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Enhanced Bioremediation of Soil Artificially Contaminated with Crude Oil Using Box – Behnken Experimental Design.

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ABSTRACT

Introduction of toxic substances from anthropogenic activities in the environment has associated pollution and adverse human health impacts. Oil spills and accidental discharges from oil and gas operations are typical examples that are sequestered by soil which serves as the sink. The study evaluates the effects of process variables on the degradation of crude oil in contaminated soils. Response Surface Methodology (RSM) using Box-Behnken Design (BBD) was used to evaluate and optimise the effects of NPK fertilizer (0.5 - 3.0 g), amount of inoculum (5 – 20 ml) and the degradation time (24 – 72 hr) on the percentage degradation of crude oil in the contaminated soil. *Pseudomonas aeruginosa* was used for this ex situ bioremediation study while the statistical design expert program (version 9.0.2) was used for the experimental design and data analysis. The coefficient of determination (R^2) of the model of the percentage degradation of crude oil in terms of the three factors was 0.99 while the optimum percentage crude oil degradation of 73.88 % was obtained when volume of inoculum, mass of NPK and degradation time were 12.50 ml, 3.00 g and 72.00 hr, respectively. Results obtained from the study can serve as guide for relevant stakeholders and regulatory agencies in the oil and gas industry in choosing good process parameters to achieve a better degradation of crude oil in contaminated sites.

Keywords: sequestered; bioremediation; degradation; optimization; toxicity; Box-Behnken

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1. INTRODUCTION

Environmental contamination of air, water and soil has been known to wreak havoc on the ecosystem worldwide and several remediation procedures have been employed ranging from, physical to chemical and biological techniques depending on the degree of saturation and aeration of the contaminated area (Holliger *et al.*,1997; AdeOluwa *et al.,* 2015). Rise in crude oil production worldwide is accompanied with increase in the contamination of soil in the oil producing area and this has led to a need to clean up polluted areas. Several methods of cleanup such as adsorption, remediation, disinfection and chemical coagulation have been employed to ensure further ecological damage is prevented (Aghamiri *et al.,* 2011). With increasing demands of fossil fuel energy, extensive exploration of natural resources has caused a number of large scale accidental spills of crude oil and this has resulted in some significant environmental disasters (Wang *et al.,* 2010; Aghamiri *et al.,* 2011; AdeOluwa *et al.,* 2015). Remediation as a cleanup technique is the removal, destruction, restoration or transformation of contaminants to less harmful substances and, it is the most common technology employed for cleaning up contaminated areas. (Beskoskia *et al.,* 2011; Dadrasnia and Agamuthu, 2013). This may be chemical remediation or bio remediation. It has been noted that, even in small concentrations, hydrocarbon pollutants could kill or inhibit soil organisms thereby disrupting the balance of the soil ecosystem hence, the need for remediation (Lotfinasabasl *et al.,* 2013).

Bioremediation could be classified as in-situ or ex situ depending on the location of treatment. It exploits various naturally occurring mitigation processes: natural attenuation, biostimulation and bioaugmentation and, can be defined as, any process that uses microorganisms, green plants, or their enzymes to return the environment altered by contaminants to their original state (Boopathy, 2000; Rimmer *et al.,* 2006; Nie *et al.,* 2009). It has been used as an effective means of mitigating hydrocarbons, halogenated organic solvents, halogenated organic

compounds, non – chlorinated pesticides, herbicides and others. It is not only an attractive approach of cleaning up petroleum hydrocarbons, it is cost effective and easy to maintain (Bento *et al.,* 2005; Roy *et al.,* 2014). Bioremediation of petroleum hydrocarbon polluted soil relies on the petroleum – degradation ability of microbial consortium which is resident in the soil. A combination of microorganisms may sometimes be necessary to carry out the degradation (Ghazali *et al*., 2004; *Stroud et al*., 2007; Joo *et al.,* 2008; Abioye *et al*., 2009).

Biostimulation involves the addition of substrates, oxygen and other compounds (organic or inorganic) that stimulates the activity of microorganisms to catalyze the natural degradation of petroleum hydrocarbons (Das and Chandran, 2010; Crissafi *et a*l, 2016). Addition of organisms leads to the rapid degradation of the hydrocarbons while fertilizer acts as the source of nitrogen and phosphorus (Rimmer *et al*., 2006; Nie *et al.*, 2009; Roy *et al*., 2014). Bioaugmentation involves the introduction of exogenic microorganisms capable of detoxifying a particular contaminant (MacNaughton *et al*, 1999; Langer *et al*, 2004; Beskoskia *et al*, 2011).

