Biostimulation of Hydrocarbon-Utilizing Bacteria in Soil Amended with Spent Engine Oil Using *Citrullus lanatus* and *Citrus sinensis* Peels Agro-Wastes

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Abstract: Soil contamination by spent engine oil from mechanical parts of machines is a growing concern in many countries. Biostimulation of contaminated soil with agro-waste like *Citrullus lanatus* (water melon) and *Citrus sinensi* (orange) peels offers an environmentally friendly and cost-effective method for remediating the contaminated soil. In this study, biostimualtion of 200g soil contaminated with 20 ml spent engine oil was enhanced using water melon peel (WP), orange peel (OP) and water melon and orange peels compost (WP+OP). This was undertaken for a period of 56 days under room condition. The result showed that the hydrocarbon utilizing bacteria (HUB) counts obtained from the nutrient-treated sample when compared with those from the oil-amended-untreated were statistically significant ($P < 0.05$). The presence of *Micrococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp. and *Proteus* spp. were detected after amendment with watermelon and orange peels agro-wastes from the contaminated soil. The result also showed that amendment with 40g orange peels had higher percentage degradation (75%) compared to the watermelon peels (56%). The half-life (t½) of the amended soil sample treated with combined agro-waste was observed to be 8.45 days as compared to 9 days and 10.66 days observed after individual treatments with orange and watermelon peels respectively. This study has proven that water melon and orange peel samples have stimulating potential in the biodegradation of spent engine oil from the contaminated soil.
Keywords: Agro-wastes, biostimulation, contaminated soil, spent engine oil.

INTRODUCTION

Spent engine oil are sourced from automobile workshops, and industries which contains mixtures of hydraulics oil, turbine oils, processing oil and metal working fluids after servicing the machines (Olugboji and Ogunwole, 2008). The oil is often disposed into open vacant plots, farms and drainages which easily migrates into the environment and eventually pollutes both the terrestrial and aquatic ecosystem (Godleads *et al*., 2015; Anwesha *et al*., 2016). Spent engine oil causes significant change in the physical, chemical and microbiological properties of the natural soil environment (Nilanjana and Preethy, 2010). According to Omoni *et al*. (2015), spent engine oil increases bulk density, decreases water holding capacity and aeration propensity of soil in addition to decreased nitrogen, phosphorus, potassium, magnesium, calcium, sodium and increased levels of heavy metals in soils contaminated with spent oil.

The spent-engine oil has led to widespread contamination of both the aquatic and terrestrial ecological systems (Odokuma and Ikpe, 2003). Soil contamination with oil spills is the major global concern today (Adams *et al*., 2015). Additionally, the contaminant results in organic pollution to ground water which limits the water usage and causes economic loss, environmental problems, and decreases the agricultural productivity (Wang *et al*., 2008). The concern stems primarily from health risks, direct contact with the contaminants, vapors from the contaminants, and secondary contamination of water supplies within the underlying soil (Thapa *et al*., 2012).

Different amendment technologies such as solvent addition and chemical oxidation have been developed for the removal of toxic contaminants and enhancing soil aeration (Adams *et al*., 2015). However, these technologies are disadvantaged by several limitations such as high cost of operation, not environmentally favourable as they tend to destroy soil texture and other characteristics and can only temporarily neutralize the priority pollutants. Bioremediation has currently gained acceptance in cleaning up contaminated soil and water through natural biological activity (Victor *et al*., 2015).

Interestingly, biostimulation of hydrocarbon-contaminated soil can enhance the contaminants removal (Abioye *et al.*, 2009; Gladvin *et al.*, 2017). This study is aimed at evaluating two different organic wastes (biowastes); *Citrullus lanatus* (water melon) and *Citrus sinensi* (orange) peels as supplements for bioremediation stimulants.

