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Bacterization of Biostimulant (Brewers Spent Grains) on Hydrocarbon Degradation of Crude Oil Contaminated Garden Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author USI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BMP and USI managed the analyses of the study. Author UMP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The influence of bio-stimulants (Brewers Spent Grains - BSG) on hydrocarbon degradation of bacteria used in the remediation of oil-contaminated soil was evaluated using standard microbiological techniques. The result revealed that the test soil contained 2.0 $x10^7$cfu/g total heterotrophic bacteria counts (THBC); of which 4.0 $x10^2$ cfu/g were oil degrading bacteria (ODB). The values of ODB/THBC ratio recorded for soil sample was less than one; and indication with a system with low level of hydrocarbon pollution. However, results obtained when microbially *(B. subtilis*) -augmented bio-stimulants were used in a Bacterization-Biostimulation (BB) remediation protocol revealed enhanced degradation of crude oil and it components. Viable cell measurement showed that the higher the biostimulants/contaminant (BC) - ratio employed the more the heterotrophic activity but less hydrocarbonoclastic activity. However, results have shown that soil contamination with crude oil drastically reduced the population of denitrifying bacteria but increased the population of oil degrading bacteria in soil but has concentration-dependent effects on the densities of heterotrophic bacteria. For soils remedied for 8 weeks with bacterized - BSG, the degradation rates were remarkably high and near 100% as against 44.02% recorded for the control

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(treatment with spent grains alone). This shows that biostimulation was better when "cropped" with oil degrading bacterium. The best degradation (99.09%) was achieved when 1% of BSG was applied (2.08%) at a BC ratio of 0.48: 1 which induced oil degraders' growth rate and generation time of 0.00077 and 898.83 h^{-1} in 8 weeks respectively. Beyond these ratios the treatments created "diauxic influence", retarding the growth and activities of hydrocarbonoclastic bacteria while heterotrophic bacteria proliferate. The study revealed that augmenting biostimulants with strong strains of hydrocarbons degrading bacteria would stimulate the activities of indigenous degraders and ensure a hasten natural attenuation process in contaminated ecosystems. Longer incubation would certainly have led to complete or higher hydrocarbon degradation when hydrocarbonoclastic degradation enters the second log phase. Enhanced remediation with brewer's spent grains using BB protocol is strongly recommended but will be sustainable if the organic amendment stabilized with a fibre-rich carrier.

Keywords: Bacterization; biostimulant; brewers spent grains; hydrocarbon and degradation.

1. INTRODUCTION

The oil industry in Nigeria has contributed immensely to changing the state of the country's economy and environment. The petroleum sector though has increasingly provided the bulk of current government revenue, on which economic growth largely depends; their operations have impacted adversely on the environment. Crude oil exploration and exploitation of its refined products in Nigeria has resulted in massive degradation and deterioration of soil, water, sediment and the atmosphere due to pollution. Hydrocarbons are amongst the known components of crude oil that is of much concern during spills.

The tremendous increase in the production, refining and distribution of crude oil and other petroleum products are accompanied by increasing problem of environmental pollution. A major part of this problem results from the fact that massive movements of petroleum have to be made from area of high production to those of high consumption [1]. Even small releases of petroleum hydrocarbons in the acquifers can lead to concentrations of dissolved hydrocarbon far in excess of regulatory limits [2]. Crude oil pollution often results in serious effects on both the biotic and abiotic components of the ecosystem. Also, some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organopollutants [3].

The recalcitrant nature, ease of biomagnification along food chain and health risk of petroleum hydrocarbons are attributed to its hydrophobicity, lipophilic nature and low water solubility [4,5]. This is posing great threats and danger to environmental and public health [4] resulting in biodiversity losses especially in the Niger Delta region. These concerns have led to the development of various remediation technologies including bioremediation – which mainly depends on microorganisms to degrade, transform, detoxify or breakdown the contaminant.

Bioremediation is the optimization of natural biodegradation in which organisms chemically alter and breakdown organic molecules into other substances such as carbon dioxide, fatty acids and water in order to obtain energy and nutrients. Biostimulation and bioaugmentation are the two approaches to bioremediation. Biostimulation involves the addition of adequate microbial nutrient to the polluted site to increase nutrients and microbial activities of indigenous microbial flora while bioaugmentation involves the external microbial population to the polluted site.

Various substances and materials when applied to plants or growing substrates demonstrate capacity to modify the physiology of plants, promoting their growth and enhancing their growth response. Their action is different from that of nutrients and plant pesticides thus the term bio-stimulant has been coined to describe the function. Bio-stimulant can be defined as organic substances and material, that when applied in small quantities enhance plants growth and development that such response cannot be attributed to application of the traditional nutrients [6]. Biostimulants have been shown to influence several metabolic processes such as respiration, photosynthesis, nucleic acid synthesis and ion uptake. They are not fertilizers meant to correct a severe nutrients deficiency but they are mixtures of one or more things such as microorganisms, trace elements, enzymes/ plants hormones and seaweeds extracts. They contain substances and or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance nutrient uptake, nutrient

efficiency, tolerance to abiotic and biotic stress biostimulants have no direct action against pest therefore do not fall with the regulatory frame work of pesticides [7].

In nature, microbial cells are confronted with a wide range of environmental conditions that change over time. These conditions often elicit specific metabolic responses that increase the division rate of a cell. For example, when bacterial cells are exposed to multiple sugars, they do not metabolize all sugars simultaneously, but rather use the sugar that allows the highest cell-division rate. Cells switch to the less-preferred sugar when the most-preferred one (in many cases glucose) is depleted. The word "diauxie" was coined by Jacques Monod [8,9]. This phenomenon is characterized by two exponential growth separated by lag phase called diauxic lag [10]. The most well-known example of diauxie is the growths of *Escherichia coli* on mixture of glucose and lactose. The two exponential phases reflect the sequential consumption of glucose and lactose. Moreover, only glucose is consumed in the first exponential phase because synthesis of the peripheral enzyme is somehow abolished in the presence of glucose. These enzymes include lactose permease, β-galactosidase and lactose transacetylase. The diauxic lag reflects the time to buildup these enzymes to sufficient levels. After the lag phase, the second exponential phase corresponding to the lactose consumption is observed.

Recent studies have shown that hydrocarbons degrading microorganisms are widely distributed in contaminated ecosystems [11] and may adapt to interact with heterogeneous materials which serve as primary environmental sorbent for PAHs and hydrophobic hydrocarbons in ways that facilitate these pollutants and their subsequent metabolism [12]. Most studies carried out on bioremediation of soils impacted in the Niger Delta of Nigeria are majorly on enhanced remediation using biostimulation and bioaugmentation protocols. A combination of both protocols (Biostimulation - Bioaugmentation [BB] protocol) has not been attempted. This study is focused on the bacterization of biostimulants and its diauxic influence on the degradation of hydrocarbons in garden soil. This would be achieved by isolating crude oil degrading bacteria from humic ecosystem using the enrichment culture technique, determining the microbiological, physico-chemical properties of test soil and bio-stimulants (Brewers spent grains), and analyzing the residual load of hydrocarbons in remediated soils.