Several studies have been carried out and more are ongoing on this subject matter. Bioremediation of soil contaminated with Premium Motor Spirit (PMS) using compost as nutrient to facilitate the biodegradation was carried out and, it was found that the soil which recorded nil growth of amaranths initially had improved yield after 2 years of treatment of the contaminated soil with compost (AdeOluwa *et al*., 2015). Roy *et al*. (2014) have demonstrated that the combined use of oil degrading bacteria along with nutrient supplements could revive crude oil contaminated soil. Bioremediation of crude oil contaminated soil has been studied using activated sludge and compost in Isfahan, Iran (Aghamiri *et al.*, 2011); melon seeds (Abioye *et al*., 2009), all these wastes that would ordinarily have constituted additional environmental nuisance now put to targeted positive use. The effect of soil amendments on diesel fuel degradation in soil has been studied using soy cake, potato skin and tea leaf and, it was found that diesel fuel utilizing bacteria were significantly high in all organic wastes amended treatments. The soil used was obtained from the garden section of Asia Europe Institute, University of Malaysia, Kuala Lumpur (Dadrasnia and Agamuthu, 2013).

Similarly, soil contaminated with spent lubricating oil has been remediated using cow dung and sewage sludge as nutrients in Kuala Lumpur, Malaysia and Agamuthu *et al*.(2013) reported that the soil amended with cow dung had 94% oil biodegradation, soil amended with sewage sludge 82% while the un-amended soil showed 56% biodegradation after 98 days. The ability of a mixture or consortia of bacteria to degrade hydrocarbon in the environment has been investigated using soil samples contaminated with diesel, crude oil and engine oil. The consortium consisting predominantly of *Bacillus and Pseudomonas spp* had reduced the alkanes in the sand to below detection limit (Ghazali *et al*., 2004).

In Nigeria, crude oil and its derivatives constitutes the bulk of Nigerian economy (AdeOluwa *et al*., 2015; Emodi, 2016;) and, accidents and vandalism of oil production facilities have resulted in spillages that had adversely affected the soil especially in the oil producing areas. Most of the agricultural practices in these areas have been adversely reduced to the barest minimum. Also, this contamination of soil is very hazardous to man, animal and the ecosystem. It has also been discovered that majority of the pollutants are from petroleum hydrocarbon. Thus, the study of bioremediation of the soil contaminated with crude oil is vital. The aim of this study is to evaluate the

combined effects of process variables of bioaugmentation and biostimulation on the degradation of crude oil in contaminated soils.

2. MATERIALS AND METHODS.

2.1 Collection of materials.

Crude oil was obtained from Port Harcourt Refining Company (PHRC) Nigeria and, this was used as the sole carbon and energy source while the NPK fertilizer used as the biostimulating agent was purchased from a local laboratory store. Loamy soil sample was obtained from the agricultural farm in University of Ilorin. Nigeria. Surface decaying leaves and other non-biodegradable litters were cleared off before the collection of samples using a hand trowel. The soil was sieved into clean black polythene bags and labeled clearly as control and test samples and then stored in the laboratory at room temperature prior to analysis. Properties of the soil determined include, pH, moisture content, percentage of silt and sand.

2.2 Determination of moisture content of soil.

Two crucibles were dried in an oven at 105 ^{o}C to constant weight. Ten grammes (10 g) of soil sample was then weighed into each crucible and their weight were recorded separately. Each sample was dried in an oven and weighed continuously until constant weight was obtained. The difference between their weights before and after the soil samples were dried was calculated and their percentages were calculated respectively as shown in equation (1):

$$\frac{Loss\ in\ weight\ of\ sample\ after\ drying}{Initial\ weight\ of\ sample\ before\ drying} \times \frac{100}{1} \quad (1)$$

This was done in quadruplicates and the average value of the percentages was calculated and reported as the moisture content of the soil.