MATERIALS AND METHODS

Samples Collection and Processing

Auger was used to collect soil samples at 15 cm depth from the Botanical Garden of Biological Science Department, Bayero University, Kano, Nigeria. The samples were collected from three different points, thoroughly mixed in a clean plastic container and sieved with 2 mm sieve before transferred into clean polyethylene bags for analysis. The water-melon (WP) and orange (OP) peels were collected from different fruit seller locations in Kano metropolis and washed thoroughly. The peels were then cut into smaller units to increase surface area for drying within 14 days, homogenized by grinding and sieved through 2 mm mesh as described by Akpoveta *et al.* (2011). Spent engine oil were aseptically collected from a road-side mechanic workshop and transported to the laboratory in a clean plastic container.

Experimental Design for Biostimulation Studies

At least 200g of Soil samples were measured in six different manson's jar (A, B, C, D, E and F) with each being amended with 20 ml of spent engine oil (SEO), mixed thoroughly and left undisturbed for two days. Thereafter, the prepared agro-wastes were added to the amended soil samples separately and in combined forms at various concentrations and mixed thoroughly. All treatment were incubated aerobically with perforated aluminum foil on top of the bottles at room temperature for 56 days (Bahadure *et al*., 2013). The treatments are shown in the table below.

Samples	Treatment
A	200g soil + 20ml SEO + 20g WP
B	200g soil + 20ml SEO + 40g WP
C	200g soil + 20ml SEO + 20g OP
D	200g soil + 20ml SEO + 40g OP
E	200g soil + 20ml SEO + 40g WP and OP at 1:1 ratio
Control (F)	200g soil + 20ml SEO

Hydrocarbon Utilizing Bacteria (HUB) Counts

The HUB counts in the agro-waste treated soil was determined by plating a serially diluted 1 g of the soil on mineral salt agar plate supplemented with oil [1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.1 g NaCl, 20 g agar, 20 ml spent engine oil in 1000 mL distilled water, pH 7.4]. Inoculated plates were incubated at 30 °C for 72 h. The colonies on each plate were counted and recorded as colony forming unit per gram of soil (CFU/g). The pure culture of the bacterial isolates was identified by Gram staining technique and API 20NE for Gram negative bacteria and BBL Crystal rapid identification kit for Gram positive bacteria.

Total Petroleum Hydrocarbon Determination

The total petroleum hydrocarbon (TPH) content of the soil samples was extracted using solvent extraction method of Adesodun and Mbagwu (2008). For each filtrate, 1ml of the extract is diluted into 50 ml *n*-hexane. The absorbance of this solution was measured with spectrophotometer (HACH DR/2010) at a wavelength of 460 nm using *n*-hexane as blank. The total petroleum hydrocarbon was determined at two weeks intervals for eight weeks. Percent degradation (D) was calculated using the following formula:

$$D = \frac{TPH_i - TPH_r}{TPH_i} \times 100$$

Where, TPH_i and TPH_r are the initial and residual TPH concentrations respectively.

Assessment of Agro-wastes Effectiveness

The effectiveness of each agro-wastes was tested, through evaluation of untreated amended soil (natural attenuation) and amended soil treated with the selected agro-wastes (biostimulation). Percentage efficiency (% B.E) was calculated at the end of the 56 days remediation period using the equation as described by Zahed *et al.* (2011):

$$\%B.E = \frac{\%TPH_{dp} - \%TPH_{dup}}{\%TPH_{dp}} \times 100$$

Where; %B.E = Percentage Biostimulant Efficiency; $\%TPH_{dp}$ is the removal of spent engine oil in the amended soil, and $\%TPH_{dup}$, the removal of spent engine oil in the untreated soil.

Bioremediation Kinetics

Kinetic analysis is a key factor for understanding biodegradation process, bioremediation speed measurement and development of efficient clean up for a hydrocarbon contaminated environment. The information on the kinetics of soil bioremediation is of great importance because it characterizes the concentration of the contaminant remaining at any time and permit prediction of the level likely to be present at some future time (Agarry, 2017). Biodegradability of hydrocarbon is usually explained by first order kinetics (Agarry *et al*., 2013). This is given as:

$C_t = C_o e^{-kt}$

Where: C_o - the initial TPH content in soil (mg/g),

C_t - the residual TPH content in soil at time t, (mg/g),

K - the biodegradation rate constant (day^{-1}),

t - time (day).

