Hydrocarbons Degrading Potential of Stimulated Cultures of Bacteria Isolated from Humic Fresh Water Sediment of Eniong River in the Niger Delta of Nigeria

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Authors' contributions

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

Original Research Article

ABSTRACT

The Hydrocarbons degrading potential of stimulated cultures of bacteria isolated from humic fresh water sediment of Eniong River in the Niger Delta of Nigeria was studied. Preliminary screening of the humic hydrocarbonoclastic bacterial isolates revealed that among the 5 bacterial isolates (HSC1, HSC2, HSC3, HSC4 and HSC5), isolate HSC1 exhibited the strongest ability to utilize crude oil and was characterized to be *Bacillus subtilis*. *In-vitro* analysis of crude oil utilization of *Bacillus subtilis*- HSC1 when energized with various concentrations of sugar (glucose and sucrose) revealed variable levels of hydrocarbons utilization by monoculture of *B. subtilis* and enhanced degradation with biostimulation. The results of indirect assessment using the total viable cells revealed that the

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microbial biomass increased over time during degradation. The rate of increase was apparently higher in cultures stimulated with various concentrations of sugar (glucose and sucrose) than in the control and best growth were recorded on the 12th day when treated with 10% of glucose. Analysis of the optical density of the *Bacillus subtilis* during the degradation process revealed that the optical density increased with time. The pH of the test substrates decreased over time indicating a higher catabolic activity. The increase in acidity was higher in 15% and 20% glucose supplemented medium. The nutrient addition increased the bacterial cell numbers, optical density as well as the acidity (high decrease in the pH) of the test media between days 9 and 15 when compared with the rate derived from the test medium. *In vitro* degradation study carried out for the 15 days showed that, the degradation of crude oil and its component by *B. subtilis* was faster when stimulated with the different concentrations of sugar than when un-stimulated. The result showed a remarkable reduction in the total petroleum content of the test substrates treated with glucose and sucrose. The best results were obtained by treatment with 1 and 5% levels of the stimulants. At this level, the TPH content was reduced from 15.81 mg/kg observed for the control to 10.19 mg/kg (49.92% degradation) and 8.03 mg/kg (60.52% degradation) obtained from substrates stimulated with 5% glucose and 1% sucrose respectively. The high hydrocarbon degradation by stimulated culture of *Bacillus subtilis*- HSC1 implies that biostimulation can be harnessed for bioremediation purposes.

1. INTRODUCTION

The wide increase in industrialization and indiscriminate discharge of wastes, accidental spills or deliberate release of hydrocarbons into the aquatic ecosystem has led to the pollution of this ecosystem.Sediments are an important sink for hydrocarbons especially in river mouth ecosystems. It is known that hydrocarbons accumulate on sediment surface, in benthic living things, planktonic organisms and other living matter and is enhanced through food chain. Fish accumulate xenobiotic compounds, especially those with high water solubility because of the very intimate contact with the medium that carries the compounds in solution, suspension and also because fish have to extract oxygen from the medium by passing the enormous volumes of water over gills. For fish, the gills, skin and digestive tract are potential sites of absorption of water soluble chemicals. The chemical once absorbed is transported by the blood to either a storage point, such as bone or to the liver for transportation. If transported by the liver it may be stored there, excreted in bile or passed back into the blood for possible excretion by kidney or gills or stored in extra hepatic tissues such as fat [1].

The release of these pollutants has led to ecological and toxicological problems especially with the release of those that are persistent, hydrophobic, lipohylic and electrochemically stable. Of these pollutants, crude oil is known to be carcinogenic, mutagenic, recalcitrant and toxic [2,3] while humic substances are highly refractory [4]. These pollutants can affect the availability and toxicity of organic chemicals in the environment and almost any biochemical and biogeochemical pathway in fresh water organisms including microorganisms. Hydrocarbon pollution has destructive effects on the ecological balance of the recipient environment and diversity of aquatic organisms [5]. Fishes are the most affected sea animals by hydrocarbons contaminations. It is pertinent to note that hydrocarbons have toxic effects even at places away from the source of pollution as they have the property of biological accumulation. Toxic substances may knock down immune reproductive, nervous and endocrine systems in animals and these effects can be at organ, tissue, and cell levels [6]. Pollutants remain in the environment and can become concentrated up in the food chain. Lee et al. [7] have reported that pollutants may be bioconcentrated, bioaccumulated and biomagnified within food chains, causing higher tropic organisms to become contaminated with higher concentrations of chemical and metal contaminants than their prey.

