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OPTIMIZATION OF THE EFFECTS OF PROCESS PARAMETERS ON BIOREMEDIATION OF CRUDE OIL CONTAMINATED SOIL BY BIO-AUGMENTATION AND BIO-STIMULATION

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Abstract - One of the environmental concerns in recent decades is the prevalence of different pollutants in soil. Hence, the importance of remediation has led to the development of various methods to remediate crude oil polluted soil. Among these methods, bio-augmentation and bio-stimulation have gained significant attention to treat crude oil polluted soil. This study investigated the rate of bioremediation of artificial crua Surface Methodology. The effects of process variables on rate of bioremediation by bio-augmentation and bio-stimulation were also investigated. The fresh soil was obtained and characterized to determine the physiochemical properties; it was then contaminated with crude oil. The contaminated soil was characterized to ascertain the effect of crude oil on the physiochemical properties. The contaminated soil was augmented with cow dung (inoculum carrier) and stimulated with NPK nutrients to degrade the crude oil. The effects of process variables on rate of bioremediation by bio-augmentation and bio-stimulation were studied and optimized. The predicted optimum parameters were amount of inoculums: 15%wt/wt, amount of crude oil: 16.8%wt/wt and time: 21days with optimal percentage reduction of 85.64% for bio-augmentation and amount of nutrient (NPK): 16.75%wt/wt, amount of crude oil: 19%wt/wt and time: 20days with optimal percentage reduction of 76.53% for bio-stimulation.Therefore bioremediation by bio-augmentation and bio-stimulation resulted in the crude oil degradation.

Keywords: Bioremediation, Bio-Augmentation, Bio-Stimulation, Crude Oil, Cow Dung, Inoculum, NPK nutrients, Optimization.

1 Introduction

Worldwide oil pollution has been hazardous and problematic. Crude oil hydrocarbons are some of the most widely distributed pollutants resulting from oil exploration, mechanic sites, spills, tankers, ballast water, fuels and garages (Okerentugba and Ezeronye, 2003). The problem has been compounded in the oil producing areas of Nigeria due to both sabotage and vandalization of well heads and flow lines, oil bunkering and negligence (Salam *et al.,* 2011; Etuk *et al., 2013*; Onuoha et al., 2014). The endless demand for, indiscriminate use and the subsequent discharge of petroleum and its derivatives frequently result in pollution with its concomitant environmental impacts on both living and non-living components of the environment (Salami and Elum, 2010; Salam *et al.,* 2011).

Soil is the key recipient of crude oil spills as well as many different types of product and chemicals. Once these substances enter the soil, they become part of the biological cycle, thereby affecting all life forms supported by the environment (Mbah *et al.,* 2009). Therefore, soil as an essential component of our ecosystem must be protected and sustained in order to achieve sustainable ecosystem and improve the livelihood of the people in the crude oil pollution risk areas (Adedokun and

Ataga, 2007; Adenipekun, 2008; Obasi *et al.,* 2013*)*

Remediation as a cleanup technique is recommended, which is the removal, destruction, restoration or transformation of contaminants to less harmful substances. It is the most common technology employed for cleaning up contaminated areas. (Beskoskia *et al*., 2011; Dadrasnia and Agamuthu, 2013).

A whole lot of literature has reported bioremediation techniques as alternatives and or supplements to these technologies. This is because of their cost effectiveness, simplicity in technology, environmental friendliness and conservation of soil texture and characteristics (Vidali*,* 2001; Yerushalmi *et al.,* 2003; Adams and Guzman-Osorio, 2008; Fouépé *et al.,* 2009). Bioremediation is the use of microorganisms to remove or detoxify organic and inorganic xenobiotic compounds from the environments (Onuoha *et al.,* 2014).Addition of pre-grown microbial cultures to enhance the degradation of unwanted compounds (bio-augmentation) and injection of nutrients and other supplementary components to the native microbial population to induce propagation at a hastened rate (bio-stimulation), are the most common approaches for in situ bioremediation of accidental spills and chronically contaminated sites worldwide.

This work will study and determine the rate of bioremediation, the effect of process parameters and optimizationby the application of bio-stimulation and bio-augmentation techniques.