2. MATERIALS AND METHODS

2.1 Sources of Materials

2.1.1 Biostimulating agents

The spent grains were obtained from Champions Breweries, Uyo Akwa Ibom State, Nigeria. The spent grains were air dried and stored at room temperature, while Bonny light crude oil was obtained from Oil Company operating in the Niger Delta Region of Nigeria.

2.1.2 Crude oil degrading bacteria

Bacteria with potentials to effectively degrade crude oil were isolated from the humic collected from Eniong River in Itu LGA of Akwa Ibom State, Nigeria.

2.1.3 Physicochemical analysis of garden soil and spent grain samples

Physicochemical parameters of the garden soil and spent grain samples were determined using standard analytical procedures recommended in `1998 by APHA

2.2 Microbiological Analysis

Standard microbiological techniques were employed in this study.

2.2.1 Determination of microbiological loads of the garden soil and bio-stimulants

2.2.1.1 Serial dilution

This was done according to the method of Collins and Lyne [13]. Ten grams of the samples was measured and introduced into beaker containing 90ml of distilled water. It was shaken for even distribution; 1ml of the aliquot was aseptically transferred into sterile test tube containing 9ml of diluents to give a dilution of 10^{-1} (10-fold dilution). Further 10-fold serial dilution was carried out up to factor 10^{-9} dilution factor.

2.2.1.2 Analytical media

Nutrient Agar (NA) was used to determine total heterotrophic bacterial counts (THBC),

Ammonium Carbonate Medium was used to determine *Nitrosomonas* counts, Nitrite carbonate Medium was used to determine *Nitrobacter* counts, Sabouraud Dextrose Agar was used to determine total Fungal counts while Mineral salt agar supplemented with crude oil was used to isolate hydrocarbon utilizing bacteria. The media were prepared according to manufacturer's instructions and sterilized by autoclaving at 121°C at 15 minutes.

2.2.1.3 Enumeration of microbial densities

The density of the total heterotrophic bacteria (THBC) in the biostimulants (Brewer's spent grain) and garden soil was assessed using the pour plate technique [13] while the population of oil degrading bacteria (ODB) was estimated by the vapour phase transfer technique as described by Okpokwasili and Amanchukwu [14]. The inoculation of selected media was done with the desired diluent.

For THBC, Nutrients agar a general purpose medium was used. The density was estimated by the pour plate technique of Collins and Lynes [13]. The inoculated plates were incubated inverted at room temperature for 24 hours and colonies formed were counted and expressed as cfu/g of the sample. On the other hand the density of oil degrading bacteria in the soil and biostimulant was measured by vapour phase technique.

2.2.2 Isolation of crude oil degrading bacteria from humic sediment

2.2.2.1 Enrichment and isolation of bacterial isolates

The enrichment culture technique was employed. Presicely 1 g of humic sediment sample from Eniong River was inoculated into three sets of conical flask containing 50 ml of sterile Mineral Salt Medium [K_2HPO_4 – 6g, NaCl – 12g, KH_2PO_4 – 6g, $(NH_4)_2SO_4$ – 6g, $MgSO_4.7H_2O$ – 2.6g, $CaCl_2.2H_2O$ – 0.16g, per liter (pH 7.0 ±0.2)] (MSM) enriched with 1% crude oil as carbon source. The medium was incubated at 28°C in shaker incubator (100 rpm) for 7 days. After 7 days of incubation, the samples were serially diluted using sterile water and plated on Nutrient Agar (NA) to obtain viable cells of bacteria. Discrete colonies obtained were sub-cultured using streak method as described by Cheesbrough [15] to obtain pure cultures.

2.2.2.2 Screening for crude oil utilizing potential of the bacterial isolates

Crude oil utilizing potential of the bacteria isolates was determined using the hydrocarbon overlay method. Precisely 15 g of agar-agar was added to mineral salt medium, sterilized and allowed to set. The solidified plates were overlaid with 1% (v/v) sterile crude oil, allowed for about 15 to 30 minutes then the test isolates were streaked on the surface of the plate.

All inoculated plates were incubated at room temperature for 5-15 days with periodic observation. Colonies that eventually developed showing area of clearing were selected and rated. The utilization was rated based on the diameter and luxurious nature of the developed colonies, i.e., '+', '++' or '+++' indicating the magnitude of the oil degrading potentials as described by Ekundayo and Obire [16].

2.2.3 Characterization of bacterial isolate

The best crude oil utilizing bacterial isolates were characterized based on their cultural and morphological attributes as well as their responses to standard biochemical test as described by Cheesbrough [15].

2.2.4 *Ex situ* analysis of the influence organic amendments (biostimulants) on the remediation of crude oil contaminated soil

The garden soil was carefully and manually sorted to remove debris. Thereafter 4 kg of the soil contained in perforated 30 x 30 cm wooden boxes was contaminated with different concentrations of crude oil. Graded concentrations (1, 5, 10, 15 and 20%) of the biostimulants was bacterized with 20ml broth culture of the oil degrading bacterium (isolated from humic fresh water sediments) and then used to amend the contaminated soil. Un-bacterized but contaminated soil served as control.

The influence of the organic amendment on the physicochemical parameters of the amended and un-amended soil was assessed at

the end of the degradation course (2 months after treatment) while microbial properties of the test soils and rate of crude oil biodegradation was carried out for a period of two months at 7 days interval (ie 0, 7th, 14th, 21st, 28th, 35th, 42nd, 49th and 56th day after amendment).

2.3 Assessing the Influence of Biostimulants on the Physico-chemistry of Amended Soil

Standard analytical techniques were employed to determine the changes in the physicochemistry of the amended soil. The total hydrocarbon content of the amended soil was measured at 460 nm after extraction with 50 ml hexane with the aid of Mamotte 701 Spectrophtometer using n-hexane as blank [17]. On the other hand the residual hydrocarbons loads (hydrocarbon fractions) remaining after degradation in the treated soil were determined using the methods of ASTDM 3921 and UDEPA 8270B [18]. The total residual petroleum hydrocarbons (TRPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted from the soil samples and quantified using Gas chromatograph HP5890.

2.4 Data Analysis

The data collected were subjected to correlation matrix analysis to establish relationships between the microbial groups.