Crude oil pollution can be as a result of accidental or deliberate action. Other sources of crude oil pollution include sabotage, corrosion of pipes automobile operation, petroleum refining activities and seepage. The presence of crude oil/hydrocarbon pollution in the environment especially the aquatic ecosystem regardless their amount is potentially dangerous to living organisms including plants and animals and has a harmful effect on the ecosystem [8,9]. The

impact, such as severe ecosystem in-balances caused by these pollutants is of great public concerns about remediating and restoring the environment of crude oil or hydrocarbon pollution. Interest in biodegradation mechanism and environmental fate of crude oil constituent especially polycyclic aromatic hydrocarbons is motivated by their ubiquitous distribution, their low bioavailability and high persistence in environment and their potentially carcinogenic properties [10]. The chemical properties and hence the environmental fate of crude oil is dependent on their molecular size/composition.

Microbial biodegradation is an important process of crude oil decomposition in the environment [9]. It plays a vital role in the restoration and remediation of hydrocarbon-contaminated sites. The ability of microorganisms to degrade organic contaminants (crude oil) into harmless constituent has been explored as a means to biologically treat contaminated environments thus; this has been the basis for emergent field of bioremediation [4,11]. Biological degradation, a safe, effective and economic alternative method is a complete microbial mineralization of complex materials into simple inorganic constituents such as carbon dioxide [12]. This technology uses the metabolic potential of microorganism to clean up polluted environments. Due to its simple application it can be used on large areas, it is cost effective and removes the contaminants completely [13]. Strategies used in bioremediation of polluted ecosystem revolves around either stimulating indigenous microbial population by environmental modification or inducing exogenous microbial population that are known degraders to a contaminated site, a process known as seeding [14,15]. In aquatic and terrestrial environments, the biodegradation of crude oil and other petroleum components predominantly revolves around the action of bacteria and fungal populations (14). The continuous input of petroleum based pollutants has resulted in an enriched microbial community capable of surviving toxic contamination. Microorganisms are sensitive to fluctuations/changes in the environment. Whenever their chemical or physical environment is suddenly altered, there is a lag period during which the microbial community adapts to the new conditions [16,17]. This lag period is also called acclimatization period and enables microorganisms to acquire the metabolic repertoire necessary for their survival. The common occurrence of metabolically active bacterial and fungal populations in areas that are

contaminated with hydrocarbon strongly suggests that these microorganisms are able to utilize hydrocarbon as their carbon and/or energy sources [4]. Some members of these populations degrade alkanes, some aromatics, while others decompose both paraffinic and aromatic hydrocarbons, transforming them into products such as carbon dioxide, water and biomass or other less harmful end products [18].

Due to the complex nature of crude oil, biodegradation involves the interaction of many different microbial species. In aquatic environment, biodegradation is largely carried out by diverse bacterial populations, which are ubiquitously distributed in the water. The most commonly reported genera of hydrocarbon degraders include *Pseudomonas*, *Acinetobacter*, *Nocardia*, *Vibrio* and *Achromobacter* [19].

Most studies carried out on bioremediation of soils impacted in the Niger Delta of Nigeria are majorly on identification of hydrocarbon degrading bacteria although few researches have also carried out on the bioremediation of different hydrocarbons. Despite all these researches information is still lacking on the hydrocarbons degrading potential of stimulated cultures of bacteria isolated from humic freshwater ecosystem. This study is focused on providing information on the hydrocarbons degrading potential of stimulated cultures of bacteria isolated from humic fresh water sediment of Eniong River in the Niger Delta of Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

The humic freshwater of Eniong River in Itu Local Government Area of Akwa Ibom State is a "blackwater" aquatic ecosystem that flows as a tributary of the middle course of Cross River. The River lies between latitude $5^o12'N – 5^o22'N$ and longitude $7^o54' E – 8^o2'E$. it is characterized by intense coloration due to humic substance and possibly soluble iron. The river serves as a source of water, means of transport and fishing for the communities within its catchment.

2.2 Sources of Materials

2.2.1 Biostimulating agents

Processed sugars (Glucose and Sucrose) were purchased from a laboratory in Uyo, Akwa Ibom State, Nigeria, while Bonny light crude oil was

obtained from Oil Company operating in the Niger Delta Region of Nigeria.

