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Enhancing Biodegradation of Spent Oil in Soil through Autochthonous Bioaugmentation

Chinenye Nna Orji [a*]

[a] *National Mathematical Center (NMC), Mathematics Programme, Kaduna-Lokoja Expressway Sheda, Abuja, Nigeria.*

Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

The study used autochthonous bio-augmentation for the bio-degradation of spent oil in soil via standard non-molecular analysis. These organisms were reintroduced individually and in synergy into microcosms of the contaminated soil so they can use the spent oil as food, carbon and energy sources. The results revealed the presence of seventeen (17) bacteria in the contaminated soil. The performance of the individual bacteria accounted for 67.60% of the total variance in the extracted residual oil following bio-augmentation with the inherent microbes while the days of bio-augmentation accounts for 23.16% of the total variance, $p< 0.001$. However, the days allowed for autochthonous bioaugmentation with microbes in synergy to occur , caused 47.89% of the total variance in the amount of residual oil extracted, $p<0.001$ while the different mixed bacteria used accounted for 35.03% of the total variance, $p <0.001$. At the end of the 42-day incubation period, t*he performance of Bacillus spp. >Clostridium spp. >Staphylococcus spp. in* their microcosms were significantly better than the rest of the bacteria isolated from the contaminated soil. Also, *Micrococcus spp. + Citrobacter spp., Clostridium spp. + Citrobacter spp. and Mycobacterium spp. + Salmonella species in their respective microcosms performance significantly better than the rest*

**Corresponding author: E-mail: nenyenna@gmail.com;*

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mixed bacteria, p<0.05. The individual bacteria and in synergy performed significantly better than the natural attenuated microcosms in reducing the amount of residual oil obtained with *p* values< 0.001. Increasing the days of bioaugmentation will also increase the effectiveness and efficiency of the bacteria. Thus, the application of autochthonous bio-augmentation with these tried species of bacteria for further studies in a larger scale may be helpful to reduce the pollution of the environment with spent oil.

Keywords: Autochthonous bioaugmentation; biodegradation; bioaugmentation; spent-oil; pollution management; mechanic workshop.

1. INTRODUCTION

Pollution is the introduction of harmful substances called pollutants into the environment [1], which causes notable health issues to man and his environment [2] and can occur in form of soil, air, water, noise pollution [3,4] and could be natural or of anthropogenic origin [5]. Anthropogenic pollutions are due to Urbanization, industrialization, technological development and various other economic activities toxins [2,6]. These pollutant may be agricultural chemicals; radioactive waste; industrial waste; biological agents; domestic waste, waste from timber, accidental oil spills, leakages of chemicals and oil, spent oil from automobiles workshop [5], burning of fossil fuels, plastics, heavy metals, acid rain [6].

Spent oil, which is drawn from engines of vehicles, generators, hydraulic machines, following servicing of such engine, are disposed indiscriminately on land and farmland, which is common among auto-mechanics [7]. These wastes are distributed by run-off water into lakes, streams, rivers, lands, ground water, farmland with its constituent hazardous substances such as heavy metals, mono-aromatic and Poly-aromatic hydrocarbons [8-10], which are detrimental to the health of plants, animal and man [11,12], being toxic and carcinogenic [13]. The management of spent oil pollution in the environment through eco-friendly methods will reduce the toxicity on man and his environment and reclaim the land for agricultural purposes.

Biodegradation, a promising approach for the degradation of spent oil in soil, is a process whereby microorganisms breakdown organic pollutants into non-toxic form via bio-deterioration, bio-fragmentation, assimilation and mineralization [14,15]. Strategies under bio-degradation include biostimulation, bioventing, biosparging, bio-augmentation, and biofiltration [16,17]. Bio-augmentation is a widely accepted biodegradation process that involves the introduction and use of highly concentrated natural or genetically engineered organisms; such as, bacteria, fungi, bacteria, fungi, yeast, and actinomycetes [16], in contaminated environment such as soil, water, air, to breakdown organic pollutants into non-toxic [18]. This can be achieve by enhancing the strains of microbes from or outside the contaminated site via isolation, sub-culturing, optimization of the inoculum size, and genetic engineering of the microbes and reintroducing them into contaminated site [16,19]. Biodegradation via bioaugmentation has proven to be a reliable process of treating contamination in the environment. In the literature, bacteria isolated from Greek sites were used to degrade Petroleum [20]; the role of fungal metabolites in remediation of polluted environment [3] was discussed; the effectiveness of crude oil degraders via bio-augmentation was monitored via their percentage oil degradation [21]. Bio-venting and brewery waste effluents were used in bio-stimulation-bio-augmentation for the biodegradation of diesel oil in unsaturated soil [22,23] examined Allochthonous bio-augmentation in ex situ treatment of crude oil-polluted sediments in the presence of an effective degrading indigenous micro-biome. Also, Arezoo and Salmah [24] investigated bio-enrichment with *bacillus 139SI* and organic waste in crude oil polluted soil; Research has discussed the use of bio-augmentation for the removal of important pollutants from industrial wastewater [25,26], while Chen et al [27] studied the treatment of full-scale diethylene glycol monobutyl ether (DGBE) wastewater by *Serratia spp.*

The advantages of bio-augmentation include cost effectiveness, safer and cleaner, easy to handle and eco-friendly [3,19,28]. However, its disadvantages are reduced survival rate of microbe in highly toxic contaminated site, substrate competition, climatic conditions and remediation cycles [18]. Autochthonous bio-augmentation is an approach in which inherent microorganisms from a specific polluted site are isolated, purified, cultured and used as inocula for degradation of contaminant in the same

polluted site [20]. This approach of bioaugmentation overcomes the possible difficulties experienced when isolates from a foreign soil is introduced into another environment. The benefits of bio-augmentation has earned the strategy great acceptance in degradation of contaminants in the environment. Hence, the aim of study was to use autochthonous bio-augmentation to enhance the biodegradation of spent oil in soil via non-molecular analysis by individual or mixed culture in microcosms experimental setup. The non-molecular analysis was achieved via serial dilution, isolation, purification, gram staining and IMVIC test of the isolates. The scope did not cover the DNA extraction of the isolated microbes and GC/MS analysis of the extracted residual oil.

