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Research article

Crude oil degradation potential of bacteria isolated from oil-polluted soil and animal wastes in soil amended with animal wastes

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Abstract: The influence of animal wastes on crude oil degradation potential of strains of *Proteus vulgaris* and *Bacillus subtilis* isolated from animal wastes (poultry and pig droppings) and petroleum-polluted soil was compared in laboratory studies. Both bacterial strains were selected for high crude oil degradation ability after screening many isolates by the 2,6-dichlorophenol indophenol method. Analyses by gas chromatography (GC) showed that degradation of crude oil was markedly enhanced (88.3–97.3% vs 72.1–78.8%) in soil amended with animal wastes as indicated by the reduction of total petroleum hydrocarbon (TPH). TPH reduction by animal waste bacterial strains in animal waste-amended soil was more than the reduction by strains from soil contaminated with petroleum ($P < 0.001$). The greatest reduction of TPH (96.6–97.3% vs 80.4–95.9%) was by poultry waste strains and it occurred in soil amended with poultry waste. GC analyses of n-alkanes showed that although shorter chains were preferentially degraded [32.0–78.5% (C8–23) vs 6.3–18.5% (C24–36)] in normal soil, biodegradation of longer chains increased to 38.4–46.3% in animal waste-amended soil inoculated with the same animal wastes' strains. The results indicate that these animal waste strains may be of potential application for bioremediation of oil-polluted soil in the presence of the wastes from where they were isolated.

Keywords: poultry wastes; pig wastes; crude oil; biodegradation; *Proteus vulgaris*; *Bacillus subtilis*

1. Introduction

Oil exploration and exploitation activities in addition to refining and distribution often lead to spillages that pollute the aquatic and terrestrial environment. The ecological impact of petroleum

pollutants have been well documented [1-4]. Decontamination of oil-polluted environment involves mechanical, chemical and biological measures. Apart from cost, mechanical and chemical methods of treatment can lead to incomplete removal of the hydrocarbons [5] and the chemical dispersants and surfactants often used, may be toxic to the environment [6]. Microbiological techniques involve bioremediation which is a less expensive and ecologically friendly alternative for restoring the integrity of contaminated soils. It is a natural process that relies on the metabolic activities of microorganisms to degrade pollutants. A variety of microorganisms are known to be capable of degrading hydrocarbons [7], but the extent depends on the availability of nutrients such as nitrogen and phosphorus [8]. Studies have shown that addition of nitrogen and phosphorus in form of fertilizers enhances biodegradation of hydrocarbons [9]. Animal wastes (poultry droppings, cow and pig dung) as less costly source of nutrients [6] have also been used because they contain nitrogen, phosphorus and trace elements needed for microbial metabolism [10-12]. The animal wastes also contain a variety of microorganisms that may combine with the resident microflora of oil-polluted environment to degrade hydrocarbons.

However, researchers often ignore the possibility that the microflora of the animal may degrade hydrocarbon as much as the resident microflora of oil-polluted environments. This study was therefore designed to test two bacterial isolates from animal wastes (poultry and pig droppings) for crude oil degradation potential; and compare their crude oil degradation capability with that of the same bacterial species isolated from soil with history of petroleum contamination in the presence and absence of the animal wastes.

2. Materials and Methods

2.1. Isolation of hydrocarbon-utilizing bacteria

Soil samples were obtained from the oil-producing Niger Delta soil in an area with a history of petroleum spillage based on personal knowledge. The soil samples were collected with polythene bags that were previously sterilized by immersion in 3.5% (w/v) sodium hypochlorite solution for 12 hours. The soil was collected at depths of 5, 10 and 15 cm and bulked together for homogeneity. Thereafter, 1 mL aliquot of the serially diluted soil was used to streak mineral salts agar incorporated with crude oil (crude oil, 10 mL; KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; NH_4NO_3, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_2$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; distilled water, 1000 mL, pH 7.0). The crude oil (Escravos light) used was obtained from Warri Refining and Petrochemical Company, Delta State, Nigeria. After 72 h incubation at room temperature (30 ± 2 °C) emerging colonies were transferred to Nutrient Agar plates and further sub-cultured for purification. The same procedure was used to isolate hydrocarbon-utilizing bacteria from poultry and pig wastes collected from Mannes Farms, New- layout, Ekpan, Delta State, Nigeria.