2.2.2 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. The sediment sample was obtained from three different stations (upper, middle and lower course of the river) with the aid of a metal grab sampler, stored in clean glass bottles and then preserved in ice packed coolers which were transported to the laboratory for further analysis.

2.3 Microbiological Analysis

Standard microbiological techniques were employed in this study.

2.3.1 Isolation of crude oil degrading bacteria from humic sediment

The enrichment culture technique was employed. Precisely 1 g of each composite humic sediment sample from Eniong River were 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 $\pm$ 0.2)] (MSM) enriched with 1% crude oil as carbon source. The medium was incubated at 28 ℃ 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 [20] to obtain pure cultures.

2.3.2 Maintenance of pure cultures of oil-degrading bacterial isolates

Distinct colonies of the oil degrading bacteria isolated from the humic sediment were sub-cultured into McCartney bottles containing freshly prepared Nutrient Agar slants and incubated at 30 ± 2^oC for 24 hours before storage at 4^oC for characterization.

2.3.3 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 [21].

2.3.4 Characterization of bacterial isolate

The best crude oil utilizing bacterial isolate was characterized based on its cultural and morphological attributes as well as it responses to standard biochemical test as described by Cheesbrough [20]. Twenty four hours old mono-cultures of bacteria obtained were subjected to Gram's and endospore staining and several biochemical test such as Catalase test, Citrate Utilization test, Oxidase test, Motility test, Endospore, Methyl red and Vogues Proskauertest and Indole test, as well as sugar fermentation test.The results obtained were compared with characteristics described in Bergey's manual of determinative bacteriology [22].

2.3.4 *In vitro* determination of the influence of processed-stimulants (Glucose and Sucrose) on the Crude Oil Degradation by Bacteria Isolated from Humic Sediment

Exactly 2.0 ml of the filter sterilized crude oil was incorporated into conical flask containing 100 ml of sterile mineral salt medium. This was then inoculated with 1ml amount of active inocula from 24 hours old nutrient broth culture of the best oil degrading isolate. The two processed-stimulants were added to each flask in graded proportions of 1%, 5.0%, 10.0%, 15.0% and 20.0% respectively and were incubated aerobically in a rotator shaker. The flasks without the test organism served as control.

All tubes were monitored 72 hourly for 15 days for the rate of crude oil degradation based on viable plate counts, turbidity and pH indices. The viable plate count was estimated by the spread plate technique while the degree of turbidity and

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changes in pH were determined using spectrophotometer and pH meter respectively.

2.4 Determination of Total and Residual Petroleum Hydrocarbons Content

Before and after treatment with test isolates, 100 ml of the set up sample was measured into a separating funnel and 10 ml of Dichloromethane Haxane (1:1) was added into it. The mixture was shaken gently and vented for 5 minutes. The aqueous layer was allowed to separate and was decanted. The extracts was concentrated by rotary evaporator into 1ml. Precisely 1.0µL of the extracts was injected into a programed Hewlett-Packard H.P 5890 GC-FID. The concentration of TPH was calculated from the peak area of the calibration standards. The concentration of TPH was calculated from the peak area of the calibration standards. The GC operational conditions employed for determining the TPH is as follows: initial oven temp-50°C, initial hold time- 2.0mins, Ramp-10°C/min to 300°C, Final oven temp-320°C, Detector temp-250oC, Carrier gas – helium, ignition gas- hydrogen and air [23].

2.5 Data Analysis

Simple percentage was used to express the hydrocarbon degradation potential of the bacterial isolate where necessary.

3. RESULTS

3.1 Hydrocarbon Utilization Potential of Bacteria Isolated from Humic Sediment

The results of the screening test for capability of bacteria isolated from humic freshwater sediment of Eniong River to utilize crude oil are presented in Table 1. The results revealed the presence of bacterial isolates (HSC1, HSC2, HSC3, HSC4 and HSC5) with crude oil utilization potentials. However isolate HSC1 exhibited the strongest ability to utilize crude oil and was characterized for identification (Table 2).