Estimation of Biodegradation Half-life Times

The biological half-life is the time taken for a substance to lose half of its amount. Biodegradation half-lives are needed for many applications such as chemical screening, environmental fate modeling and describing the transformation of pollutants (Agarry *et al*., 2015). Biodegradation half-life times (t½) can be estimated as described by (Agarry *et al*., 2013).

Half-life = ln(2)/k

Where: k – the biodegradation rate constant (day^{-1}). The half-life model is based on the assumption that the biodegradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in soil (Yeung *et al.*, 1997).

Statistical Analysis

Statistical analysis of the data was carried out using one-way ANOVA with SPSS Statistics version 20.

RESULTS

Bacterial Counts

The result of the microbial analysis showed that the hydrocarbon utilizing bacteria (HUB) increased significantly ($P< 0.05$) from the 14th day of incubation to the 56th day of incubation in amended soil samples treated with watermelon and orange peels (agro-wastes) (Figure 1).

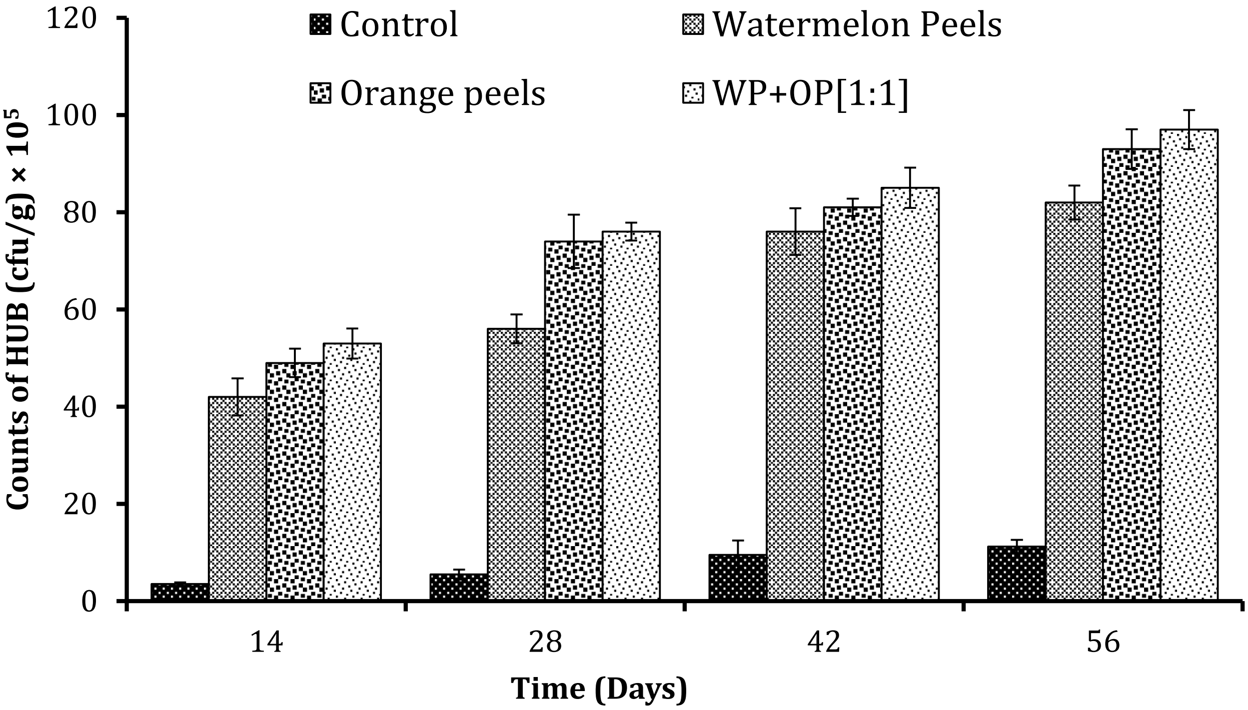

Figure 1: Counts of hydrocarbon utilizing bacteria in amended soil samples. Bars indicate standard deviation (n = 2). CFU: colony forming unit; Control (soil + spent engine oil); WP: watermelon peels, OP: orange peels.

