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ENHANCED BIOREMEDIATION OF SOIL CONTAMINATED WITH ANTHRACENE: OPTIMIZATION OF BIOSTIMULANT LEVELS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Response Surface Methodology (RSM) with Box Behnken Design (BBD) was used to study the effects of time, organic fertilizer (composted from 25% cattle dung, 25% goat dung, 25% pig dung and 25% poultry manure), palm kernel oil (PKO) and commercial activated carbon (CAC) as independent biostimulating agents on the enhanced bioremediation of soil contaminated with anthracene, a polycyclic aromatic hydrocarbon (PAH) consisting of three fused benzene rings. The BBD consisted of three levels and four factors with anthracene reduction and total hydrocarbon utilizing bacteria (THUB) count as dependent variables (responses) in a six week remediation period. The results indicated that the rate of anthracene removal and THUB count generally increased as time progressed and with increase in the level of organic fertilizer, PKO and CAC amended. A statistically significant ($P < 0.0001$) second-order quadratic regression model for anthracene removal (using design-expert statistical program (v. 6.0.8) with coefficient of determination, R^2 (0.9818 and 0.9866) for anthracene reduction and THUB count were obtained respectively. A multi objective numerical optimization technique based on desirability function was carried out to optimize the bioremediation process. The predicted optimum values of time, organic fertilizer, PKO and CAC were correspondingly found to be 5 weeks and 6 days, 25.87 g, 29.63 g and 29.83 g to achieve 91.04% and 19.57×10^5 cfu/g maximum anthracene reduction and THUB count. In the optimized condition, 90.85% anthracene reduction and 19.49×10^5cfu/g THUB were obtained respectively. The statistical analyses and the closeness of the experimental results and model predictions show the reliability of the regression model. Thus, biostimulation of indigenous microbial community can enhance remediation of PAH contaminated environment.

KEYWORDS: Anthracene, BBD, Bioremediation, Biostimulating Agents, PAH, RSM, Second-Order Quadratic Regression Model

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) also known as polyarenes, polynuclear aromatic hydrocarbons) are a product of incomplete combustion. They are a class of organic compounds that consist of two or more fused benzene rings that are arranged in various structural configurations (Sims and Overcash, 1983; Dabestani and Ivanov, 1999; Harvey, 1997). They are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility (Cerniglia, 1992). The toxicity of PAHs was first recognized in the second half of the 18th century. In 1761 the physician John Hill documented a high incidence of nasal cancer in tobacco snuff consumers and in 1775 Percival Pott reported a high rate of scrotal skin cancer in chimney sweeps (Cerniglia, 1984). It is known today that low molecular weight (LMW) PAHs are acutely toxic and high molecular weight (HMW) PAHs are considered genotoxic.

Epidemiological studies show direct evidence of the carcinogenic effects of PAHs in occupationally exposed persons and demonstrate that the risk of lung and bladder cancer is dose related (Mastrangelo *et al.*,1996). Due to their mutagenic, carcinogenic and genotoxic activities, PAHs are classified as priority environmental pollutants (Wattiau, 2002).Various petroleum products are common soil contaminants and often contain potentially hazardous chemicals, particularly the polycyclic aromatic hydrocarbons (Huang et al., 2004; Okon and Mbong, 2013).

Anthracene is a solid polycyclic aromatic hydrocarbon consisting of three fused benzene rings. Its molecular formula is $C_{14}H_{10}$ It is a component of coal tar. Anthracene is colorless but exhibits a blue (400-500 nm peak) fluorescence under ultraviolet light (Iglesias-Grothy *et al.,* 2010). Common ways anthracene can enter the body are through breathing contaminated air, eating or drinking food and water that are contaminated with PAHs. Anthracene forms during incomplete combustion of organic compounds (Faust, 1993). Like most PAHs, anthracene is used to make dyes, plastics and pesticides. Smoking cigarettes can lead to exposure to anthracene since it has been found in tobacco and cigarette smoke. Exposure can also occur by eating foods grown in contaminated soil or by eating meat or other food that is grilled. Grilling and charring food actually increases the amount of PAHs in the food. Exposed to anthracene could also occur by eating smoked fish or meats. Anthracene has also been found in surface water and drinking water. Anthracene has been detected in coal tar so working at a business that makes or uses coal tar could also lead to exposure to anthracene and other PAHs (ATSDR, 1990).

Bioremediation is the use of microorganisms to degrade organic pollutant present in water, waste water, sludge, soil, aquifer material. It is cost effective and environmental friendly and it is presumed to play an increasing important role in the cleanup of soils, sediments and ground water contaminated with hazardous contaminants like PAHs. Bioremediation of contaminated soils offers a number of advantages over conventional treatments on the basis of its environmental friendliness and low costs. The interest in this technology has increased over the last few years (USEPA, 2001; Sayed *et al*., 2011). For bioremediation process to be effective, environmental condition must permit microbial growth and activity and therefore manipulation of environmental parameters to allow microbial growth and degradation process must be done in order to make the process proceed at a faster rate (USEPA, 2012). For bioremediation to be successful, the bioremediation methods depend on having the right microbes in the right place with the right environmental factors for degradation to occur. The right microbes are bacteria or fungi, which have the physiological and metabolic capabilities to degrade the pollutants. Bioremediation offers several advantages over conventional techniques such as land filling or incineration. Bioremediation can be done on site, is often less expensive and site disruption is minimal, it eliminates waste permanently, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods (Caplan, 1993).

Several factors may however limit the biodegradation of PAHs in contaminated soils including limited supply of bacterial, nutrient or carbon sources, nonoptimal abiotic conditions of temperature, pH, salts, oxygen concentration and toxins, lack of bacterial species that can degrade PAH compounds or low microbial biomass in general, low PAH bioavailability to degrading organisms and physiochemical characteristics of PAH compound(Alexander, 1999; Olson *et al*., 2003; Straube *et al.*, 2003; Harmsen *et al*., 2007; Ghaly *et al*., 2013).

Manipulations of these limitations are the basis for bioremediation of PAHs in this study with the subsequent goals of improving soil microbial habitat through biostimulation technique, overcoming the toxicity of organic pollutants to indigenous microorganisms by the use of adsorptive biostimulation and increasing the bioavailability of the PAH.

Response surface methodology (RSM) was used to study the bioremediation of soil contaminated with anthracene using organic fertilizer, PKO and CAC as biostimulating agents. Also, 2^3 full factorial Box-Behnken designs of experiment were implemented in order to evaluate the interaction effects of the biostimulating agents and time on the biodegradation rate of anthracene as well as to optimize the anthracene removal.

2. MATERIALS AND METHOD

2.1. Collection of Samples

The soil sample used for the study was collected from the top surface (0 – 15cm) of Teaching and Research farm of LadokeAkintola university of Technology (LAUTECH), Ogbomoso, Nigeria. The soil samples were air dried, homogenized, passed through a 2 mm (pore size) sieve and stored in a polyethylene bag and kept in the laboratory prior to use(Agarry *et al*., 2010). The anthracene and commercial activated carbon, (manufactured by Sigma-Aldrich, St. Louis, MO, USA) were of analytical grade while the Palm Kernel Oil was purchased from a local producer in Ogbomoso, Nigeria. The cattle dung (CD), goat dung (GD), pig dung (PD) and poultry manure (PM) were obtained from LAUTECH Teaching and Research farm, Ogbomoso, Nigeria.

2.2. Preparation of Organic Fertilizer

The animal wastes were each sun dried for two weeks, grinded and sieved to obtain uniform size particles. The animal wastes were then each weighed in the laboratory using digital weighing equipment to 300g each. The dungs were afterwards crushed and mixed together with water added to ensure thorough mixing. The mixture was allowed to compost for two weeks with regular mixing after every 3 days and also water was added to allow proper mixing (Agamuthu, *et al*., 2013; Chijioke –Osuji, *et al.*, 2014).

