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Optimization of Bioremediation of Crude Oil Polluted Soil through Variation in Moisture Content and Proportion of Augmenting Bacterium and Fungus

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Authors' contributions

This work was carried out in collaboration among all authors. Author PLP designed the study, and performed the statistical analysis. Author FM wrote the protocol, managed the analyses of the study, and wrote the first draft of the manuscript. Author AO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to enhance bioremediation of crude-oil polluted soil through optimization procedures.
Study Design: The Box-Benkhen coded values format and experimental design were used in the design of the study.

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Place and Duration of Study: The study was carried out in the Microbiology laboratory of the Department of Microbiology, Rivers State University, Nigeria, between February 2024 and July 2024.
Methodology: Hydrocarbon utilizing bacteria (HUB) & fungi (HUF) were isolated from crude-oil polluted soil, and screened for hydrocarbon degrading ability (HCDA). HUB and HUF with the highest HCDA were identified and used for bioremediation optimization experiment. In the experiment, different combination of moisture content (MC), and proportion of HUB (pHUB) & HUF (pHUF) were investigated for their effect on extent of hydrocarbon reduction (EoHR). EoHR obtained from the different combinations were fitted using a generalized polynomial model so as to obtain a polynomial equation for predicting EoHR. The equation was used in generating prediction profiles from which the combined values of the 3 parameters that will lead to the highest EoHR was determined. The predicted combined values were implemented in a new setup. A control and a setup enhanced with fertilizer were also prepared. The setups were maintained for 3 weeks. On day 0, 14, and 21 samples were collected and analyzed for total hydrocarbon concentration (THC).
Results: The results obtained showed that among the coded isolated HUB and HUF, HB5 and HF1 had the highest HCDA; 36.4% and 4.1% respectively, and were identified phenotypically as *Klebsiella ornithinolytica* and *Aspergillus flavus* respectively. The results of the optimization experiment and prediction profiles showed that the highest (68.6%) EoHR was achievable at MC = 20%, pHUB = 10%, and pHUF = 1%. The actual EoHR on day 21 in the optimized, enhanced optimized, and control setups were 60.3, 58.9, and 39.9% respectively.
Conclusion: Bioremediation optimization studies is advantageous and should be carried out on crude-oil polluted sites before carrying out bioremediation.

Keywords: Bioremediation; hydrocarbon-polluted soil; hydrocarbon-utilizing microorganisms; box-behnken experimental design matrix; prediction profiles.

1. INTRODUCTION

Bioremediation involves the use of microorganisms or their products in degrading, mineralizing or transforming pollutants in an environment [1]. Some microorganisms have high capacity to utilize or degrade hydrocarbon pollutants and a broad spectrum of petroleum products [2]. Microorganisms that utilize hydrocarbons are commonly referred to as hydrocarbon utilizing microorganisms; hydrocarbon utilizing bacteria (HUB) and hydrocarbon utilizing fungi (HUF). They include *Bacillus*, *Pseudomonas, Klebsiella, Penicillium, Aspergillus,* and *Trichoderma* [3, 4]. Hydrocarbon utilizing microorganisms (HUMs) can be isolated from environments polluted with crude-oil using mineral salts medium supplemented with crude-oil [5]. Individual HUMs metabolizes a limited range of hydrocarbon fractions [6]. Thus a consortium of HUB and HUF will be required to degrade the different fractions of hydrocarbons in a crude-oil spill. Addition of HUMs to crude-oil impacted soil may therefore be necessary for effective bioremediation.

Hydrocarbon utilizing microorganisms are the primary agents in bioremediation of crude-oil polluted environment. However, there are other factors that are also important and influence the success of bioremediation. They include nutrients, air, microbial load, and moisture content [6-8]. For effective bioremediation of crude-oil polluted soils, moisture content values between 14 and 19% have been cited by Chorom *et al* [9] to be appropriate. As a means of air provision, tilling rates of 2 to 5 times a week have been suggested to be necessary for effective bioremediation of crude-oil polluted soils [9, 10]. It is therefore evident that some factors influencing bioremediation can be adjusted, and thus their optimum values for enhance bioremediation can be deciphered.

Bioremediation has the drawback of requiring more time compared to other remediation options. This issue is of concern where there is an urgent need to clean up crude-oil polluted environments within the shortest possible time due to the threat posed to habitats exposed to the pollution. Therefore it is necessary to optimize bioremediation processes so as to enhance the hydrocarbon biodegradation process. Optimization has been applied in other aspects of Microbiology including biosurfactant and enzyme productions [11, 12]. During optimization, a mathematical model is generated which can be used to determine the maximum output of a system resulting from combinations of values of adjustable variables

of the system [13]. Combinations of adjustable variables that influence biodegradation of hydrocarbons in crude-oil polluted soils can therefore be adjusted so as to achieve maximum hydrocarbon reduction during bioremediation of crude-oil polluted soils. Therefore the aim of this study was to enhance bioremediation of crude-oil polluted soil through optimization procedures with focus on moisture content, proportion of augmenting hydrocarbon utilizing bacterium and fungus.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection

About 2 kg of soil was collected from a crude-oil polluted farm (4.673 N, 7.297 E) in Gokana Local Government Area of Nigeria, and transported to the Microbiology laboratory of the Rivers State University, for analysis, isolation of hydrocarbon utilizing bacteria and fungi, and bioremediation optimization study.

2.2 Determination of Total Hydrocarbon Concentration

The total hydrocarbon concentration (THC) in the soil and subsequent optimization experiment was determined using the spectrophotometric method described in Peekate *et al* [14]. A quantity of 5 g soil was placed in a 150 ml capacity beaker, followed by the addition of 10 ml hexane. The resulting mixture was agitated for about 30 seconds and filtered using Whatman No. 1 filter paper. The filtrate was subjected to absorbance measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set at 420 nm. Absorbance reading was used to determine THC through extrapolation from a previously obtained calibration graph.