Table 1 shows the identified bacterial isolates in the soil samples before and after it amendment with spent engine oil. Eight bacteria isolates; *Micrococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Corynebacterium* spp., *Nocardia* spp., *Achromobacter* spp. and *Proteus* spp. were identified and isolated in the unamended soil samples. In contrast, five bacterial isolates; *Micrococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp. and *Proteus* spp. were identified and isolated after amendment and treatment with watermelon and orange peels agro-wastes indicating five bacterial isolates had the potential to utilize spent engine oil in the amended soil sample.

Table 1: Bacterial isolates identified in soil before and after amendment with spent engine oil and agro-waste during the bioremediation experiment

Bacteria genera	Soil sample before amendment	Soil sample after amendment	
		Watermelon Peels (WP)	Orange Peels (OP)
Micrococcus spp.	+	+	+
Pseudomonas spp.	+	+	+
Bacillus spp.	+	+	+
Corynebacterium spp.	+	-	-
Nocardia spp.	+	-	-
Achromobacter spp.	+	-	-
Klebsiella spp.	-	+	+
Proteus spp.	+	+	+

Positive (+), seen; negative (-), not seen.

Total Petroleum Hydrocarbon (TPH) Content

Table 2 shows the TPH content of the amended soil. There was linear decrease in TPH with increase in incubation period at the various concentrations of the agro-waste. The TPH of the amended soil treated with 20g watermelon peels decreased from 8409.55±15.87 mg/g to 646.15±78.84 mg/g, a decrease from 8427.25±38.40 mg/g to 313.70±17.25 mg/g was observed in treatment with 20g orange peels. The result revealed the TPH content after the 56 days treatment period using agro-waste at 40g concentrations to be in the order control (7061.55± 59.18 mg/g) > watermelon peels (440.43±13.15 mg/g) > orange peels (263.20±62.93 mg/g). The result of the combined agro-wastes (watermelon peels + orange peels) (1:1) treatment showed lower TPH than the 20g and 40g treatment with individual agro-waste.

Table 2: Total Petroleum Hydrocarbon (TPH) recovered from the amended soil during the period of study

Conc.	Sampling Time (Day)	Total Petroleum Hydrocarbon (mg/g) ± SD					
		WP	**ln(C) TPH**	**OP**	**ln(C) TPH**	**Control**	**ln(C) TPH**
20g	0	8409.55±15.87	9.04	8427.25±38.40	9.04	8454.25±30.48	9.04
	14	6072.90±50.35	8.71	5668.00±47.38	8.64	8157.70±55.15	9.01
	28	4249.70±65.20	8.35	3553.80±68.02	8.18	7866.95±50.13	8.97
	42	1195.15±20.15	7.09	973.60±18.67	6.88	7552.65±83.79	8.93
	56	646.15±78.84	6.47	313.70±17.25	5.75	7061.55±59.18	8.86
40g	0	8400.50±41.30	9.04	8361.10±65.48	9.03	8454.25±30.48	9.04
	14	6044.55±52.11	8.71	5271.70±76.37	8.57	8157.70±55.15	9.01
	28	4008.45±14.07	8.30	3162.05±81.25	8.06	7866.95±50.13	8.97
	42	932.70±80.75	6.84	882.50±14.28	6.78	7552.65±83.79	8.93
	56	440.43±13.15	6.09	263.20±62.93	5.57	7061.55±59.18	8.86
		Combination (Watermelon + Orange peels) (1:1)					
		Treatment	**ln(C) TPH**	**Control**	**ln(C) TPH**		
	0	7985.05±35.85	8.99	8454.25±30.48	9.04		
40g (1:1)	14	4265.30±81.32	8.36	8157.70±55.15	9.01		
	28	2059.15±54.38	7.63	7866.95±50.13	8.97		
	42	473.60±18.53	6.16	7552.65±83.79	8.93		
	56	74.75±32.74	4.31	7061.55±59.18	8.86		

Keys: ln(C): Natural logarithm of the concentration, WP: Watermelon peels, OP: Orange peels, TPH: Total petroleum hydrocarbon

Percentage Degradation and Agro-waste Efficiency

Figure 2 shows the percentage biodegradation of spent engine oil in soil throughout the period of study. The control sample had the lowest percentage biodegradation (19%) of spent engine oil compared to amended samples treated with 20g and 40g agro-wastes. At the end of 56 days, spent engine oil amended soil treated with 40g orange peels (OP) showed the highest percentage of oil biodegradation with 75%. The percentage biodegrading potential and efficiency of combined agro-waste treatment were 89% and 55% respectively at the end of the 56 days incubation period (Figure 3).