2.3. Characterization of Soil Sample and Organic Fertilizer

The soil sample and amendment agents were characterized for total carbon (TOC), total nitrogen (N), total phosphorus, moisture content, and pH according to standard methods. Total nitrogen was determined by kjedahl digestion and steam distillation method of Bremner and Mulvaney, (1982). Available phosphorus was determined through the method used by Olsen and Sommers (1982). Available potassium was determined using the flame photometer (Chapman and Pratt, 1978). Available micro nutrientswere determined by the (diethylenetriaminepentaacetic acid) DTPA micronutrient extraction method, developed by Lindsay *et al.*, 1978, Total Heterotrophic Bacteria (THB) and Total Hydrogen Utilizing Bacteria (THUB) present in the soil were determined according to the methods of Odokuma and Okpokwasili, 1993; Odokuma and Ibor, 2002; Amanchukwu *et al*., 1989; and Mills *et al*., (1978). The pH was determined according to the modified method of McLean (1982); total organic carbon was determined by the modified wet combustion method (Nelson and Sommers, 1982) and moisture content was determined by the dry weight method. The physicochemical characterized parameters are presented in Table 1.

Table 1: Soil Sample and Organic Fertilizer Physicochemical and Microbiological Analysis

Parameter	Soil	Organic Fertilizer
pH	6.8±0.1	7.5±0.1
Organic Carbon (%)	1.15±0.02	26.5±0.01
Total Nitrogen %)	0.75±0.02	2.5±0.03
Phosphorus (%)	0.06±0.01	0.34±0.01
Potassium (%)	0.09±0.01	0.21±0.01
Moisture Content (%)	10.41±0.2	9.5±0.2
Residual Anthracene (mg/kg)	0.19	
Sand (%)	14.2±0.2	
Silt (%)	78.2±0.2	
Clay (%)	7.6±0.2	
THUB	$0.68 \times 10^5 \pm 0.2$	$1.93 \times 10^5 \pm 0.3$
THB	$14.8 \times 10^5 \pm 0.1$	$21.2 \times 10^5 \pm 0.2$

Data presented are means of triplicate determination ± standard deviation.

2.4. Enumeration and Identification of THUB in Soil

The total hydrocarbon utilizing bacteria (THUB) in the soil samples were enumerated using modified mineral salt medium of Mills *et al.*, (1978) 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgS0_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeS0_4.7H_2O$, 0.1 g NaCl, 20 g agar, in 1000ml distilled water, pH 7.4). The vapour phase transfer method (Amanchukwu *et al*., 1989) was used. A filter paper saturated with anthracene was aseptically placed on the inside of the inverted Petri dishes and the culture plates were incubated at (28±2°C) for 7 days (Odokuma and Okpokwasili, 1993; Odokuma and Ibor, 2002). Plates yielding 30 - 300 colonies were enumerated. Colonies of different hydrocarbon utilizing bacteria were randomly picked and pure isolates were obtained by repeated sub-culturing on nutrient agar. The bacteria isolates were characterized using microscopic techniques and biochemical tests. The identities of the isolates were determined by comparing their characteristics with those of known taxa as described by Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1994).

2.5. Determination of Residual Anthracene in Soil Sample

Samples were taken before contamination and after contamination at the stipulated days from each of the experimental runs. The residual anthracene content in the anthracene polluted soil during the study period was determined gravimetrically by toluene cold extraction method of Adesodun and Mbagwu (2008). Soil samples (10 g) were weighed into 50 ml flask and 20ml of toluene was added to extract the anthracenein the soil. After shaking for 30 min, the mixture was allowed to stand for 10 min and it was then filtered through whatman No1 filter paper. The liquid phase of the extract was measured at 420 nm absorbance using a spectrophotometer (Model 6100 PYE UNICAM Instrument England). The anthracene content in the soil was estimated with reference to standard curve derived from fresh anthracene diluted with toluene

2.6. Preparation of Contaminated Soil

200 mg of anthracene was dissolved in 50 ml of ether and added to 1 kg of soil present in a plastic bucket. After capping for 24 h, the cap was opened and evaporated for 24 h in a hood. The final concentration of the soil was then 200.19 mg /kg, which is in the concentration range found in contaminated sites (Zemanek *et al*., 1997; Fung *et al*., 2010).

2.7. Bioremediation Experiment

To optimize the range of experimentation for 2^3 full factorial Box-Behnken design, the following experiments were performed in containers (used as bioreactors) maintained at room temperature. Soil samples (1000 g) were placed in containers (microcosm) and were contaminated with anthracene as described in section 2.6. The anthracene contaminated soil in each container was amended with different amounts of organic fertilizer (10, 15, 20, 25 and 30 g), PKO (10, 15, 20, 25 and 30 g) and CAC (10, 15, 20, 25 and 30 g), respectively. Soils used as controls were not amended with any nutrient. In total, 17 microcosms were settled and incubated for 42 days. All bioreactors were mixed manually once per week to enhance oxygenation and kept moist during the 42 days experimental period. Samples were withdrawn at intervals of one week for residual anthracene and THUB count analysis.

2.8. Experimental Design and Data Analysis

The experimental design was done using Response Surface Methodology (RSM) via the Box Behnken Design (BBD) and the factors (time in the range 2-6 weeks, organic fertilizer in the range 10-30 g, PKO in the range 10-30 g and CAC in the range 10-30 g) with their ranges were set as given in the Table 2. The number of experimental runs generated by RSM was 30 as shown in Table 3 and two responses namely percentage anthracene removal and THUB counts were considered. Each of the independent amendment variables was studied at three levels of −1, 0, +1 (Table 2), the levels were selected based on the preliminary study results discussed in section 2.7. Anthracene contaminated soil without amendment served as control 1 while anthracene contaminated autoclaved soil served as control 2. The statistical software Design Expert 6.0.8, (Stat-Ease Inc., Minneapolis, USA) was used to evaluate the analysis of variance ($P < 0.05$) to determine the significance of each term in the fitted equations and to estimate the goodness of fit in each case.

Table 2: Experimental Range and the Levels of the Variables

Dependent Variable	Unit	Level		
		Low (-1)	Medium 0	High (+1)
Time	wks	2	4	6
Organic Fertilizer	g	10	20	30
Palm Kernel oil	g	10	20	30
Commercial Activated Carbon	g	10	20	30

Table 3: Coded and Uncoded Box-Behnken Design for the Four Independent Variables for Anthracenebiore mediation

Run	Time (A)		Organic Fertilizer (B)	Value (g)	Palm Kernel Oil (C)		Activated Carbon (D)	Value (g)
	Code	Value (wk)	Code		Code	Value (g)	Code	
1	0	4	0	20	1	30	1	30
2	-1	2	-1	10	0	20	0	20
3	-1	2	1	30	0	20	0	20
4	0	4	0	20	0	20	0	20
5	1	6	1	30	0	20	0	20
6	1	6	-1	10	0	20	0	20
7	0	4	0	20	1	30	-1	10

8	0	4	0	20	0	20	0	20
9	0	4	0	20	-1	10	-1	10
10	0	4	0	20	-1	10	1	30
11	0	4	0	20	0	20	0	20
12	1	6	0	20	0	20	1	30
13	0	4	1	30	1	30	0	20
14	0	4	1	30	-1	10	0	20
15	1	6	0	20	0	20	-1	10
16	0	4	-1	10	1	30	0	20
17	-1	2	0	20	0	20	-1	10
18	0	4	0	20	0	20	0	20
19	0	4	-1	10	-1	10	0	20
20	-1	2	0	20	0	20	1	30
21	-1	2	0	20	-1	10	0	20
22	-1	2	0	20	1	30	0	20
23	0	4	-1	10	0	20	1	30
24	0	4	0	20	0	20	0	20
25	0	4	1	30	0	20	-1	10
26	1	6	0	20	1	30	0	20
27	1	6	0	20	-1	10	0	20
28	0	4	0	20	0	20	0	20
29	0	4	1	30	0	20	1	30
30	0	4	-1	10	0	20	-1	10
Control 1	-	-	-	-	-	-	-	-
Control 2	-	-	-	-	-	-	-	-

3. RESULTS AND DISCUSSION

3.1. Natural Bioattenuation and Enhanced Bioremediation

The results of the statistical experiment were analyzed with regard to the coded design matrix after performing 30 runs of the Box-Behnken Design (BBD) and 2 controls. The regression equation shows that the anthracene degradation rate was an experimental function of test variables in coded units. Table 4 shows that at the end of the 6th week, anthracene concentration had decreased in all the containers and THUB counts also had increased in all the containers. Natural biodegradation (natural bioattenuation) removed 15.21% anthracene in control 1 and 6.62% in control 2 while THUB count in control 1 was 9.2 x 10^4cfu/g and that in control 2 was 7.1 x 10^1cfu/g. The reduction in anthracene content of containers with amendments was much higher as shown in Table 4 in the same period.