2.3 Isolation of Hydrocarbon Utilizing Bacteria and Fungi

A quantity of 1 g of soil was inoculated into 10 ml nutrient broth (NB) and potato dextrose broth (PDB), separately. Inoculated NB and PDB were incubated at 37 °C for 24 hours and ambient temperatures (27 - 32 °C) for 5 days respectively for enrichment of bacterial and fungal populations. After incubation, the NB and PDB cultures were subjected to ten-fold serial dilution using tubes of sterile normal saline to obtain 10^{-5} and 10^{-4} dilutions respectively. Volumes 0.1 ml of 10^{-3}, 10^{-4}, and 10^{-5}dilutions of the NB culture were inoculated, in duplicates, on plates of mineral salt agar supplemented with 1000 $\mu g.ml^{-1}$ Ketoconazole (MSAK) using the spread plate technique. The composition ($g.L^{-1}$) of the mineral salt agar is as follows: $MgSO_4.7H_2O$ - 0.42, KH_2PO_4 - 0.83, NaCl - 10.0, KCl - 0.29, Na_2HPO_4 -1.25, $NaNO_3$ - 0.42, Agar – 15.0 [7]. Volumes of 0.1 ml of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the PDB culture were inoculated, in duplicates, on plates of mineral salt agar supplemented with 100 $\mu g.ml^{-1}$ tetracycline (MSAT). Crude-oil hydrocarbons were supplied into the inoculated MSAK and MSAB plates using the vapour phase transfer technique [15]. In the method, filter paper was placed in the lid of the plates and saturated with 1 ml crude-oil. The plates were incubated at ambient temperatures (27 – 32 °C) for 7 days. After incubation, bacterial colonies (hydrocarbon-utilizing bacteria) on MSAK plates were isolated onto nutrient agar (NA) plates, while fungal colonies (hydrocarbon utilizing fungi) on MSAT plates were isolated onto potato dextrose agar (PDA) plates. Stock cultures of the hydrocarbon-utilizing bacteria (HUB) and hydrocarbon-utilizing fungi (HUF) were prepared on NA and PDA slants respectively.

2.4 Selection of Hydrocarbon-Utilizing Bacteria and Fungi

Broth cultures were prepared from stock cultures of HUB isolates, while plate cultures were prepared from stock cultures of HUF isolates. The broth cultures of the HUB isolates were inoculated onto NA plates. Inoculation was carried out by spreading with the aid of sterile swab sticks. Growth on plate cultures of HUF isolates was inoculated, with the aid of sterile forceps, onto PDA plates. Inoculation was carried out by picking and placing fungal growth onto several places on the agar surface. Inoculated NA and PDA plates were incubated at previously specified conditions. Resulting bacterial growth after incubation were scooped with the aid of sterile spatula and transferred into 100 ml sterile normal saline (NS). The bacterial-NS suspensions were standardized to 0.5 McFarland standard [16]. Resulting fungal growths were scraped with the aid of sterile spatula, placed on aluminum foiled, and weighed. Standardized bacterial-NS suspensions and weighed fungal growths were assessed for hydrocarbon degrading ability as follows. A number of beakers containing 100 g wet soil (moisture content; 20%) was prepared according to the number of HUB and HUF isolates. The setups of wet soil were

polluted with 10 ml crude-oil, partially sealed with aluminum foil, and allowed undisturbed for 7 days. After this period, THCs in the setups were determination, and then 10 ml standardized bacterial-NS suspensions, and 0.1 g fungal growths added to the setups separately. The setups were stirred once in 3 days, and maintained for 14 days after which THC was determined, and the extent of hydrocarbon reduction (EoHR) calculated as $\frac{\text{THC,day 0} - \text{THC,day 14}}{\text{THC,day 0}} \times 100$. Setups which had the highest EoHR were noted, and the isolate added into them were identified, and used for the bioremediation optimization experiment.

2.5 Identification of the Selected HUB and HUF Isolates

The selected HUB isolate was subjected to the following Morphological and biochemical tests: Gram-stain reaction, motility, Catalase production, Oxidase production, citrate utilization, Indole production, Methyl Red-Vogues Proskauer (MRVP), casein hydrolysis, starch hydrolysis, and fermentation tests using Glucose, Lactose, Maltose, Xylose, and Glycerol. The procedures used for these tests are as described by Peekate [17]. Result patterns obtained from the tests was used in deciphering the identity of the HUB isolate. The selected HUF isolate was subjected to macroscopic and microscopic examination, and the colonial features and microscopic morphology described. The description was compared with fungal descriptions in a textbook by Zafar *et al*[18] so as to obtain a probable identity of the fungus.

2.6 Bioremediation Optimization Experiment

Value ranges of moisture content, and relative proportion of the chosen HUB and HUF was selected based on the Box-Behnken coded value levels [19]as presented in Table 1. Combinations of the values according to the Box-Behnken experimental design matrix are presented in Table 2. In line with the experimental design matrix, 13 experimental bioremediation units were prepared as follows: 1.5 kg soil was polluted with 150 ml crude-oil, and allowed for 7 days; at the end of the 7 days the polluted soil was divided into 13 experimental units (setups), 105 g per unit; 5 g soil sample was then collected for THC determination; each setup was then treated as presented in Table 3. The soil in the setups was stirred once in 3 days, and maintained for 14 days after which THC was determined and the EoHR calculated.