2 Materials and Methods

2.1 Materials

Agricultural soil sample was obtained from a farmland in Emene Industrial Layout Enugu. Raw crude oil sample was gotten from NNPC lab Port-Harcout. Cow dung was collected from slaughter house in Gariki Enugu.NPK nutrient was purchased from an agro-chemical store in Ogbete Enugu.

2.2 Methods

2.2.1 Soil sample preparation.

Soil samples for laboratory microcosm studies were collected from a farmland in the address above. The soil samples were left to dry in ambient conditions for 3 days. The samples were then sieved using a 2 mm sieve mesh to remove any unwanted large debris or particles. After sieving, the samples were placed into storage containers and cooled to 4ºC in a freezer prior to the experiments.

2.2.2 Characterization of the soil sample

The soil was characterized to evaluate thepH, moisture content, total nitrogen, total phosphorous, total potassium, total carbon and conductivity by using method employed by Eletta Omodele (2016). FTIR and GCMS analysis were also done to determine the quantitative components of the soil samples.

2.3 Bioremediation experiment.

2.3.1 Soil activation/ contamination

1kg of the prepared soil was treated with crude oil according to the method of Omosun et al., (2008); Ekpo and Ebeagwu (2009) to obtain 5%, 10%, 15%, 20% and 25 %(w/w) of crude oil contaminated soil. This was then incubated at room temperature for 21 days to develop acclimated microbial consortium and thereafter called activated soil (Ikuesan, 2015).

2.3.2 Bio-augmentation experiment (using cow dung)

This study was undertaken to investigate bioremediation of 5%, 10%, 15%, 20% and 25%(w/w) crude oil contaminated soil using cow dung for 35 days. Bioremediation of the crude oil contaminated agricultural soils were carried out through the introduction of cow dung (inoculum carrier). The cow dung was added at 5%, 10%, 15%, 20%, and 25% (w/w). The plastic vessels containing the experimentally crude oil contaminated soils were left undisturbed for 2days to allow the volatilization of toxic components of the oil (Abioye et al., 2012). The inoculum carrier (cow dung) was dissolved in the sterilized water used for adjusting the moisture content of the soil sample. The experimental plastic containers were not covered and the contaminated soil moistened weekly by the addition of 2ml sterile distilled water until the end of the study (Bento et al., 2005). Crude oil loss in the samples was estimated by the weight loss method using n-hexane as extractant. Periodic sampling for crude oil loss from each container was carried out at 7days intervals (Onuoha et al., 2014), for 5 weeks.

2.4 Bio-stimulation experiment (using inorganic NPK nutrient)

Nutrient media comprising of nitrogen, phosphorus and potassium in the ratio 2:1:1 was used as stimulant on the crude oil contaminated soil at 5%, 10% 15, 20% and 25% w/w for 35 days. This was used to investigate bioremediation of 5%, 10%, 15%, 20% and 25% crude oil contaminated soil. The plastic vessels containing the crude oil contaminated soils were left undisturbed for 2days to allow the volatilization of toxic components of the oil (Abioye et al., 2012). The sterilized water used for adjusting the soil moisture was used to dissolve the nutrient mix. The same method as above was applied.

2.5 Determination of percentage reduction of crude Oil

The weight loss method described by Nwaogu *et al*., (2008) and Njoku *et al*., (2009) was used to determine the amount of crude oil degraded in the soil sample. The amount of crude oil in soil samples was determined using air-dried sample to which crude oil had been added. 5 g portion of each soil sample was mixed with 40ml of n- hexane as extractant in a 250 ml Erlenmeyer flask. The flask was then shaken vigorously with mechanical shaker for 30 min to extract the oil (Njoku *et al*., 2009). The soil-crude oil- n-hexane mixture was allowed to stand for 10 min and then slowly filtered into a pre-weighed beaker through a WhatmanNo.1 filter paper. Anhydrous sodium sulphate was spread over the filter paper to remove any moisture present in the mixture. The solvent (n - hexane) was allowed to evaporate by gentle heating at 40°C to a constant weight and the residual crude oil determined. The amount of crude oil loss from the soil was then determined as the amount of crude oil added to the soil minus that recovered in the soil at the time of analysis (Njoku et al., 2009). This was then expressed as percentage of crude oil degraded in sample.