3.2 Crude Oil Index of Hydrocarbon Utilization Potential of *Bacillus subtilis* when stimulated with Different Concentrations of Sugar *in Vitro*

The oil utilizing strain of *Bacillus subtilis*- HSC1 was further subjected to *in-vitro* analysis of crude

oil utilization when energized with various concentrations of sugar (glucose and sucrose) because it exhibited the strongest ability to utilize crude oil. The results of the utilization study have revealed variable levels of hydrocarbons utilization by monoculture of *B. subtilis* and enhanced degradation with biostimulation. The ability to degrade the hydrocarbons (measured indirectly by determination of the density of viable cells produced, the optical density and changes in pH of culture medium; and directly by estimating the rate of degradation of petroleum hydrocarbon contents in Bonny Light Crude oil) varied overtime and with the concentrations of sugars added.

Table 1. Hydrocarbonoclastic bacteria isolated from humic sediment

Isolate code	Growth on crude oil after 7 days	Growth on crude oil after 14 days
HSC1	+++	+++
HSC2	+	+
HSC3	+	+
HSC4	++	++
HSC5	-	+

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

3.2.1 Changes in total viable count, optical density and ph levels of *bacillus subtilis* culture stimulated with glucose

The results of indirect assessment using the total viable cells are presented in Fig. 1. The result of the total viable count revealed that the microbial biomass increased over time during degradation. The rate of increase was apparently higher in cultures stimulated with glucose than in the control and best growth were recorded on the 12th day when treated with 10% of glucose, however viable count decreased by the 15th day which could be attributed to the depletion of nutrient in the medium.

Analysis of the optical density of the *Bacillus subtilis* during the degradation process revealed that the optical density increased with time. The attenuance levels varied with the concentrations of sugar introduced. Slightly higher levels were recorded for 20% glucose concentration (Fig. 2).

There was a sharp increase in the acidity of the test media at different levels of glucose supplementation. The pH of the test substrates decreased over time indicating a higher catabolic

Table 2. Morphological, cultural and biochemical characteristics of isolates from test samples

Morphological characteristics					Citrate	Catalase	Coagulase	Indole	Oxidase	Methyl Red	VorgesPas keur	Sugar fermentation				Spore	Motility	Probable organism
Surface	Colour	Shape	Gram's Reaction									Glucose	Manitol	Sucrose	Lactose			
Rough	Milky `	Irregular	+ rod		-	+	-	-	-	-	+	AG	A	AG	AG	+	+	*Bacillus subtilis*

Key: A=Acid production, G= Gas production, +=Positive, -=Negative reaction, VP =VogesProskauer, sp =species

activity (Fig. 3). The increase in acidity was higher in 15% and 20% glucose supplemented medium.

3.2.2 Changes in total viable count, optical density and pH levels of *Bacillus subtilis* culture stimulated with sucrose

Similarly, the addition of sucrose supplement to the growing culture of *B. subtilis in vitro* resulted in increase in bacterial biomass over time during oil degradation. The rate of increase was apparently higher in cultures stimulated with sucrose than in the control (Fig. 4). The optical density of the test culture also increased with time. The attenuance levels increased steadily with the different concentrations of sugar and slightly higher levels were recorded for 15% sucrose concentration in days 12 and 15 (Fig. 5). The nutrient addition increased the bacterial cell numbers, optical density as well as the acidity (high decrease in the pH) of the test media between days 9 and 15 when compared with the rate derived from the test medium (Fig. 6).

3.3 Petroleum Hydrocarbons Degradability of the Test Isolate

In vitro degradation study carried out for the 15 days showed that, the degradation of crude oil and its component by *B. subtilis* was faster when stimulated with the different concentrations of sugar than when carried out alone. Summary of the petroleum hydrocarbon components remaining after degradation is presented in Table 3. The result has showed a remarkable reduction in the total petroleum content of the test substrates treated with glucose and sucrose. The

best results were obtained by treatment with 1 and 5% levels of the stimulants. At this level, the TPH content was reduced from 15.81 mg/kg observed for the control to 10.19 mg/kg and 8.03 mg/kg obtained from substrates stimulated with 5% glucose and 1% sucrose respectively.