Table 4: Experimental Design and Results for Bioremediation of Anthracene

Run	Anthracene Reduction (%)		THUB(cfu/g x10^5)	
	Actual Value	Predicted Value	Actual Value	Predicted Value
1	79.32	80.41	10.32	10.13
2	66.33	66.27	3.90	3.25
3	71.13	70.23	4.80	4.82
4	73.23	73.94	6.20	6.43
5	84.86	85.13	15.20	15.27
6	82.32	82.36	12.60	11.26
7	79.64	79.93	11.00	10.67
8	81.27	79.89	12.00	11.21
9	78.42	78.03	9.70	9.80
10	78.42	78.03	9.70	9.80
11	76.22	76.57	8.40	8.34
12	88.76	88.06	18.20	18.26
13	80.11	81.37	11.20	11.09

14	76.45	76.29	8.70	9.07
15	83.47	83.23	13.70	11.83
16	75.37	75.34	8.20	7.99
17	64.43	65.59	3.60	3.65
18	78.97	79.55	9.90	10.20
19	78.42	77.80	9.70	9.24
20	72.13	72.45	5.40	5.07
21	67.78	67.97	4.20	3.96
22	68.73	69.09	4.40	4.56
23	78.42	77.80	9.70	9.24
24	77.72	79.66	9.50	10.77
25	74.22	73.95	7.90	7.69
26	86.91	88.06	17.50	18.14
27	84.96	84.45	16.60	16.52
28	74.17	75.42	5.40	5.23
29	78.42	79.43	9.70	10.07
30	78.42	79.43	9.70	10.07
Control 1	15.21		0.92	
Control 2	6.62		0.00071	

These results indicate that the addition of biostimulants increased the rate of biodegradation. A considerable decrease in anthracene concentration was observed in runs 5, 6, 12, 15, 26 and 27 all at six weeks. The comparison of percentage anthracene reduction in enhanced bioremediation and natural bioattenuation for each run is shown in Figure 1 and the comparison of THUB count enhanced bioremediation and natural bioattenuation for each run is shown in Figure 2.

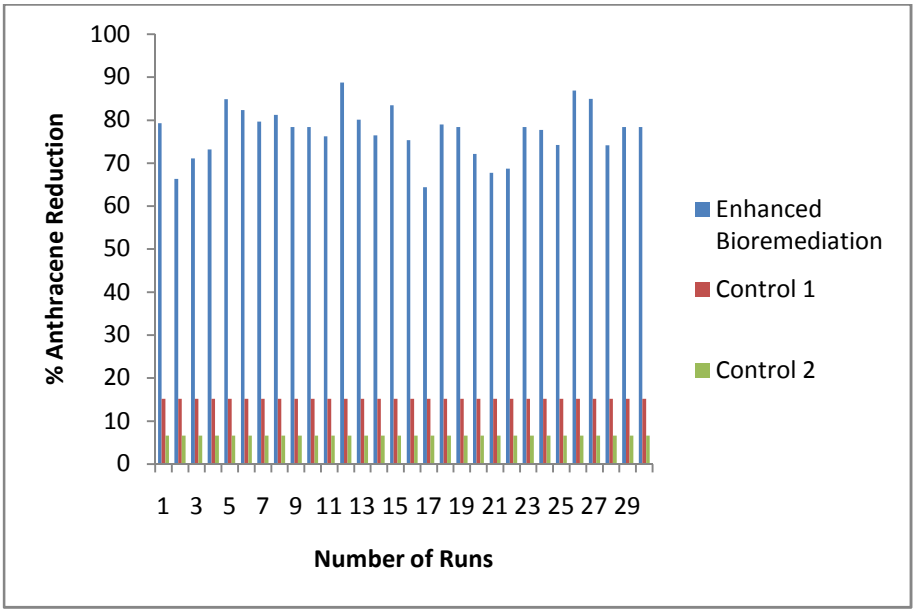


Figure 1: Percentage Anthracene Reduction from Soil in 30 Runs of Complete Factorial Design Samples In Comparison To Natural Bioattenuation

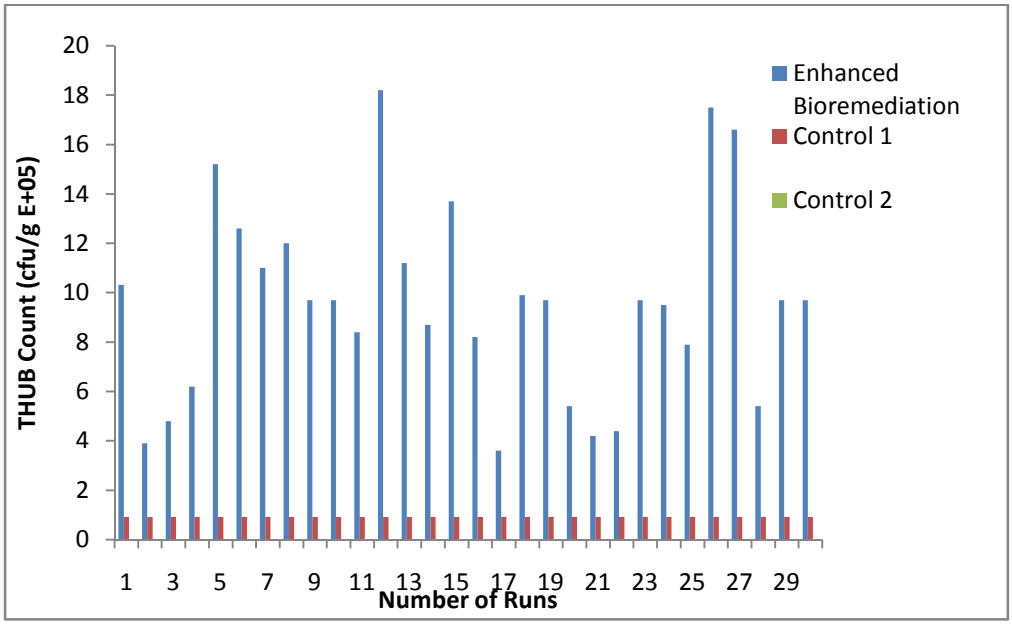


Figure 2: THUB Count in Soil in 30 Runs of Complete Factorial Design Samples in Comparison to Natural Bioattenuation

The effects of different concentrations of organic fertilizer were investigated at the same condition of time, PKO and CAC (run numbers 2 and 3, run numbers 5 and 6, run numbers 14 and 19 and run numbers 25and 30). The findings demonstrated that the addition of organic fertilizer can enhance the biodegradation process of anthracene contaminantion in soil. Abioye *et al.*, (2009), Liu *et al*., (2010) and Akpoveta *et al*., (2011) in their respective works have demonstrated the positive effect of organic wastes on enhanced biodegradation of petroleum hydrocarbons. Abioye *et al*., (2009) reported the positive effect of organic waste (brewery spent grain, spent mushroom compost, and banana skin) on enhanced biodegradation of used motor oil, Liu *et al*. (2010) used organic manure made up of rice straw and pig dung to biostimulate the degradation of an oily sludge and obtained a Total Petroleum Hydrocarbon(TPH) reduction of 58.2% in a remediation period of 360 days while Akpoveta *et al*. (2011) made use of the mixture of cow dung, pig dung and poultry dung to biostimulate crude oil biodegradation in soil and obtained 81.7% TPH reduction in a remediation period of six weeks.

Likewise, run numbers 13 and 14, run numbers 16 and 19, run numbers 21 and 22 and run numbers 26 and 27 had the same condition of time, organic fertilizer and CAC but different concentrations of PKO and the results show that extra addition of PKO improved anthracene biodegradation. A similar observation has been reported for bioremediation of different PAHs like phenanthrene and fluoranthene, naphthalene, fluorine, pyrenee.t.c. using vegetable oils such as PKO and soybean oil (Zongqiang *et al*., 2006; Lau *et al*., 2009; Fung *et al*., 2010) and it has been demonstrated that vegetable oil can be used as an effective solvent to extract organic contaminants from soils for remediation purpose (Isosaari *et al*., 2001; Bragato and El Seoud, 2003; Pannu *et al*., 2004).

