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Full Length Research Paper

Biodegradation of *Lagoma* crude oil using pig dung

Yakubu, M. Bello

School of Science, College of Education, PMB 39, Minna, Nigeria. E-mail: bellyms@yahoo.com. Tel: +234-66-222205, +234-805-046-7560.

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Pig dung bacteria were isolated and screened for crude oil degrading capabilities. The pig dung was also investigated for enhancement of crude oil biodegradation. Addition of chicken manure to oil polluted soil (at 10% (v/w) pollution level) stimulated the biodegradation of lagoma crude oil used in the present study. In the soil amended with pig dung, 68.2% of the crude oil was degraded, whereas only 50.7% of same oil was degraded in the unamended soil. The pH of the amended soil rose from 6.2 to 7.2. Pig dung was found to contain 1.7 x 10^6 cfu g^{-1} crude oil degrading bacteria, and 1.8 x 10^8 cfu g^{-1} aerobic heterotrophs. The crude oil utilizing bacteria were identified as species of *Pseudomonas, Proteus, Klebsiella, Bacillus* and *Micrococcus*. Pig dung could therefore be an option for crude oil pollution mitigation project.

Key words: Pig dung, biodegradation, crude oil, pollution, nutrients, amended soil.

INTRODUCTION

Oil released into the environment is a well-recognized problem in today's world. Oil spills affect many species of plants and animals in the environment, as well as humans (Plohl et al., 2002). The involvement of microorganisms in the degradation of petroleum and its products in the environment has been established as an efficient, economic, versatile, and environmentally sound treatment (Margesin and Schinner, 2001). The microorganisms implicated in oil degradation are widely distributed in nature and have been isolated from soil and water ecosystems with their oil degrading potentials investigated.

The search for effective and efficient methods of oil removal from contaminated sites has intensified in recent years, because microbial degradation that is responsible for clearing untreated oil spills is slow (Grangemard, et al., 2001). One promising method that has been researched into is the application of chemical fertilizers to augment for the mineral elements, particularly nitrogen and phosphorous' limitations in the soil during biodegradation (Margesin and Schinner, 1999). These fertilizers, especially in developing countries are not sufficient for agriculture, let alone for clearing oil spills.

They also tend to result in soil hardening and fertility decline. It therefore, necessitates the search for cheaper and environmentally friendly options of enhancing crude oil biodegradation. The present study is therefore to investigate the potential of pig dung bacteria for crude oil degradation, as well as utilizing the pig dung for the probable stimulation of crude oil biodegradation in the soil.

MATERIALS AND METHOD

Collection and processing of samples

The crude oil used was lagoma light crude oil, which was collected in sterile sampling bottles from Kaduna Refining and Petrochemical Company, Kaduna, Nigeria. The soil sample used was collected from the main campus of Wuhan University, Wuhan, PR China, transported to the laboratory and processed for use in the bioremediation studies. The sample was air dried and sieved through 2 mm mesh size.

The pig dung used was collected fresh from a pigsty at Eastlake, Wuhan, China. The dung was collected in thick polythene sheets and transported to the laboratory, and then sun dried for several days. After drying, the samples were ground and stored in the laboratory for further use. The biological and physicochemical properties of the pig dung used in this work were determined (Ijah and Antai, 2003)

Isolation and identification of bacteria

Isolation of bacteria was done following the modified methods of Solano-Solano et al. (2000). Ten grams of air-dried pig dung were thoroughly suspended in 100 ml sterile tap water, and then the supernatant was serially diluted with sterile tap water. Zero point one milliliter each of a serially diluted (10^{-7}, 10^{-5}, 10^{-3} and 10^{-1}) pig

Table 1. Biological and physicochemical properties of pig dung.