2. MATERIALS AND METHODS

2.1 Sampling

The spent oil contaminated soil samples were collected from a mechanic workshop at Jude garage Gwagwalada, Abuja, at 0-15 cm depth, using soil auger that was previously washed to prevent contamination. The soil samples were stored in dark sterile waterproofs, conveyed immediately to the laboratory and stores the refrigerator at 4 ℃.

2.2 Serial Dilution, Isolation *and Identification of* autochthonous *microorganisms*

The serial dilution and isolation of the inherent microbes in the soil sample was performed using Nna Orji's method [21]. The apparatuses and media for this study were sterilized in the autoclave for 20 minutes at 121°C. The 1 g of the homogenized soil sample was serially diluted in 9 ml of sterile distilled water in a test tube. This was agitated to loose the microbes from the soil particles, to give 10^{-1} dilution, suspension was allowed to settle down and further ten-fold serial dilutions of up to 10^{-6} was done. Approximately 0.1 ml from 10^{-4} to 10^{-6} dilutions were inoculated on nutrient agar plates and spread with an L-shaped spreader. The plates were labelled accordingly after inoculating in duplicates and plates incubated at 35°C for 24 h in an incubator. Thereafter, the isolates were sub-cultured severally, purified accordingly and preserved on agar slant at 4 ºC [29].

The Bergey's manual of determinative bacteriology was used for the identification of the bacterial isolates based on their cultural, morphological and biochemical characteristics [30].

A modified method was used for the autochthonous bioaugmentation of the contaminated soil [21]. About 40 g of contaminated soil samples were weighed into 54 pieces of 250 ml conical flasks, sterilized accordingly in the autoclave and allowed to cool overnight in the laminar-flow hood to avoid contamination. The bacteria were revived from the agar slant, cultured by streak inoculating the microorganisms on freshly prepared nutrient agar and the inoculums of 18-24 h introduced individual and in synergy into 20 ml sterile nutrient broth in duplicate and incubated at 37°C for 24 h. After 24 h growth, the 20 ml cultures were introduced into the sterilized contaminated soil, the flasks/microcosms labeled accordingly and in duplicates as individual bacteria and mixed bacteria. The conical flasks were capped with cotton wool and wrapped with a foil paper to avoid contamination. A duplicated control was kept without the introduction of any isolate and were incubated for 42 days in the incubator.

2.3 Preparation of Standard Curve

Standards curves to ascertain the concentration of isolates spiked into the contaminated soil prior to bioaugmentation were prepared [31]. A loopful of each 24 hrs culture was added to 9 ml sterile distilled water and the absorbance determined spectrophotometrically using UV spectrophotometer at 320 nm. The suspension was diluted with distilled water and the optical density of each dilution was measured, recorded and plotted. Ten-fold serial dilutions using test tubes were also carried out using a loopful of 24 hours culture of the different isolates from their respective nutrient agar plates. Thereafter, approximately 0.1 ml of the each test tube were inoculated on plates using spread plate method. This was carried out to determine the Colony Forming unit (CFU) of each isolate following the serial dilution. The absorbance of each dilution was also determined at 320 nm. The corresponding absorbances and the CFU of every Isolate were used to plot a standard curve from which the number CFU of the isolate used for bio-augmentation was obtained by interpolation using Microsoft office excel 2007.

2.4 Cold Extraction of the Residual Oil

The microcosms flasks containing sterilized soil and inoculum of individual and mixed bacteria

were incubated for a period of 42 days. At intervals of 14, 28 and 42 days after incubation, oils were cold extracted using dichloromethane from the 54 microcosms [21]. About 56 weighing boats were labelled, weighed and approximately 2 g of the soil sampled from each of the 54 conical flask after proper and adequate mixing and 2 g of Na_2SO_4 was added to it. These were properly mixed and transferred quantitatively into a burette blocked previously with glass wool. The burette was also blocked with extra glass wool after the transfer of the soil mixture. Then, extraction was carried out with 5 ml of dichloromethane. The extraction continued until the glass wool at the tip of the burette was free of oil. The weighing boats used for the extraction were weighed again following complete evaporation of the solvent. Then, the dry weight of the oil was determined and the residual oil in 1 g of the soil was determined.

Gain in weight of flask (mg) = (weight Bijou bottle and residue after evaporation of extraction solvents) – (weight of empty Bijou bottle)

$$\text{Residual oil (g/g)} = \frac{\text{gain in weight of flask (g)}}{\text{weight of wet solid (g)}}$$

2.5 Method of Data Analysis

Analysis of variance (ANOVA) was used to determine if the relationships between treatment conditions were statistically significant ($p < 0.05$) at various time points during the experiments. Tukey and Dunnett's multiple comparisons test at $\alpha = 0.05$ simultaneous confidence level were used for this analysis and results were generated using the Graph pad Prisms 7 Statistical Software ® Program.