2.3 Determination of percentage of silt and sand

Weight of dried soil that has been broken up to loosen particles was measured and the soil sample was sieved to separate silt from sand. Percentages of sand and silt were calculated using equations [2] and [3], respectively:

$$\frac{Mass\ of\ sand}{Total\ mass}\ x\ 100 = \%\ Sand \quad (2)$$

$$\frac{Mass\ of\ silt}{Total\ mass}\ x\ 100 = \%\ Silt. \quad (3)$$

2.4 Inoculum Preparation and Experimental Design for Soil Treatment.

The inoculum was prepared following standard methods from literature (Sood *et al.,* 2011).Seventeen (17) earthen pots were prepared for the treatment and each pot contained 42.5 g soil sample with 7.5 ml of crude oil. Different quantities of NPK fertilizer was then added to each earthen pot (the quantities were determined using BBD in the RSM as shown in Table 1). All the earthen pots with contents were sterilized at 121 ^{o}C for 15 min. After sterilizing, the contents were amended with different volumes of inoculum as suggested in the BBD (Table 1).The range for the three level factors; amount of NPK, amount of inoculum and time are shown in Table 2. To correlate the response variable to the independent factors, multiple regressions was used to fit the coefficient of the polynomial model of the response. The quality of the fit of the model was evaluated using test of significance and analysis of variance (ANOVA) using statistical design expert program (version 9.0.2). The fitted quadratic response model is given by equation [4]:

$$Y = a_0 + \sum_{i=1}^{k} biXi + \sum_{i=1}^{k} biiXi^2 + \sum_{i=1}^{k} bijXij + e \quad (4)$$

where, Y is response variable, a_o is the intercept value, b_i (i = 1, 2,., k) is the first order model coefficient, b_{ij} is the

interaction effect, and b_{ii} represents the quadratic coefficients of X_i, and *e* is the random error.

2.5 Total hydrocarbon content determination

Crude oil of different concentration was prepared using n – hexane as the solvent and, the absorbance of each was determined using a UV Spectrophotometer at a wavelength of 420 nm This was used to prepare the calibration curve (Aghamiri *et al.,* 2011).

Table 1. Box- Behnken design matrix for the experiment.

Run	Factor 1:Inoculum (ml)	Factor 2:NPK (g)	Factor 3:Time (hr)
1	05.00	0.50	48.00
2	12.50	1.75	48.00
3	12.50	3.00	24.00
4	05.00	1.75	72.00
5	12.50	0.50	72.00
6	20.00	3.00	48.00
7	12.50	0.50	24.00
8	05.00	3.00	48.00
9	05.00	1.75	24.00
10	12.50	1.75	48.00
11	12.50	3.00	72.00
12	20.00	0.50	48.00
13	12.50	1.75	48.00
14	12.50	1.75	48.00
15	20.00	1.75	24.00
16	12.50	1.75	48.00
17	20.00	1.75	72.00

Table 2. Factor coding and their levels

Factor	Name	Units	-1	0	1
X_1	Volume of inoculum	ml	5.00	12.50	20.00
X_2	Mass of NPK	G	0.50	1.75	3.00
X_3	Time	hr	24.00	48.00	72.00

3. RESULTS AND DISCUSSION

3.1 Experimental results for the soil characterization

The average pH, percentage moisture content, percentage silt and sand and their standard deviation are 7.5 (±0.03), 18.97%(±0.08), 38.26 %(±0.11) and

61.74%(±0.06), respectively. Soil pH is important because most microbial species would survive only within a certain pH range and, soil pH can affect the availability of nutrients. Range of pH between 6 – 9 have been successfully used (Thavasi *et al.,* 2007, Roy *et al.,* 2014) The pH of the soil used for this study of 7.5 is within the acceptable range.

3.2 BBD biodegradation experimental result

The factor design of the three input variables (X_1: Volume of inoculum (ml);

X_2: Quantity of NPK (g); X_3: Time (hours) and the response: Percentage Degradation (%) obtained from the experimental work using Box-Behnken experimental design approach and response surface methodology in Design Expert version 9.0.2 is shown in Table 3. The effect of combining two variables at a time while keeping the third constant on the percentage degradation of crude oil based on the analysis of Table 3 using RSM are as shown on Figure 1 to 3.