Similarly, run numbers 12 and 15, run numbers 17 and 20, run numbers 23 and 30 and run numbers 25 and 29 had the same condition of time, organic fertilizer and PKO but different concentrations of CAC. The results obtained indicate that addition of CAC improved anthracene biodegradation. This is corroborated by the findings of Galina *et al*., (2006) and Ademiluyi *et al*., (2009) who achieved remediation of contaminated soil and polluted industrial waste waters respectively through the use of activated carbon as the use of activated carbon helps to overcome the toxicity of organic pollutants to microbes and plants during bioremediation.

In addition, efficiency of bioremediation is a function of the microbial viability in the natural environment (Joo *et al*., 2008). Factors, such as nitrogen, phosphorus and microorganism presence have been reported to affect bioremediation (Odokumaand Dickson, 2003; Mohan *et al*., 2009). Abdulsalam *et al.*, (2011) and Abioye *et al*. (2009) showed that natural attenuation removed 50% of oil and grease and 68% of TPH in petroleum contaminated sites after 70 and 84 day incubations respectively. When the soil was supplemented with nutrients (nitrogen and phosphorus), 66% and 92% to 95% of the contaminant was respectively removed. The results suggest that high dose of nutrient amendment can accelerate the initial PAH degradation rate and may shorten the period to clean up contaminated environments. The accelerating effect of amendment is stronger when nutrient availability is a limiting factor in the biodegradation process (Pala *et al*., 2006).

3.2. Second-Order Polynomial Regression Model and Statistical Analysis

The experimental data were fitted to a second-order polynomial regression model (Equation 1), containing four linear, four quadratic and six interaction terms (Montgomery, 2008) using the same experimental design software to derive the equation for anthracene removal from contaminated soil.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

where β_0 is the value of the fixed response at the center point of the design; β_1,β_2, β_3 and β_4, the linear coefficients; β_{11},β_{22}, β_{33} and β_{44}, the quadratic coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}, the interaction effect coefficient regression terms, respectively; and A, B, C and D the levels of independent amendment variables. The significance of each coefficient in the equation was determined by F test and P values.To test the fit of the model, the regression equation and determination coefficient (R^2) were evaluated. The regression equation obtained after analysis of variance gives the level of percentage PAH reduction and THUB count as a function of the different amendment variables: Organic fertilizer, PKO, CAC and time. From Tables 5 below, Model F-value of 50.11 and 68.48 for percentage anthracene reduction and THUB count in anthracenecontaminated soil respectively implied that the models are significant and there is only a 0.01% chance that "Model F-Values" this large could occur due to noise. Values of "Prob > F" less than 0.0500 i.e. ($P < 0.05$) at the 95% confidence level indicate model terms are significant and values greater than 0.1000 indicate the model terms are not significant. Values of "Prob > F" less than 0.0500 i.e.($P < 0.05$) at the 95% confidence level indicate model terms are significant and values greater than 0.1000 indicate the model terms are not significant. For percentage anthracene reduction, A, B, C, D, B^2 and D^2 are the significant model termswhile for THUB count in anthracene contaminated soil, A, B, C, D, A^2, B^2 and $D^2$are the significant model terms.

Also, Standard deviations of 1.13 and 0.7, mean of 78.09 and 10.11, C.V of 1.45 and 7.09, PRESS of 116.45 and 49.96 for percentage anthracene reduction and THUB in anthracene contaminated soil respectively were obtained. The value of the determination coefficient R-Squared of 0.9818 and 0.9866 for percentage anthracene reduction and THUB in anthracene contaminated soil repectively is a measure of goodness of fit to the model. Adjusted (Adj) R-Squared of 0.9622 and 0.9722, Predicted (Pred) R-Squared of 0.8735 and 0.9001, and Adequate (Adeq) Precision of 25.507 and 27.795 were obtained for percentage anthracene reduction and THUB in anthracene contaminated soil respectively. The Predicted (Pred) R-Squared of 0.8735 and 0.9001 are in reasonable agreement with the Adjusted (Adj) R-Squared of 0.9622 and 0.9722. "Adeq Precision" measures the signal to noise ratio and a ratio greater than 4 is desirable. The ratio of 25.507 and 27.795 obtained for percentage anthracene reduction and THUB in anthracene contaminated soil respectively indicate an

adequate signal and this model can be used to navigate the design space. The fitted model is considered adequate if the F test is significant ($P < 0.05$). The analysis of variance (ANOVA) quadratic regression model demonstrated that the model was highly significant for percentage anthracene reduction and THUB in anthracene contaminated soil as was evident from the very low probability ($P < 0.0001$) of the F test and insignificant result from the lack-of-fit model (Table 5). The model F-values for percentage anthracene reduction and THUB count in anthracene contaminated soil (50.11 and 68.48 respectively) were significant at the 99% level. On this basis, it can be concluded that the selected models adequately represent the data for percentage anthracene reduction and THUB count in anthracene contaminated soil.

The lack-of-fit test is performed by comparing the variability of the current residual model to the variability between observations. The coefficient of variation (CV) as the ratio of the standard error of estimate to the mean value of the observed response is a measure of reproducibility of the model; generally, a model can be considered reasonably reproducible if its CV is not greater than 10%. Hence, the low variation coefficient value (CV = 1.45% for % anthracene reduction and 7.09% for THUB count in anthracene contaminated soil obtained indicates a high precision and reliability of the experiments at replicate settings of the factors.

The final equation in terms of coded factors for the percentage anthracene reduction for the bioremediation of anthracene including both the significant and insignificant terms is given by:

$$Y_{PercentageAnthracene\ \ reduction} = + 78.42 + 7.26A + 2.17B + 2.78C + 2.47D - 0.94A^2 - 1.58B^2 + 0.46C^2 + 1.24D^2 + 0.19AB - 0.9AC + 0.22AD + 0.72BC - 0.5BD - 0.52CD \quad (2)$$

and the final equation in terms of coded factors for total hydrocarbon utilizing bacteria for the bioremediation of anthracene including both the significant and insignificant terms is given by:

$$Y_{TotalHydrocarbon\ utilizing\ bacteria} = + 9.70 + 6.07A + 0.91B + 1.08C + 0.96D + 1.01A^2 - 0.70B^2 + 0.083C^2 + 0.65D^2 + 0.13AB - 0.050AC + 0.22AD + 0.45BC - 0.45BD - 0.050CD \quad (3)$$

Where A is time (wks), B is organic fertilizer (g), C is PKO (g) and D is CAC (g).

Table 5: ANOVA for the Quadratic Response Surface Model Fitting to the Bioremediation Data of Anthracene

Source	SS	DF	MS	F Value	Probability < F (P Value)
Anthracene Reduction					
Residual Model	16.75	13	1.29	50.11	
Lack of Fit	16.75	10	1.67		< 0.0001
Pure Error	0.000	3	0.000		
Total Correlation	936.13	29			
					$R^2 = 0.9818$
					Adjusted $R^2 = 0.9622$
					Predicted $R^2 = 0.8735$
					Adequate Precision = 25.507
THUB					
Residual Model	6.69	13	0.51	68.48	
Lack of Fit	6.69	10	0.67		< 0.0001
Pure Error	0.000	3	0.000		
Total Correlation	503.47	29			
					$R^2 = 0.9866$
					Adjusted $R^2 = 0.9722$
					Predicted $R^2 = 0.9001$
					Adequate Precision = 27.795

The coefficient of the model (parameter estimation) and the corresponding P values are presented in Tables 6, the significance of regression coefficients was considered at a significance level of 95%. A, B, C, D, B^2 and D^2 are the significant model terms for percentage anthracene reduction and for THUB count in anthracene contaminated soil, A, B, C, D, A^2, B^2 and D^2. Thus, statistical analysis of all the experimental data showed that time, organic fertilizer, PKO, and CAC had a significant effect on the percentage anthracene reduction and THUB count in this study. Moreover, it was observed that PKO and organic fertilizer concentrations exerted more pronounced linear effect (higher coefficient values) on percentage anthracene reduction and THUB count.

