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OPTIMIZATION OF PROCESS CONDITIONS FOR THE BIODEGRADATION OF CRUDE OIL IN A CRUDE OIL CONTAMINATED SOIL BY MICROCOCCUS LUTEUS

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Abstract - Optimal conditions that affected the biodegradation rate of crude oil in a crude oil contaminated soil by Micrococcus luteus was studied. The factors studied are the moisture content, pH and nutrient concentration. Three different bacterial strain were isolated and identified as Bacillus pumilus, Ecterobacter and Micrococcus luteus from a crude oil polluted soil sample obtained from Ogoni town in Gokana Local Government Area, Rivers State of Nigeria. Micrococcus was chosen and used for the study. The biodegradation experiment lasted for twenty-one (21) days. Optimization of the process factors was done and the result obtained were analysed using Design expert v.7.04 which was used in designing the experiment. There was significant increase in the viable microbial cell count in all the runs. The biodegradation rate of the crude oil was found to have its optimum rate at the pH of 7.98, nutrient concentration of 5.74gm/ml oil and at the percentage moisture content of 36.68%. The results showed significant increase in all the runs of the microbial cell population which also signifies decrease in the crude oil concentration. Above these optimum conditions obtained, microbial activity on the crude oil showed a downward trend.

Keywords: ***Micrococcus luteus*****, Optimization, Biodegradation, pH, Moisture and Nutrient.**

1. Introduction

Crude oil is a natural occurring mixture from the conversion of organic matter under the influence of high temperature, pressure and bacterial activity spanning over a long period of time. Jain et al. (2011) defined crude oil as a complex mixture of hydrocarbons composed of aliphatic, aromatic and asphaltine fractions along with nitrogen, sulphur and oxygen containing compounds. The constituents of these hydrocarbon compounds are present in varied proportions which accounts for high variability in crude oil from different sources (Speight, 1999).

Crude oil plays very important role in the modern industrial society; it helps and propels world's technological production. The increase in demand and dependence of man on fossil fuels especially petroleum hydrocarbons and its products has led to increase in exploration and exploitation of crude oil. The increase in exploration and exploitation to meet up with demands has resulted in an increasing pollution of the environment (water, air and land) more so when there is oil spill. The exploration, production and consumption of this nature's gift (oil and petroleum) products are increasing worldwide, and the threat of oil production increases accordingly. The movement of petroleum from the oil field to the consumer involves as many as 10 to 15 transfers between different modes of transportation including tankers, pipelines, railcars and tank trucks. In Nigeria's Niger Delta region, all the stages of oil exploitation, impact negatively on the environment, and the greatest single intractable environmental problem caused by crude oil exploitation is oil spillage, (DPR, 1997). Oil

spillage is an unintentional release of the liquid petroleum hydrocarbon into the environment as a result of human activities. These could be accidents involving oil tankers, barges, refineries, pipelines and oil storage facilities. The accidents could be as a result of human mistakes or carelessness and sometimes could be by natural disaster or deliberate acts by terrorists or vandals or militants as is the case in Nigeria. Lack of regular maintenance of pipelines and storage tanks can also cause oil spill.

Oil pollution has caused enormous harm to the people of the Niger Delta Region of Nigeria. Oil spills have degraded most agricultural lands in the region and have turned hitherto productive areas into wastelands (Odjuvwuederhie et al., 2006). Enormous ecological problems has been observed as a result of oil pollution ranging from brownish vegetation, soil erosion, diminishing resources of the natural ecosystem, to turning fertile lands barren, the health and economy of the people are adversely affected (Roberts, 1977). The vegetation is affected by oil pollution, there is defoliation and loss of the productive cycles and most times there is outright death of the affected plants. Essien and John, (2010) reported that plants normally germinate, develop and grow in soil medium where air, water and other nutrients are available for health growth and for productive and profitable agriculture. They are of the opinion that the frequent crude oil spillage in the Niger Delta region of Nigeria on agricultural soils and the consequent fouling effect on all forms of life render the soil, especially the biological active surface layer, toxic and unproductive. The oil reduces the soils fertility such that most of the essential nutrients are no longer available for the plant and crop to utilize (Abii and Nwosu, 2009). Brain, (1979), reported that spilled crude oil reduces and restricts soil permeability; the organic hydrocarbons that fill the soil pores prevent water and air from reaching the soil, thus depriving the plants roots of the much needed water and air. With increasing soil infertility due to the destruction of soil microorganisms and dwindling agricultural productivity, farmers have been forced to abandon their land to seek for non-existent alternative means of livelihood. Thus, these problems motivated this research work which involves the use of bioremediation technology to remedy the situation. The optimal conditions of pH, nutrient concentration and % moisture content of the soil for the microorganism to biodegrade the crude oil were studied.

