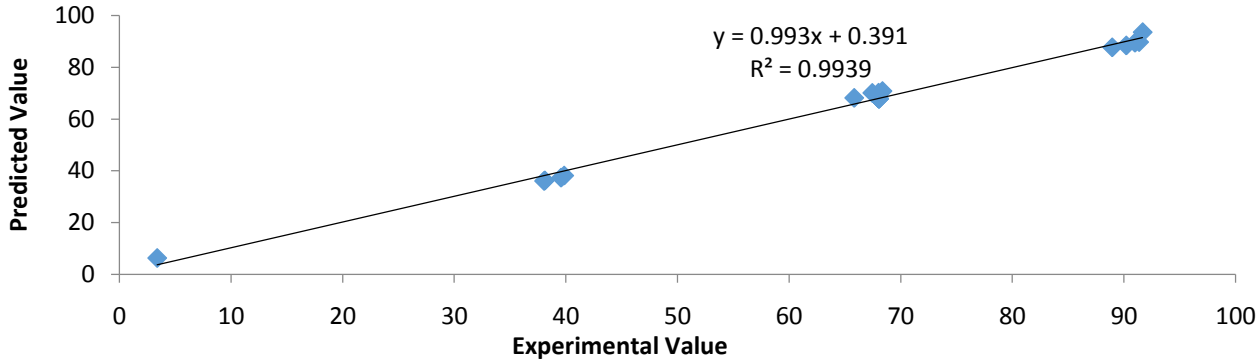

Figure 2: Predicted Value against experimental Value for Anthracene degraded by *Pseudomonas putida*

R^2indicates how much of the observed variability in the data was accounted for by the model, while adj R^2modifies $R^2$by taking into account the number of covariates or predictors (model terms without the constant term) in the model as shown in the equation (6).

$$R^2 = 1 - \frac{SS_r}{SS_m + SS_r} \quad (5)$$

$$R^2_{adj} = 1 - \frac{n-1}{n-p}(1 - R^2) \quad (6)$$

Where SS_m and SS_r is the sum of the squares for the model and residual, *n*the number of runs or experiments, and *p* the number of predictors (term) in the model.

Table 3: ANOVA for response surface quadratic model of biodegradation of Anthracene

Source	sum of	Degree of	Mean	F-value	Prob>F	R^2	Adj R^2	Pred	Adeq

	square	freedom	Square					R^2	Precision
			Biodegradation of Anthracene by Corynebacterium sp						
Model	11000	9	1222.2	31.5029	< 0.0001	0.9659	0.9353	0.7371	21.937
Residual	387.97	10	38.8						
Lack of Fit	387.97	5	77.59						
Pure error	0	5	0						
Total	11388	19							
			Biodegradation of Anthracene by Pseudomonas putida						
Model	9837.1	9	1093	182.0334	< 0.0001	0.9939	0.9885	0.9542	50.387
Residual	60.045	10	6.004						
Lack of Fit	60.045	5	12.01						
Pure error	0	5	0						
Total	9897.2	19							

The response surface models developed in this study with values of R^2higher than 0.9000, say 0.9659 and 0.9939 for *Corynebacterium sp* and *Pseudomonas putida* respectively. Furthermore, the magnitude of R^2 are closed to $adj\ R^2$which insures a satisfactory adjustment of the quadratic models to the experimental data for both anthracene degradation using *Corynebacterium sp* and *Pseudomonas putida* as presented in the Table 3. Therefore, the regression models explained the microbial degradation efficiency well. Moreover, the ANOVA on these models, as shown in Table 3, demonstrates that the models were highly significant, as evident from the very low probability of $p > F$ values in the regression less than 0.0001 for anthracene degradation by the activity of *Corynebacterium sp* and *Pseudomonas putida*. The "PredR^2" of 0.7371 is in reasonable agreement with the "Adj R^2" of 0.9353. Adeq Precision of magnitude 21.937 (> 4) for activity of *Corynebacterium sp* and 50.387(> 4) for *Pseudomonas putida* indicated that the signal to noise or error ratio is desirable. This model can be used to navigate the design space.