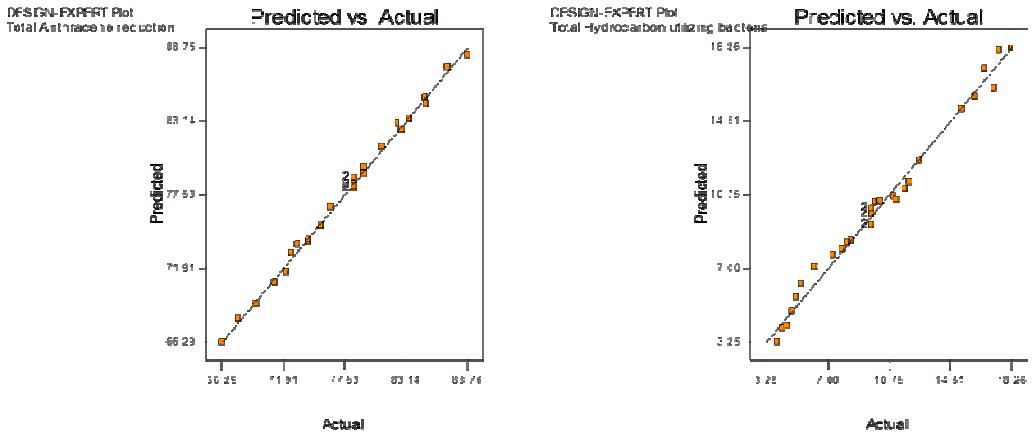
Furthermore, time exerted the highest positive linear effect (due to higher coefficient) than the interaction effect between the amendment variables. The strong influence of time on petroleum hydrocarbon degradation has been shown in the works of Atagana, (2008); Beesley *et al.*, (2010); Liu *et al.*, (2010) and Agarry and Jimoda, (2013) who all obtained increased bioremediation rates as time progressed. Considering the quadratic effect of the independent variables on percentage anthracene reduction and THUB count, the quadratic effect of time and organic fertilizer were negative for percentage anthracene reduction andonly the quadratic effect of organic fertilizer was negative for THUB count in anthracene contaminated soil. The quadratic effect of time though negative is significant.

Table 6: Coefficient of the Model for Bioremediation of Anthracene

Factor	Coefficient Estimate	Standard Error	F Value	P Value	Remark
Anthracene Reduction					
β_0	78.42	0.46	50.11	< 0.0001	Significant
β_1	7.26	0.33	490.95	< 0.0001	Significant
β_2	2.17	0.33	43.81	< 0.0001	Significant
β_3	2.78	0.33	72.24	< 0.0001	Significant
β_4	2.47	0.33	56.95	< 0.0001	Significant
β_{11}	-0.94	0.43	4.67	0.0500	Significant
β_{22}	-1.58	0.43	13.28	0.0030	Significant
β_{33}	0.46	0.43	1.11	0.3121	Not significant
β_{44}	1.24	0.43	8.14	0.0136	Significant
β_{12}	0.19	0.57	0.11	0.7466	Not significant
β_{13}	-0.94	0.57	2.72	0.1228	Not significant
β_{14}	0.22	0.57	0.16	0.6985	Not significant
β_{23}	0.72	0.57	1.63	0.2242	Not significant
β_{24}	-0.50	0.57	0.77	0.3947	Not significant
β_{34}	-0.52	0.57	0.85	0.3722	Not significant
THUB					
β_0	9.70	0.29	68.48	< 0.0001	Significant
β_1	6.07	0.21	858.50	< 0.0001	Significant
β_2	0.91	0.21	19.25	0.0007	Significant
β_3	1.08	0.21	27.38	0.0002	Significant
β_4	0.96	0.21	21.42	0.0005	Significant
β_{11}	1.01	0.27	13.55	0.0028	Significant
β_{22}	-0.70	0.27	6.61	0.0233	Significant
β_{33}	0.083	0.27	0.093	0.7658	Not significant
β_{44}	0.65	0.27	5.56	0.0347	Significant
β_{12}	0.13	0.36	0.12	0.7330	Not significant
β_{13}	-0.050	0.36	0.019	0.8913	Not significant
β_{14}	0.22	0.36	0.39	0.5413	Not significant
β_{23}	0.45	0.36	1.57	0.2316	Not significant
β_{24}	-0.45	0.36	1.57	0.2316	Not significant
β_{34}	-0.050	0.36	0.019	0.8913	Not significant

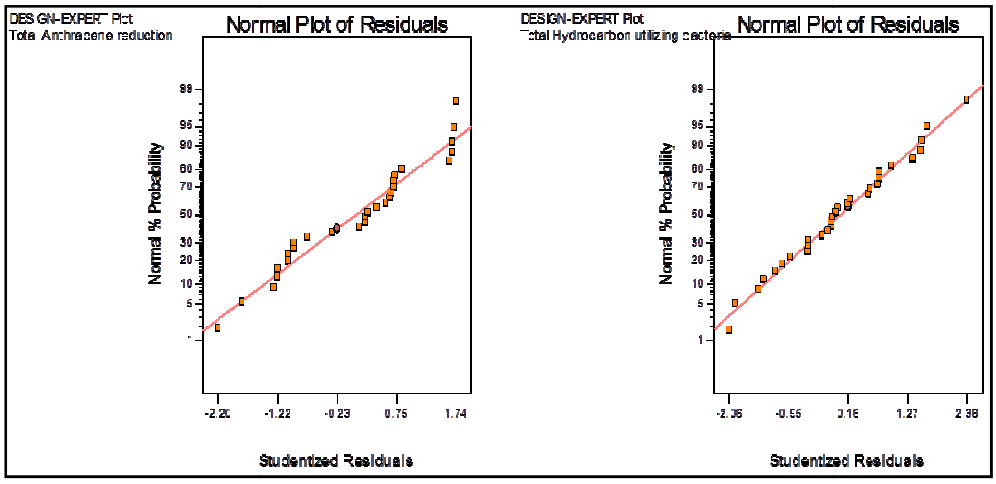
The predicted versus actual plot of total anthracene reduction and THUB count are shown in Figures 3(a) and 3(b). Actual values were determined for a particular run, and the predicted values were calculated from the approximating function used for the model. The normal plot of residuals for total anthracene reduction and THUB countare shown in Figures 4(a) and 4(b). Residual shows the difference between the observed value of a response measurement and the value that is fitted under the theorized model and thus the closeness of the actual value to the predicted value. Small residual values indicate that model prediction is accurate. Negative value of the residual indicates that the actual value is greater than the predicted value while a positive value implies that the predicted value is greater than the actual value and a predicted value of zero means that the actual value is tantamount to the standard value on which it comparison is based.

The Cooks distance and studentized residuals illustrate the normal distribution and constant variance of the residuals, the goodness of fit, linearity of the fitted model, and the independence. Cooks distance plotare shown in Figures 5(a) and 5(b), according to this plot there were no points that were potentially powerful due to their location in the factor.



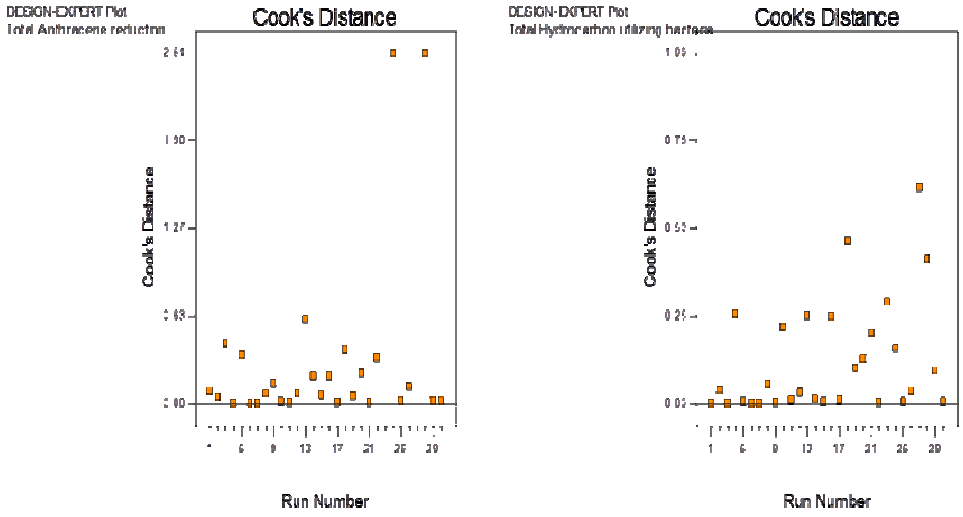
(a) **(b)**

Figure 3: Predicted Versus Actual Plot of (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