2.2. Selection of isolates with the best hydrocarbon-utilizing capacity

The 2,6-dichlorophenol indophenol method [13] was used to screen the crude oil degradation ability of the isolates. A loopful of each isolate was introduced into 7.5 mL of mineral salts medium containing 50 µL of crude oil before 40 µL of 2,6-dichlorophenol indophenol (DCPIP) was added and incubated at room temperature (30 ± 2 °C) for five days. The isolates, (two from each animal wastes and two from oil-polluted soil) that discoloured the medium in the shortest time were selected. The selection was confirmed by spectrophotometric analyses at 600 nm where the isolates that

caused the lowest absorbance were matched with those selected by the visual method. The organisms were subsequently identified by cultural, microscopic and biochemical tests based on Bergeys Manual of Determinative Bacteriology [14]. For the purpose of the comparative experiment on biodegradation of crude oil in soil, two identical bacterial species from each of the animal wastes and oil-polluted soil were chosen. This brought the number of bacterial strains used for the experiment to six.

2.3. Determination of some physical and chemical characteristics of test soil and animal wastes.

The pH of the garden soil and animal wastes used for the experiments was determined in distilled water using a digital pH meter. It was standardized with buffer solutions of pH 4, 7 and 9. Moisture content was determined by gravimetry based on oven dry weight. Total organic carbon, total nitrogen and phosphorus were analyzed by potassium dichromate, Kjeldhal and hypochlorate, methods, respectively [15].

2.4. Biodegradation of crude oil in soil with and without amendment with animal wastes

Garden soil samples weighing 500 g were placed in flasks and sterilized by autoclaving at 121 °C for 30 mins. Upon cooling, the soil in six replicate flasks was mixed with filter-sterilized crude oil at 50 mL/flask. Thereafter, the flasks were inoculated with 10 mL normal saline suspension of 10^6 test bacterial isolate. This inoculum size was determined by plate count on Nutrient Agar plates. The set-up was repeated for each of the six test bacterial strains and set aside on the laboratory bench at room temperature for six weeks. The flasks were manually turned over for aeration and moistened with 10 mL sterile tap water at weekly intervals. The control flasks were not inoculated. After incubation for six weeks, the flask contents were extracted with hexane and subsequently analysed for TPH by gas chromatography. The concentrations were recorded in ppm. The reduction of TPH was calculated by subtracting the concentration (ppm) in test flasks from that of control and expressing it as % loss. The n-alkanes were quantified with a range of C8–C36 and the reduction of each chain was similarly expressed as % loss. The above experiment was repeated with soils mixed separately with 50 g poultry or pig wastes. The animal wastes were also sterilized by autoclave at 121 °C for 30 mins. The control flasks were not inoculated with the test bacterial strains. The differences in TPH concentrations in all inoculated flasks and un-inoculated control flask were analyzed by one-way Analysis of Variance (ANOVA) and Tukey post hoc multiple comparison tests using SPSS version 22.

2.5. Growth of selected bacterial isolates in sterilized garden soil amended with animal wastes

Freshly obtained poultry and pig wastes samples were separately mixed with garden soil at a ratio of 1:1 and dispensed into 50 mL flasks in 10 g quantities before sterilisation by autoclave at 121 °C for 30 mins. The control flask contained garden soil only. Each of the six bacterial strains from the three sources was used to inoculate three separate flasks as illustrated in Table 1. This brought the total number of flasks inoculated to 18. A 1 mL normal saline suspension of 10^6 bacterial strains served as inoculum. The flasks were manually shaken to ensure even mixture. The overall set up was in three replicates. The flasks were incubated at room temperature for 14 days and moistened with 1 mL sterile tap water on the 7^{th} day. At intervals of two days, 1 g samples were aseptically withdrawn and analysed for bacterial population by plate counts on Nutrient Agar after appropriate serial dilutions.

Table 1. Inoculation arrangement for determining the growth of test bacteria in soil amended with animal wastes.

Bacteria	Source of bacteria	Medium		
		Soil + poultry waste	Soil + pig waste	Soil only (control)
P. vulgaris	Petroleum-contaminated soil	+	+	+
	Poultry waste	+	+	+
	Pig waste	+	+	+
B. subtilis	Petroleum-contaminated soil	+	+	+
	Poultry waste	+	+	+
	Pig waste	+	+	+

+, present

3. Results and discussion

Over 35 bacterial isolates were screened for crude oil degradation ability and 12 were selected based on their high identical crude oil degradation ability. The identity of the 12 strains is presented in Table 2. However, *Proteus vulgaris* and *Bacillus subtilis* were the only strains selected for subsequent experiments because they were the only identical isolates encountered in both petroleum-polluted soil and animal wastes (Table 2). It was considered more reliable to use identical strains for the purpose of comparing the influence of their sources (animal waste, petroleum-contaminated soil) on their crude oil biodegradation potential.