2.7 Development of Mathematical Model and Prediction Profiles

Extent of hydrocarbon reduction (EoHR) obtained in the different setups and values of the varied parameters (moisture content, relative proportion of HUB and HUF isolates) were fitted using the generalized polynomial model for 3-factor design [20]: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 + \beta_{2,3} X_2 X_3 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2$. In the equation, Y represents the predicted response; X_1, X_2, and X_3 represent the values for the three parameters; β_0 represents the value of fitted response at the centre point of the design; β_1, β_2, and β_3 represent the linear coefficients; $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ represent the interaction coefficients; and $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ represent the quadratic coefficients. Polynomial equations obtained from the model fitting was resolved through matrices so as to derive the single polynomial equation that was used to predict EoHR obtainable from different combine values of the parameters. The solution of the resolved model was then used in generating prediction profiles. From the prediction profiles, the combined values of the 3 parameters that could lead to the highest extent of hydrocarbon reduction were determined.

2.8 Experimenting the Predicted Optimized Combination

A new experimental setup of 150 g polluted soil was prepared. In the new setup, combination of values of the 3 parameters that could lead to the highest extent of hydrocarbon reduction was implemented. A control and an enhanced setup were also prepared. In the control setup, the only treatment was maintenance of moisture content at 20%. In the enhanced setup, in addition to the implementation of the optimized condition, 1.5 g of nitrogen-phosphorus-potassium (NPK) fertilizer was added. The setups were maintained for 3 weeks; stirring was carried out once in a week. On day 0 and 14, soil samples were collected from the setups and analyzed for concentration of total hydrocarbons (THC), and populations of total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB), total fungi (TF), & hydrocarbon utilizing fungi (HUF). On day 21, soil samples were collected and analyzed for THC, and then THC on day 0, 14, and 21 were used to calculate the EoHR.

2.9 Determination of Microbial Populations

A quantity of 1 g soil was placed in 10 ml sterile normal saline, and the soil-saline mixture serially diluted to 10^{-5} dilution. Aliquots of 0.1 ml of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions was inoculated on plates of NA, in duplicates; 0.1 ml of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions on plates of PDA, MSAK, and MSAB, in duplicates. Inoculated NA plates were incubated at 35 °C for 24 hours; Inoculated PDA plates were incubated at ambient temperatures for (27 - 32 °C) for 5 days; Inoculated MSAK and MSAB plates were supplied with crude-oil hydrocarbons using the vapour phase transfer technique, and incubated at ambient temperatures for 7 days. After incubation, counts of ensuing colonies on NA, PDA, MSAK, and MSAB plates were used to calculate the populations of THB, TF, HUB, and HUF respectively.

Table 1. Parameters value selection based on Box-Behnken coded value levels

Parameters	Coded Levels		
	-1	**0**	**+1**
MC (%)	20	25	30
PHBS (%)	0	5	10
PHF (%)	0	0.5	1.0

MC: moisture content, PHBS: Proportion of hydrocarbon-utilizing bacteria normal-saline suspension, PHF: Proportion of hydrocarbon-utilizing fungi

Table 2. Box-Behnken Experimental design matrix for the optimization experiment

EUN	MC (%)			PHBS (%)			PHF (%)		
	(X_1)			(X_2)			(X_3)		
1	-	1	(20)	-	1	(0)		0	(0.5)
2	-	1	(20)	+	1	(10)		0	(0.5)
3	+	1	(30)	-	1	(0)		0	(0.5)
4	+	1	(30)	+	1	(10)		0	(0.5)
5	-	1	(20)		0	(5)	-	1	(0)
6	-	1	(20)		0	(5)	+	1	(1.0)
7	+	1	(30)		0	(5)	-	1	(0)
8	+	1	(30)		0	(5)	+	1	(1.0)
9		0	(25)	-	1	(0)	-	1	(0)
10		0	(25)	-	1	(0)	+	1	(1.0)
11		0	(25)	+	1	(10)	-	1	(0)
12		0	(25)	+	1	(10)	+	1	(1.0)
13		0	(25)		0	(5)		0	(0.5)

EUN: Experimental unit number, MC: moisture content, PHBS: Proportion of hydrocarbon-utilizing bacteria normal-saline suspension, PHF: Proportion of hydrocarbon-utilizing fungi.

Table 3. Additions to the polluted soil setups according to the experimental design

EUN	HBNS (ml)	HUF (g)	VW (ml)
1	0	0.5	20
2	10	0.5	10
3	0	0.5	30
4	10	0.5	20
5	5	0	15
6	5	1.0	15
7	5	0	25
8	5	1.0	25
9	0	0	25
10	0	1.0	25
11	10	0	15
12	10	1.0	15
13	5	0.5	20

EUN: Experimental unit number, HBNS: Hydrocarbon-utilizing bacteria normal-saline suspension, HUF: hydrocarbon utilizing fungi, VW: volume of water added so as to achieve the estimated moisture content based on total volume of liquid added and dry soil ratio.

3. RESULTS

3.1 Hydrocarbon Enhancing Degrading Ability of the Hydrocarbon-Utilizing Microorganisms

The number of hydrocarbon-utilizing bacteria and fungi isolated from the crude-oil polluted soil presenting with different colonial characteristics were 5 and 4 respectively. They were coded accordingly as HB1 – HB5, HF1 – HF4 respectively. The result (Table 4) of the hydrocarbon enhancing degrading ability (HEDA) test carried out on the isolates showed that of the HUB isolates, HB5 had the highest (36.4%) HEDA; of the fungal isolates, HF1 had the highest (4.1%) HEDA.