(a) **(b)**

Figure 4: Normal Plots of Residuals of (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



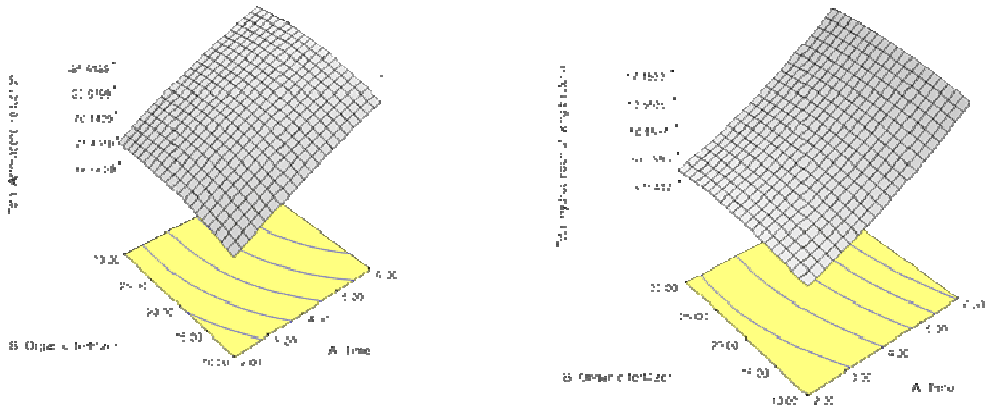
(a) **(b)**

Figure 5: Cook's Distance Plots of (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria

3.3 Influence of Variable Interaction on Bioremediation of Anthracene

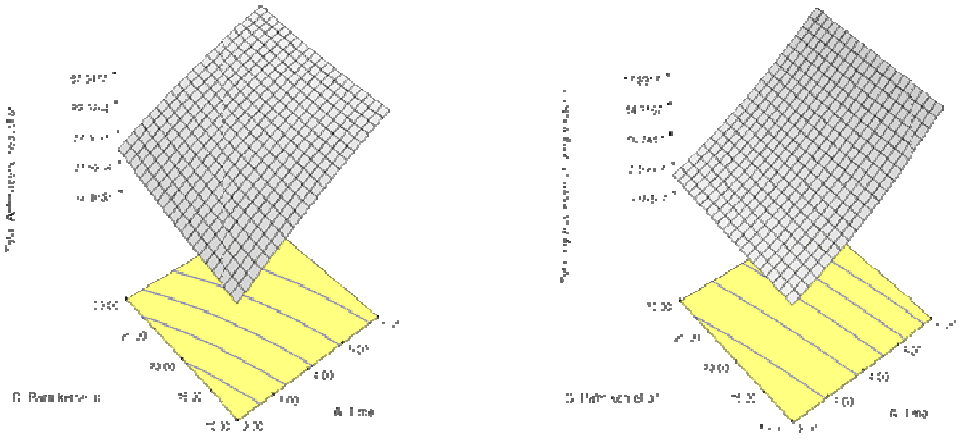
It can be observed from Table 6 that anthracene bioremediation was influenced positively by the interactions of time (A) and organic fertilizer (B); time (A) and activated carbon (D); and organic fertilizer (B) and activated carbon (D) for both percentage anthracene removal THUB in anthracene contaminated soil respectively. The interaction effects of all the independent variables considered exerted less positive influence due to lower coefficient. However, the interaction effect of organic fertilizer and PKO exerted more pronounced positive influence (due to higher coefficient) on anthracene removal (i.e. percentage anthracene reduction and THUB in anthracene contaminated soil) than the quadratic effect of organic fertilizer and PKO. Several works have reported successful removal of hydrocarbon contaminants from soil with efficiencies above 80% when vegetable oil like PKO was used (Song *et al*., 2002; Enell *et al*., 2004; Pannu *et al*., 2004; Gong *et al*., 2006; Zhou and Zhu, 2007). In addition, Atagana et al., (2003); Agarry *et al*. (2010); Onuoha *et al*. (2014) have all reported that the biodegradation of crude oil in soil was more enhanced by the addition of organic fertilizer.

The graphical representations of the responses are shown in Figure 6 to 11 help to visualize the interactive effects on the bioremediation process.



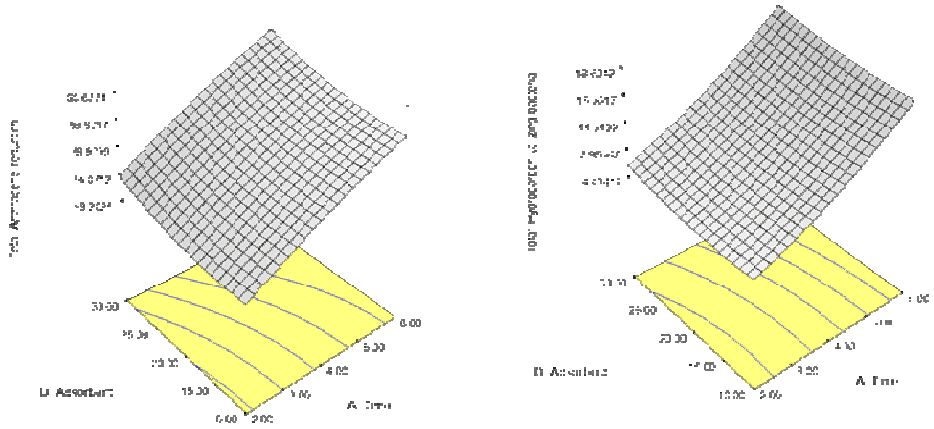
(a) **(b)**

Figure 6: Effect of Organic Fertilizer and Time on Anthracene Bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



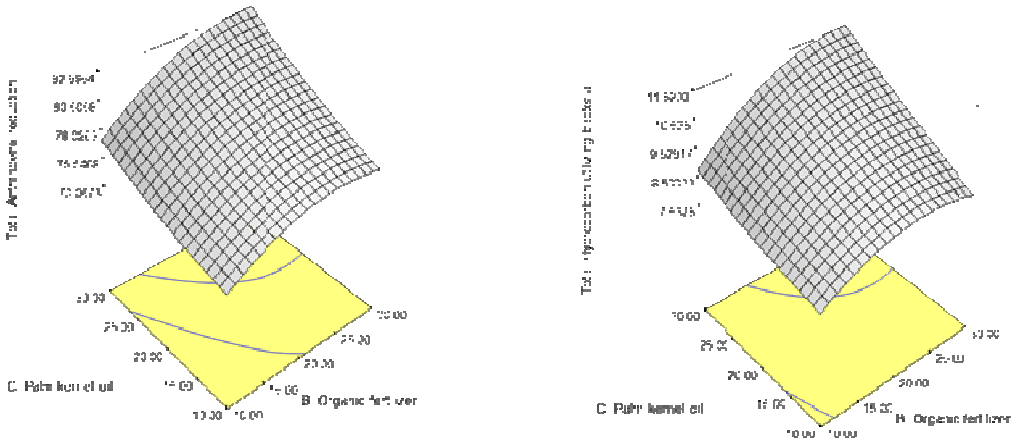
(a) **(b)**

Figure 7: Effect of PKO and Time on Anthracene bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