3. RESULTS

3.1 Physicochemical Properties of Untreated Soil

The physical and chemical properties of the garden soil used for the remediation study are as presented in Table 1. The results have revealed an acidic soil (pH of 5.05±0.18) characterized with low levels of conductivity (45.7±0.34 µS/cm) and total organic carbon content (2.46±0.02%). The total nitrogen content of 0.39±0.01% and cation levels of 2.76±0.41 mg/kg, 0.98±0.01 mg/kg, 1.54±0.31 mg/kg and 0.86±0.03 mg/kg recorded for calcium, potassium, magnesium and sodium respectively revealed a comparatively fertile ultisol with a sandy loam texture and relatively low petroleum hydrocarbons content (7.13 mg/kg).

3.2 Chemical Properties of the Bio-stimulants (Brewer's Spent Grains)

Table 2 shows the chemical properties of the bio-stimulants used for the remediation study. The spent grain shows moisture content of 4.4±0.17%. The caloric values (Kcal) estimated for the bio-stimulants was 399.39±0.35% The spent grain had a concentration of 1.12±0.12 mg/kg, 1.36±0.07 mg/kg, 35.45±0.50 mg/kg recorded for sulphate, nitrate and chloride respectively but lower concentration of phosphate (0.21±0.04). Slightly higher concentrations of 76.0±0.84 and 2.33±0.05 were recorded for total organic carbon and nitrogen respectively in spent grains.

Table 1. Physicochemical properties of test soil

Parameter	Samples			Mean±SD
	1	2	3	
pH	5.00	5.25	4.9	5.05±0.18
Conductivity (µS/cm)	45.3	45.9	45.9	45.7±0.34
Redox Potential (mV)	100.0	101.5	101.5	101.1±0.87
Moisture (%)	1.45	1.40	1.41	1.42±0.03
Total organic carbon(mg/kg)	2.47	2.44	2.47	2.46±0.02
Calcium (mg/kg)	2.29	2.98	3.01	2.76±0.41
Potassium (mg/kg)	0.98	0.99	0.98	0.98±0.01
Magnesium (mg/kg)	1.36	1.90	1.36	1.54±0.31
Total Nitrogen (%)	0.39	0.40	0.38	0.39±0.01
Sodium (mg/kg)	0.83	0.86	0.89	0.86±0.03
Exchangeable Acidity(Cmol/kg	1.09	1.18	1.00	1.09±0.09
TPH (mg/kg)	8.89	4.66	7.84	7.13
PAH (mg/kg)	0.57	0.57	0.57	0.57
Sand	63.40	66.20	63.60	64.4±1.56
Slit	19.14	19.16	19.00	19.10±0.09
Clay	16.03	16.55	16.92	16.5±0.44
Textural class				Sandy loam soil

Source: Own research

Table 2. Chemical properties of biostimulants (brewer's spent grains) used in the remediation of contaminated soils

Parameter	Spent grain			Mean±SD
	1	2	3	
Sulphates (mg/kg)	1.12	1.00	1.24	1.12±0.12
Phosphate (mg/kg)	0.18	0.25	0.20	0.21±0.04
Nitrate (mg/kg)	1.43	1.35	1.30	1.36±0.07
Chloride (mg/kg)	35.04	35.31	36.0	35.45±0.50
Moisture (%)	4.3	4.3	4.6	4.4±0.17
Ash (%)	3.7	3.5	3.3	3.5±0.20
Fibre (%)	2.2	2.4	2.6	2.4±0.20
Protein (%)	27.9	27.4	27.2	27.5±0.36
Lipid (%)	4.9	5.0	5.1	5.0±0.10
CHO (%)	61.8	61.8	62.4	62.0±0.35
Caloric value (kcal)	399.6	399.6	399.0	399.4±0.35
PAH (mg/kg)	1.05	1.05	1.05	1.05
TPH (ppm)	3754.43	3754.43	3754.43	3754.43
pH	5.1	5.2	5.0	5.1±0.1
Total organic carbon (%)	75.85	76.90	75.25	76.0±0.84
Nitrogen	2.28	2.37	2.34	2.33±0.05

Key: CHO = carbohydrates, PAH = Polycyclic aromatic hydrocarbon, TPH = total petroleum hydrocarbons
Source: Own Research

3.3 Microbiological Properties of Test Soil, Biostimulant and Humic Sediment

The results presented in Table 3 revealed the microbiological properties recorded for the garden soil and organic amendment (Brewer's spent grains) used for the enhanced remediation study. The results have revealed the rich microbial assemblage and diversity of the garden (test) soil used. The result in Table 4 revealed a rich bacteriological diversity in soil than in organic biostimulant.

Table 3. Microbiological properties of test soil and biostimulants

Microbial group	Soil	Spent grain	Sediments
Heterotrophic bacterial counts	2.0×10^7	2.67×10^4	2.7×10^5
Hydrocarbonoclastic bacterial counts	4.0×10^2	2.1×10^1	2.0×10^2
Nitrosomonas counts	3.3×10^3	-	ND
Nitrobacater counts	1.7×10^3	-	ND
Fungal Loads	1.3×10^5	5.5×10^4	ND

Values are mean of three determinations; Key: - = not detected; ND = not done; Source: Own Research

Table 4. Diverse species of bacteria isolated from the test soil and biostimulants.

Isolate	Humic sediment	Soil	Spent grain
P. aeruginosa	-	+	+
Corynebacterium sp	-	+	-
Alcaligenes sp	-	+	-
Bacillus subtilis	+	+	+
Micrococcus sp	-	+	+
Bacillus cereus	-	-	+
Staphylococcus albus	-	-	+
Staphylococcus aureus	-	+	-
Nitrosomonas sp	-	+	-
Nitrobacter sp	-	+	-
Enterobacter sp	-	+	-
Kelbsiella sp	-	+	-
Enterococcus	-	+	-

Key: - = not present, + = present; Source: Own Research

3.4 Hydrocarbons Utilization Potential of Bacteria Isolated from Test Soil *in situ*

The capability of indigenous bacterial species isolated from test soil to utilize crude oil is presented in Table 5. The results revealed the presence of bacterial isolates with strong crude oil utilization potentials. *P. aeruginosa*, and *Bacillus subtilis* were the strongest degraders, *Micrococcus* and *Corynebacterium* were moderate degraders, *Alcaligenes* sp, *Enterobacter* sp and *Klebsiella* sp exhibited very weak crude oil utilization while *Nitrosomonas* sp, *Nitrobacter* sp, *Enterococcus* sp and *Staphylococus aureus* were unable to degrade hydrocarbons.

3.5 Influence of biostimulation with Brewer's spent grains and Augmentation with Oil Degrading Strain of *Bacillus subtilis* on the Activities of Bacteria in Crude Oil Contaminated Soil

The Influence of biostimulant (spent grain) and application oil degrading strain of *Bacillus subtillis* on the activities of heterotrophic bacteria in garden soil contaminated with crude oil is presented in Figs. 1-7.