3.2 Identity of the Selected HUB and HUF Isolates

HB5 and HF1, been the HUB and HUF isolates with the highest hydrocarbon degrading ability were selected for the optimization study. The results of the Morphological and biochemical tests carried out on HB5 are presented in Table 5. Use of the result patterns of the Morphological and biochemical tests in searching the database of ABIS (https://www.tgw1916.net/bacteria_abis.html) revealed that HB5 is possibly *Klebsiella ornithinolytica*. Descriptions of the macroscopic and microscopic characteristics of HF1 are presented in Table 6. Base on comparison with information on fungal characteristics in Zafar *et al.* 2017, HF1 is suspected to be *Aspergillus flavus*.

3.3 Extent of Hydrocarbon Reduction in the Optimization Experimental Units

The extent of hydrocarbon reduction (EoHR) in the optimization experimental units is presented in Table 7. In the Table, it can be seen that the lowest (24. 1%) EoHR occurred in experimental unit number (EUN) 11 where the combination of moisture content (MC), proportion of hydrocarbon-utilizing bacteria normal-saline suspension (PHBS), and proportion of hydrocarbon-utilizing fungi (PHF) was 25, 10, and 0% respectively. The highest (73.7%) EoHR occurred in EUN 12 where the combination of MC, PHBS, and PHF was 25, 10, and 1% respectively.

3.4 Developed Mathematical Model and Prediction Profiles

The outcome of fitting EoHR in Table 7 and the corresponding values of MC, PHBS, and PHF using the generalized polynomial model for 3-factor design is presented in Table 8. Resolving the 13 polynomial equations derived from the information in Table 8 through matrices revealed the values of the coefficients β_0, β_1, β_2, β_3, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$, $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ to be -40.98, 7.4, 2.44, 46.07, -0.13, 0.22, 2.73, -0.16, -0.12, and -38.60 respectively. The regression model derived from these worked out coefficients is thus given as $Y = -40.98 + 7.4X_1 + 2.44X_2 + 46.07X_3 - 0.13X_1X_2 + 0.22X_1X_3 + 2.73X_2X_3 - 0.16X_1^2 - 0.12X_2^2 - 38.6X_3^2$. Analysis of variance (ANOVA) of the regression model indicated that at least 1 coefficient out of the 9 coefficients is significant. In other words, a regression model exists between EoHR and one or more of the factors (MC, PHBS, and PHF). A summary of the ANOVA is presented in Table 9. In the Table, it can be seen that F calculated is greater than F tabulated, indicating the significance of at least one of the coefficients.

The prediction profiles developed from the regression model is presented in Table 10a – 10c. From the prediction profiles, it can be seen that the theoretical highest (68.6%) EoHR is achievable at MC = 20%, PHBS = 10%, and PHF = 1%.

3.5 Extent of Hydrocarbon Reduction in the Optimized Bioremediation Setups

The extent of hydrocarbon reduction (EoHR) in the optimized bioremediation setup (setup in which predicted optimized combination was employed), enhanced optimized bioremediation setup, and control setup is presented in Table 11. In the Table, it can be seen that the lowest (35.1%) EoHR occurred in the control on day 14, while the highest (60.3%) EoHR occurred in the optimized bioremediation setup on day 21.

3.6 Change in Microbial Population in the Optimized Bioremediation Setups

The population of total heterotrophic bacteria (THB) in the control setup was $3.90\pm0.10 \times 10^7$ CFU/g on day 0 and $1.40\pm0.53 \times 10^7$ CFU/g on day 14, indicating that there was slight decrease in THB (Fig. 1). There was also slight decrease in THB in the optimized bioremediation setup (OPT) (from $5.45\pm3.47 \times 10^7$ to $3.0\pm1.3 \times 10^7$ CFU/g) and enhanced optimized

bioremediation setup (EOP) (from 4.23±2.84 × $10^7$to 3.0±0.7 × 10^7CFU/g), though the decrease was minimal compared to the control. The population of hydrocarbon-utilizing bacteria (HUB) in the control setup increased from 2.78±0.74 × 10^4 CFU/g on day 0 to 4.05±1.34 × 10^5 CFU/g on day 14. On the other hand, there was slight decrease in HUB in OPT (from 3.99±3.69 × 10^5 to 2.35±1.91 × 10^5 CFU/g) and EOP (from 5.25±5.10 × 10^5 to 2.75±0.35 × 10^5 CFU/g).

The population of total fungi (TF) in the control setup increased from 5.60±3.39 × 10^4 CFU/g on day 0 to 4.45±3.61 × 10^6 CFU/g on day 14 (Fig. 2). On the other hand, there was decrease in TF in OPT (from 2.25±0.25 × $10^7$to 5.58±5.55 × 10^6 CFU/g) and EOP (from 3.48±1.58 × 10^7 to 3.70±3.26 × 10^6 CFU/g). The population of hydrocarbon-utilizing fungi (HUF) in the control setup increased from 1.65±4.45 × 10^2 CFU/g on day 0 to 2.15±0.21 × 10^5 CFU/g on day 14. There was also increase in HUF population in OPT; from 3.30±0.42 × 10^5 on day 0 to 5.15±3.50 × 10^6 CFU/g on day 14. In EOP there was slight decrease in HUF from 3.52±1.64 × 10^5 to 2.75±1.06 × 10^5 CFU/g.

The percentage of THB that were HUB from day 0 to day 14 increased from 0.1% to 3% in the control setup, but decreased from 1% to 0.6% in the optimized setup, and from 2% to 1% in the enhanced optimized setup (Fig. 3). The percentage of TF that were HUF increased from 0.3 to 5.0% in the control setup from day 0 to day 14. Similar trend was observed in the optimized setup (1.5 to 92.3%), and in the enhanced optimized setup (1 to 7.4%). Evidently, the optimized setup had the highest increase.