$$\%\text{Reduction}=\frac{initial\ amount\ of\ crude\ oil\ in\ the\ soil - amount\ of\ crude\ oil\ recovered}{Initial\ amount\ of\ crude\ oil\ in\ the\ soil}\text{x}100 \qquad \textbf{(1)}$$

2.6 Statistical Analysis and Optimization of Bioremediation Processes

The optimization of the bioremediation processes was done using central composite design of response surface methodology (RSM) with Design Expert software (version 9.0 version). The experimental design employed in this work was a two-level-three factor factorial design, including 20 experiments. The empirical equation is represented as shown below:

$$Y = \beta_0 + \sum_{i=1}^{5}\beta_i X_i + \sum_{i=1}^{5}\sum_{j=i+1}^{5}\beta_{ij}X_iX_j + \sum_{i=1}^{5}\beta_{ii}X^2{}_i \qquad \textbf{(2)}$$

Where Y = Percentage reduction,
$\sum_{i=1}^{5}\beta_i X_i$ = coefficient of single terms,
$\sum_{i=1}^{5}\sum_{j=i+1}^{5}\beta_{ij}X_iX_j$ = coefficient of interactive terms,
$\sum_{i=1}^{5}\beta_{ii}X^2{}_i$ = coefficient of quadratic terms,
And β_0 = constant term.

Table 1: Range of each factor in actual and coded form for bio augmentation.

Independent variables	Symbols	Range and levels				
		-α	-1	0	+1	+α
Amount of inoculums (%wt/wt)	A	5	10	15	20	25
Crude oil (%wt/wt)	B	5	10	15	20	25
Time (Days)	C	7	14	21	28	35

2.7 Design of Experiment for Bio-Augmentation Process

As shown in Table 1, amount of inoculums, crude oil and time were selected as independent factors for the optimization study. The response chosen was the percentage reduction obtained from degradation of contaminated soil sample. Six replications of centre points were used in order to predict a good estimation of errors and experiments were performed in a randomized order.

Table 2: Range of Each Factor In Actual And Coded Form for Bio-Stimulation.

Independent variables	Symbols	Range and levels				
		-α	-1	0	+1	+α
Amount of NPK (%wt/wt)	A	5	10	15	20	25
Crude oil (%wt/wt)	B	5	10	15	20	25
Time (Days)	C	7	14	21	28	35

2.8 Design of Experiment for Bio-Simulation Process

As shown in Table 2, amount of NPK nutrients, crude oil and time were selected as independent factors for the optimization study. The response chosen was the percentage reduction obtained from degradation of contaminated soil sample. Equation in coded values is used to study the effect of the variables on the response.

3 **Results and Discussions**

Table 3:Physiochemical Properties of the Soil Samples

S/N	Soil Properties	Uncontaminated soil sample Parameters	Contaminated soil sample Parameters	Bio-augmented soil sample Parameters	Bio-stimulated soil sample Parameters
1	pH	6.47	8	7.2	6.94
2	Conductivity (us/cm)	34	23	32	31
3	Moisture (%w/w)	0.8	0.8	0.9	1.0
4	Phosphorus (mg/kg)	3.69	3.60	3.64	3.61
5	Potassium (mg/kg)	6.22	6.20	6.21	6.21
6	Nitrogen (%w/w)	0.092	0.09	0.091	0.092
7	Organic carbon (%w/w)	0.72	0.68	0.71	0.70

The physiochemical properties of the uncontaminated, contaminated and degraded soil samples are shown in Table 3above. It could be seen that the crude oil affected all the properties of soil tested and most importantly made the soil alkaline which may aid in the activity of the micro-organisms that degrade crude oil. This inference is supported by the reports of Ijah and Abioye, (2003); Njoku et al. (2009) that bacteria thrive better in alkaline medium than acidic medium. There was also reduction in the phosphorous content of the soil; this could be that the phosphate ion reacted with some metallic ions present in the soil to form less soluble compounds. It was observed from the table that the biodegradation of the contaminated soil by both bio-augmentation and bio-stimulation restored the soil properties.