3. RESULTS AND DISCUSSION

3.1 Bio-augmentation of Oil Contaminant with Individual Isolates

The sterilization of the contaminated soil was to ensure that no other microbe was involved in the enhancement of the bio-degradation except those introduced for the auto-augmentation process. The results of gram staining and IMVIC test are shown in Table 1. From the table, all the isolates were bacteria and seventeen in number. About ten of the bacteria were gram positive rods, five were gram positive cocci and two were gram negative cocci. From the results, no gram negative rods was isolated. These bacteria include *Pseudomonas spp.*, *Corynebacterium spp.*, *Actinomyces spp.*, *Clostridium spp.*, *mycobacterium species*, *Bacillus spp.*, *Neisseria spp., Shigella species, Enterococcus spp.*, *Staphylococcus species, Micrococcus spp.*, *Azomonas* spp., *Neisseria spp., Azotobacter chroococcum*, *Yersinia spp., Salmonella species*, *and Listeria species.*

Table 1. The results of gram staining and IMVIC test

Isolates	Codes	Names	Shape	Gram staining	Indole	MR	VP	Citrate
1.	i	*Pseudomonas spp.*	Rod	+	-	-	-	-
2.	ii	*Micrococcus spp.*	Cocci	+	-	-	-	-
3.	iii	*Corynebacterium spp.*	Rod	+	-	-	-	+
4.	Iv	*Actinomyces spp.*	Rod	+	-	-	-	-
5.	v	*Staphylococcus species*	Cocci	+	-	-	-	-
6.	Vi	*Salmonella species*	Cocci	+	-	+	-	-
7.	Vii	*Clostridium spp.*	Rod	+	-	-	-	-
8.	Viii	*Mycobacterium spp.*	Rod	+	-	-	-	-
9.	Ix	*Azotobacter chroococcum*	Cocci	-	-	-	-	-
10.	X	*Listeria spp.*	Rod	+	-	+	-	+
11.	xi-a	*Bacillus species*	Rod	+	-	-	-	-
12.	xi-b	*Neisseria spp.*	cocci	+	-	-	-	+
13.	xi-c	*Yersinia species*	Rod	+	+	+	-	+
14.	Xii	*Shigella species*	Rod	+	-	-	-	+
15.	Xiii	*Citrobacter spp.*	Rod	+	-	-	-	-
16.	Xv	*Enterococcus spp.*	cocci	+	-	-	-	-
17.	xvi	*Azomonas* spp.	cocci	-	-	-	-	-

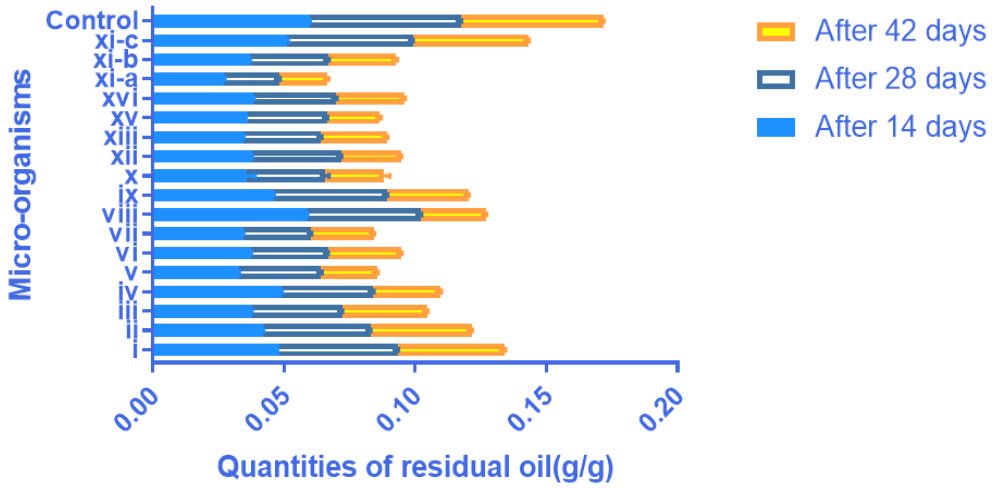

Fig. 1. The residual oil (g/g) obtained after 42 days autochthonous bio-augmentation of spent oil in with mixed individual microbes

Table 2. Two-way ANOVA of bioaugmentation with autochthonous individual isolates at α = 0.05

Source of Variation	% of total variation	P-value	P value sum.	Sign.?	
Interaction	9.198	<0.0001	****	Yes	
Row Factor	67.6	<0.0001	****	Yes	
Column Factor	23.16	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0009968	32	3.12E-05	F (32, 51) = 393.9	P<0.0001
Row Factor	0.007327	16	0.0004579	F (16, 51) = 5790	P<0.0001
Column Factor	0.00251	2	0.001255	F (2, 51) = 15870	P<0.0001

From the results, among the entire isolates, only brown coloured gram positive cocci tested positive to the Mannitol test, having changed the colour of the agar from red to yellow and grew despite the high salt concentration of the agar. Even though isolates: *Micrococcus spp.*, *Staphylococcus species*, *Clostridium spp.*, *mycobacterium spp.*, *Azotobacter chroococcum*, *Yersinia spp.* and *Shigella species*, grew on the Mannitol salt having withstood the high salt concentration, they did not change the colour of the red agar to yellow. Meanwhile, isolate *Corynebacterium spp.*, *Actinomyces spp.*, *Bacillus spp.*, *Neisseria spp.*, *Shigella species*, *Enterococcus spp.* and *Azomonas species* were not able to withstand the high salt concentration of the mannitol salt, thus they either grew on or changed the colour of the agar. Most of these microbes were also isolated in the literature [21,32]. These seventeen autochthonous microorganisms were used for the bio-degradation process. The residual oil extracted from the microcosm setup after 14, 28 and 44 days following bio-augmentation process is displayed in Fig. 1.