Table 4. Extent of hydrocarbon reduction resulting from augmentation with HUB and HUF isolates

N		THC, Day 0 (mg/kg)	THC, Day 14 (mg/kg)	EoHR (%)
1	Control	40,767	40,588	0.44
2	HB1	55,995	51,429	8.15
3	HB2	65,468	55,714	14.90
4	HB3	63,909	63,896	0.02
5	HB4	49,760	49,729	0.06
6	HB5	56,115	35,714	36.36
7	HF1	59,592	57,143	4.11
8	HF2	57,554	56,571	1.71
9	HF3	48,441	48,300	0.29
10	HF4	49,640	49,557	0.17

THC: Total Hydrocarbon Concentration, EoHR: Extent of hydrocarbon reduction.

Table 5. Morphological and Biochemical characteristics of HB5

Tests	Characteristic/reaction
Morphology	Rods
Gram stain	-
Catalase	+
Oxidase	-
Motility	-
Citrate utilization	+
Casein hydrolysis	-
Starch hydrolysis	+
Indole production	+
Methyl red	+
Voges-Proskauer	+
Glucose fermentation	AG
Lactose fermentation	AG
Maltose fermentation	AG
Xylose fermentation	AG
Glycerol fermentation	A
SB.ABIS (similarity, Probability index)	*Klebsiella ornithinolytica* (90.8%, 54.2%)

A: Acid, AG: Acid and gas, SB.ABIS: Suspected bacteria as determined using ABIS online tools.

Table 6. Macroscopic and Microscopic characteristics of HF1

Macroscopic characteristics	Microscopic morphology	Suspected fungus
Green lawn-like growth with reverse yellow colour.	Septate hyphae, long conidiophores, and numerous conidia.	*Aspergillus flavus*

Table 7. Extent of hydrocarbon reduction in the Box-Behnken tailored experimental units

EUN	THC on day 0 (mg/kg)	THC on day 14 (mg/kg)	EoHR (%)
1	34875	14448	58.6
2	36456	18430	49.4
3	45939	17008	63.0
4	43094	25484	40.9
5	42251	21729	48.6
6	47203	16610	64.8
7	28764	21047	26.8
8	35824	19625	45.2
9	31398	20535	34.6
10	40144	17292	56.9
11	33190	25199	24.1
12	59741	15700	73.7
13	45939	18373	60.0

EUN: Experimental unit number, THC: Total hydrocarbon concentration, EoHR: Extent of hydrocarbon reduction.

Table 8. Design matrix of fitted treatment factors and Extent of hydrocarbon reduction using the generalized model for 3-factor

$.\beta_0$	$.\beta_1$	$.\beta_2$	$.\beta_3$	$.\beta_{1,2}$	$.\beta_{1,3}$	$.\beta_{2,3}$	$.\beta_{1,1}$	$.\beta_{2,2}$	$.\beta_{3,3}$	
	MC (%)	PHBS (%)	PHF (%)							
Design Matrix [X]										**Y**
	X_1	X_2	X_3	$X_1 X_2$	$X_1 X_3$	$X_2 X_3$	X_1^2	X_2^2	X_3^2	**EoHR (%)**
1	20	0	0.5	0	10	0	400	0	0.25	58.6
1	20	10	0.5	200	10	5	400	100	0.25	49.4
1	30	0	0.5	0	15	0	900	0	0.25	63.0
1	30	10	0.5	300	15	5	900	100	0.25	40.9
1	20	5	0	100	0	0	400	25	0	48.6
1	20	5	1.0	100	20	5	400	25	1	64.8
1	30	5	0	150	0	0	900	25	0	26.8
1	30	5	1.0	150	30	5	900	25	1	45.2
1	25	0	0	0	0	0	625	0	0	34.6
1	25	0	1.0	0	25	0	625	0	1	56.9
1	25	10	0	250	0	0	625	100	0	24.1
1	25	10	1.0	250	25	10	625	100	1	73.7
1	25	5	0.5	125	12.5	2.5	625	25	0.25	60.0

Table 9. Summary of the Analysis of Variance of the optimized model

SV	DF	SS	MS	$F_{Cal.}$	$F_{Tab.}$
Regression	3	2209.9	736.63	12.64	3.86
Error	9	524.54	58.28		

SV: Source of Variation, DF: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, $F_{Cal.}$: F calculated, $F_{Tab.}$: F tabulated at α = 0.05, DF1 = 3, DF2 = 9.

Table 10a. EoHR (%) prediction profile for combinations of values of PHBS and PHF at MC = 20%

X_2 (%)	X_3 (%)				
	0.0	**0.5**	**1.0**	**1.5**	**2.0**
0	43.0	58.6	54.9	31.9	-10.4
5	39.2	61.6	64.7	48.6	13.1
10	29.4	58.7	***68.6***	59.2	30.6
15	13.6	49.7	66.4	63.9	42.1
20	-8.2	34.7	58.3	62.6	47.6

X_2:proportion of hydrocarbon-utilizing bacteria normal-saline suspension (PHBS), X_3: proportion of hydrocarbon-fungi (PHF).