Descriptor	Value[m]
pH	7.3 ± 0.2
Moisture (%)	8.6 ± 0.9
Nitrogen (%)	2.1 ± 0.6
Phosphorous (%)	1.6 ± 0.3
Calcium (%)	0.2 ± 0.1
Magnesium (%)	0.5 ± 0.1
Sodium (%)	1.9 ± 0.9
Potassium (%)	1.8 ± 0.3
Aerobic heterotrophic bacteria	1.8×10^8 CFU g^{-1}
Crude oil utilizing bacteria	1.7×10^6 CFUg^{-1}

[m]Mean of three determinations.

dung samples were spread inoculated onto nutrient agar (NA) and mineral salts medium supplemented with the crude oil (oil agar, OA) for the isolation of bacteria and enumeration of crude oil degrading bacteria respectively. The mineral salts medium (MSM) of Bushnell and Haas (1941) was employed, which has the following composition: (1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2.2H_2O$ in 1000 ml of distilled water at pH 7.0). The inoculated NA plates were incubated at room temperature (28 ± 2°C) for 48 h, while the OA plates were incubated for 3 to 5 days at the same temperature. The bacterial colonies that appeared on the plates were counted, and distinct colonies were picked and purified by repeated sub-culturing.

A number of microscopic and biochemical tests were carried out for identification purposes. The tests included gram reaction, shape, spore, motility, catalase, indole production, oxidase, methyl red-voges proskauer (MR-VP), gelatin liquefaction, nitrate reduction, urease, citrate, starch hydrolysis, O/F of glucose, pigmentation and utilization of the following carbohydrates – glucose, sucrose, fructose, maltose, xylose, arabinose, mannitol and inositol. The isolates were identified by comparing their characteristics with those of known taxa, as recommended by Cowan and Lisbon (Ijah and Antai, 2003).

Utilization of crude oil by the bacterial isolates

The ability of the bacterial isolates to utilize crude oil as the only source of carbon and energy was determined by the method of Okpokwasili and Okorie (1988). 0.1 ml of 24 h old nutrient broth culture was inoculated into each test tube containing 10 ml of sterile MSM of Bushnell and Haas and 1% (v/v) crude oil. Control test tubes were set up containing 10 ml of MSM with 1% (v/v) crude oil, but had no added bacteria. The tubes were incubated at room temperature for sixteen days on an orbital shaker (HYA, Scientific Instrument Manufacturer, Wuhan) at 120 rpm. At the end of the incubation period, the growth of the isolates was determined by visual observation of the oil medium for turbidity, as compared to the control tubes (Olesnicky et al., 2002; Okpokwasili and Okorie, 1988).

The extent of degradation of the incorporated crude oil by the bacterial isolates was determined by the gravimetric analysis method of Odu (Ijah and Antai, 2003). The amount of crude oil left after the incubation time was determined by extracting the residual oil with 50 ml of toluene from the 10 ml culture. The mixture was separated using separatory funnel and then filtered off with What man filter paper. The optical density (absorbance) was read on a spectrophotometer (UV-120-02, Shimadzu, Japan) at 410 nm wavelength. Using a previously prepared standard curve, the weight of the crude oil was determined. The amount of crude oil degraded was calculated by subtracting the weight of residual crude oil from weight of the added (initial) crude oil, divided by the weight of the initial crude oil and then multiplied by 100.

Measurement of crude oil biodegradation in soil amended with pig dung

The rate of bacterial utilization of crude oil in the soil was assessed by using the the gravimetric method. 100 g of soil contained in screw-capped bottles in triplicates were each treated with 10% (v/w) crude oil. 10 g of pig dung were added to each bottle, and the soil moisture corrected by the addition of 10 ml of sterile double distilled water (Roman et al., 1996). A control experiment, without added chicken manure was set up. All the bottles were incubated for 16 days at room temperature. At four days' interval, the amount of weight loss of the crude oil was determined. 20 g of oil-polluted soil were weighed into sampling bottle and 100 ml of CCl_4 added to each bottle. The oil-solvent mixes were separated using a separatory funnel. The extracts were dried by adding 0.1g anhydrous Na_2SO_4 (Facundo, 2000; Mulligan et al., 2001), and then filtered through Whatman No. 1 filter paper. The CCl_4 was allowed to evaporate off at room temperature in a fume hood. Weight loss was determined as described above.