2. Materials and Methods

2.1 Sample collection

The contaminated soil sample was obtained from Ogoni town in Gokana Local Government Area of Rivers State, Nigeria. The crude oil used was Bonny light gotten from Kokori field during drilling by Heritage Energy/NNPC JV in Kwale, Delta State.

The urea (nutrient) used was obtained from Ogbete Market, Enugu. Pure and analytical grade of Microbiological and Biochemicals were obtained from De-Cliff Integrated Company Enugu, Nigeria. The microbial culturing was done at His Grace Diagnostic Laboratory while the biodegradation study was carried out at Pymotech Laboratory, all at Abakpa Nike, Enugu State, Nigeria.

2.2 Isolation and Identification of the Isolates

Mineral salt medium (MSM) was prepared and used as basal medium for the study of crude oil degrading bacteria. This was prepared by measuring and mixing the following: 1.25g of $(NH_4)_2SO_4$, 0.001g of $CaCl_2$, 1.95g of $(NH_4)_2HPO_4$, 0.85g of KH_2PO_4 and 0.09g of $MgSO_4.7H_2O$ in a litre of distilled water. The mineral salt medium was divided into two.

50ml of the solution was measured into two Erlenmeyer flasks. The mineral salt medium was subjected to sterilization by autoclaving at a temperature of 121^OC for 15 minutes. After cooling the medium, 2g of the crude oil contaminated soil was added to each of the two flasks containing 50ml of the mineral salt medium. 1.0ml of kerosene filtered through a

filter paper was added into each of the flasks and incubated in an orbital shaker at 30^{O}C and shaken for one hour so as to make the nutrients available to the microorganism in the soil sample (Nwosu et al., 2018).

2.3 Agar medium

The agar medium prepared contained one litre of distilled water, 2.0g of Na_2SO_4, 1.0g of NH_4Cl, 0.5g KH_2PO_4, 0.001g $CaCl_2$, 2.0g KNO_3 and 1.0g $MgSO_4$ with 15g of agar in mineral salt medium. 20ml of this was poured into petri-dishes and allowed to cool and solidify. Sterilized wire loop was used to collect samples from the mineral salt earlier prepared and inoculated into the agar medium by streaking method. Filter papers impregnated with kerosene was placed on the cover of the petri dishes and served as the only carbon source for the microorganisms. After 48 hours of incubation, the colonies formed were streaked separately on prepared agar media. Resulting colonies were repeatedly sub cultured in this manner twice and pure isolates were obtained and stored in nutrient agar slants.

2.4 Sub-Culturing of Pure Cultures and Characterization of the Isolates

The isolated pure cultures were sub-cultured and incubated at temperature of 37^{O}C for 72 hours. All the cultures that showed proper growth were identified using conventional Microbiological and Biochemical procedures and reference to the Berge's Manual of Determinative Bacteriology. The following identification tests were carried out in addition to cultural and morphological properties: Gram reaction, spore stain, motility, catalase, oxidase, starch hydrolysis, nitrate reduction, growth on 7% NaCl, Arginine hydrolysis, tests for the fermentation of sucrose, dextrose, mannitol, lactose, maltose, mannose and xylose.