From the results, the range of the residual oil extracted after 14-, 28- and 42-day incubation period were 0.0326, 0.0364 and 0.0359, respectively. After 14-day incubation, the microcosms bioaugmented with *Bacillus species* had the lowest mean residual oil, 0.0273 ± 0.0003 g/g, followed by 0.0329 ± 0.0001 of *Staphylococcus species*, and thirdly by 0.0346 ± 0.0001 of *Clostridium spp.* The microorganism with the highest residual oil, 0.0589 ± 0.0001 g/g, was Mycobacterium *spp.*, while the attenuated microcosm had the highest residual oil, 0.0599 ± 0.0002 g/g, of all the microcosms of control. After 28-day biodegradation, *Bacillus species* had the lowest mean residual oil, 0.0211 ± 0.0002 g/g, seconded by *Clostridium spp.* with 0.0259 ± 0.0002 g/g mean residual oil and thirdly by *Salmonella species*, with 0.0295 ± 0.0001 g/g residual oil. The natural attenuated microcosm had the highest mean residual oil of 0.0575 ±

0.0003 g/g. After 42-day bioaugmentation, the order of the mean residual oil extracted from the microcosms was *Bacillus species* < *Enterococcus spp.*< *Staphylococcus species*< *Listeria spp.*< *Shigella species* < *Clostridium spp.*< *Mycobacterium spp.* < *Citrobacter spp.*< *Actinomyces spp.*< *Neisseria spp.*< *Azomonas* spp.< *Salmonella species* < *Azotobacter chroococcum* < *Corynebacterium spp.*< *Micrococcus spp.*< *Pseudomonas spp.*< *Yersinia species*< attenuated microcosm.

From the multiple comparison test conducted following 14 days bioaugmentation process, there was statistical difference between the performance of *Bacillus species, Staphylococcus spp.*, a gram-positive cocci and *Clostridium spp.,* gram-positive rod, in the reduction of the residual oil. However, there was no statistical difference between the performance of *Clostridium spp.,* and *Citrobacter spp.*, in the bioaugment after 14-day incubation period. However, there was no statistical difference between the performance of *Clostridium spp.* and *Citrobacter spp.* in the bioaugmentation process following 14-day incubation period. After 28 days of bio-augmentation, there was a statistical difference between the performance of *Bacillus spp., Clostridium spp.* and *Salmonella spp.* in using the spent oil as their sole source of food, energy and carbon. However, *Salmonella spp., Enterococcus spp. and Citrobacter spp.*, performed significant alike without any statistical difference. At the end of the incubation period of 42 days, *Bacillus spp.* was the best performing bacteria as it significantly reduced the residual oil lower than *Clostridium spp. although there was no statistical difference between the performance of Clostridium spp. and Staphylococcus* species in the reduction of the residual oil. From the results, after the 14th, 28th and 42nd day extractions following bio-augmentation with individual bacteria, the mean residual oil, 0.0273±0.0003, 0.0211±0.0002, 0.0184±0.0004 g/g, respectively extracted from *Bacillus spp. microcosm*, were significantly lower than those extracted from the other bioaugmented microcosms. Thus, *Bacillus spp.* performed excellently well as it reduced the quantity of oil contaminant progressively and this indicates that *Bacillus spp.* isolated from the contaminated soil had abilities to bio-augment the inherent microbes for the bio-degradation of the spent oil [29]. This demonstrates that microbes are crucial for the reclamation of contaminated soil for agricultural purposes. In addition, most of the isolates that performed significantly well were gram-positive bacteria. Hence, *Bacillus spp.* proved the best bacteria for the bio-degradation of the spent oil in the soil, as observed by [21,32,33].

3.2 The Performance of the Individual Bacteria and the Natural Attenuated Microcosms

The mean residual oil of 0.0599±0.0002, 0.0575±0.0003 and 0.0543±0.0003 g/g extracted at 14, 28 and 42nd incubation period, respectively from the attenuated microcosm experiment were significantly the highest residual oil during the bio-augmentation process. Table 2 indicates the ANOVA results of the bioaugmentation with autochthonous individual isolates for the degradation of spent oil.

From Table 2, the isolated microbes accounted for 67.60% of the total variance in the quantities of residual oil extracted following the bio-augmentation process, with p value < 0.001 and the effect extremely significant. This tells that the significant percentage performance of 67.60 % by the bacteria isolates, in the reduction of the quantities of residual oil obtained during the entire process was accounted by the presence of the microbes. This implies that the isolates played a major role more than the days in the process of the bio-augmentation and without the microbes, the rate at which the biodegradation occurred would not have been possible. From the multiple comparison tests between the residual oil extracted from the control and the isolates' microcosm after 14, 28 and 42-day of bio-augmentation, all the bio-augmented microcosms performed significant better than the natural attenuation microcosm, p-values< 0.05, having yielded residual oil lower than that extracted from the control microcosms. This shows that the presence of the bacterial isolates introduced into the contaminated soil enhanced the biodegradation of the spent oil in the soil [29,34]. This enhancement increased progressively as the days of biodegradation increased, which reduced the residual oil extracted from the first extraction day to the last day of incubation period.