The results showed variation in densities of different bacterial groups. At 2.08% of crude oil contamination the application of 1% spent grain "cropped" with 20 ml (2.9×10^4 cfu/ml) of broth culture of *B. subtilis* - HSC 1 resulted in a sharp increase in the density of oil degrading bacteria within two weeks of exposure (Fig. 1).

At 2.28% level of contamination the application of 5% spent grain "cropped" with 20 ml (2.9×10^4 cfu/ml) of broth culture of *B. subtilis* - HSC 1 (Fig. 2), the density of heterotrophic bacteria was neither increasing nor decreasing depicting stationary phase bacteria activities throughout the remediation period.

In soil contaminated 2.6% of crude oil, the application of 10% spent grain "cropped" with 20 ml (2.9×10^4 cfu/ml) of broth culture of *B. subtilis* - HSC 1, the results presented in Fig. 3 showed remarkable increase in density of heterotrophic bacterial count between the 6th and 8th weeks of exposure while the growth of oil utilizing bacteria, *Nitrosomonas* and *Nitrobacter* showed a decreasing trend throughout the remediation study.

Fig. 4 shows that when 15% spent grain and 20 ml (2.9×10^4 cfu/ml) of broth culture of *B. subtilis* - HSC 1 were introduced into soil contaminated with 3.12% crude oil, the *Nitrobacter* and *Nitrosomonas* activities were not detected after 4 and 5 weeks of exposure respectively and till the end of the remediation period.

3.6 Total Petroleum Hydrocarbon Contents of Soil after Remediation with Organic Amendments "Cropped" with *B. subtilis*

The *in situ* degradation study carried out for 8 weeks showed that, the degradation of crude oil and its components was faster when enhanced with biostimulant "cropped" with oil degrading strain of *B. subtilis* -HSC 1 than when carried out with biostimulants alone. For contaminated soils amended with bacterized - spent grains (Table 6), the degradation rates were remarkably high and near 100% within 8 weeks. But when compared with the rate (44.02%) recorded for treatment with spent grains alone (Control 2) without *B. subtilis* –HSC 1, it shows remediation was more enhanced by the activity of the "cropped" oil degrading bacterium.

Fig. 8 shows the efficiency of the adopted biostimulants/crude oil ratios on the remediation of oil contaminated soils. Crude oil degradation rates were found to decrease with increase in percentage of organic amendment applied. In soil remedied with microbially cropped spent grains, the best degradation rate (99.09%) was achieved when 1% of stimulant was applied to soil contaminated with 2.08% of crude oil at a BC - (BC) ratio of 0.48: 1 beyond which the treatment supported more heterotrophic than hydrocarbonoclastic activities.

Analysis of growth rate and generation time revealed that the best degradation rate was attained when 1% of brewer's spent grain was employed (Table 7). This coincides with heterotrophic bacterial growth rate and generation time of 0.0008 and 860.87 h^{-1} in 8 weeks respectively as against 0.0006 and 1123.18 h^{-1} in 8 weeks observed for 15% biostimulants application.

3.7 Effect of the Organic Amendment on Soil Properties

The pH of the treated soil samples increased gradually from 5.05 towards neutrality with 8.15

average value during the degradation studies. The soil conductivity decreased in samples treated with 1% spent grains with 2.08% crude oil and 10% spent grains with 2.6% spent grain but increased in 20% spent grains with 4.16% crude oil (Table 8). The organic carbon content showed an increasing trend from 2.47 to 10.61, 10.64, 10.76 mg/kg in soils treated with different concentrations of crude oil and amended with spent grains.

Table 5. Hydrocarbonoclastic potential of bacteria isolated from garden soil

Isolate code	Growth on crude oil after 7 days	Growth on crude oil after 14 days
P. aeruginosa	+++	+++
Corynebacterium sp	+	++
Alcaligenes sp	-	+
Bacillus subtilis	+++	+++
Micrococcus	++	++
Staphylococcus aureus	-	-
Nitrosomonas sp	-	-
Enterococcus sp	-	-
Klebsiella	-	+
Nitrobacter sp	-	-
Enterobacter sp	+	+

Key: - = no growth, + = 1-5mm (weak) + = 6-10 mm (moderate), ++ = 11-15 mm (strong), +++ =16-20 mm (strongest)
Source: Own Research

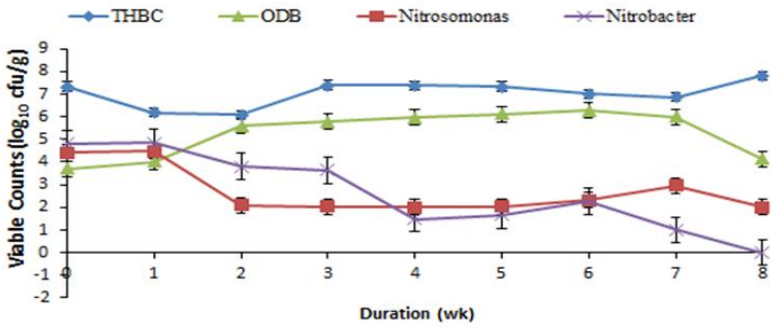

Figure1: Influence of 1% biostimulant (spent grain) plus *B. subtilis*-HSC1 application on the bacterial activities in garden soil contaminated with 2.08% crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

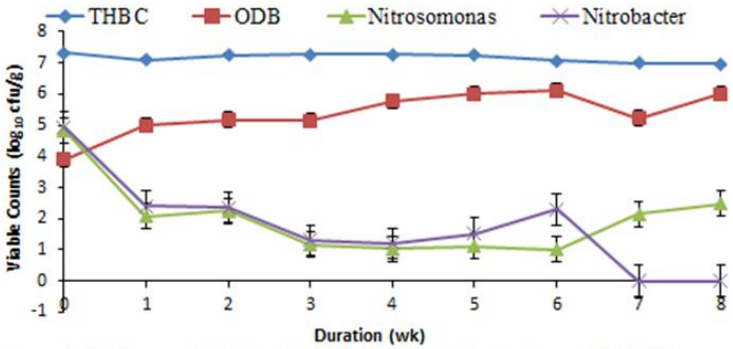

Figure 2: Influence of 5% biostimulant (spent grain) plus *B. subtilis*-HSC1 application on the bacterial activities in garden soil contaminated with 2.28% crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