Table 3. Box-Behnken responses for soil bioremediation

Run	Factor 1: Inoculum (ml)	Factor 2: NPK (g)	Factor 3: Time (hr)	Response 1: Percentage Degradation (%)
1	05.00	0.50	48.00	64.06
2	12.50	1.75	48.00	52.45
3	12.50	3.00	24.00	69.94
4	05.00	1.75	72.00	61.98
5	12.50	0.50	72.00	73.05
6	20.00	3.00	48.00	61.63
7	12.50	0.50	24.00	66.74
8	05.00	3.00	48.00	58.24
9	05.00	1.75	24.00	60.88
10	12.50	1.75	48.00	50.10
11	12.50	3.00	72.00	73.88
12	20.00	0.50	48.00	52.92
13	12.50	1.75	48.00	53.65
14	12.50	1.75	48.00	52.45
15	20.00	1.75	24.00	55.52
16	12.50	1.75	48.00	52.22
17	20.00	1.75	72.00	63.17

The combined effect of volume of inoculum applied and the mass of NPK fertilizer on the bioremediation of soil contaminated with crude oil is presented in Figure 1. Degradation rate decreased from 64.06 – 52.92 % as the amount of inoculum added was increased from 2 – 20 ml when the mass of NPK fertilizer added was 0.50 g. However, degradation rate increased from 58.24 – 61.62 % when the amount of NPK added was 3.00 g and as the amount of inoculum added increased from 5 – 10 ml. When bioremediation time was 24 hr, slight increment of degradation rate was observed when mass of NPK was 3.00 g and sharp decrease of degradation rate was obtained at low level of NPK (0.50 g) as the volume of inoculum was increased from 5.00 – 20.00 ml

The interaction effect of the volume of inoculum and bioremediation time is represented in a surface plot Figure 2. Higher degradation rates were obtained when the bioremediation time was 72 hr. At 72 hr and when the amount of NPK fertilizer was at midpoint (1.75 g), degradation rate increased slightly from 61.98 – 63.17 % as the volume of inoculum added increased from 5 – 20 ml. However, low bioremediation time (24 hr) did not favor degradation rate; which decreased from 60.87 – 54.88 over the range of the investigated volume of the inoculum. `At low level of the added NPK fertilizer (0.50 g), degradation rate decreased as the

amount of inoculum increased from 5 – 20 ml for both lower and upper limits of the bioremediation time.

Response surface plot of the effects of mass of NPK and bioremediation time is presented in Figure 3. It shows high bioremediation time favored degradation rate more than at lower bioremediation times as higher degradation rate was obtained with increase in the amount of inoculum added and time. All soil microorganisms require moisture for cell growth and function; however, the availability of water affects the diffusion of water soluble nutrients in and out of the cells of the microorganisms. Excess moisture, such as in saturated soil, is undesirable because it reduces the amount of available oxygen for aerobic respiration. Soil moisture content "between 45 and 85 percent of the water-holding capacity of the soil or about 12 percent to 30 percent by weight" is optimal for crude oil degradation. The finding from this study 18.97 % falls within acceptable range (Das and Mukherjee, 2007; Das and Chandran, 2010).

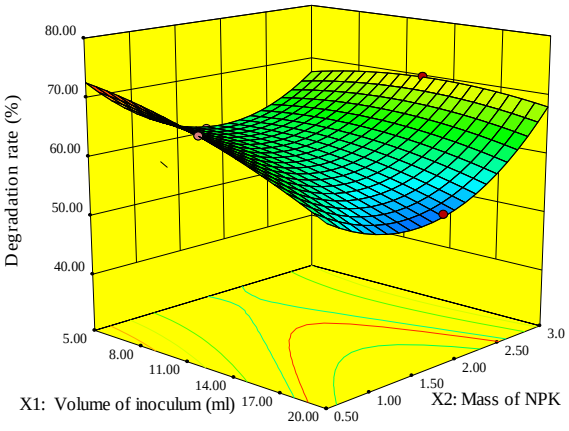

Figure 1. Effects of volume of inoculum and mass of NPK on percentage crude oil degradation

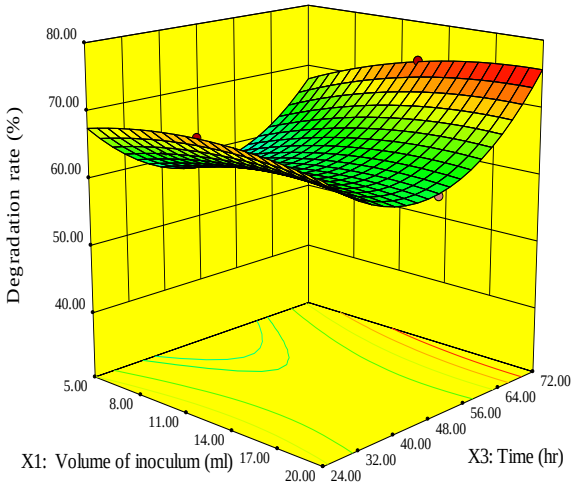

Figure 2. Effects of time and volume of inoculum on percentage crude oil degradation