Days of bio-augmentation accounted for 23.16% of the total variance with p value< 0.001 and the effect was extremely significant. This implies that the days allowed for bio-augmentation process to take place, was important in the reduction of the quantities of the residual oil obtained during the study. Multiple comparison test revealed that

Pseudomonas species, *Corynebacterium species*, *Actinomyces spp.*, *Clostridium* spp., *Listeria species*, *Shigella species*, *Citrobacter spp.*, *Bacillus spp.*, Neisseria spp., *Yersinia species, Enterococcus species*, *Azomonas* species and even the attenuated microcosm had significant effect in the reduction of the residual oil obtained at all the days of bio-augmentation. This showed that there was a progressive improvement in the reduction of the quantities of residual oil extracted from the first day of incubation to the last day of incubation. These isolates were all gram positive rods except *Enterococcus spp.*, a gram positive cocci and *Azotobacter chroococcum, Neisseria spp. and Azomonas* sp*p.*, which were gram negative cocci. Thus, these gram positive rods, gram positive and negative cocci bacteria had progressive ability to reduce the quantities of oil contaminant in the soil at long as the right environmental condition of increased aeration and addition of sterilized water for effective microbial growth were provided. *Salmonella species*, *Staphylococcus species and Micrococcus spp.,* which were all gram positive cocci, did not make that progressive impact. Rather, there was a statistical difference between the residual oils extracted after bioaugmentation for 42 days with *Staphylococcus species and Micrococcus spp.* and those extracted after 14 and 28 days of bio-augmentation. This means that both microbes need more than 28 days to make a positive impact on the bio-augmentation process. While the residual oil extracted from gram positive isolate *Salmonella species* after 28 days and 42 days were significantly lower than that extracted after 14 days of bio-augmentation.

3.3 Performances and Comparisons of the Mixed Bacteria at 14, 28 and 42-day Incubation Period

The result of the bioaugmentation process with bacteria in synergy is shown in Fig. 2. From the figure, the residual oil extracted after 14 days of bioaugmentation ranged from 0.0447±0.0007 g/g of *Mycobacterium spp.* **+** *Salmonella species* to 0.05987±0.0002 g/g of the attenuated microcosms and range from 0.03385±0.0012 g/g of *Corynebacterium spp.* **+** *Citrobacter spp.* to 0.057525±0.0003 g/g of natural attenuated microcosms after 28 days. Whereas, the residual oil ranged from 0.02145±0.0006 g/g of *Micrococcus spp.* **+** *Citrobacter spp. to* 0.054305±0.0003 g/g of the control microcosms, following 48-day of incubation period.

The ANOVA test result for the bioaugmentation with autochthonous isolates in synergy at α = 0.05 is displayed in table.

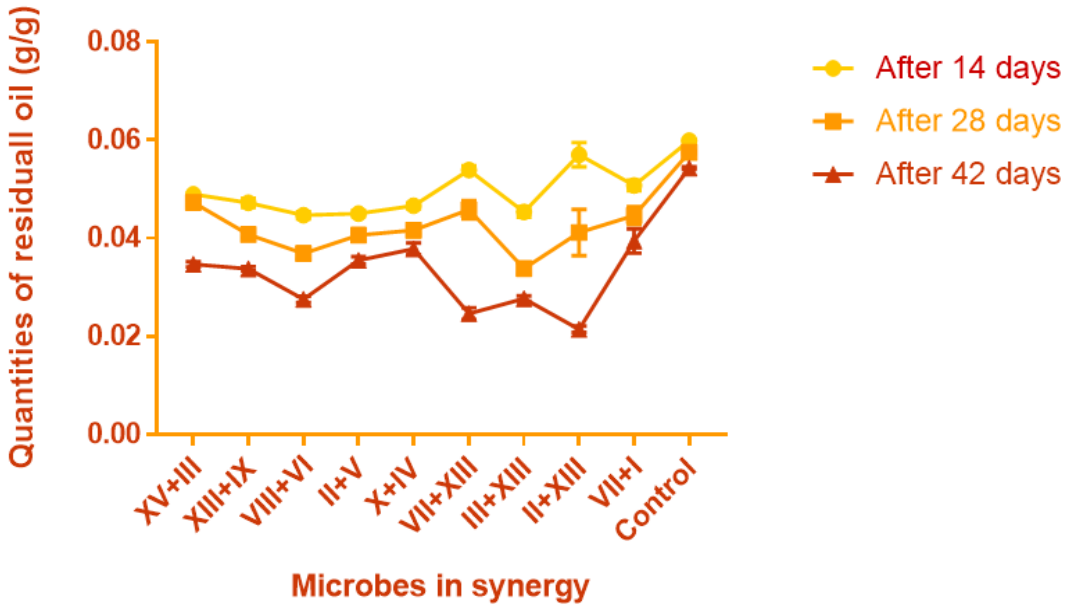

Fig. 2. The residual oil (g/g) obtained after 42 days of biodegradation of spent oil via autochthonous bioaugmentation with mixed microbes in synergy

Table 3. Two-way ANOVA of bioaugmentation with autochthonous isolates in synergy at α = 0.05