Optimization

The *p* value is used as a tool to check the significance of each factor and interaction between factors. In general, the smaller the value of p, the more significant is the corresponding coefficient term (Mason et al, 2003). From Table 4, it was found that the intercept of the model have significant effect with a magnitude of p < 0.0001 with the activity of *Corynebacterium sp* and *Pseudomonas putida.* Variable with the largest effect on anthracene degradation was time with *p* value of < 0.0001, followed by concentration (quadratic) of the anthracene, aeration and aeration (quadratic) for *Corynebacterium sp* with *p*values of 0.0014, 0.0038 and 0.0056 respectively while time and time (quadratic) for *Pseudomonas putida* with the same *p* values of < 0.0001 respectively.

Table 4: Model Coefficient Estimated by Linear Regression

Model Term	Anthracene degraded by *Corynebacterium spp*			Anthracene degraded by *Pseudomonas putida*		
	Model Coefficient	F -Value	P> F	Model Coefficient	F - Value	P> F
Intercept	199.10	31.5	< 0.0001	17.3647	182.03	< 0.0001
X_1	-0.8398	0.469	0.5089	-0.0951	0.0008	0.9775
X_2	0.7255	230.3	< 0.0001	1.6188	1532.3	< 0.0001
X_3	-123.13	14.01	0.0038	-11.0670	1.3949	0.2649
X_1^2	0.0141	19	0.0014	0.00157	1.519	0.2460
X_2^2	-0.0037	4.078	0.0710	-0.00705	96.716	< 0.0001
X_3^2	23.035	12.32	0.0056	2.2842	0.7827	0.3971
$X_1 X_2$	0.0028	0.729	0.4133	0.0002	0.0252	0.8771
$X_1 X_3$	-0.2004	1.049	0.3300	-0.022	0.0816	0.7810
$X_2 X_3$	0.1308	0.794	0.3938	-0.0005	7E-05	0.9933

It can be deduced from the statistical linear regression presented by Table 4 for the proxy model of response surface of anthracene degradation for both *Corynebacterium sp* and *Pseudomonas putida* that all interaction and concentration model terms are insignificant and aeration, aeration (quadratic), and concentration (quadratic) are inclusively insignificant model terms in *Pseudomonas putida* activity on anthracene that can be truncated in the use of the model for any design space. The proxy model reduces to equation (7) and (8) for *Corynebacterium sp* and *Pseudomonas putida respectively*:

$$Y_1 = 199.10 + 0.7255X_2 - 123.13X_3 + 0.0141X_1^2 + 23.035X_3^2 \quad (7)$$

$$Y_2 = 17.365 + 1.6188X_2 - 0.00705X_2^2 \quad (8)$$

This data analysis also substantiates the inference that can be drawn from three dimensional (3-D) graphs of anthracene by *Corynebacterium sp* and *Pseudomonas putida* with suggested optimum range of the variables as shown in the Figure 3 and 4 respectively. The proxy model of equation (7) and (8) illustrated by Figure 3 and 4 respectively. It was also observed that each of the three variables used in the present study has its individual effect on anthracene degradation by microbes used.