3.1 Effects of Process Parameter for Bioremediation

Figure 1 shows the effect of amount of inoculums on percentage reduction of crude oil by bio-augmentation process. It could be observed that the degradation rate increased as quantity of inoculums increased and decreased when the amount of inoculums was above 15%wt/wt. The increase could be as a result of efficiency of the microorganism in degrading the crude oil while the decreased could be as a result of suffocation of the microorganisms due to congestion by increased microorganism.

Bio-augmentation

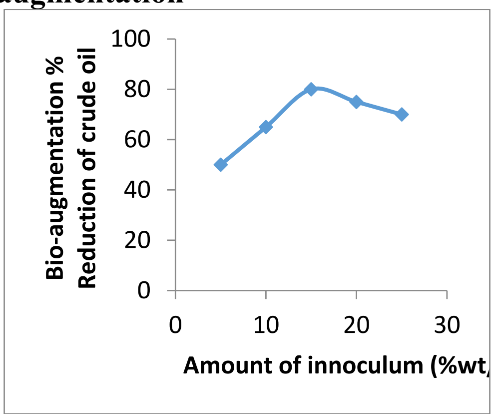

Fig 1; Effect of amount of inoculums onreduction of crude oil

Bio-stimulation

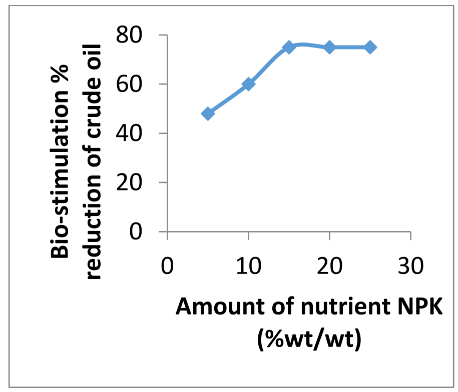

Fig 2: Effect of amount of nutrient on percentagereduction of crude oil.

Figure 2 shows the effect of amount of nutrient (NPK) on percentage reduction of crude oil by bio-stimulation process. It could be observed that the degradation rate increased as quantity of stimulant increased and remained constant when the amount of stimulant was above 15%wt/wt. This observation showed that the addition of bio-stimulants increased the rate of crude oil degradation in the soil. This is in agreement with the report of Ogunleye& Agarry, (*2012*) that there is an increase in the rate of biodegradation with addition of bio-stimulants such as NPK

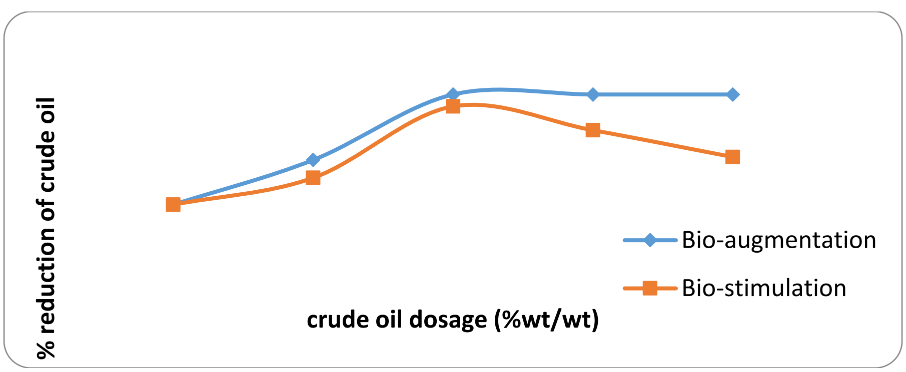

Fig 3: Effect of crude oil dosage on percentage reduction of crude oil.

Figure 3 shows the effect crude oil dosage on bioremediation percentage reduction of crude oil. It could be observed that for bio-augmentation, percentage reduction increased as the amount of crude oil increased and approximately became constant after 15%wt/wt of crude oil addition. This could be the quantity of crude oil the microorganism can degrade at the subjected conditions.