Table 10b. EoHR (%) prediction profile for combinations of the values of HBNS and HUF at MC = 25%

X_2 (%)	X_3 (%)				
	0.0	**0.5**	**1.0**	**1.5**	**2.0**
0	44.0	60.2	57.0	34.5	-7.2
5	37.0	59.9	63.6	48.0	13.0
10	23.9	53.7	***64.2***	55.4	27.3
15	4.9	41.5	58.8	56.8	35.5
20	-20.2	23.3	47.4	52.2	37.8

Table 10c. EoHR (%) prediction profile for combinations of the values of HBNS and HUF at MC = 30%

X_2 (%)	X_3 (%)				
	0.0	**0.5**	**1.0**	**1.5**	**2.0**
0	37.0	53.7	51.1	29.2	-12.0
5	26.7	50.2	***54.4***	39.4	5.0
10	10.4	40.8	51.8	43.5	16.0
15	-11.9	25.3	43.1	41.7	21.0
20	-40.2	3.8	28.5	33.9	20.0

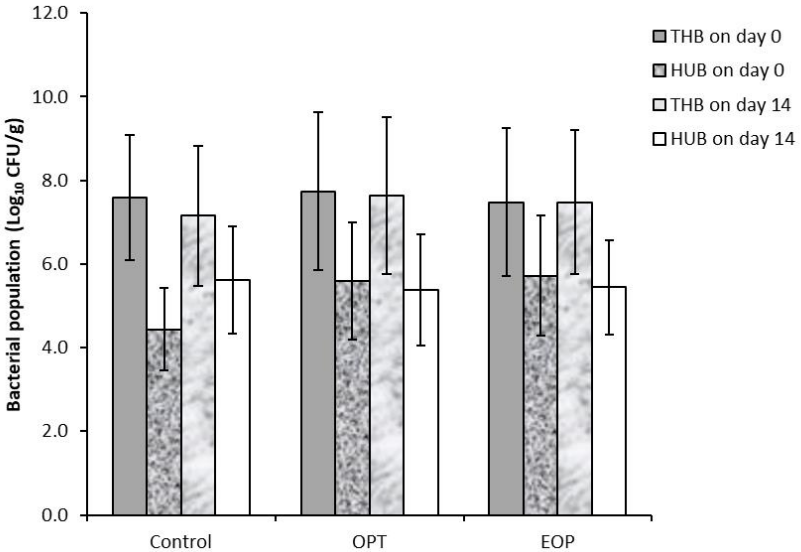

Fig. 1. Populations of Total Heterotrophic Bacteria (THB) and Hydrocarbon Utilizing Bacteria (HUB) in the setups

OPT: Optimized bioremediation setup, EOP: Enhanced optimized bioremediation setup

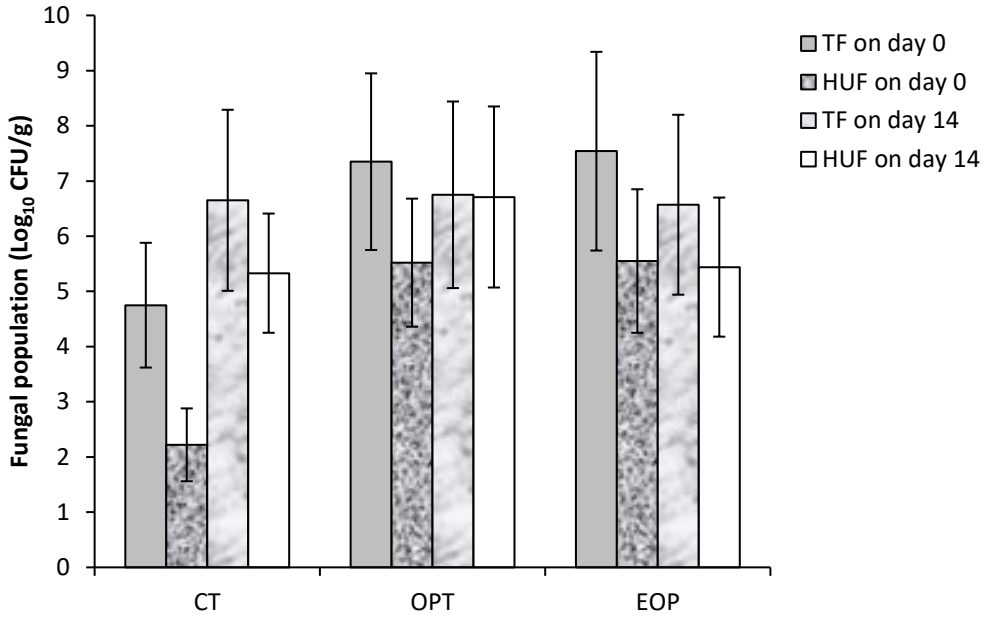

Fig. 2. Populations of Total Fungi (TF) and Hydrocarbon Utilizing Fungi (HUF) in the setups