Source of Variation	% of total variation	P value	P-value sum.	Significant?	
Interaction	16.06	<0.0001	****	Yes	
Micro-Organisms In synergy	35.03	<0.0001	****	Yes	
Days Of Bio-Augmentation	47.89	<0.0001	****	Yes	
Subjects (matching)	0.1024	0.9911	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0008919	18	4.955e-005	F (18, 20) = 19.19	P<0.0001
Micro-organisms in synergy	0.001946	9	0.0002162	F (9, 10) = 380.2	P<0.0001
Days of bio-augmentation	0.00266	2	0.00133	F (2, 20) = 515.2	P<0.0001
Subjects (matching)	5.687e-006	10	5.687e-007	F(10,20) = 0.2203	P=0.9911
Residual	5.164e-005	20	2.582e-006		

From the Table 3, the indication is that the interaction between the effect of the isolates and the days allowed for the bioaugmentation process to take place accounted for 16.06% of the total variance of the entire residual oil obtained, *p*< 0.001 and was considered extremely significant. More so, the days allowed for the process of bioaugmentation to occur affected the result, having caused 47.89 % of the total variance, *p*-value< 0.001 and was considered extremely significant. The different mixed microorganisms used, accounted for 35.03% of the total variance, *p*-value< 0.001. The effect was extremely significant. From the results, the number of day allowed for the bio-augmentation process to take place had more effect, 47.89 %, on the quantities of residual oil extracted from each microcosms experiment containing the mixed isolated. This effect was higher than effects caused by the degrading ability of the mixed isolates. Therefore, increasing the number of days for bio-augmentation will also increase the reduction of the oil contaminant in the soil, having the right mixed bacteria in the soil, thereby increasing bio-degradation. This also could means that the individual isolates play better roles in the bio-augmentation process than the mixed isolates or the isolates in synergy. It could also imply that mixed isolates acted antagonistically to each other since they performed better individually in using the autochthonous bio-augmentation while using the spent oil as the sole source of food, carbon and carbon in the oil contaminated soil.

After 14 days incubation, the multiple comparison test results showed that the performance in synergy of *Enterococcus spp.*+ *Corynebacterium spp.* was statistically different from the performances of *Clostridium spp.*+ *Citrobacter spp.* and *Micrococcus spp.* + *Citrobacter spp., p*=.034 and *p*-value<0.001 at *α = 0.05*, respectively. In addition, the following paired bacteria showed statistical difference between their performances in synergy: *Citrobacter spp.* + *Azotobacter chroococcum* and *Clostridium spp.* + *Citrobacter spp.*, p<0.001, *Citrobacter spp.* + *Azotobacter chroococcum* and *Micrococcus spp.* + *Citrobacter spp. p-value*<0.001, lastly *Micrococcus spp.* + *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp.* p=.003. *Also, the performance of Mycobacterium spp.* + *Salmonella species* differed statistically from *Clostridium spp.* + *Citrobacter spp., Micrococcus spp.* + *Citrobacter spp. and Clostridium spp.* + *Pseudomonas spp.* with *p*<0.001, *p*<0.001 and *p*= 0.005 at *α = 0.05, respectively*. While the synergy in performance of *Micrococcus spp.* + *Staphylococcus species* was statistically different from *Clostridium spp.* + *Citrobacter spp., Micrococcus spp.* + *Citrobacter spp.,* and *Clostridium spp.* + *Pseudomonas spp.*, *p-value*<0.001, *p-value*<0.001 and *p*=.009, respectively. The performance in synergy of *Listeria spp.* + *Actinomyces spp.* in degrading the spent oil following 14-day incubation period was statistically difference from Clostridium *spp.* + *Citrobacter spp.* and *Micrococcus spp.* + *Citrobacter spp., p-values*<0.001. More so, performance of *Corynebacterium spp.* + *Citrobacter spp.* differed statistically from *Clostridium spp.*+ *Citrobacter spp., Micrococcus spp.* + *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp., p*<0.001, *p*<0.001 and *p*=.015, respectively. These results showed that the combined bacteria have abilities to use up the spent oil as their source of food, carbon and energy but their abilities varies depending on the interaction between the different combinations of bacteria in the microcosms within the 14-day

incubation. These depicted the relevance of autochthonous bioaugmentation in which spent oil is degraded and the residual oil reduced.

After 28 days of bioaugmentation, the comparison test results revealed that the performance of *Enterococcus spp.* + *Corynebacterium* in synergy was statistically different from those of *Citrobacter spp.*+ *Azotobacter chroococcum, Mycobacterium spp.* + *Salmonella species, Listeria spp.* + *Actinomyces spp., Corynebacterium spp.* + *Citrobacter spp. and Micrococcus spp.*+ *Citrobacter spp., p*<0.05 at α = 0.05. Also, the performance of *Citrobacter spp.* + *Azotobacter chroococcum* differed statistically from *Clostridium spp.* + *Citrobacter spp. and Corynebacterium spp.* + *Citrobacter spp.*, *p*=.028 and *p*< 0.001, respectively. In addition, *Mycobacterium spp.* + *Actinomyces spp.* performance was statistically different from *Listeria spp.* + *Actinomyces spp., Clostridium spp.* + *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp., p*=.047 and *p*<0.001, *p*< 0.001, respectively. More so, the performance of *Micrococcus spp.* + *Staphylococcus species* differed statistically from *Clostridium spp.* + *Citrobacter spp.* and *Corynebacterium spp.* + *Citrobacter spp*., *p*=.024 and *p*< 0.001, respectively. Also, there was a statistical significant difference between the performance of *Listeria spp.* + *Actinomyces spp.* and *Corynebacterium spp.* + *Citrobacter spp.*, p< 0.001 at α = 0.05. The comparison test of the performance in synergy also revealed a statistical significant different between the performance of *Clostridium spp.* + *Citrobacter spp.* and *Corynebacterium spp*. + *Citrobacter spp.*, *p*< 0.001. Furthermore, there was a statistical difference between the performance of *Corynebacterium spp.* + *Citrobacter spp.* and those of *Micrococcus spp.* + *Citrobacter spp. and Clostridium spp.* + *Pseudomonas spp., p*< 0.001 at α = 0.05. The performances of the bacteria in synergy signifies the in inherent variations in the abilities of the different combined bacteria in the bioaugmentation process. Most of these isolates had lower residual oil when they worked individually. Therefore, they may have worked antagonistically with each other in their microcosms. Whereas, Micrococcus spp. + *Shigella species* had a lower residual oil as they worked in synergy than working as a singular entity.