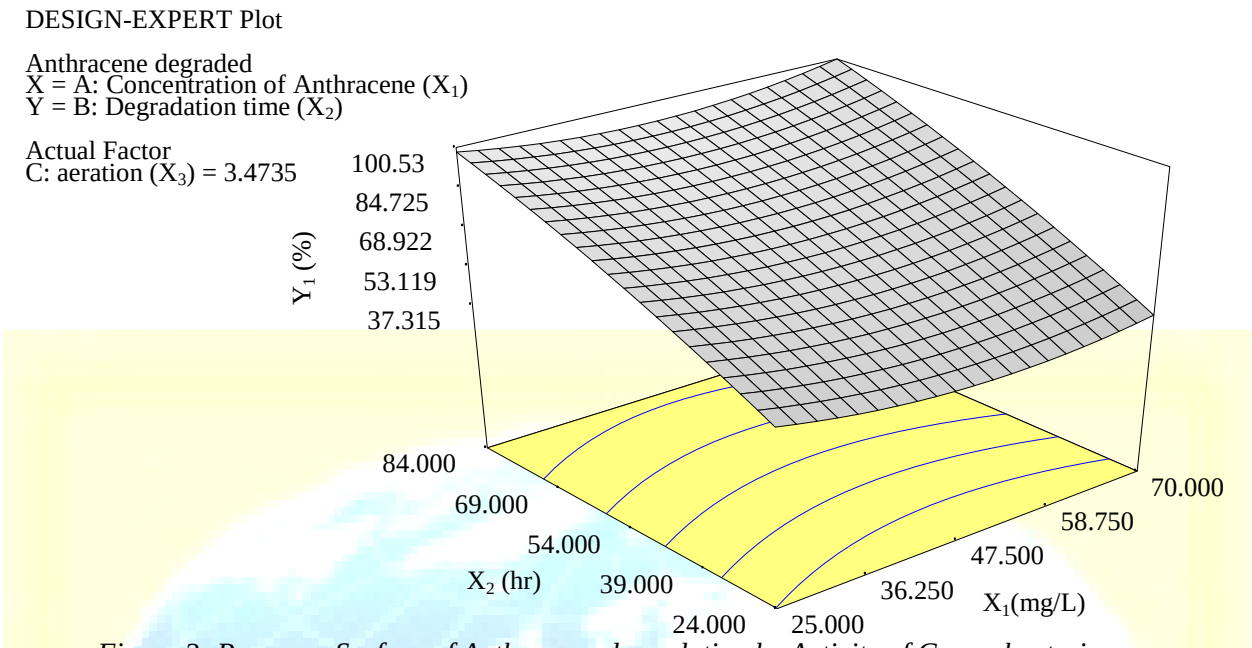

Figure 3: Response Surface of Anthracene degradation by Activity of Corynebacterium sp