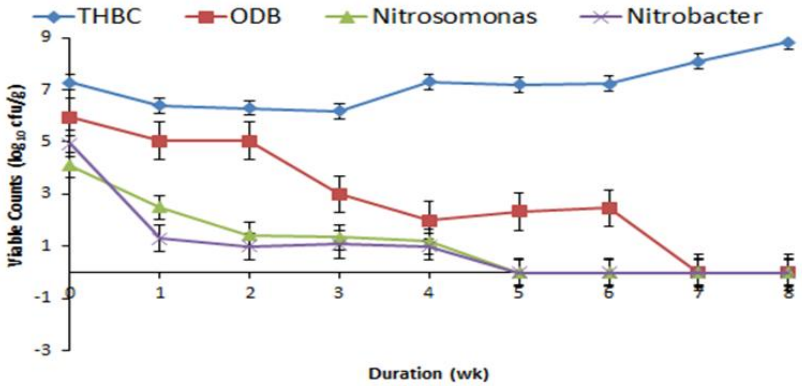

Figure 3: Influence of 10% biostimulant (spent grain) plus *B. subtilis* -HSC 1 application on the activities of bacteria in garden soil contaminated with 2.6 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

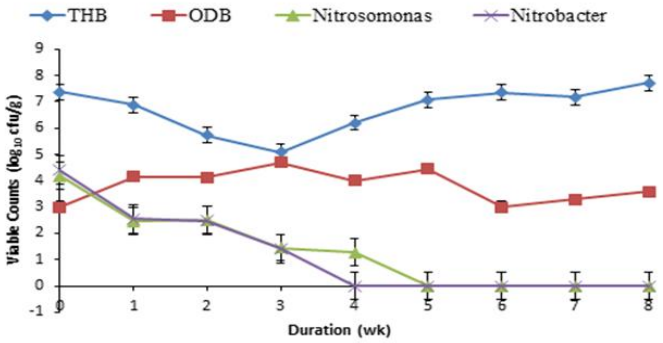

Figure 4: Influence of 15% biostimulant (spent grain) plus *B. subtilis* -HSC 1 application on the activities of bacteria in garden soil contaminated with 3.12 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

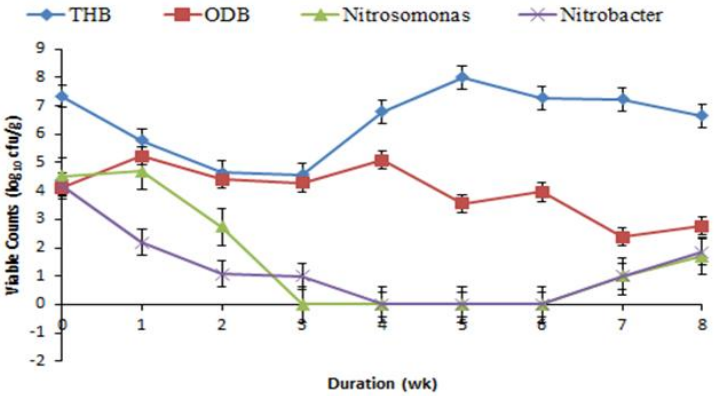

Figure 5: Influence of 20% biostimulant (spent grain) plus *B. subtilis* -HSC 1 application on the activities of bacteria in garden soil contaminated with 4.16 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

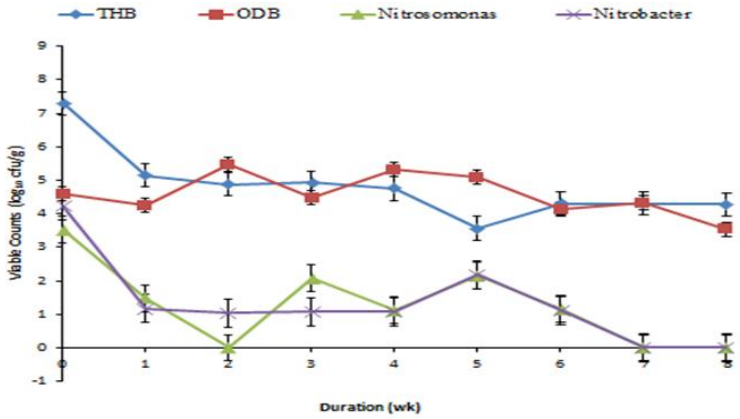

Figure 6: Changes in microbial counts of unpolluted soil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

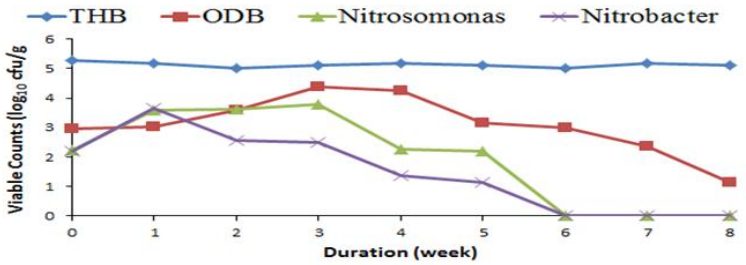

Figure 7: Changes in bacterial count of polluted soil remedied with un-bacterized biostimulants (spent grains) (control 2)
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

Source: Own Research

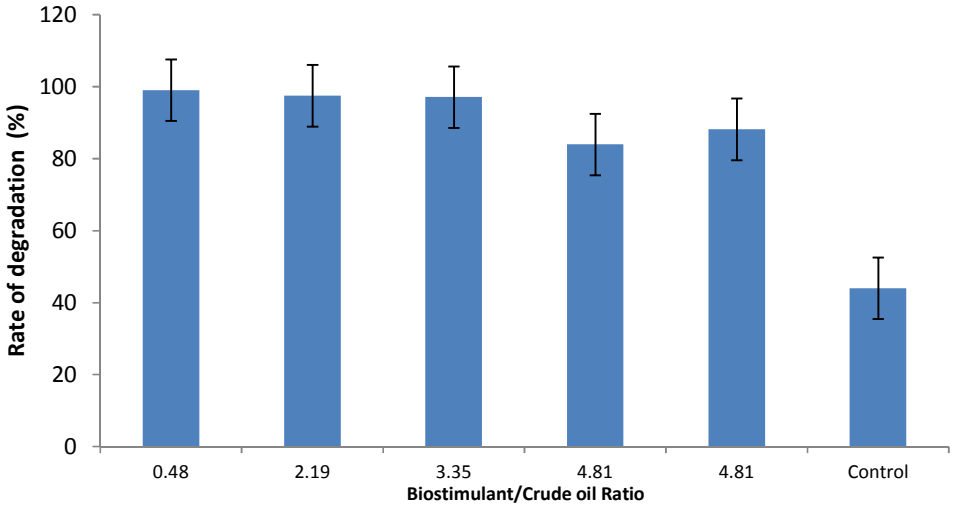

Fig. 8. Influence of biostimulant/crude oil ratio on the rate of hydrocarbons degradation in garden soils

Source: Own research

Table 6. The rate of degradation obtained from the various bioremediation treatments using spent grains