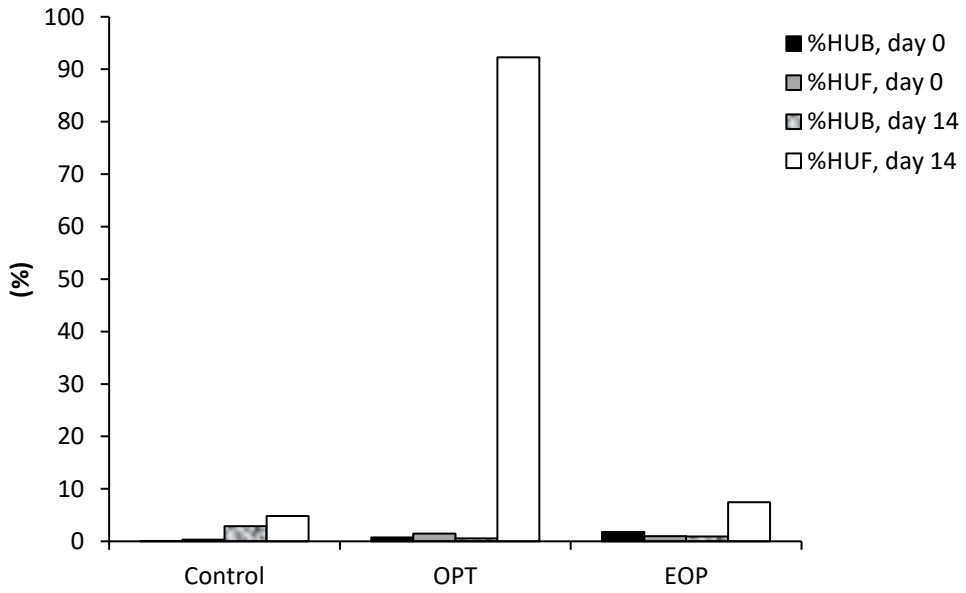

Fig. 3. Proportion (%) of THB and TF that are HUB and HUF respectively

Table 11. Hydrocarbon reduction in the optimized, enhanced optimized, and control setups

	Control	OPT	EOP
THC (mg/Kg) on Day 0	59,075	54,623	60,959
THC (mg/Kg) on Day 14	38,311	24,530	26,534
EoHR (%)on Day 14	35.1	55.1	56.5
THC (mg/Kg) on Day 21	35,521	21,694	25,043
EoHR (%)on Day 21	39.9	60.3	58.9

OPT: Optimized bioremediation setup, EOP: Enhanced optimized bioremediation setup, THC: Total Hydrocarbon Concentration, EoHR: Extent of hydrocarbon reduction

4. DISCUSSION

Hydrocarbon degrading potential of the highest hydrocarbon degrading fungal isolate (*Aspergillus flavus*) in this study was 4.1% in 14 days. In other related studies [21, 22], fungi that have been shown to degrade hydrocarbons include *Aspergillusflavus*, *A. fumigates*, *A. versicolar*, *Penicillium* sp., and *Mucor* sp. Hydrocarbon degrading ability of *Aspergillusflavus*, *A. fumigates*, and *A. versicolar* in a mineral salt medium containing 1% crude oil was revealed to be 60, 36, and 25% respectively in 15 days [21]. Hydrocarbon degrading ability of *Penicillium* and *Mucor* species in a mineral salt medium containing 1% crude oil was revealed to be 47 and 58% respectively in 14 days [22]. The relatively high hydrocarbon degrading ability of the fungi in the other studies compared to the low hydrocarbon degrading ability obtained in this study can be attributed to the crude-oil concentration and biodegradation matrix used. In this study, the biodegradation matrix used was soil, while a liquid (mineral salt solution) was used in the other studies. Also in this study, the concentration of crude-oil in the soil matrix was approximately 10%; in the other studies, the concentration of crude-oil in the liquid was 1%.

Hydrocarbon degrading potential of the highest hydrocarbon degrading bacterial isolate (*Klebsiella ornithinolytica*) in this study was 36.4% in 14 days. In other related studies [23, 24], bacteria that have been shown to degrade hydrocarbons include *Acinetobacter* sp., *Enterobacter* sp., *Klebsiella oxytoca*, *Klebsiella pneumonia*, *and Pseudomonas* sp. Hydrocarbon degrading ability of *Acinetobacter* sp., *Enterobacter* sp., and *Pseudomonas* sp. in liquid medium containing 4% diesel oil was shown to be 8.2, 9.6 and 14.2% respectively in 7 days [23]. *Klebsiella oxytoca* and *Klebsiella pneumonia* were shown to degrade hydrocarbons in industrial wastewater polluted with oil waste by 67.8 and 45.8% respectively in 7 days [24]. The hydrocarbon degrading ability (36.4%) of the highest hydrocarbon utilizing bacteria isolated in this study was higher than some bacteria and lower than some others identified in the other studies. It should be noted that the bacteria and biodegradation conditions for the different studies were different, and therefore the differences in the results.

The regression model obtained in this study for different combination of moisture content level (MC), proportion of hydrocarbon utilizing bacteria (pHUB), and proportion of hydrocarbon utilizing fungi (pHUF) was $Y = -40.98 + 7.4X_1 + 2.44X_2 + 46.07X_3 - 0.13X_1X_2 + 0.22X_1X_3 + 2.73X_2X_3 - 0.16X_1^2 - 0.12X_2^2 - 38.6X_3^2$, where Y is the extent of hydrocarbon reduction, X_1 = MC, X_2 = pHUB, and X_3 = pHUF. The prediction profiles developed from the regression model showed that the theoretical highest (68.6%) extent of hydrocarbon reduction (Y) is achievable at MC = 20%, pHUB = 10%, and pHUF = 1%. This combination led to an actual extent of hydrocarbon reduction of 55.1% and 60.3% on day 14 and 21 respectively. In other related works, other factors/parameters that affect biodegradation of hydrocarbons during bioremediation were investigated with promising results obtained. In the work of Itam*et al* [25], variation in pH and biochar blends were investigated using the Response surface experimental design. From the optimized combination, a maximum of 46% total petroleum hydrocarbon (TPH) removal was achieved in 30 days. In another related study [26], variations of bioremediation enhancement factors including soil moisture content, mixing, and carbon, nitrogen & phosphorus ratios on hydrocarbon degradation was investigated. The optimized combination yielded crude oil degradation of 91% after 36 days of remediation. Relatively high extent of hydrocarbon reduction was achieved in these studies ranging from 46 – 91% over a period of 30 – 94 days. In this current study, 55.1 – 60.3% hydrocarbon reduction was achieved in 14 – 21 days. This is an indication that the factors and model chosen was better than those in the other studies. However, it could have turned out this way due to differences in soil type, soil quantity, and initial pollutant concentration.