4. DISCUSSION

Atlas and Bartha [24] opined that microbial distribution in the biosphere is based on natural selection. These microorganisms play a vast number of important roles in the decomposition of organic matter, mineralization, elements recycling and transformation due to their versatile metabolic potentials. These metabolic potentials enable the organisms to adapt and survive even in the presence of environmental pollutants. Humic substances (HS) represent the main carbon reservoir in the biosphere, estimated at 1600×10^{15} g C. Due to their crucial role and control of biogeochemistry of organic carbon in the global system, HS are therefore extremely important to the environmental processes [25]. Several literatures have also shown that humic substances have a modulating effect on microorganisms as well as increase growth rate of many forms of beneficial microorganisms in part by stimulating enzyme activities and assist in their nutrition by complexing and delivering trace elements like iron to microbial cell surfaces [26-28]. The microbial inoculant used in this study was isolated from a humic ecosystem using enrichment culture technique. It is a *Bacillus subtilis*- HSC1 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,
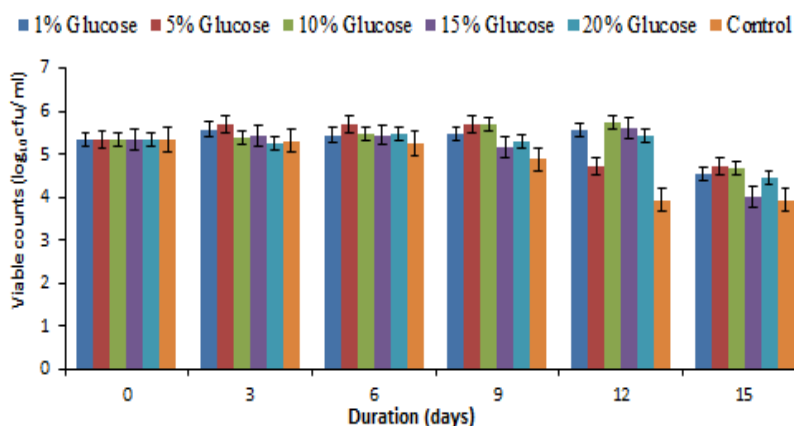

Fig. 1. Changes in total viable count of Bacillus subtilis stimulated with glucose during a 15 day-hydrocarbon degradation course
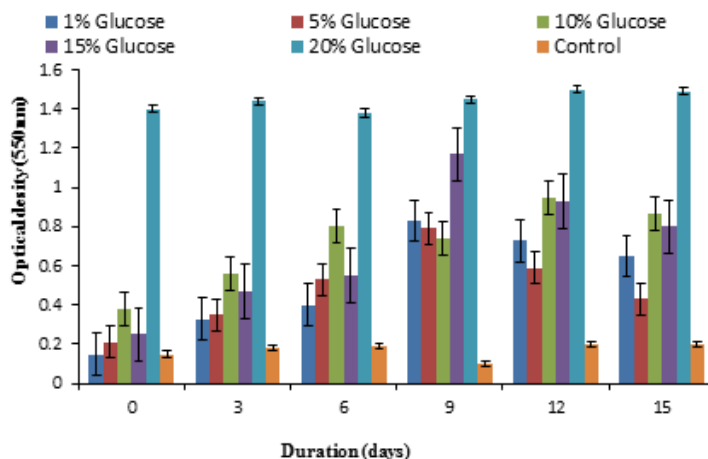
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Fig. 2. Changes in optical density of *Bacillus subtilis* growth substrate stimulated with glucose during a 15 day-hydrocarbon degradation counse
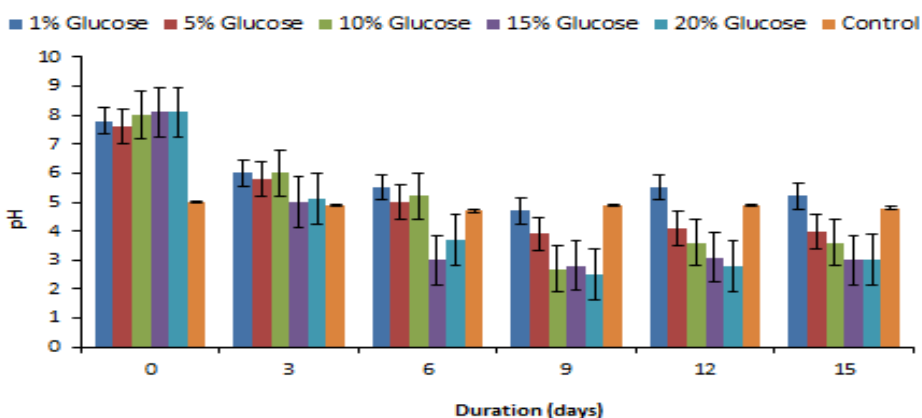