For bio-stimulation percentage reduction of crude oil increased as the amount of crude oil increased and decreased after 15%wt/wt quantity of crude oil. This could be the nutrient was no longer effective to stimulate the microorganism to degrade the crude oil at that conditions.

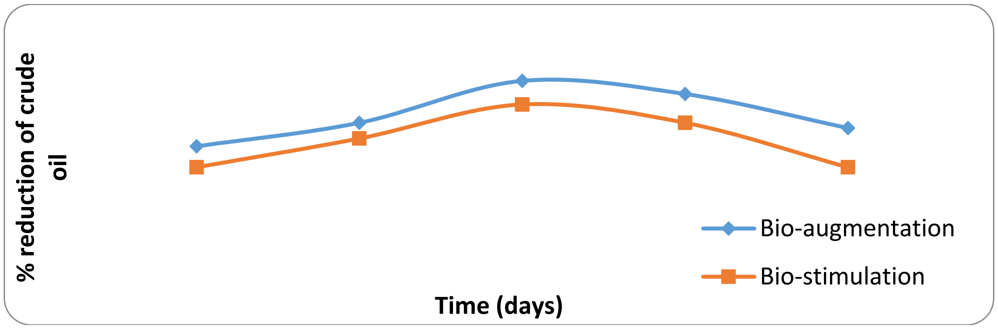

Fig 4: Effecst of time on percentage reduction of crude oil.

Figure 4 shows the effect of time on bioremediation percentage reduction of crude oil. For bio-augmentation, it could be seen that the percentage reduction of crude oil increased as time increased and decreased after 21 days. This could be that the activity of the microorganisms has reduced after 21days, thereby resulting in lower degradation of the crude oil.For bio-stimulation, the percentage reduction of crude oil increased as time increased and decreased after 21 days. This could be that the stimulation of activity of the microorganisms have reduced after 21days thereby resulting in lower degradation of the crude oil.

3.2 Statistical Analysis and Optimization of Bioremediation of Crude Oil by Bio-augmentation and Bio-stimulation processes Using Central Composite Design (CCD)

To statistically analyze and optimize the bio-augmentation and bio-stimulation percentage reduction of crude oil, Central Composite Design, a type of Response Surface Methodology, was used to determine the significance of bio-augmentation and bio-stimulation variables on response and the optimum values of the process variables. The full factorial design was used to obtain a quadratic model, consisting of factorial trials to estimate quadratic effects. To examine the combined effect of the three different factors (independent variables), amount of inoculums, quantity of crude oil and time for bio-augmentation and amount of nutrient (NPK), quantity of crude oil and time for bio-stimulation on percentage reduction of crude oil and derive models, a two-level- three – factor (2^3 + (2×3) + 6) Central Composite Design which gave 20 experiments. The factors levels are shown in Tables 1 and 2 above. The matrix for the three variables was varied at two levels (-1 and +1). The lower level of variable was designated as "-1" and higher level as "+1". The experiments were performed in random order to avoid systematic error. The response was expressed as the percentage reduction.

3.3 Statistical Analysis and Optimization of Bio-Augmentation Process

The coded and un-coded values of the test variables were used to optimize the variables namely amount of inoculums, amount of crude oil and time on percentage reduction of crude oil. The percentage reduction of crude oil depends on the results if there is significant variation for combination of process parameters. The empirical relationship between percentage reduction (Y) and the three variables in coded values obtained by using the statistical package Design-Expert 9.0.1 version for determining the levels of factors which gave optimum percentage reduction was given by the equation below. A quadratic regression equation that fitted the data is shown in Equation 3:

$$Y = 84.99 - 2.05A + 4.19B + 1.09C + 9.65AB + 1.23AC - 3.95BC - 7.35A^2 - 7.18B^2 - 8.77C^2 \quad \text{...........(3)}$$