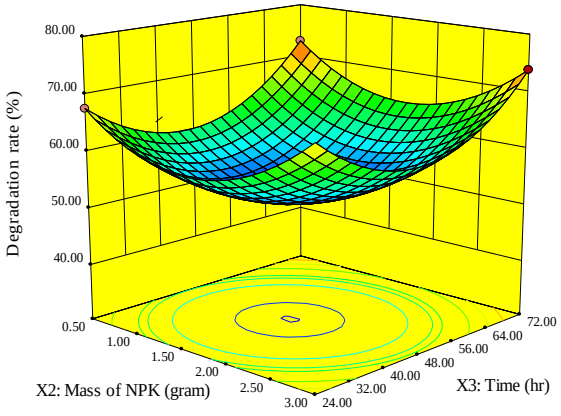

Figure 3. Effects of mass of NPK and time on crude oil degradation

3.3 Statistical analysis and Optimization

The analysis of variance (ANOVA) for estimating the coefficients of response surface quadratic model in Equation 4 is presented in Table 4 while the estimation of all the coefficients of terms in equation 4 with their statistics are presented in Table 5. From the Table 4, the obtained F-value of the quadratic model for bioremediation was 612.83 which showed that the obtained model is highly significant with the evidence of p-value (2.68E-09) which is far lower than 0.05. The "Lack of Fit F-value" of 1.13 implies the Lack of Fit is not significant

relative to the pure error. (Aremu *et al.,* 2014, Olajide *et al.,* 2014a, 2014b).Considering Table 5, the coefficients of X_1, X_2, X_3, X_1X_2, X_1X_3, X_1^2, X_2^2,and X_3^2 are significant model terms with all the p-Values less then 0.05 and high F- values. The factor with the highest F-value and minimum prob > F value is input X_3 which is time. This means time is the most influential factor on the model. The best two input combination is the one with highest F-value which is 336.28 and that is input X_3^2 which is square of the time factor (Table 5).

The equation in terms of coded factors obtained can be used to make predictions about the response for given levels of each factor. The obtained equation as presented in Equation 5 is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 4. ANOVA table for the quadratic model

Source	Sum of Squares	dF	Mean Square	F- Value	p-value	Remark
Model	928.86	9	103.21	612.83	2.69E-09	significant
Residual	1.18	7	0.17			
Lack of Fit	0.87	3	0.68	1.13	0.32	
Pure Error	0.09	4	0.02			
Total	930.04	16				

Table 5. Coefficients of the bioremediation model

Variable	Coefficient Estimates	Standard Error	F-Values	P-Values	Remark
b_0	52.29	0.50	82.26	0.0001	Significant
b_1	-1.57	0.39	14.32	0.0069	Significant
b_2	0.75	0.39	4.83	0.0064	Significant
b_3	2.34	0.39	36.39	0.0005	Significant
b_{11}	-1.99	0.54	10.26	0.0150	Significant
b_{22}	8.92	0.54	261.56	0.0001	Significant
b_{33}	9.93	0.54	336.28	0.0001	Significant
b_{12}	3.63	0.56	42.56	0.0003	Significant
b_{13}	1.79	0.56	8.65	0.0217	Significant
b_{23}	-0.36	0.56	5.42	0.0003	Significant

$$Y = 52.29 - 1.57X_1 + 0.75X_2 + 2.34X_3 + 3.63X_1X_2 + 1.79X_1X_3 - 0.36X_2X_3 - 1.99X_1^{`2} + 8.92X_2^2 + 9.93X_3^2 \quad (5)$$

Where Y = Degradation rate (%); X_1 = Volume of inoculum; X_2 = mass of NPK; X_3 = Time

The characteristics of the model in equation (5) are given in Table 6. The experimental results were close to the predicted values as R squared value of 0.9987 was obtained. The obtained predicted R-squared of 0.9811 is in reasonable agreement with the obtained adjusted R-squared of 0.9971; the difference is less than 0.2. Adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The obtained ratio of 68.538 (Table 6 indicates an adequate signal which indicates that, the obtained model can be used to navigate the design space (Aremu *et al.,* 2014). The statistical analyses and the closeness between the experimental and predicted result shows the reliability of the

regression model as shown on Table 7 while the plot of the predicted value against the experimental values are as shown on Figure 4. There is a close agreement between the predicted values and the experimental values with R^2 value of 0.9987.