Though there was slight decrease in the population of total heterotrophic bacteria (THB) in the control, optimized bioremediation, and enhanced optimized bioremediation setups, the populations were maintained at values in the magnitude of 10^7 CFU/g. On the other hand, the population of hydrocarbon-utilizing bacteria (HUB) increased in the control setup from values in magnitude of 10^4 to 10^5 CFU/g. The HUB populations in the optimized and enhanced-optimized bioremediation setups were already at values in magnitude of 10^5 CFU/g; even though there was slight decrease, the decrease was to values still in magnitude of 10^5 CFU/g. Relatively, the percentage of THB that were HUB increased in the control setup from 0.1 to 3%; in the optimized and enhanced optimized setups the percentage was already at a relatively high value

of 1 and 2% respectively, however it decreased slightly to 0.6% and 1% respectively. There was increase in the population of total fungi (TF) in the control setup from values in magnitude of 10^4 to 10^6 CFU/g. The TF populations in the optimized and enhanced-optimized bioremediation setups were at values in magnitude of 10^7 CFU/g and it decrease to values in magnitude of 10^6 CFU/g which is still at the same level of which the TF population in the control increased to. On the other hand, the population of hydrocarbon-utilizing fungi (HUF) increased in the control and optimized bioremediation setups from values in magnitudes of 10^2 to 10^5 CFU/g, and 10^5 to10^6 CFU/g respectively. The HUB population in the enhanced optimized bioremediation setup was already at a magnitude of 10^5 CFU/g, and though there was slight decrease, the decrease was still to values in magnitude of 10^5 CFU/g. Relatively, the percentage of TF that were HUF increased in the control, optimized, and enhanced optimized setups from 0.3 to 5%; 1.5 to 92.3%, and 1 to 7.4% respectively; the highest increase occurred in the optimized setup, and the least in the control setup. In other related works [27-29], HUB and HUF populations in bio-augmentation setups were shown to surpass HUB and HUF populations in the controls. Also HUB populations were shown to be lesser than THB populations in treatment and control setups, while HUF populations were either slightly lesser or equal to TF populations in treatment and control setups. In the work of Nrior & Onwuka [27], THB was shown to decrease from values in magnitude of 9.4 $\log_{10}$ CFU/g to 8.0 $\log_{10}$ CFU/g in the control setup, and from 9.8 $\log_{10}$ CFU/g to 8.0 $\log_{10}$ CFU/g in the various treatment setups, while HUB decreased from values in magnitude of 8.3 $\log_{10}$ CFU/g to 4.7 $\log_{10}$ CFU/g in the control, and from 8.5 $\log_{10}$ CFU/g to 4.3 $\log_{10}$ CFU/g in the various treatment setups. HUF was shown to decrease from values in magnitude of 8.0 $\log_{10}$ CFU/g to 4.6 $\log_{10}$ CFU/g in the control setup, and 8.9 $\log_{10}$ CFU/g to 4.6 $\log_{10}$ CFU/g in the various treatment setups. In the work of Peekate *et al* [29], THB increased but later decreased in the range of values in magnitude of 10^6 CFU/g in the control, and increased but later decreased in the range of values in magnitude of 10^6 – 10^8 CFU/g in the treatment setups. This also happened for HUB, in the range of values in magnitude of 10^3 – 10^4 CFU/g in the control setup, and 10^4 – 10^6 CFU/g in the treatment setups. Increase and subsequent decrease in THB, HUB, TF, and HUF was also observed in the work of Peekate & Ogolo [28], with THB in magnitude of 10^6 – 10^7 CFU/g in both the control and treatment setups, and HUB in the range of magnitude of 10^4 CFU/g – 10^6 CFU/g in both setups. Also, the percentage of THB that were HUB in the control setup ranged from 0.7 – 6.6%, and 0.9 to 8.8% in the treatment setup. TF ranged from values in magnitude of 10^5 – 10^6 CFU/g in the control setup, and from 10^5 – 10^7 CFU/g in the treatment setup, while HUF ranged from values in magnitude of 10^5 CFU/g – 10^6 CFU/g in both setups. The percentage of TF that was HUF in the control setup ranged from 5 – 85%, and 20 - 98% in the control and treatment setup respectively. The relative higher percentage of total bacteria and fungi that are hydrocarbon utilizers, or population of hydrocarbon utilizing bacteria and fungi in treatment setups than those in control setups as observed in these studies, is in agreement with what was observed in this present study.

5. CONCLUSION

In this study, the combination of moisture content level (MC), proportion of hydrocarbon utilizing bacteria (pHUB), and proportion of hydrocarbon utilizing fungi (pHUF) that will lead to maximum hydrocarbon degradation in crude-oil polluted soil after 14 days was determined through optimization procedures. Of the hydrocarbon utilizing bacteria and fungi isolatedfrom crude-oil polluted soil, the ones with the highest hydrocarbon degrading potential were selected for the optimization experiment. The optimization experiment culminated into creation of a regression model which could be used to predict the combined values of the factors (MC, pHUB, and pHUF) that will lead to the highest extent of hydrocarbon reduction. Analysis of variance of the regression model indicated that at least one coefficient in the model was significant. In other words, a regression model exists between extent of hydrocarbon degradationand one or more of the factors (MC, HBNS, and HUF). However, the actual highest extent of hydrocarbon reduction which was a little bit lesser than the theoretical highest extent of hydrocarbon reduction was achievable after 21 days which was just one week beyond the theoretically targeted 14 days. It is therefore concluded that application of optimization models in bioremediation studies can lead to elucidation of combined values of investigated factors that will lead to maximum hydrocarbon degradation within minimal duration.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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