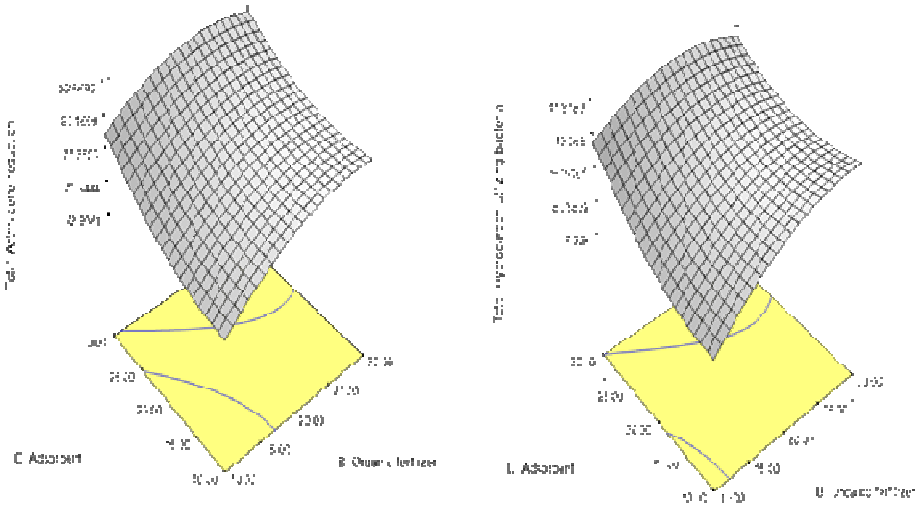
(a) **(b)**

Figure 8: Effect of Activated Carbon and Time on Anthracene Bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



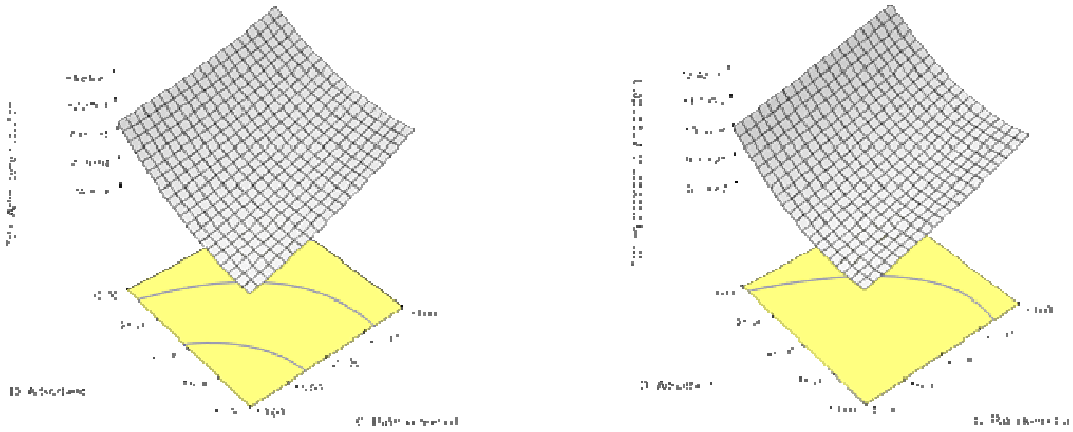
(a) **(b)**

Figure 9: Effect of PKO and Organic Fertilizer on Anthracene Bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



(a) **(b)**

Figure 10: Effect of Activated Carbon and Organic Fertilizer on Anthracene Bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria



(a) **(b)**

Figure 11: Effect of Activated Carbon and PKO on Anthracene Bioremediation for (a) Total Anthracene Reduction and (b) Total Hydrocarbon Utilizing Bacteria

The interaction effects of organic fertilizer and time on anthracene bioremediation are illustrated in Figure 6(a). The plot showed that higher rates of anthracene reductions were attained with increase in organic fertilizer and time. The maximum anthracene degradation yield of 85.62% was obtained with 30 g of organic fertilizer and at 6 weeks and at a fixed PKO mass of 20 g and activated carbon mass of 20 g. A similar effect was observed for THUB counts as shown in Figure6(b) where it could be noted that increasing organic fertilizer and time also caused an increase in THUB counts. This may be due to high concentrations of both macro and micro nutrients in organic fertilizer needed for metabolism by the intrinsic microorganisms as reported by Njokuet al., (2008); Ghaly *et al*. (2013) and Onuoha *et al*. (2014).

The 3D response surface plots of the interaction effect between PKO and time for percentage anthracene reduction is shown in Figures 7(a).Thisthree dimensional plot indicates that both PKO and time had individual impact on

anthracene removal. The maximum anthracene degradation yield of 87.04% was obtained with 30 g of PKO and at 6 weeks and at a fixed organic fertilizer mass of 20 g and activated carbon mass of 20 g. However, the impact of time was more pronounced than the impact of PKO as the individual coefficient value was higher for time than for PKO. A similar effect was observed for THUB counts shown in Figure7(b) with a maximum count of $17.89x10^5$cfu/g.

The 3D response surface plot of the effect of interaction between activated carbon and time on percentage anthracene reduction is shown in Figures 8(a). This plot indicates that both activated carbon and time had positive mutual impact on the biodegradation process. At a fixed concentration of PKO and organic fertilizer of 20 g each, it was observed that increase in activated carbon and time yielded higher percentage anthracene reduction and THUB count. The maximum percentage anthracene reduction of 88.68%was obtained with activated carbon dose of 30 g and time of six weeks while maximum THUB count of $18.60x10^5$ cfu/g was obtained as shown in Figure8(b).

The 3D response surface plot of the interaction effect between PKO and organic fertilizer on percentage anthraceneis shown in Figure9(a).This three dimensional plot indicates that both PKO and organic fertilizer had individual impact on anthracene removal. The maximum anthracene degradation yield of 82.99% was obtained with 30 g of PKO and 30 g of organic fertilizer and at a fixed activated carbon dose of 20 g and a time of four weeks. However, the impact of PKO was more than the impact of organic fertilizer as the individual coefficient valuewas higher for PKO than for organic fertilizer. A similar effect was observed for THUB counts as shown in Figures 9(b) with a maximum count of $11.52x10^5$cfu/g.

The interaction effects of activated carbon and organic fertilizer on anthracene bioremediation is illustrated in Figure10(a). The plot indicates that higher rates of anthracene reductions were attained with increase in organic fertilizer and activated carbon. The maximum anthracene degradation yield of 82.56% was obtained with 30 g of organic fertilizer and 30 g of activated carbon at a fixed PKO dose of 20 g and and time of four weeks. The same effect was observed for THUB counts as shown in Figures 10(b) where it can be seen that increasing organic fertilizer and activated carbon also caused an increase in THUB counts. This agrees with the findings of Vasilyeva et al., (1996); Vasilyeva et al., (2003) and Pizzul *et al*., (2007).

Finally, the 3D response surface plots of the effect of interaction between activated carbon and PKO is shown in Figure11(a). This plot indicates that both activated carbon and PKO had positive mutual impact on the biodegradation process. At a fixed dose of organic fertilizer and time of 20 g and four weeks respectively, it was observed that increase in activated carbon and PKO yielded higher percentage anthracene reduction and THUB count. The maximum reduction in percentage anthracene of 84.85% was obtained with activated carbon dose of 30 g and PKO dose of 30 g while a maximum THUB count of $12.42x10^5$ cfu/g was obtained as shown in Figures 11(b).