2.5 Biodegradation Studies/ Optimization of process parameters

The biodegrading experiment was done following the matrix developed which contained 20 runs and some constant variables. A constant volume of crude oil (10ml) was added to a known mass of sterile soil sample (20g) to contaminate it. A constant volume of bacterial isolate (5ml) kept in saline condition was inoculated into the crude oil and soil sample. Five different pH solutions were prepared (5, 6, 7 8 and 9 pH values). The urea used as nutrient supplement ranging from 2, 4, 6, 8, 10g were measured out. The five different pH solutions were prepared in five different volumes of water that served as the moisture content of the experimental set up (Nwosu et al., 2018). These constant variables prepared were added to the soil sample with crude oil and the bacteria isolate following the matrix developed. After preparing the twenty runs, the samples were kept at room temperature for a period of 21 days, after which the volume of residual oil and final microbial cell population were determined.

2.6 Extraction of Residual Oil

Twenty millilitre of n-hexane was poured into a bottle containing crude oil contaminated soil with the organisms. The extraction was done twice with 10ml of n-hexane each time. It was shaken vigorously and decanted into sterile test tube. The total extract which contains the residual crude oil was then heated over a hot water bath to evaporate the solvent (n-hexane). The residual crude oil left in the test tube was then weighed and the volume calculated based on the fractional recovery rate (FRR).

2.7 Fractional Recovery Rate (FRR)

Most extraction procedures fall short of 100% efficiency. In most cases, these percentages lost are not considered and this in fact affects the final result. Except the fractional loss is known in biodegradation experiments, false assessment of the biodegradation can be recorded. It was on this premise that the crude oil extraction method of Toogood and McGill (1977) was used to determine the FRR of the crude oil in this study. Four (4ml) of the crude oil was introduced into 20g of soil, the crude oil was extracted from the soil with n-hexane. It was discovered that the percentage recovery rates was consistently 62.5±2% (meaning that

from the 4ml introduced into the soil, 2.5±0.16 was consistently recovered with the remaining adsorbed to the soil particles). There was a constant percentage loss of about 37.5% which amounts to 1.5ml of the 4ml used. From the above, it was possible to calculate the fractional lost per ml of the crude oil as:

- Amount of oil introduced = 4ml
- Amount of oil recovered =2.5/4 = 0.625 per ml
- The fractional recovery rate (FRR) = 0.625
- The fractional lost per ml = 0.375

The implication of FRR is that after the biodegradation study, the actual quality of oil degraded will be gotten by adding the residual oil and the fractional lost and subtracting the sum from the total crude oil used in the biodegradation experiment.

2.8 Estimation of the Bacterial Number (cfu/ml)

Ten-fold serial dilution was done by taking 1ml of the isolated microbial sample and added it to the first of the ten test tubes containing 9ml sterile distilled water and shaken thoroughly. 1ml was then pipetted from this and transferred into another test tube containing 9ml sterile distilled water to give a 10^{-1} dilution. The sample was diluted serially up to 10^{-10}. The enumeration proper was done by inoculating 0.1ml aliquot from an appropriate serially diluted sample into mineral salt agar medium. Kerosene supplied carbon by the vapour phase transfer method. Inoculated plates were incubated for 48 hours at room temperature (25 – 30^{O}C) after which the resulting colonies were counted and the bacterial number estimated as colony forming units (cfu) using the equation:

(cfu) = average number of colonies x original dilution
Volume of inoculum x dilution factor

$$= \frac{Y \times 1}{V \times 10^{-x}} \qquad (2.1)$$

V = Volume of inoculum
10^{-x} = Dilution factor
Y = Average number of colonies
$10^{o} = 1$ = Original dilution

3.0 Results and Discussion

3.1 Optimization of Biodegradation Process

Optimization of process conditions for biodegradation of soil sample contaminated with crude oil was studied using central composite design. The central composite design consists of axial factorial and centre points. The process factors considered were moisture content, pH and Nutrient with the biodegraded oil in percentage as the response. These process conditions were studied at five levels with five centre point giving a total of twenty runs. The experimental design was randomized to control the effect of lurking variables ruining the experiment. The experiment was strictly based on the design matrix on Table 1.