Fig. 3. Changes in pH of *Bacilius subtilis* growth substrate stimulated with glucose during a 15 day-hydrocarbon degradation course

oxidase, mannitol, methyl and indole. *B. subtilis* has been previously reported to be a good crude oil degrader [29]. It has been hypothesized that *Bacillus* generally adapts to nutrient-limited conditions and consequently do not fluctuate in response to hydrocarbon enrichment [30,31] which normally causes nutrient deficiencies. Also, similar findings have revealed that *Bacillus* species are more tolerant to high levels of hydrocarbons in soil due to production of resistant endospores [32].

In this study, the potential of *Bacillus subtilis* HSC-1 to utilize hydrocarbons was assayed in the laboratory using indirect (the optical density, pH and total viable count) and direct procedures as indices of degradation. The results obtained revealed increase in total viable counts and

corresponding optical density during the degradation of crude oil. Under aerobic condition, terminal oxidation of hydrocarbons is most common metabolic pathway employed by bacteria [33]. Following the terminal oxidation, alcohol is oxidized to corresponding aldehyde and fatty acid by means of pyridine nucleotide linked dehydrogenases. Therefore, microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products [34-36]. This implies that utilization of the crude oil as sole carbon and energy source by the oil degrader resulted in their growth with a concomitant production of acid. These acidic metabolic products might account for the decrease in pH of the cultures. The reduction in pH of the culture fluids in the experimental flasks within the 15-day incubation period further

confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes [37].

The result of crude oil index of *in vitro* hydrocarbon degradation potential of *Bacillus subtilis* HSC-1 when stimulated with different
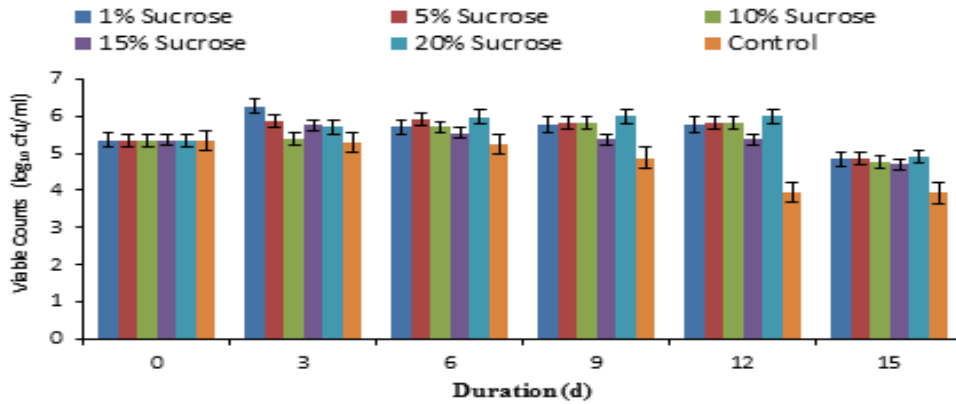

Fig. 4. Changes in total viable count of *Bacillus subtilis* stimulated with sucrose during a 15 day-hydrocarbon degradation course
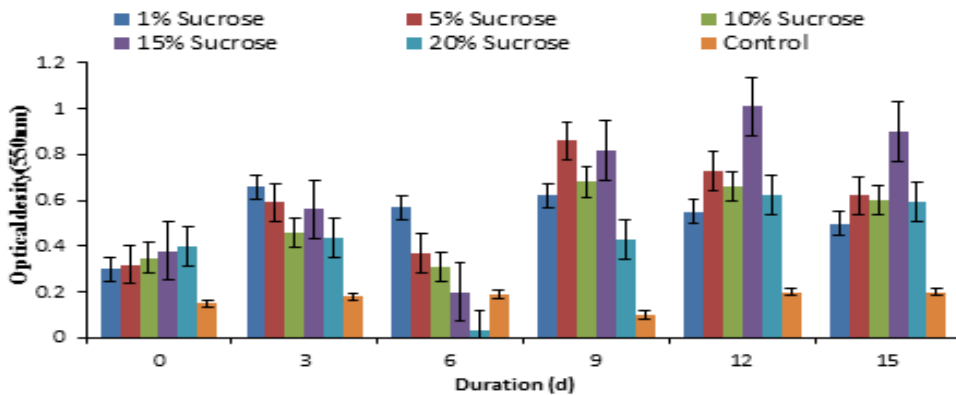