where Y is the response variable (percentage reduction of crude oil) and A-C are the coded values of the independent variables. The above equation represents the quantitative effect of the factors (A, B, and C) upon the response (Y). Coefficients with one factor represent the effect of that particular factor while the coefficients with more than one factor represent the interaction between those factors. Positive sign in front of the terms indicates synergistic effect, while negative sign indicates antagonistic effect of the factor. The adequacy of the above proposed model was tested using the Design Expert sequential model sum of squares and the model test statistics. From the statistics test, the regression coefficient (R^2 = 0.9993) is high, and the adjusted R^2 (0.9986) is in close agreement with the predicted R^2 (0.9961) value. The coefficient of variance (CV) is the ratio of the standard error of the estimate to the mean value of the observed response and is considered reproducible once it is not greater than 10%. In this work, the CV obtained was 0.86%. The "Adeq Precision" value measures the signal-to-noise ratio. A ratio greater than 4 is desirable (Myers and Montgomery, 2002). From this experiment, a ratio of 112.636 was observed, which indicates an adequate signal. This model can be used to navigate the design space. This test is shown in Table 4.

Table 4: Significance of regression coefficients of bio-augmentation percentage reduction of crude oil.

Source	**Degree of freedom**	**Sum of square**	**Mean Square**	**F-value**	**P-value (Prob >F)**
Model	9	4005.67	445.07	647.18	< 0.0001
A-Amount of inoculum	1	67.91	67.91	98.75	< 0.0001
B-Crude Oil	1	222.96	222.96	324.21	< 0.0001
C-time	1	18.11	18.11	26.34	0.0004
AB	1	780.13	780.13	1134.38	< 0.0001
AC	1	10.13	10.13	14.72	0.0033
BC	1	120.13	120.13	174.67	< 0.0001
A^2	1	1098.83	1098.83	1597.80	< 0.0001
B^2	1	848.10	848.10	1233.21	< 0.0001
C^2	1	1381.99	1381.99	2009.54	< 0.0001
Residual	10	6.88	0.6877		
Cor. Total	19	4012.55			

Std. Dev. = 0.57; Mean = 66.36; C.V.% = 0.86; PRESS = 17.64; R^2 = 0.9993; Adj. R^2 = 0.9986; Pred. R^2 = 0.9961; Adeq. Precision = 112.636

The ANOVA results for the model terms are given in Table 4. Analysis of variance (ANOVA) was applied for estimating the significance of the model at 5% significance level and shown in Table 4. A model is considered significant if the p-value (significance probability value) is less than 0.05. From the p-values presented in Table 4, it can be stated that all the linear terms A, Band C and interaction terms AB, AC, and BC with all the quadratic terms A^2, B^2 and C^2 are significant model terms. Based on this, the quadractic model remained as in Equation (3)

Table 5:Results of the model validation (experiment to validate the optimum percentage crude oil degradation by bio-augmentation)

Experiment	Amount of inoculums (%wt/wt) A	Amount of crude oil (%wt/wt) B	Time (days) C	Experimented percentage crude oil reduction (%)	Predicted percentage crude oil reduction (%)
1	15.5	16.8	21	84.3	85.64

The percentage crude oil reduction was optimized with the design expert giving 85.64% at optimum conditions of amount of inoculums, 15.5%wt/wt; amount of crude oil, 16.8%wt/wt and time, 21days, with desirability of 0.992. The bioremediation by bio-augmentation under the obtained optimum operating conditions was carried out in order to evaluate the precision of the quadratic model; the experimental value and predicted values are shown in Table 5. Comparing the experimental and predicted results, it can be seen that the error between the experimental and predicted is less than 2%, therefore it can be concluded that the generated model has sufficient accurancy to predict the degradation of crude oil via bio-augmentation.

3.4 Statistical Analysis and Optimization of Bio-Stimulation Process

The coded and uncoded values of the test variables were used to optimize the variables namely amount of nutrient (NPK), quantity of crude oil and time on percentage reduction of crude oil. A quadratic regression equation that fitted the data shown in equation 4.

$$\mathbf{Y = 75.06 - 1.46A + 5.19B + 3.08C + 7.71AB + 0.54AC - 5.71BC - 6.10A^2 - 5.70B^2 - 7.22C^2} \quad .... (4)$$

From the statistics test, the regression coefficient (R^2 = 0.9995) is high, and the adjusted R^2 (0.9991) is in close agreement with the predicted R^2 (0.9983) value In this work, the CV obtained was 0.69%. From this experiment, a ratio of 147.172 was observed, which indicates an adequate signal. This test is shown in Table 5.