Quantity of soil treated	Amount of oil added	Level of contamination	Amount of spent grains added	Biostimulant/ Crude oil ratio	Residual load of TPH in remedied soil (mg/kg)	Rate of biodegradation	% Degradation
4 kg Bio-stimulated & Bacterized	83.2 g (20.8 g/kg)	2.08 %	40 g (1 %)	0.480	188.54	20611.46	99.09
4 kg Bio-stimulated & Bacterized	91.52 g (22.88 g/kg)	2.28 %	200 g (5 %)	2.185	567.98	22312.02	97.51
4 kg Bio-stimulated & Bacterized	104 g (26 g/kg)	2.6 %	400 g (10 %)	3.346	745.12	25254.88	97.13
4 kg Bio-stimulated & Bacterized	124.8 g (31.2 g/kg)	3.12 %	600 g (15 %)	4.807	4999.03	26,200.97	83.97
4 kg Bio-stimulated & Bacterized	166.4 g (41.6 g/kg)	4.16 %	800 g (20 %)	4.807	4913.92	36687	88.18
4 kg Con. 2a Bio-stimulated No Bacterization	83.2 g (20.8 g/kg)	2.08 %	40 g (1 %)	0.480	11643.66	9156.34	44.02

Source: Own research

Table 7. Growth rate and viable cells generation of bacteria during the remediation with BSG

Treatment	Growth rate/Decimal death rate (h^{-1})	Generation time (h^{-1})
1% BSG plus *B. subtilis*-HSC1 on 2.08% crude oil contamination		
THBC	0.000805	860.8696
ODB	0.000771	898.8327
Nitrosomonas	-560	-
Nitrobacter	-	-
5% BSG plus *B. subtilis*-HSC1 on 2.28% crude oil contamination		
THBC	-3536.84	-
ODB	0.003598	192.607
Nitrosomonas	-567.089	-
Nitrobacter	-	-
10% BSG plus *B. subtilis*-HSC1 on 2.6% crude oil contamination		
THBC	0.002639	262.5995
ODB	-	-
Nitrosomonas	-	-
Nitrobacter	-	-
15% BSG plus *B. subtilis*-HSC1 on 3.12% crude oil contamination		
THBC	0.000617	1123.177
ODB	0.000977	709.3142
Nitrosomonas	-	-
Nitrobacter	-	-
20% BSG plus *B. subtilis*-HSC1 on 4.16% crude oil contamination		
THBC	-1947.83	-
ODB	-995.556	-
Nitrosomonas	-476.596	-
Nitrobacter	-576.824	-

Key: Negative values = decimal death rate
Source: Own research

Table 9 shows the PAH profiles of the brewer's spent grains - stimulated and un-stimulated (control) crude oil contaminated soils as well as that of organic amendments used for the remediation. The result shows remarkable reduction in residual load of the recalcitrant component of the crude oil within the 8 weeks of remediation. The microbially activated spent grains reduced the total PAH load of the crude oil polluted soil from 0.57 mg/kg to 0.09 mg/kg within 8 weeks of enhanced remediation.

4. DISCUSSION

The soil physical and chemical characteristics of the test soil (Garden soil) are presented in Table 1. The particle size arrangement of soil determines the texture of the soil, while soil texture determines the water absorption capacity, water storage, ease of tilling as well as soil aeration and fertility [19]. The results of the present analysis indicate that the test soil was predominantly sandy. The mean pH of 5.05 ± 0.18 revealed a strongly acid soil and acid soils are known to exhibit intensive leaching, low exchangeable basic cation content and slow microbial activity [20]. This soil pH level reported here corroborates with the results of previous work by Nkereuwem et al. [21], who reported a slightly acidic pH of 6.6 for loamy sand soil. The mean conductivity of the soil was 45.7 ± 0.34 μScm^{-1} and was consistent with findings of the soils of the Niger Delta [22]. With mean of 0.46±0.02%, the TOC level is less than 12% reported for organic carbon levels derived from mineral sources [19].

Table 8. Physicochemical properties of treated soil samples after 8 weeks of amendment

Parameter	1% Spent grain plus 2.08% Crude oil	10% Spent grain plus 2.6% Crude oil	20% Spent grain plus 4.16% Crude oil	Soil plus 2.08% Crude oil
pH	7.26±0.03	8.21±0.03	8.05±0.03	8.35±0.04
Conductivity (µS/cm)	3.08±0.03	1.77±0.02	160.0±2.61	9.82±0.07
Redox Potential (mV)	90.0±0.1	76.0±0.35	54.0±0.2	22.0±0.2
Moisture (%)	14.97±0.06	16.47±0.45	16.52±0.43	1.32±0.22
Organic carbon (mg/kg)	10.61±0.02	10.64±0.04	10.76±0.05	10.68±0.51
Calcium (mg/kg)	3.86±0.76	3.11±1.04	3.95±0.06	3.03±0.90
Potassium (mg/kg)	1.03±0.03	1.93±0.17	2.01±0.02	1.95±0.05
Magnesium (mg/kg)	2.02±0.02	3.22±0.02	4.07±0.18	2.09±0.01
Total Nitrogen (%)	1.06±0.20	1.16±0.01	0.98±0.01	1.52±0.34
Sodium (mg/kg)	1.05±0.06	2.83±0.07	1.99±0.16	2.04±0.35
Exchangeable Acidity(Cmol/kg)	3.01±0.04	1.96±0.08	3.54±0.04	2.09±0.12
Sand (%)	64.40±1.56	63.75±2.38	59.30±2.33	64.40±1.56
Slit	19.10±0.09	19.75±0.67	19.98±0.83	19.10±0.09
Clay	16.5±0.45	16.5±0.45	20.72±0.15	16.5±0.45
Textural class	Sandy loam soil	Sandy loam soil	Sandy loam soil	Sandy loam soil

Source: Own research

Table 9. Polycyclic aromatic hydrocarbons profile of the biostimulants, control and biostimulated soil samples

Parameter	Results (mg/kg)		
	Spent grains	Bio-Soil (Spent grains)	Con. Soil
Naphthalene	0.02	0.03	0.02
2-Methylnaphthalene	0.02	0.02	0.02
Acenapthylene	0.02	0.02	0.02
Acenaphthene	0.02	0.03	0.02
Fluorene	0.03	0.03	0.03
Phananthrene	0.03	0.04	0.03
Anthracene	0.02	0.02	0.02
Flouranthene	0.04	0.04	0.01
Pyrene	0.03	0.02	0.02
Benzo(a)anthracene	0.09	0.04	0.02
Chrysene	0.08	0.03	0.03
Benzo(b)flouranthene	0.07	0.03	0.02
Benzo(k)flouranthene	0.09	0.04	0.03
Benzo(a)pyrene	0.09	0.03	0.03
Dibenzo(a,h)anthracene	0.12	0.04	0.02
Benzo(g,h,i)perylene	0.11	0.02	0.02
Indeno(1,2,3-d) pyrene	0.30	0.11	0.05
Total	1.05	0.57	0.09

Source: Own research

The total hydrocarbon content (THC) of the soil is a measure of the hydrocarbon content of the soil. Sources of hydrocarbon accumulation in an environment include natural sources (e.g. plant and animal matter, oil seeps); the atmosphere; accidents during transportation, storage, or use of petroleum products, inland oil exploration and exploitation, as well as municipal/industrial wastes. The THC content of the garden soil used for the study was below method detectable limit of 10 mg/kg, indicating the absence of hydrocarbon contamination. Hence the level

(0.09 mg/kg) of polycyclic aromatic hydrocarbons (PAHs) was negligible.