After 42-days autochthonous bioaugmentation, the multiple comparison test results showed that the performance in synergy of *Enterococcus spp.* + *Corynebacterium spp.* was significantly different from *Mycobacterium spp.*+ *Actinomyces spp.*, *Clostridium spp.*+ *Citrobacter spp.*, *Corynebacterium spp.* + *Citrobacter spp., Micrococcus spp.*+ *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp.*, *p*<0.05. Also, there was a statistical significant difference between the performance of *Citrobacter spp.* + *Azotobacter chroococcum* in synergy and those of *Mycobacterium spp.*+ *Actinomyces spp.*, *Clostridium spp.* + *Citrobacter spp.*, *Corynebacterium spp.* + *Citrobacter spp.*, *Micrococcus spp.* + *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp.*, p<0.01. More so, *Mycobacterium spp.* + *Actinomyces spp. showed a statistically significant difference between their performance and those of Micrococcus spp.* + *Staphylococcus species*, *Listeria spp.* + *Actinomyces spp.*, *Micrococcus spp.*+ *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp.*, p<0.01. In addition, the performance of *Micrococcus spp.* + *Staphylococcus species* in synergy, differed significantly from the performances of *Clostridium spp.* + *Citrobacter spp.*, *Corynebacterium spp.* + *Citrobacter spp.*, *Micrococcus spp.*+ *Citrobacter spp.*, p<0.001

Performance of each mixed bacteria: The following combination of microorganisms: *Citrobacter spp.* + Azotobacter chroococcum, *Clostridium spp.* + *Salmonella species*, *Micrococcus spp.* + *Staphylococcus species*, *Clostridium spp.* + *Citrobacter spp.*, *Corynebacterium spp.* + *Citrobacter spp.*, *Micrococcus spp.* + *Citrobacter spp.* and *Clostridium spp.* + *Pseudomonas spp.* used for bioaugmentation, showed progressive and significant reduction in the amount of residual oil extracted from their microcosms following autochthonous bio-augmentation in synergy. Their performance increased as the days of incubation period progressed. Therefore, these microbes in synergy were able to use the oil contaminant as sole carbon source from the beginning of incubation and continued to the 42 days. The residual oil extraction from *Listeria*