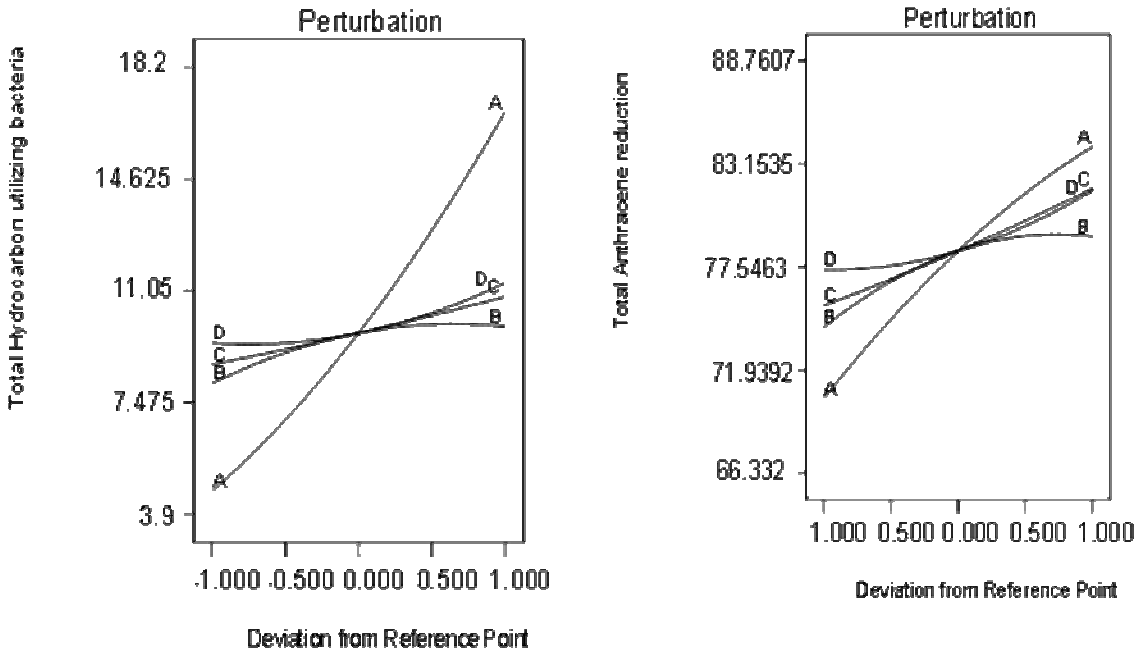
In all the doses amended, the percentage anthracene reduction and THUB count increased. It should be noted that this increase cannot continue indiscriminately because when equilibrium is attained further addition of amendment will lead to a decline in the percentage PAH reduction and THUB counts. Moreover, eutrophication and harmful algal blooms may occur due to excessive nutrient concentration (APHA, 1985; Atlas, 1995; Tam *et al*., 2009).

3.4 Factor Plot

The factor effect function plots shown on Figure 12 (a) and 12 (b) for total anthracene reduction THUB in anthracene contaminated soil respectively were used to assess the effect of each factor graphically. These figures show the

comparative effects of each of the factors considered on bioremediation of anthracene. The steeper the slope of the plot, the more profound the effect of the factor (Ravanipour, *et al.*, 2015).The slopes of time and CAC show that the response of anthracene removal and also the response of THUB was sensitive to these factors and the slopes of the other three factors for each of the responses confirm their possibly significant roles in the bioremediation process. Atagana(2004); Atagana (2008); Beesley *et al.* (2010); Liu *et al.*, (2010) and Agarry and Jimoda, (2013) have also reported the strong influence of time on petroleum hydrocarbon degradation while Carmichael and Pfaender, 1997; Huang *et al.*, 2004; Zimmerman, *et al.*, (2004); Tang and Weber, (2006) and Mellendorf*et al.*, 2010 have all reported the strong positive effect of activated carbon on petroleum hydrocarbon removal.

It can be observed that each of the four variables used in the present study has its individual effect on anthracene removal and THUB count in the soil. It can be observed from the figures that over the range of –1 (2 wks) to +1 (6 wks) of time and CAC -1 (10 g) to +1 (30 g) each,anthracene degradation and THUB count changed in a wide range. However, for organic fertilizer and PKO, it did not change over a wide range. This indicates that keeping organic fertilizer and PKO at the optimum levels, a change in time and CAC will affect the bioremediation process more profoundly. In this study application of high level of CAC (30 g) for the highest remediation period of 6 wks considered in run 12 resulted in 88.76% removal of anthracene and 18.20 10^5 cfu/g THUB count compared to low level of CAC (10 g) for the lowest remediation period of 2 wks considered in run 17 which resulted in 64.43% removal of anthracene and 3.6 10^5 cfu/g THUB count. Vasilyeva *et al.* (2001) in their work have also shown the potential of activated carbon to decrease 2,4,6-trinitritoluene toxicity and accelerate soil decontamination with the positive effect of activated carbon becoming more pronounced with time.



(a) **(b)**

Figure 12: Perturbation Plot for (a) Total Anthracene Reduction and (b) THUB

3.5. Optimization and Validation

A multi objective numerical optimization technique based on desirability function was carried out to determine the workable optimum conditions for anthracene bioremediation process. In order to provide an ideal case for

biodegradation, the goal for time, organic fertilizer, PKO and CAC was set in range based upon the requirements of anthracene biodegradation, percentage anthracene removal and THUB count were set to maximum. The predicted optimum (uncoded) values of time, organic fertilizer, PKO and CAC were correspondingly found to be 5 weeks, 6 days, 25.87 g, 29.63 g and 29.83 g to achieve 91.04% and $19.57x10^5$ cfu/g maximum anthracene reduction and THUB count in anthracene contaminated soil respectively while desirability was 1.000 for the experiments (Figures 13).

However, validation experiments were conducted to determine the optimum anthtracene removal when the amendment variables were set at the favorable optimum levels established above, through BBD and RSM. Standard deviation and percentage error were investigated for validation of the experiments. Errors between predicted and actual values were calculated according to Equation 4.

$$Error = \frac{actual\ value - predicted\ value}{actual\ value}\ x100 \quad (4)$$

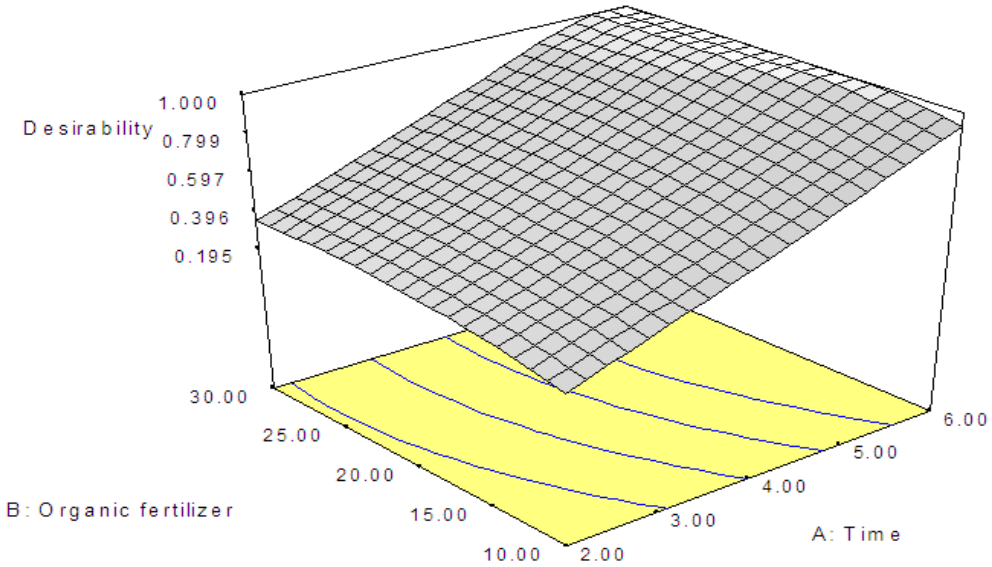


Figure 13: Desirability Plot to Optimize the Bioremediation Process of Anthracene

In the optimized condition for the bioremediation of anthracene concentration of 200.19 mg/kg, 90.85% anthracene reduction and $19.49x10^5$ cfu/g THUB count in anthracene contaminated soil were obtained, respectively. The percentage error between the predicted and actual values were found to be 0.4 for both percentage anthracene reduction and THUB count in anthracene contaminated soil. The results indicate that no significant differences were observed between the predicted values and the actual values.

4.CONCLUSIONS

In this study, the effect of four factors (time, organic fertilizer, palm kernel oil and commercial activated carbon) on the bioremediation of anthracene in contaminated soil was established employing the Box Behnken design embedded in RSM using Design Expert software (Version 6.0.8). Analysis of variance resulted in high coefficient of determination, R^2 values of 0.9818 and 0.9866 for total anthracene removel and THUB count respectively thus ensuring a satisfactory adjustment of the second order regression model with the experimental data. Under the optimized conditions of 5 weeks, 6 days, 25.87 g, 29.63 g and 29.83 g of time, organic fertilizer, PKO and commercial activated carbon respectively, the optimal experimental yield of 90.85% anthracene reduction and $19.49x10^5$ cfu/g THUB count in anthracene contaminated

soilobtained agreed closely to the model predicted yield of91.04% and 19.57x10^5 cfu/g. This study clearly shows that RSM is a reliable and powerful tool for modeling and optimization of PAH bioremediation processes. Also, the results indicate that biostimulation of PAH contaminated soil resuls in the enhancement of PAH degradation.

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