Table 2. Bacterial isolates with substantial crude oil degradation potential.

Source of bacteria	Bacteria
Oil-polluted soil	*Bacillus subtilis*
	Proteus vulgaris
	Acinetobacter sp.
	Arthrobacter sp.
	Bacillus cereus
	Klebsiella sp.
Poultry waste	*Proteus vulgaris*
	Bacillus subtilis
	Enterobacter aerogenes
Pig waste	*Bacillus subtilis*
	Proteus vulgaris
	Enterococcus faecalis

The degradation of crude oil by the three strains of *Proteus vulgaris* was significant when compared to control. However, there was no significant difference in the degradation caused by the three strains (poultry waste, pig waste, petroleum-polluted soil) in normal soil (Table 3). Over 75.5% of the total petroleum hydrocarbon (TPH) in both normal and amended soil was degraded by the bacterial strains. However, degradation of TPH in soil amended with animal wastes was significantly

greater than in normal soil. This was not unexpected, because previous reports show that biodegradation of petroleum hydrocarbon is enhanced in soil treated with animal wastes [11,12,16,17]. The results in Table 3 further show that degradation of crude oil by animal waste strains of *P. vulgaris* was significantly enhanced in soil amended with animal wastes when compared to degradation by the strain from oil-polluted soil. Degradation of crude oil in poultry waste-amended soil by the poultry waste strain of *P. vulgaris* was significantly more enhanced than degradation by the pig waste strain. However, such significant difference did not occur in soil amended with pig waste. The above trend was similarly encountered in the degradation of crude oil by the three strains of *Bacillus subtilis* as shown in Table 4.

Table 3. Biodegradation of crude oil in normal and animal waste-amended soil by *Proteus vulgaris* isolated from three sources.

Crude oil medium	Source of *P. vulgaris*	Mean TPH (ppm ± SD) after 6 weeks	Reduction of TPH (%)
Normal soil	Petroleum-contaminated soil	^{ab}20.5 ± 0.1	75.5
	Poultry waste	^{ab}19.2 ± 0.1	78.1
	Pig waste	^{ab}19.0 ± 0.1	8.8
	Control	104.5 ± 3.8	0.0
Poultry waste-amended soil	Petroleum-contaminated soil	^{ac}11.7 ± 0.02	88.6
	Poultry waste	^{ac}2.8 ± 0.01	97.3
	Pig waste	^{ac}4.8 ± 0.01	95.3
	Control	102.5 ± 4.0	0.0
Pig waste-amended soil	Petroleum-contaminated soil	^{ac}10.4 ± 0.05	89.9
	Poultry waste	^{abc}4.5 ± 0.02	95.6
	Pig waste	^{abc}4.2 ± 0.03	95.9
	Control	^{a}103.4 ± 3.6	0.0

Control = Not inoculated with *P. vulgaris* (See Materials and Methods). Significant difference: from control, $^{a}P < 0.0001$; between sources of *P. vulgaris*, $^{b}P > 0.05$, $^{c}P < 0.001$.

The observation that degradation of crude oil by the animal waste isolates was enhanced in the presence of animal wastes when compared to strains from petroleum-polluted soil suggests the influence of ecological niche adaptation. Going by this concept, highly enhanced biodegradation ability was expected from the petroleum-polluted soil strains given their frequent exposure to petroleum contaminants. But it was not the case. The plausible explanation lies in the adaptation ability of the bacterial isolates to exact nutrients from the animal wastes as biostimulants. The attendant population growth would therefore, result in greater attack on the petroleum hydrocarbon. The animal waste strains can therefore, be seen as better placed to exact the nutrients. The observation that degradation of crude oil by animal waste strains was enhanced in soil amended with the wastes lends credence to this inference. This deduction is supported by the results presented in Figure 1, which showed that the growth of the animal waste bacterial strains was markedly greater than that of petroleum-polluted soil strains in soil mixed with animal wastes. The influence of the nutrients in animal wastes and the greater degradation ability of the bacterial strains from animal wastes were further demonstrated by the results of biodegradation of n-alkane chains (Figures 2). All the bacterial strains expectedly preferentially degraded the shorter chains (C8–C23) in normal and animal

waste-amended soil as shown in Figure 2. The pattern of degradation of the alkane chains in normal soil by all the bacterial strains were not markedly different. However, there was marked increase in the degradation of the longer chains in soils amended with animal wastes when compared to biodegradation in normal soil. Compared to other strains, degradation of longer chains of n-alkane (C24–C36) by poultry waste strains in soil amended with poultry waste markedly increased. Degradation of longer chains by pig waste strains followed a similar pattern (Figures 2).