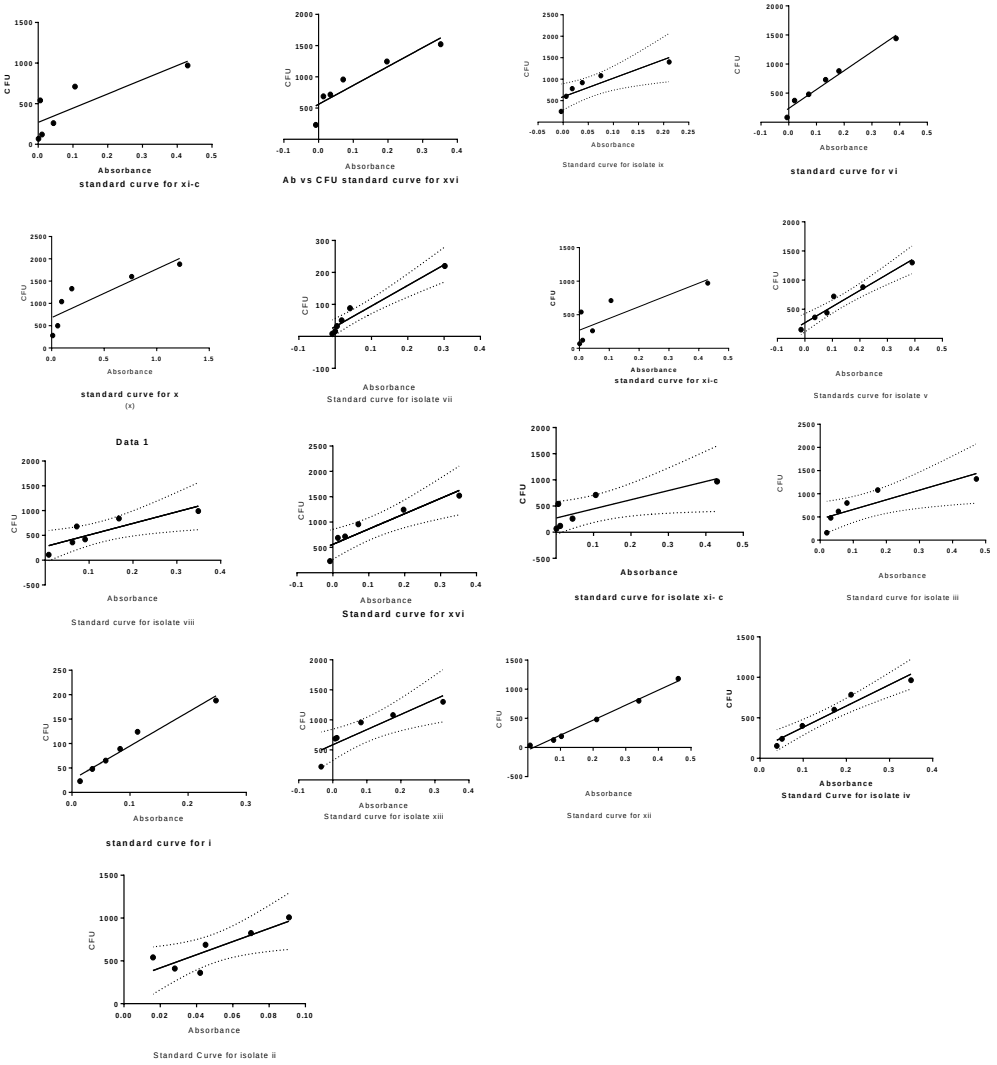

Fig. 3. The absorbance and corresponding CFU of all the isolated microorganisms

spp. + *Actinomyces spp.* microcosms after 28 and 42-day incubation period were significant lower than that extracted after 14 days of bio-augmentation while the oil extracted after 28 and 42 days were not significantly different from each other. This means that the two microbe performed better in the degradation of the spent oil as the days of incubation increased. More so, in the microcosms bioaugmented with *Enterococcus spp.* + *Micrococcus spp.*, the residual oil extracted after 42 days of incubation was significantly lower than that extracted after 14 and 28 days, though the extracted oil after 14 and 28 days did not differ significantly with each other. Notwithstanding, the quantities of residual oil extracted from the microcosms containing individual microorganisms were significantly a lower compared to their performance in synergy. It seems they acted antagonistically when in synergy and as such, individual application of these microbes in autochthonous bioaugmentation was preferred to bioaugmentation in synergy. The control only made significant reduction of the residual oil after 42 days with p=.0066, implying that for there to be any significant degradation and reduction in the oil contaminant, adequate time must be given, of at least 42 days with improved environmental conditions in place.

Comparison of autochthonous bioaugmentation in synergy with control microcosms: After the 14 days bio-augmentation with the isolates in synergy, the performance of the microbes were found to be significantly better than that of the control experiment with p values< 0.001 each at 0.05

significant level. However, the performance of *Micrococcus spp. + Shigella species* was not significantly different from that of the control at the end of 14 days of bio-augmentation and as such performed as poorly as the control. This implies that the performance of these two microbes in synergy after 14 days of incubation seems as though no microorganisms were added and as such may be that time was required for both to make significant effort in using the spent oil as their sole source of food, energy and carbon. The control had the significantly highest residual oil of 0.057525±0.0003 g/g after 28 days of bio-augmentation, showing that natural attenuation prolongs biodegradation of oil contaminant in soil [21]. After Furthermore, after 42 days of bio-augmentation, all the isolates in synergy performed significantly better than the control in reducing the quantities of residual oil obtained with p values< 0.001 each at 0.05 level of significant, indicating the importance of the autochthonous bioaugmentation for the degradation of spent oil in soils.

Prior to the bio-augmentation process, standard curves were prepared and the concentration of the introduced isolate interpolated from the curve. Fig. 3 show the absorbance and corresponding CFU of all the isolated microorganisms

From the results, the gram positive rod bacteria with the highest colony-forming unit, 937.23 CFU/ml, was *Shigella species*; seconded by *Citrobacter spp.* with 905.5 CFU/ml and thirdly by *Listeria spp.* with 827.05 CFU/ml. Among the gram positive cocci, *Staphylococcus species* had the highest colony-forming unit of 1457.63 CFU/ml, followed by *Salmonella species* with 922.55 CFU/ml and thirdly by *Azomonas spp.* with 766.11 CFU/ml. The gram negative cocci with the highest colony-forming unit was *Azotobacter chroococcum* with 793.59 CFU/ml, followed by *Neisseria spp.* with 111.43 CFU/ml. Of all the entire bacteria, *Staphylococcus species* had the highest colony-forming unit 1457.63 CFU/ml but did not differ statistically with the colony-forming units of *Salmonella species* and *Enterococcus spp*. *Shigella species* had the second highest colony-forming unit of 937.23 CFU/ml but was not significantly different from the colony-forming unit of *Azomonas spp.* Whereas, *Citrobacter spp.,* with 905.5 CFU/ml had the third highest CFU. The least CFU was *Bacillus species* with 45.92 CFU/ml, followed by *mycobacterium spp.*, with 58.91 CFU/ml and thirdly by *Pseudomonas spp.* with 78.53 CFU/ml. Though Bacillus Species had the least colony-forming unit, it was the best performing bacteria isolated from the contaminated soil. It implies that it had the greatest ability to withstand and survive poisoning from the hazardous spent oil and was able to utilize the spent oil as sole source of food, carbon and energy during the autochthonous bioaugmentation period.

4. CONCLUSION

The study indicated that autochthonous bioaugmentation to enhance the degradation of spent oil in soil is both effective and efficient. The use of these inherent microorganisms in individual and mixed microcosms showed significant reduction of the residual oil better than natural attenuation. Time allowed for incubation and type of isolate whether individual or in synergy played major role in the rate of the degradation of the oil contaminant in the soil. The result from this study can be applied to old mechanic villages across Nigeria to reclaim the lands for agricultural purposes, especially in this season where food is scares. In addition, GC/MS analysis of the residual oil extracted at the end of the autochthonous bioaugmentation period should be done and DNA of the isolated bacteria analyzed.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author 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 manuscripts.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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