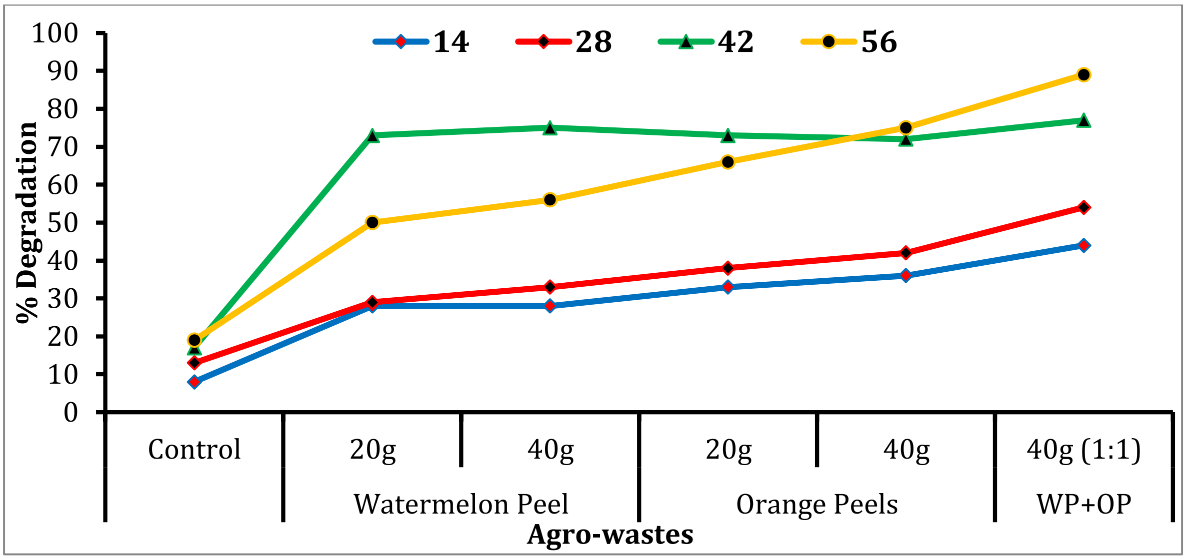

Figure 2: Percentage biodegradation of spent engine oil in soil
Keys: WP: Watermelon peels, OP: Orange peels