Table 6. Response surface quadratic model estimation results

Std. Dev.	0.4104	R-Squared	0.9987
Mean	60.2205	Adj R-Squared	0.9971
C.V. %	0.6815	Pred R-Squared	0.9811
PRESS	17.5560	Adeq Precision	68.5380

Table 7. Model analysis of performance

Run Order	Actual Value	Predicted Value	Residual
1	64.06	63.66	0.39
2	52.45	52.29	0.16
3	69.94	69.90	0.04
4	61.98	62.34	-0.36
5	73.05	73.08	-0.04
6	61.63	62.02	-0.39
7	67.66	67.68	-0.02
8	58.24	57.90	0.34
9	60.88	61.25	-0.38
10	52.15	52.29	-0.14
11	73.88	73.86	0.02
12	52.92	53.26	-0.34
13	52.18	52.29	-0.11
14	52.45	52.29	0.16
15	54.88	54.52	0.36
16	52.22	52.29	-0.07
17	63.17	62.79	0.38

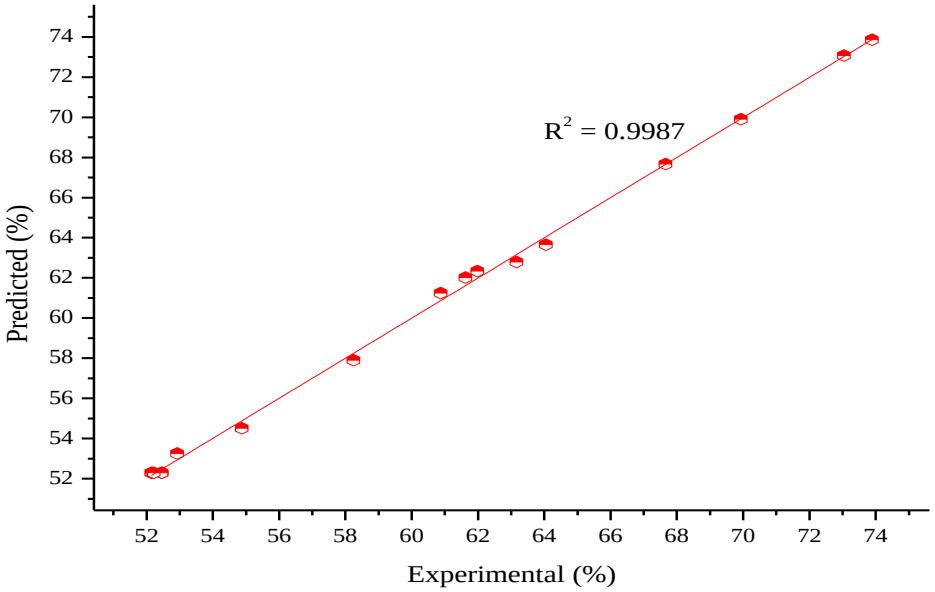

Figure 4. Plot of predicted against actual responses.

The result of the optimization showed that the factors influenced the degradation of soil. Time had the most influence on the degradation while the mass of NPK had the least influence. The coefficient of determination (R^2 square) of the model analysis was found to be 0.9987 and this is very close to the predicted R^2 square value of 0.9811. Optimum degradation rate 73.88% was obtained when volume of inoculum, mass of NPK and time were 12.50 ml, 3.00 g and 72.00 hr, respectively. The model proved effective at predicting the percentage degradation on 99% accuracy (Table 3).

4. CONCLUSION

Box – Behnken approach in Response Surface Methodology has been employed in the design of an experiment to bioremediate soil that is artificially contaminated with crude oil using *Pseudomonas aeruginosa.* The models developed can be used to represent the process behaviours, prediction for performance measure and for process optimization and, the design generated may be used for designing a treatment plant for crude oil contaminated sites where percentage degradation can be achieved on a large scale.

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