Table 6: Significance of regression coefficients of bio-stimulation percentage reduction of crude oil.

Source	**Degree of freedom**	**Sum of square**	**Mean Square**	**F-value**	**P-value (Prob >F)**
Model	9	3517.85	390.87	2320.73	< 0.0001
A amount of NPK nutreint	1	33.93	33.93	201.46	< 0.0001
B amount of crude oil	1	431.60	431.60	2562.55	< 0.0001
C time	1	151.91	151.91	901.91	< 0.0001
AB	1	475.86	475.86	2825.34	< 0.0001
AC	1	2.31	2.31	13.72	0.0041
BC	1	261.06	261.06	1550	< 0.0001
A^2	1	934.52	934.52	5548.55	< 0.0001
B^2	1	815.91	815.91	4844.35	< 0.0001
C^2	1	1311.23	1311.23	7785.22	<0.0001
Residual	**10**	**1.68**	0.17		
Lack of fit	**5**	**0.6**	0.12	0.55	0.7377

Cor. Total	**19**	**4484.61**

Std. Dev. = 0.41; Mean = 59.85; C.V.% = 0.69; PRESS = 5.95; R^2 = 0.9995; Adj. R^2 = 0.9991; Pred. R^2 = 0.9983; Adeq. Precision = 147.172

The ANOVA results for the model terms are given in Table 6. Analysis of variance (ANOVA) was applied for estimating the significance of the model at 5% significance level and shown in Table 6. A model is considered significant if the p-value (significance probability value) is less than 0.05. From the p-values presented in Table 6, it can be stated that all the linear terms A, Band C and interaction terms AB, AC, and BC with all the quadratic terms A^2, B^2 and $C^2$are significant model terms. Based on this, the model remained as in Equation (4)

Table 7:Results of the model validation (experiment to validate the optimum percentage crude oil degradation by bio-stimulation)

Experiment	Amount of nutrient (NPK) (%wt/wt) A	Amount of crude oil (%wt/wt) B	Time (days) C	Experimented percentage crude oil reduction (%)	Predicted percentage crude oil reduction (%)
1	16.7	19	20	75.4	76.53

The percentage crude oil reduction was optimized with the design expert giving a 76.53% at optimum conditions of amount of nutrient, 16.7%wt/wt; crude oil, 19%wt/wt and time, 20days, with desirability of 1.0. The bioremediation by bio-stimulation under the obtained optimum operating conditions was carried out in order to evaluate the precision of the quadratic model; the experimental value and predicted values are shown in Table 7. Comparing the experimental and predicted results, it can be seen that the error between the experimental and predicted is less than 2%, therefore it can be concluded that the generated model has sufficient accurancy to predict the degradation of crude oil via bio-stimulation.

4 Conclusion and Recommendation

This study investigated the rate of bioremediation of crude oil contaminated soil by bio-augmentation and bio-stimulation. The soil was contaminated with different concentration of crude oil and incubated for different bioremediation periods and statistical analysis was carried out. Bio-augmentation and bio-stimulation process helped in degrading the crude oil. The predicted optimum parameters were amount of inoculums: 15%wt/wt, initial concentration of crude oil: 16.8%wt/wt and time: 21days with optimal percentage reduction of 85.64% for bio-augmentation and amount of nutrient (NPK): 16.75%wt/wt, initial concentration of crude oil: 19%wt/wt and time: 20days with optimal percentage reduction of 76.53% for bio-stimulation.

Research institutions and individuals should be empowered to develop, design and establish affordable bioremediation techniques for the treatment and reclamation of crude oil contaminated soil. The environmental impact of bioremediation by bio-augmentation and bio-stimulation should be critically assessed. Further studies should be conducted to find additional locally sourced materials that can aid and improve bioremediation of crude oil contaminated soil.

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