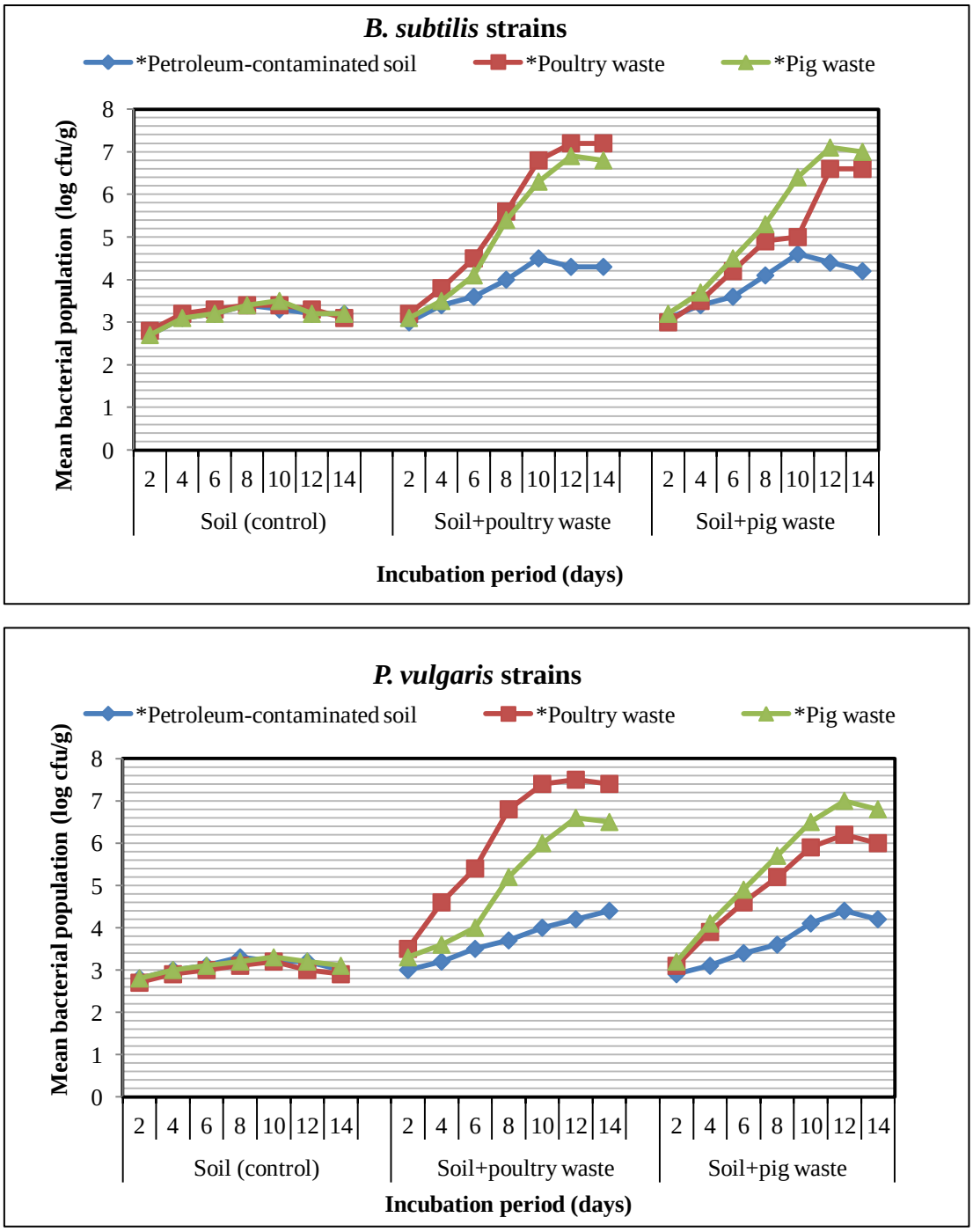

Figure 1. Growth of *P. vulgaris* and *B. subtilis* in soil and soil amended with animal wastes. *Source of strains of *P. vulgaris* and *B. subtilis*.