Table 1: Design matrix for the biodegradation process

Std Order	Run order	Moisture content (%)	PH (-)	Nutrient (g)	Biodegraded oil (%)
13	1	35.00	7.00	2.00	37.205
10	2	45.00	7.00	6.00	47.1
19	3	35.00	7.00	6.00	48.31
4	4	40.00	8.00	4.00	48.57
2	5	40.00	6.00	4.00	38.59
15	6	35.00	7.00	6.00	48.26
1	7	30.00	6.00	4.00	37.675
14	8	35.00	7.00	10.00	46.435
16	9	35.00	7.00	6.00	47.73
3	10	30.00	8.00	4.00	44.49
7	11	30.00	8.00	8.00	48.5
8	12	40.00	8.00	8.00	49.09
5	13	30.00	6.00	8.00	45.295
12	14	35.00	9.00	6.00	47.375
20	15	35.00	7.00	6.00	47.78
9	16	25.00	7.00	6.00	45.76
11	17	35.00	5.00	6.00	37.905
6	18	40.00	6.00	8.00	46.29
17	19	35.00	7.00	6.00	48.28
18	20	35.00	7.00	6.00	46.26

3.2 Selection of a good predictive model

Different models like linear, 2F1 (two factor interaction) and quadratic were tested for response prediction. The selected model was based on the lack of fit test, adjusted and predicted R-squared. The sequential model sum of squares was used to select the best model based on the highest order model that was significant (little p-value) and not aliased, non-significant lack of fit (p-value > 0.10) and good agreement between adjusted R-squared and predicted R-squared (within 0.2 from each other). Tables 2 to 4 shows the summary table,

sequential model sum of squares and lack of fit test for the models.

Table 2: Summary tables for the models

Sources	Sequential p-value	Lack of fit p-value	Adjusted R-squared	Predicted R-squared	
Linear	0.0004	0.004	0.6041	0.4580	
2F1	0.4892	0.0032	0.5929	0.4747	
Quadratic	<0.0001	0.4229	0.9584	0.8936	Suggested

Table 3: Sequential Model Sum of Squares

Sources	Sum of squares	df	Mean squares	F values	p-value prob > F
Mean vs total	4123.38	1	41123.38		
Linear vs mean	205.98	3	68.66	10.66	0.0004
2F1	16.96	3	5.65	0.85	0.4892
Quadratic vs 2FI	79.30	3	26.43	39.08	<0.0001 Suggested
Residuals	3.17	6	0.53		
Total	41432.38	16	2071.62		

Table 4: Lack of fit tests for the models

Sources	Sum of squares	Df	Mean squares	F values	p-value prob > F	
Linear	99.95	11	9.09	14.78	0.004	
2F1	82.99	8	10.37	16.88	0.0032	
Quadratic	3.69	5	0.74	1.20	<0.4229	Suggested
Pure Error	3.07	5	0.61			

From Tables 2 to 4, it was observed that the selected model was quadratic. It was selected as the highest order polynomial where the additional terms were significant and the model not aliased. The model equally had insignificant lack of fit.

3.3 Analysis of Variance for the Biodegradation Process

The variations obtained from the single interaction and quadratic effects of the process factors were studied with Analysis of variance (ANOVA) Table 5. The model F-value of 54.71 implied that the model was significant (Ejikeme et al., 2016). There was only a 0.01% chance that a 'model F-value' this large could occur due to noise. Values of 'prob >F' less than 0.05 indicated that the model term were significant. In this case, the single effect of moisture content, nutrient and pH was significant. The interaction effect of pH and nutrient was significant while the quadratic effect of pH and nutrient were significant. Values greater than 0.100 indicate the model terms were not significant. The lack of fit F-value of 1.77 implied the lack of fit was not significant relative to pure error. There was a 27.47% chance that a 'lack of fit F-value' this large could occur due to noise. The predicted R-squared of 0.9086 is in reasonable agreement with the 'Adj R-squared of 0.9443. Adequate precision measures the signal to noise ratio and a ratio greater than 4 is always desired. The observed ratio of 21.383 indicated an adequate signal.