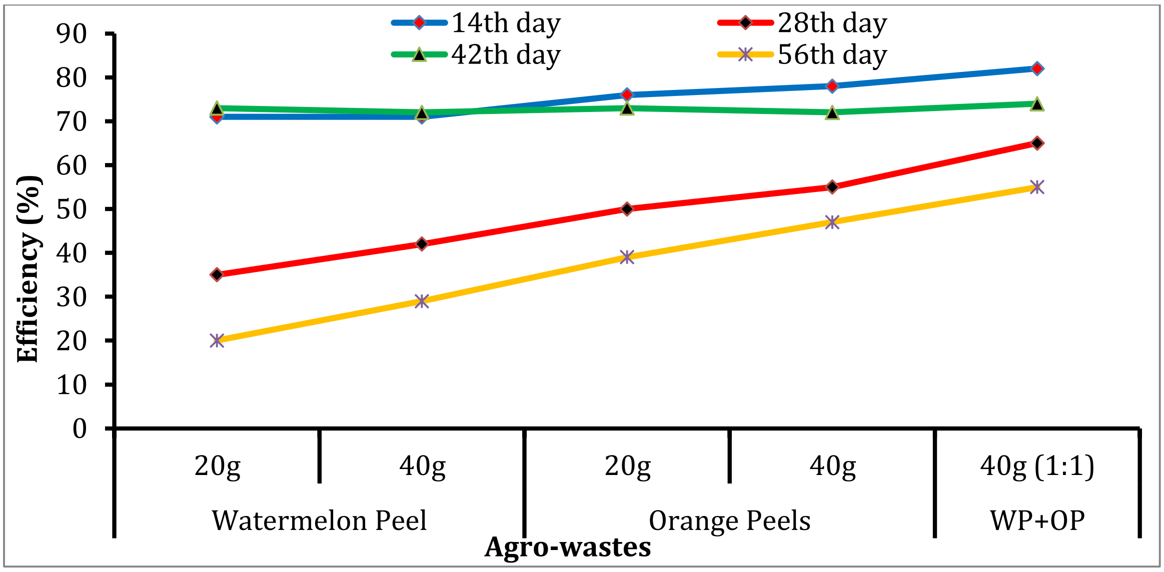

Figure 3: Percentage Agro-waste Efficiency
Keys: WP: Watermelon peels, OP: Orange peels

Biodegradation Rate Constant and Half-life of Spent Engine Oil in Soil

Table 3 shows the biodegradation rate constant and half-life of hydrocarbon amended soil treated with agro-wastes. The results obtained from the study fitted with the first order kinetic model used to determine the rate of biodegradation showed that the biodegradation constant (*k*) of amended soil samples treated with 20g watermelon and 20g orange peels were 0.058 and 0.069 day^{-1} respectively. The half-life of the spend engine oil in the soil samples was in the order, control > A > B > C > D > E.

Table 3: Biodegradation rate constant and half-life of hydrocarbon amended soil treated with agro-wastes

Samples	Biodegradation constant (k) day $^{-1}$	Half-life (days) t½	Coefficients of determination (r^2)
A	0.058	11.95	0.952
B	0.065	10.66	0.961
C	0.048	10.05	0.962
D	0.077	9.00	0.972
E	0.082	8.45	0.963
F (control)	0.018	38.51	0.915

Keys: WP: Watermelon peels, OP: Orange peels.

DISCUSSION

The microbial population of hydrocarbon utilizing bacteria (HUB) in the control soil sample was significantly low compared to those of the agro-wastes amended soil samples. In similar studies, Okolo *et al.* (2005), Akpoveta *et al*. (2011) observed that crude oil contaminated soil also supported rapid bacteria growth because the crude oil served as carbon and energy source for them. According to Victor *et al*. (2015), the reason for the higher counts of the bacteria in amended soil may be the result of the presence of appreciable quantities of nitrogen and phosphorus in the agro-wastes. The HUB isolated from the spent-engine oil amended soil were identified as species of *Micrococcus*, *Pseudomonas*, *Bacillus*, *Klebsiella* and *Proteus* spp. Previous studies (Van Hamme *et al*., 2003; Bento *et al*., 2005; Adeline *et al*., 2009; Agbor *et al.*, 2012) have implicated these bacterial species in hydrocarbon degradation. Finding of the present study showed that the amendments increased the hydrocarbon utilizing bacteria population of the amended soil. Agbor *et al.* (2012) observed that the use of agro-wastes effectively stimulated bacterial growth into utilization of spent-engine oil.

The present finding showed that contaminated soil samples amended with orange peels had significant ($P < 0.05$) higher microbial growth than contaminated soil samples amended with watermelon peels. However, the combination of watermelon and orange peels showed higher microbial counts than the single amendments. This is an indication that the combination of these amendments has stronger bio-utilization potentials because of the presence of acclimated microorganisms found in the single amendments that can significantly affect the degradation rate of the contaminants (Olabisi *et al*., 2009; Omoni *et al*., 2015). Sexton and Atlas (1997) and Agbor *et al.* (2012) reported that a combination of amendments in the right proportions would be effective in degradation of crude oil in amended soil. This is thus an indication that the higher the concentration of these agro-wastes the higher the microbial population in the soil. The result of this study shows that for a speedy degradation rate of crude oil cleanup in the soil the combination of agro-wastes would be a better option in increasing the population of crude oil utilizing bacteria in any contaminated soil.