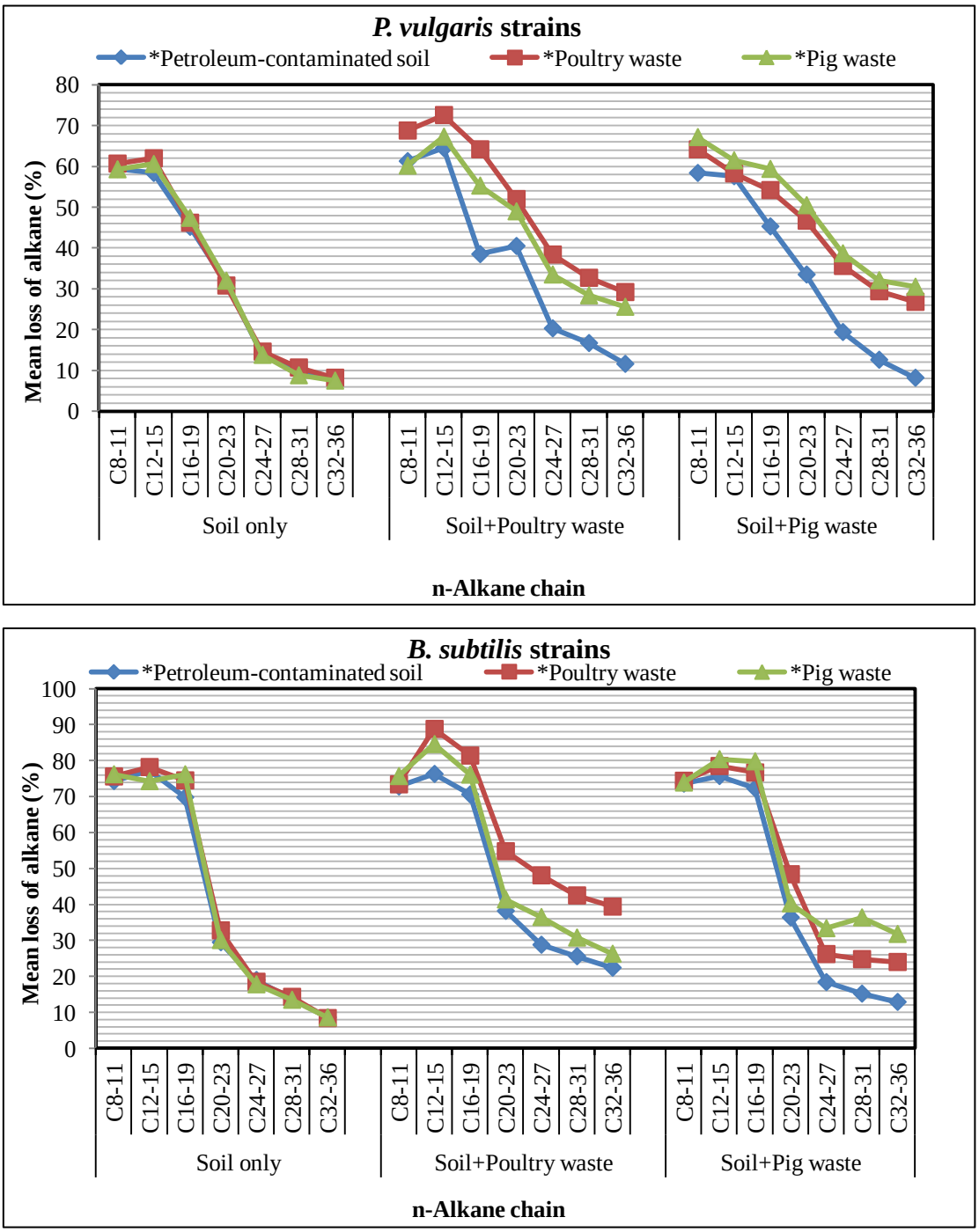

Figure 2. Loss of n-alkane after degradation of crude oil for 6 weeks by *Proteus vulgaris* and *B. subtilis* in soil and soil amended with animal wastes. *Source of strains of *P. vulgaris* and *B. subtilis*.