Fig. 5. Changesin optical density of *Bacilius subtilis* growth substratestimulated with sucrose during a 15 day-hydrocarbon degradation course
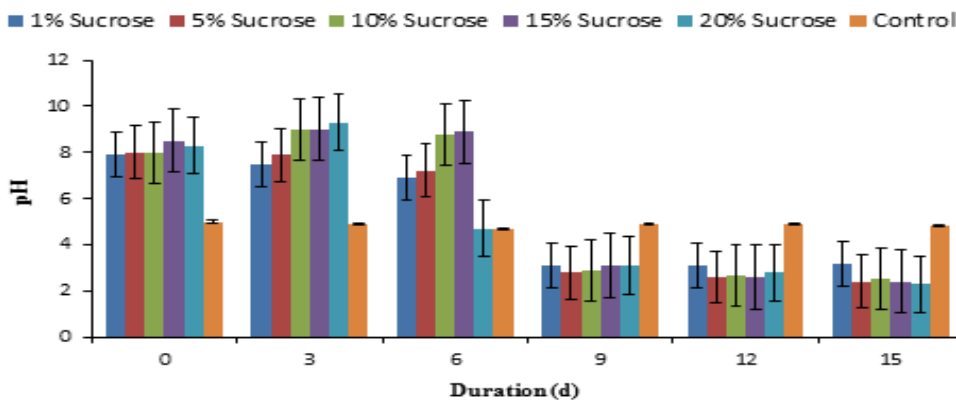

Fig. 6. Changes in pH of *Bacilius subtilis* growth substrate stimulated with sucrose during a 15 day-hydrocarbon degradation course

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concentrations of sugar showed observable differences in the bacterial counts from the process of biostimulation. The counts of *Bacillus subtilis* in the biostimulated medium were appreciably higher compared to those of non-amended control medium. The reason for higher counts of bacteria in amended medium might be as a result of presence of appreciable quantities of nutrients in the biostimulants [38]. However, although increase in amount of stimulants corresponded with increase in microbial cells density, it did not translate to higher rates of hydrocarbons degradation. It implies that the ability of *B. subtilis* HSC-1 to grow rapidly and produce high optical density might necessarily be an indication of its hydrocarbon degrading capability. Similar assertion has been made by Itah and Essien [39,40]. Itah and Essien [39] reported that the hydrocarbonclastic activities of microorgansims is greatly determined by the

ability of the organisms to elaborate the vital enzymes required for the decomposition of the recalcitrant components of hydrocarbons rather than being nutritionally fastidious.

In vitro study carried out for the 15 days showed that, the degradation of crude oil and its components by *B. subtilis* HSC-1 was faster when stimulated with the different concentrations of sugar than when carried out alone. Crude oil degradation by non-stimulate culture (control) resulted in 22.27% degradation by reducing the total petroleum hydrocarbon (TPH) content of crude oil from 20.3467 to 15.8165 mg/l within 15 days. The degradation by biostimulated cultures resulted in 60.52%, 56.41% degradation from 20.3467 to 8.0326, 8.8671mg/l for 1% and 5% sucrose respectively while glucose enhanced degradation by 47.30, 49.92, and 46.51% by reducing the TPH to 10.7219, 10.1905 and

Table 3. Level of petroleum hydrocarbons degradation by *Bacillus subtilis* (test isolate) stimulated by sugars