The soil nutrients are very essential for bioremediation process as biological agents utilize them for optimum growth and production of suitable enzymes [23,24]. The loamy sand texture observed is known to allow for the distribution of air, moisture, soil nutrients and contaminants. The test soil had a different C:N ratio of 6.31 against 100:10 ratio stipulated for optimum hydrocarbon degradation [25] coupled with low nutrient level, the acidic nature of soil can increase the chances of crude oil components becoming persistent in soil if bioremediation procedure is not applied.

The composition of the brewer's spent grains used as bio-amendments for the remediation of contaminated soil revealed waste substrates of nutritional significance. The high protein content in this study corroborates the work of Ajanakua et al. [26]. The high protein values observed in the spent grain sample may be due to the water fraction leaching of water-soluble protein during the wort separation step which is one of the processes in brewing operations. The protein content and moisture contents of spent grains make it particularly susceptible to microbial growth and degradation [27].

The presence of resident microflora initiates these processes within the shortest time, in an attempt to utilize it as sole carbon source [28]. The fibre content of the biostimulants was high but Ajanakua et al. [26] reported a higher value of fibre and ash in spent grains. The crude fibre content (3.5 ± 0.2%) of the brewer's spent grain obtained in this study was lower than the 7.6% obtained for banana peels, 32 5% for cocoyam peels and 20.97% for cassava peels [29]. Fibre contain appreciable amount of nutrients which are released slowly in further enzymic action. High fibre content reduces the rate of glucose and fat absorption in biological cells [30]. It is implied here that the low fibre content of the biostimulants may likely favour fastidious growth microorganisms. The ash content of the organic amendments is low and is a reflection of the poor mineral elements contained in the amendments. The carbohydrate content of the spent grains was very high and could serve as the main carbon source for microbial growth during remediation of oil-contaminated soils.

Oil spill impact soil fertility, affects germination, growth and yields of plants and therefore food productivity [31]. These concerns have contributed to the development of various remediation technologies including bioremediation – which mainly depends on microorganisms to degrade, transform, detoxify or breakdown the contaminants. Despite the advantages of bioremediation, its efficiency is limited majorly by the limited bioavailability of hydrocarbons to microorganisms. This is attributed to the low solubility and strong and/or irreversible sorption to soil matrix [5,32]. To solve this problem, several methods have been developed to enhance the bio-availability of hydrocarbons and the Remediation by Enhanced Natural Attenuation (RENA) is one, with preference for biological sources. This increasing health hazard and soil pollution has compelled the scientists to look for non-chemical way of supplying the fertilizers to enhance crop productivity. As a result, the application of biofertilizers in place of chemical fertilizers has been recommended. Biofertilizer means input of plant nutrients of biological origin for improved growth of plants. Biofertilizers are also known as 'microbial inoculants' or microbial preparations. They themselves do not increase the soil fertility directly, but instead they exercise their biological effect on improving nutritional conditions including enhanced microbial activities in soil [33,34]. The biostimulants (brewer's spent grains) used in this study was used basically as stimulants to microbial inoculants and indigenous microbial populations for enhanced natural attenuation of hydrocarbons in contaminated soil.

The microbial inoculant used in this study was isolated from a humic ecosystem using enrichment culture technique. It is a *Bacillus subtilis* strain with a strong crude oil degrading potential. The strain exhibited positive results for Voges-Proskauer and sporulation tests. Negative results were noted for catalase, oxidase, mannitol, methyl and indole. *B. subtilis* has been previously reported to be a good crude oil degrader [35]).

The test soil had rich microbial loads with remarkable populations of heterotrophic bacteria and but low numbers of oil degrading bacteria. Only 21 viable cells of oil degraders were obtained per gram of spent grains. The oil utilizing bacteria isolated from both soil and biostimulants in the present work have been previously implicated in crude oil biodegradation in varying degrees of crude oil degrading capabilities from different sources [36].

Stimulated biodegradation of crude oil is at present encouraged because it ensures rapid remediation of oil polluted ecosystems [37,38]. Much study have been conducted on the remediation of crude oil contaminated ecosystems where organic amendments are commonly used to enhance remediation process [39,40]. Studies have also been conducted on the bio-augmentation of remediation processes with microbes with strong hydrocarbon degrading potentials. In this study, both approaches were technically employed to enhance crude oil degradation in soil. The study used microbially augmented bio-stimulants (brewer's spent grains) for the remediation process. In this case, 20 ml (2.9 cfu/ml) of a strong crude oil degrading strain, *B. subtilis* HSC-1 isolated from a humic ecosystem, was "cropped" or introduced into the biostimulants.

This study has shown that soil contamination with crude oil drastically reduced the population of denitrifying bacteria but increased the population of oil degrading bacteria in soil but has concentration-dependent effects on the densities of heterotrophic bacteria. Our findings revealed that the higher the biostimulants/contaminant ratio added, the more the heterotrophic activities but less hydrocarbonoclastic activities. This observation correlates with the report of Atlas and Bartha [41] who reported that the application of crude oil to Arctic tundra soil caused overall increase in microbial numbers compared to un-oiled reference (control) soil. However this study reveals that heterotrophic bacterial isolates were dominant in both polluted and unpolluted soil compared to other physiological groups of microorganisms, probably because heterotrophic bacteria are more numerous in the soil. Some of them are fast-growing and capable of utilizing a wide variety of organic compounds for survival [42].