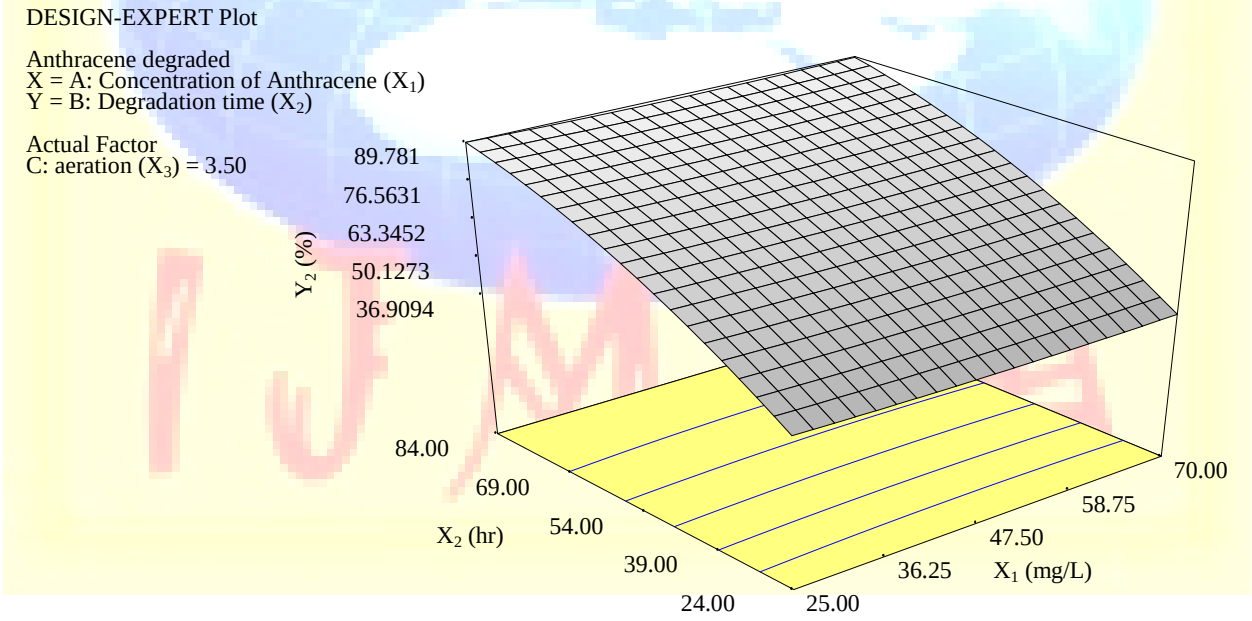

Figure 4: Response Surface of Anthracene degradation by Activity of Pseudomonas putida

The optimal conditions were obtained by solving quadratic regression equation analytically. The highest anthracene degradation was achieved by the activity of *Corynebacterium sp* compared with *Pseudomonas putida*. The results in this present study are reasonably in agreement with the degradation kinetics of anthracene as reported by Azeez (2012). The anthracene utilization using *Corynebacterium sp* (98.48%)

shows better experimental data of RSM with CCD based proxy-models for (Equation 3) compared with the result of Kumar (2010). Though, anthracene degraded by *Pseudomonas putida* (89.51%) is lesser than that of report of Azeez (2012) which degraded 93.5% of the anthracene by the activity of the *Pseudomonas putida* but higher than the result of Kumar et al (2010) as reported that *Pseudomonas sp* degraded 74.8% anthracene supplemented in BSM medium at 0.1% with Acridine orange induced plasmid. The result of this presented study is also in agreement with report of Mathew et al (2000) which isolated *Micrococcus luteus*from crude oil polluted environment for degradation of about 91.7% anthracene by the activity of *Micrococcus luteus* isolated from crude oil polluted environment.

Table 5: Validation of Biodegradation of Anthracene

	Biodegradation conditions			Response
	Concentration (mg/l)	Time (hr)	Aeration (vvm)	Anthracene degraded (%)
	Biodegradation of Anthracene by Corynebacterium spp			
Experimental	69.99	83.2	3.4735	96.87
Predicted	69.99	83.2	3.4735	98.48
Error				1.66
	Biodegradation of Anthracene by Pseudomonas putida spp			
Experimental	69.989	83.58	3.5	90.24
Predicted	69.989	83.58	3.5	89.51
Error				0.81

In validation of the experiment as shown in Table 5, error obtained was 1.66 and 0.81 percent for using *Corynebacterium sp* and *Pseudomonas putida* respectively. This which signifies using *Corynebacterium sp* and *Pseudomonas putida* respectively. This degradation of anthracene obtained from the experiments are very close to estimated using model which indicated that 2^3 full factorial Central Composite Design (CCD) with response surface methodology was appropriate for optimizing the conditions of biodegradation of anthracene.

CONCLUSIONS

This work has demonstrated the application of RSM in seeking optimal conditions for degradation of anthracene by activity *Corynebacterium sp* and *Pseudomonas putida*. In order to gain a better understanding of the three factors for optimal utilization of anthracene by *Corynebacterium sp*sp and *Pseudomonas putida*, the models were presented as 3-D response surface. Based on the statistical analyses from RSM with CCD, linear degradation time and all quadratics (concentration of the substrate (anthracene), degradation time and aeration) have significant effects on degradation of anthracene by activity of *Corynebacterium sp* while degradation time and its quadratic are the factors that have significant effect on degradation of anthracene by *Pseudomonas putida*.

The confirmed results of experiment were found to be in good agreement with the values predicted by the model. This demonstrates that maximum amount of anthracene degraded using CCD with RSM and it can be successfully applied for modeling and optimizing the biodegradation of anthracene.

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