The increased in microbial population in 14th day may be explained by the fact that when the spent engine oil and amendments were newly applied to the soil, after the initial toxicity of the oil to the soil microorganisms, they became adapted to the new environment and utilize the spent engine oil as their carbon source resulting to the increase in population (Ekanem and Ogunjobi, 2017). The adaptability of the microorganism to the polluted soil and the essential nutrient (nitrogen and phosphorus) provided from the agro-wastes led to a rapid increase in microbial population of heterotrophic and engine oil utilizing bacteria in 42 days. Sampling at 56 days recorded a significant increased ($P < 0.05$) in microbial counts, thus this was the period with the highest hydrocarbon utilizing bacteria counts (Victor *et al*., 2015). The present finding showed remarkable reduction in total petroleum hydrocarbon during the studied period in soil treated with agro-wastes (watermelon and orange peels). At the end of 56 days, amended soil treated with 20g agro-waste showed a significant reduction in TPH content with remnant of 646.15± 78.84, 313.70±17.25 and 7061.55±59.18 mg/g from the initial TPH content of 8409.55±15.87, 8427.25±38.40 and 8454.25±30.48 mg/g for the amended soil treated with watermelon peels, orange peels and untreated soil sample (control) respectively. The results clearly showed that natural attenuation occurs in the control at day 56 due to about 17% removal of TPH as a result of abiotic factors. This process tends to be slow, time consuming and depend on the nature of the soil. The results of statistical analysis showed that there was significance difference in all the treatment options. It can be stated that the high loss of petroleum hydrocarbon in unamended soil could be as a result of natural attenuation such as physical and chemical processes, such as dispersion, dilution, sorption, volatilization, and abiotic transformations (USEPA, 1999).

Similar to the present finding, Abioye *et al.* (2012) reported that the rate of biodegradation of spent engine oil polluted soil during bioremediation increased linearly with increase in incubation period. In this study the highest percentage biodegradation was observed at day 56 in soil treated with combined watermelon and orange peels (89%). It is noteworthy that the two agro-wastes were effective in biostimulating the hydrocarbons utilizers that subsequently led to a reduction in petroleum hydrocarbon in the soil polluted with spent engine oil. The result from the study revealed higher biostimulation efficiency (BE) in 40g orange peels treatment (71%) than that of 40g watermelon peels treatment (63%). However, the observed reduction in petroleum hydrocarbons in spent oil may not only be due to the biodegradation process induced by nutrient additions, but other processes such as volatilization, adsorption to organic compounds, other abiotic factors are equally implicated in the reduction (Ekpo *et al.*, 2012; Onuoha, 2013).

Finding of the present study showed higher biodegradation constant and lower half-life for agro-waste treated soil compared to the control. Since oil degradation is a natural process limited by temperature, pH, and scarcity of nutrients such as nitrogen and phosphorus (Leahy and Colwell, 1990; Ladousse and Tramier, 1991; Pathak *et al*., 2015), the higher rate of hydrocarbon reduction reported in this study with the addition of watermelon and orange peels could be due to bioavailability of the nutrients in these organic wastes to bacterial species in the oil polluted soil (Chukwudozie, 2013). In agreement to the present finding, Victor *et al*. (2015) reported that the biodegradation rate constants of oil contaminated soil samples amended with agro-waste increased compared to the unamended (control) soil samples.

CONCLUSION

The agro-wastes (*Citrullus lanatus* and *Citrus sinensis*) considered in this study have effective potential in the remediation of soil polluted with spent engine oil. *Micrococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp. and *Proteus* spp. bacterial isolates were identified after amendment and treatment of polluted soil with watermelon and orange peels agro-wastes. The highest percentage biodegradation was observed at day 56 in soil treated with combined watermelon and orange peels (89%). Individual addition of orange peel to the contaminated soil accounted for 75% degradation compared to 56% degradation by watermelon peels in the contaminated soil. The study therefore proves the viability of using *Citrullus lanatus* (water melon) and *Citrus sinensi* (orange) peels amendment in remediating hydrocarbon-contaminated soil. This affords an alternative method in removing oil contaminants from soil while promoting the use of agro-wastes like water melon and orange peels for biodegradation.

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