The population of oil degrading microorganisms was higher in polluted soil than in the unpolluted soil. Similarly, large populations of hydrocarbon degrading bacteria were observed for oil polluted environments [43]. Population levels of hydrocarbon degraders within the microbial community appear to be an indication of environmental exposure to hydrocarbons. The nature of microbial population usually reflects the extent of exposure of a particular environmental hydrocarbon contamination [44]. Nevertheless, the reverse was the case in nitrifying bacterial isolates (*Nitrosomonas* and *Nitrobacter*). It was also observed that the polluted soil had a lower population than the unpolluted soil. This could be attributed to a rapid multiplication of some microorganisms which used up the available inorganic nitrogen for growth leaving nitrifiers at disadvantage. Among the nitrifiers, it was also observed that oil adversely affected the *Nitrobacter* more than *Nitrosomonas*. It was apparent that when the quantity of oil was increased, a smaller number of *Nitrobacter* was found than that of *Nitrosomonas*. This may be ascribed to the fact that *Nitrosomonas* oxidizes ammonia first to nitrite making use of the available oxygen leaving small amount for *Nitrobacter* to use in the oxidation of nitrite to nitrate.

The bacterial population dynamics observed during crude oil degradation could also be associated with the degradation patterns of the crude oil hydrocarbons in the different treatment options when compared with the non-amended crude oil. The influence of 1% biostimulant (spent grain) application on the activities of heterotrophic bacteria in garden soil contaminated with 2.08% crude oil augmented with oil degrading *Bacillus subtilis* showed a strong relationship (r=0.013) between the total heterotrophic count and the oil degrading bacteria. Similar result was observed between *Nitrosomonas* and oil degrading bacteria at 0.05 level of probability (r=0.702) on treatment. The influence of 5% level of biostimulant application on the activities of heterotrophic bacteria in garden soil contaminated with 2.28% crude oil augmented with oil degrading *Bacillus subtilis*, revealed a significant relationship (r=0.687) between oil degrading bacteria and *Nitrobacter* at 0.05 level in spent.

However at 10% level of biostimulants application in garden soil contaminated with 2.6% crude oil shows the application of spent grains caused a significant relationship (r=0.738) between total heterotrophic bacteria and oil degrading bacteria and also between oil degrading bacteria and *Nitrobacter* (r= 0.727) at P =0.05 At higher levels of biostimulants application, varied relationships were established between bacterial groups, nevertheless a strong relationship (r=0.23) was observed between oil degrading bacteria and *Nitrobacter* upon spent grain application.

The activities of hydrocarbonclastic microorganisms clearly influenced the hydrocarbons degradation rates in the remedied

substrates. The research results have revealed that the rate of the degradation of crude oil and it components was faster when enhanced with biostimulant (spent grain) "cropped" with oil degrading strain of *B. subtilis* -HSC 1 than when carried out alone with biostimulants.

Crude oil degradation rates were found to decrease with increase in percentage of organic amendment applied. In soil remedied with microbially cropped spent grains, the best degradation rate (99.09%) was achieved when 1% of stimulant was applied to soil contaminated with 2.08% of crude oil at a BC - (BC) ratio of 0.48: 1, beyond which the treatment supported more heterotrophic than hydrocarbonoclastic activities. However, a stronger hydrocarbonoclastic activity is expected at longer period of remediation when the simple components derived from the biostimulants are exhausted and the log phase of oil degraders is restored. Analysis of growth rate and generation time revealed that the best degradation rate was attained when 1% of brewer's spent grain was employed coinciding with of heterotrophic bacterial growth rate and generation time of 0.0008 and 860.87 h^{-1} in 8 weeks respectively as against 0.0006 and 1123.18 h^{-1} in 8 weeks observed for 15% biostimulants application For the highest hydrocarbonclastic activity the growth rate and generation time observed were 0.00077 h^{-1} in 8 weeks and 898.83 h^{-1} in 8 weeks respectively for spent grains. The highest hydrocarbonoclastic bacteria decimal rate - 3536.84 was recorded when 5% of spent grains at the B/C ratio of 2.185 and at a B/C ratio of 2.185 were applied.

The enhanced remediation using microbially "cropped" or activated on the properties of the test soil revealed improvement in the levels of plants essential components. The organic carbon and total nitrogen contents of the garden soil increased after the remediation process. This is in agreement with previous report by Nkereuwem et al. [21] that soils that have been contaminated with natural gas or crude oil exhibited large increases in organic matter, total carbon and nitrogen compared with normal (unpolluted) soils. This increase in organic carbon and total nitrogen in the polluted soils could be attributed to the activities of microorganisms in converting hydrocarbons into organic acids and salts [39]. Mineral elements have been reported to be essential for crude oil degradation [45]. The addition of organic amendments (biostimulants) to the contaminated soil may have introduced more nutrients especially nitrogen and phosphorus hitherto not found in the chemical-contaminated soils. The study has also revealed that bio-amendment with spent grains positively influenced the pH of test soil. The pH of the polluted soil was raised at the end of the remediation studies to a range between 7.26 and 8.61 which is ideal for microbial growth and hydrocarbon degradation [24,25]. This indicates that the bio-stimulants (spent grains) used for the remediation have buffering effect on the soil. Strong acidity is a limitation to biodegradation process [46]. On the other hand the available potassium of the uncontaminated soil was observed to have decreased during the remediation of the oil contaminated soil. This may be attributed to the use of this exchangeable base by the microbes in the experimental soil samples as previously reported by Atlas [47].

5. CONCLUSION

The research findings have shown that contamination of the acidic soil with crude oil altered its physicochemical and microbiological properties, hence its fertility status. Amendment of the soil with biostimulants alone enhanced more of heterotrophic bacterial activities than hydrocarbonoclastic activtiy. However treatment with biostimulants supplemented or "cropped" with 2.9 x 10^4 cfu/ml of broth culture of crude oil degrading strain of *B. subtilis* - HSC 1 resulted in increase in hydrocarbonoclastic activities and remarkable reduction of hydrocarbon content of the contaminated soil. The bio-stimulating influence however varied with the level of contamination, the quality of organic amendment and the ratio of biostimulants/contaminants (BC) ratio employed in the remediation programme. The results obtained from this study have revealed that:

(i) Biostimulation improves microbial activities in soil, also strong bio-stimulating potential of brewer's spent grain when applied in the BC ratio of 0.48 : 1.
(ii) Augmenting the biostimulants with strong strains of hydrocarbon utilization bacteria would stimulate the activities of indigenous degraders and ensure a hastened natural attenuation process in contaminated ecosystems
(iii) The bacterization-biostimulation (BB) approach adopted here, apart from encouraging the significant degradation of hydrocarbons and it's recalcitrant PAHs

component, improves soil fertility by reducing acidity and C:N ratio of the soil.

(iv) That efficiency of the BB protocol is BC ratio-dependent as higher ratios of amendment had diauxic influence on process, retarding the growth rate of oil degraders and promoting heterotrophic activities against hydrocarbonclastic activity.

This protocol is strongly recommended for the clean-up and remediation of crude oil contaminated soils in the Niger Delta of Nigeria. Moreover the requirements are readily available and relatively harmless to the environment. However the use of the fibre-poor BSG can oil be sustainable if stabilized with a fibre-rich carrier.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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