Table 5: Analysis of variance (ANOVA) table for the biodegradation process.

Source	Sum of Square	MeanF sdf SquareValue	p-value		Prob > F	
Model	297.23	6	49.54	54.71	< 0.0001	significant
A-moisture content (%)	5.361		5.36	5.92	0.0302	
B-pH	108.891		108.89	120.27	< 0.0001	
C-Nutrient (g)	91.731		91.73	101.31	< 0.0001	
BC14.551	14.55		16.07	0.0015		
B237.961	37.96		41.93	< 0.0001		
C252.031	52.03		57.47	< 0.0001		
Residual	11.77	**13**	0.91			
Lack of Fit	8.708		1.09	1.77	0.2747	not significant
Pure Error	3.075		0.61			
Cor Total	309.00	**19**				

3.4 Model Equation for the Biodegradation Process

Model equation was generated which represents the oil biodegradation process. It was represented both in coded and actual form. The two types of model equations give an approximation that leads to the proper direction, but only the coded form of it can be used for response prediction because the actual form has been scaled to accommodate their different units.

Equation 1 and 2 show the coded and actual model equations respectively.

Biodegraded oil (%) = +47.43 + 0.58A + 2.61B + 2.39C – 1.35BC – $1.20B^2$ – $1.41C^2$ …. (1)

Biodegraded oil (%) = -81.84545 + 0.11575 moisture content (%) + 23.45750 pH + 10.13335 nutrient (g) – 0.67438 pH nutrient (g) – 1.20018 pH^2 – 0.35129 nutrient $(g)^2$ …….. (2)

Table 6 shows the residuals, actual values, and predicted values presented in standard order. Residuals is the difference between the actual and predicted values. The behaviour of the residuals tells one if the generated model equation is good. Therefore, there is need to diagnose the behaviour of the generated residuals. The model equation was diagnosed with the aid of residual plots.

Table 6: Residuals, actual and predicted values

Standard Order	Actual Value	Predicted Value	Standard Order
1	37.67	37.89	-0.22
2	38.59	39.05	-0.46
3	44.49	45.81	-1.32
4	48.57	46.97	1.60
5	45.30	45.38	-0.085
6	46.29	46.54	-0.25
7	48.50	47.90	0.60
8	49.09	49.06	0.033
9	45.76	46.27	-0.51
10	47.10	48.59	-1.49
11	37.91	37.41	0.49
12	47.38	47.85	-0.47
13	37.20	37.02	0.19
14	46.44	46.60	-0.16
15	48.26	47.43	0.83
16	47.73	47.43	0.30
17	48.28	47.43	0.85
18	46.26	47.43	-1.17
19	48.31	47.43	0.88
20	47.78	47.43	0.35

It tests the assumption of constant variance. The plot should be a random scatter which shows constant range of residuals across the graph. The third plot is that of residual vs run (Fig. 3).

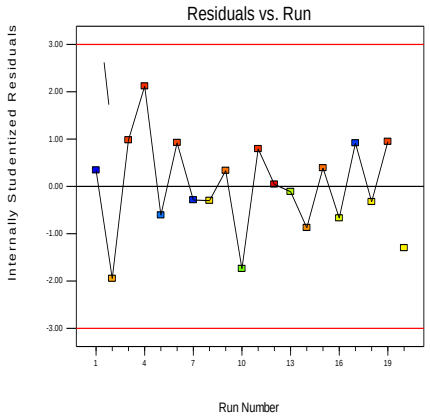

Figure 3: Plot of Residual versus Experimental Run order.

This plot allows one to check for lurking variables that may have influenced the response during the experiment. The plot should show a random scatter. The last is that of predicted vs actual Figure 4.