Parameter	Amounts (mg/l)						
	1% Glucose	5% Glucose	20% Glucose	1% Sucrose	5% Sucrose	Control (Unstimulated)	Control 2
C8							
C9							
C10							
C11	0.0241	0.0099					0.4280
C12	0.5211	0.4215		0.0145			0.4288
C13	0.9542	0.6325	0.0214	0.2145			0.5688
C14	4.0211	3.6521	0.1563	1.1024			3.1225
C15	0.9113	0.6542	0.1178	0.3652	0.2116	0.2256	1.1262
C16	0.2155	0.2044	0.1524	0.2127	0.1524	-	1.1265
C17	0.0584	0.0335	0.2058	0.0652	0.5422	0.4102	0.0568
C18	0.0045	0.0049	0.0101	0.0049	0.0063	-	1.4522
C19	3.1023	3.1598	4.0248	3.8342	3.9210	4.1122	1.9856
C20	0.6524	0.2154	1.0528	0.5228	0.6523	1.2154	0.2587
C21	0.1225	0.0141	0.3122	0.5113	0.6104	1.0217	2.0145
C22	0.0085	0.0102	0.2038	-	-	-	1.0258
C23	0.0547	0.1113	1.1048	0.4002	0.5112	0.9687	1.0699
C24	0.0365	0.1324	1.0455	0.3241	0.5009	1.0698	0.9856
C25	0.0035	0.0023	0.0254	-	-	-	0.6225
C26	0.0085	0.2567	0.5122	0.1114	0.4125	0.9614	0.521
C27	0.0101	0.2154	0.6918	0.1982	0.4217	0.9254	0.4552
C28	0.0112	0.1457	0.5922	0.1421	0.3937	0.6280	0.5114
C29	0.0036	0.0127	0.3627	0.0089	0.2046	0.5002	0.412
C30	-	-	0.1956		0.1253	0.4227	0.3245
C31	0.0015	-	0.0965		0.1025	0.2014	0.8489
C32	-	-			-	-	0.1524
C33	-	-			0.0985	0.1538	0.8489
Total	10.7219 (47.30)	10.1905 (49.92)	10.8841 (46.51)	8.0326 (60.52)	8.8671 (56.41)	15.8165 (22.27)	20.3467 (100)

Key: (Percentage degradation)

10.8841 mg/l for 1%, 5% and 20% glucose stimulated cultures respectively. The concentrations of the petroleum hydrocarbons components remaining after degradation showed a remarkable reduction in the total petroleum content of the test substrates treated with glucose and sucrose. The best results were obtained by treatment with 1% and 5% levels of the stimulants. At this level, the TPH content was reduced from 15.81 mg/kg recorded for the control to 10.19 mg/kg and 8.88 mg/kg obtained for substrates stimulated with 5% of glucose and 1% sucrose respectively.

The maximum hydrocarbon degradation by *Bacillus subtilis* was achieved in the presence of sucrose as a carbon source (60.52%, and 56.41% petroleum hydrocarbons) than in glucose. This finding has shown that the disaccharide sugar (sucrose) gave a better result as biostimulant than the monosaccharide sugar (glucose) and agrees with the report by Bayoumi et al. [41] that maximum degradation of crude oil occurred in the presence of sucrose. It however contradicts the earlier report by Batista et al. [42] that glucose is a better carbon source than fructose and sucrose for biodegradation of crude-oil by bacteria. The present research findings have shown that an effective and more sustaining remediation of crude oil-contaminated soil could be attained by the application of disaccharide-rich organic amendments.

5. CONCLUSION

The results of this study have shown that humic fresh water ecosystem of Eniong River is endowed with bacteria with strong hydrocarbonoclastic degrading potentials. Among them is *Bacillus subtilis*. The degradation of hydrocarbons by *Bacillus subtilis* stimulated cultures after 15 days were observed to be faster when stimulated with the different concentrations of sugar than when carried out alone. Crude oil degradation by non-stimulate culture (control) resulted in 22.27% degradation by reducing the total petroleum hydrocarbon (TPH) content of crude oil from 20.3467 to 15.8165 mg/l within 15 days. The concentrations of the petroleum hydrocarbons components remaining after degradation showed a remarkable reduction in the total petroleum content of the test substrates treated with glucose and sucrose. The best results were obtained by treatment with 1% and 5% levels of the stimulants. At this level, the TPH content was reduced from 15.81 mg/kg recorded for the control to 10.19 mg/kg and 8.88 mg/kg

obtained for substrates stimulated with 5% of glucose and 1% sucrose respectively.

The maximum hydrocarbon degradation by *Bacillus subtilis* was achieved in the presence of sucrose as a carbon source (60.52%, and 56.41% petroleum hydrocarbons) than in glucose. The study has also shown that the stimulation of cultures of *Bacillus subtilis* with disaccharide-rich organic amendments would readily enhanced degradation of hydrocarbons. The potential of this bacterial community coupled with the biostimulation protocol can be explored for broader use in remediating crude oil polluted environments, a condition which is inherent and of high concern in Niger Delta region of Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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