Table 4. Biodegradation of crude oil in normal and animal waste-amended soil by *Bacillus subtilis* isolated from three sources.

Crude oil medium	Source of *B. subtilis*	Mean TPH (ppm ± SD) after 6 weeks	Reduction of TPH (%)
Normal soil	Petroleum- contaminated soil	[ab]18.7 ± 0.4	72.1
	Poultry waste	[ab]17.9 ± 0.3	72.8
	Pig waste	[ab]18.0 ± 0.3	74.7
	Control	104.5 ± 3.8	0.0
Poultry waste-amended soil	Petroleum-contaminated soil	[ac]13.4 ± 0.1	89.1
	Poultry waste	[ac]3.4 ± 0.01	96.6
	Pig waste	[ac]6.5 ± 0.01	93.6
	Control	102.5 ± 4.0	0.0
Pig waste-amended soil	Petroleum-contaminated soil	[ac]12.0 ± 0.1	88.3
	Poultry waste	[ac]4.3 ± 0.02	95.8
	Pig waste	[ac]7.0 ± 0.03	93.2
	Control	103.4 ± 3.6	0.0

Control = Not inoculated with *B. subtilis* (See Materials and Methods). Significant difference from control: [a]$P < 0.0001$; between sources of *B. subtilis*, [b]$P > 0.05$, [c]$P < 0.001$.

While it is acknowledged that preferential degradation of hydrocarbons by microorganisms depends on their metabolic machinery, inadequate or absence of vital nutrients (nitrogen and phosphorus) would generally impede their metabolism [18] irrespective of their hydrocarbon specificity. It is known that alkanes of C10–C24 length are more easily degraded [19] hence microorganisms tend to begin degradation with these intermediate chain lengths before the longer chains. Depending on the environment, the vital nutrients may become exhausted before the attack on the longer chain hydrocarbons begins. Thus the ability of the organism to exact nutrients from available sources such as animal wastes is likely to promote attack on longer chains as indicated by the results of this study. Some reports [10-12] have shown that animal wastes contain nitrogen, phosphorus and minerals that can act as biostimulants. The results of the analyses of some of the physical and chemical characteristics of the test garden soil and the animal wastes showed that concentrations of nitrogen and phosphorus were markedly higher in the animal wastes than in test soil (Table 5). Indeed phosphorus was not detected in the test soil. The alkaline pH of the animal wastes would be favorable to the strains than the acidic pH of the test soil, because bacteria tend to thrive better in neutral to alkaline environment.

The results indicated that the animal waste strains may have become adapted to the physical and chemical characteristics of the wastes (Table 5) hence they thrived better than the strains from soil polluted by petroleum hydrocarbon in the presence of the animal wastes. After all, the animal waste nutrients were also available to the petroleum-polluted soil strains. The specificity of adaptation was further indicated by the observation that each of the animal waste strains tended to degrade n-alkane better in the presence of the waste from which they originated. Adaptation is a natural phenomenon that ensures the survival and perpetuation of organisms in their habitats where they face physical and chemical challenges. Survival therefore depends on the ability of the organisms to develop appropriate metabolic mechanisms that will enable them overcome the physical and chemical hurdles in their environment [20].

Table 5. Some physical and chemical properties of the soil and animal wastes used for hydrocarbon biodegradation tests.

Physico-chemical parameters	Soil	Source of animal waste	
		Poultry	Pig
pH	6.3 ± 0.11	8.7 ± 0.57	8.3 ± 0.13
Moisture content (%)	3.1 ± 0.14	20.9 ± 0.71	23.7 ± 0.16
Total organic carbon (%)	27.1 ± 0.01	44.5 ± 0.01	44.6 ± 0.00
Total Nitrogen (%)	0.05 ± 0.00	0.12 ± 0.01	0.09 ± 0.01
Phosphorus (%)	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

Values in the table represent Mean ± SD.

4. Conclusion

The results of the investigation indicate that the microflora of animal wastes are prominent in biodegradation of hydrocarbon in polluted soils where animal wastes have been applied for biostimulation. The greater growth of the animal waste strains in animal waste-amended soil when compared to petroleum-polluted soil strains increased the attack on the crude oil. Thus bioaugmentation and biostimulation may be achieved in bioremediation of petroleum-polluted sites if it includes the use of animal wastes such as poultry droppings and pig dung.

Conflict of interest

The research was self-funded. The authors declare that there is no conflict of interest.

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