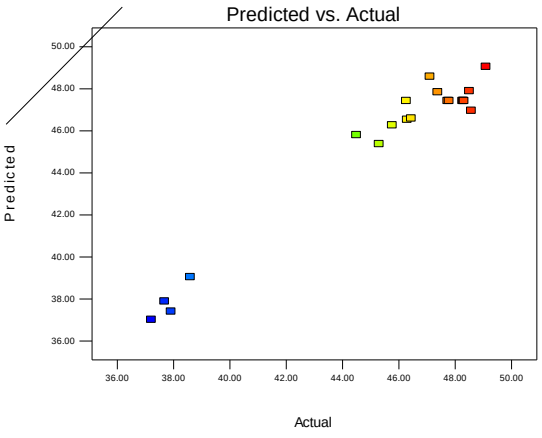

Figure 4: Plot of predicted values versus the actual values.

It helps you to detect a value or group of values that are not easily predicted by the model. The data points should be split evenly by the 45 degree line.

The behaviour of the diagnostic plots showed in Figures 1-4 confirmed that the ANOVA assumptions were met and that the model equation can be used for biodegradation prediction.

3.6 3D Surface Plot for the Biodegradation Process

It was observed from ANOVA Table that the only significant interaction effect was that of nutrient and pH, thus its 3D plot in Figure 5. From Figure 5, it was observed that biodegraded oil increased as nutrient and pH increased to a point that further increase on the two factors decreased the biodegraded oil. It is known fact that increase in pH increase the activity of microorganisms to an optimum pH after which it starts decreasing (Akpoveta et al., 2011). This is in agreement with the work done by Bossert and Barther (1984).

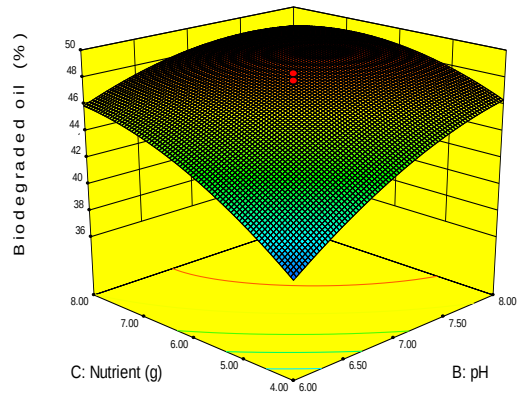

Figure 5 3D Surface plot for the interaction of nutrient with pH for biodegradation process

Also increase in nutrient is known to increase the viable organism which results in increased oil biodegradation. The decrease recorded at a higher nutrient can be attributed to substrate inhibition. Also, increase in microbial population as a result of increase in nutrient can lead to excess secretion of metabolites by the microorganism which can make the environment uncomfortable for the organism and can lead to their death thereby reducing the rate of biodegradation. This is in line with the work done by Braddock et al., 1997 where they observed that over fertilization of the soil can cause depression of microbial activity.

3.7 Optimum Condition for the Biodegradation Process

Numerical optimization was done for the biodegradation process. Table 7 shows the optimum conditions with the validation results. The validation was done using the optimum condition and the error was generated. The error shows the percentage deviation of the experimental value from the predicted values. From table 7, it was observed that little error of 0.006% was generated which shows closeness of the two values.

Table 7: Optimum conditions for the biodegradation process

			Biodegraded oil (%)		
Moisture content (%)	pH	Nutrient (g)	Predicted Value	Experimental Value	Error (%)
39.68	7.98	5.74	49.2146	49.2149	0.0006

4. Conclusion

From the results of the study, it was observed that there was a significant interaction between pH and nutrient on the level of biodegradation. This showed that increase in both pH and nutrient led to increase in biodegradation and as such decrease in level of residual oil until the optimum point of 7.98 pH and 5.74g of nutrient (urea) at 39.68% moisture content was attained, after which the level of biodegradation started to decrease.

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