Gas chromatographic analysis was employed to confirm the results of the gravimetric method. The extractable crude oil was recovered and analyzed on gas chromatograph (hp, Paw) equipped with a CHROMOSORB, P*A*-AW 80 – 100 capillary column and flame ionization detector (FID). The operational parameters were: injection temperature of 400°C, helium carrier flow of 28 ml per minute, injection 1 µl. The oven temperature was set initially at 50°C min^{-1}. The major hydrocarbon compounds of the crude oil were identified on the basis of their retention time and by comparing them to those of analytical standards.

To determine the pH, 10 g of the sample was mixed with 25 ml of sterile water in a beaker, stirred and allowed to stand for 30 min. The pH was then taken with the pH meter (HC21006, China). The mixture was stirred again, allowed to stand and pH retaken. A triplicate determination was performed.

RESULTS

The biological and physicochemical properties of the pig dung are presented in Table 1. Twenty-seven bacterial isolates were obtained from the pig dung as shown in Table 2. The identification of the isolares revealed them to belong to the species of *Staphylococcus, Pseudomonas, Proteus, Acinetobacter, Campylobacter, Streptococcus, Klebsiella, Enterobacter, Bacillus, Micrococcus* and *Escherichia*. Bacillus species were the most frequent isolated bacteria, which constituted about 15% of the total isolates. The results in Table 2 reveal the extent of growth, as well as the amount of crude oil degraded by the bacterial isolates from pig dung. Twelve (44.4%) isolates, out of the total isolates were able to utilize crude oil as sole source of carbon and energy. A *Bacillus* sp degraded the crude oil at a relatively high rate of 63.5% after the incubation time of 16 days. The other crude oil utilizers belong to the genera of *Pseudomonas, Micrococcus, Proteus*, and *Klebsiella*. It is however,

Table 2. Extent of growth and degradation of crude oil by bacterial isolates from pig dung.

Bacterial isolates	Growth in crude oil MSM	Crude oil degraded (%)[m]
PGB07 *Bacillus sp*	+++	63.5 ± 1.4
PGB12 *Pseudomonas*	++	43.4 ± 2.2
aeruginosa	++	40.6 ± 1.3
PGB19 *Micrococcus sp*	++	39.7 ± 0.5
PGB27 *Bacillus sp*	++	37.6 ± 0.6
PGB03 *Bacillus sp*	+	22.8 ± 1.5
PGB05 *Pseudomonas sp*	+	15.5 ± 0.2
PGB09 *Micrococcus sp*	+	13.2 ± 0.8
PGB11 *Proteus sp*	+	13.2 ± 0.6
PGB15 *Klebsiella sp*	+	13.0 ± 0.4
PGB24 *Micrococcus sp*	+	11.5 ± 1.2
PGB23 *Proteus sp*	+	11.5 ± 1.1
PGB17 *Klebsiella sp*	-	0
PGB01 *Escherichia coli*	-	0
PGB02 *Bacillus sp*	-	0
PGB04 *Campylobacter sp*	-	0
PGB06 *Acinetobacter sp*	-	0
PGB08 *Enterobacter sp*	-	0
PGB10 *Proteus sp*	-	0
PGB13 *Campylobacter sp*	-	0
PGB14 *Enterobacter sp*	-	0
PGB16 *Streptococcus sp*	-	0
PGB18 *Staphylococcus sp*	-	0
PGB20 *Staphylococcus sp*	-	0
PGB21 *Campylobacter sp*	-	0
PGB22 *Pseudomonas sp*	-	0
PGB25 *Streptococcus sp*	-	0
PGB26 *Staphylococcus sp*		

+++ Heavy growth; ++ moderate growth; + little growth; - no growth. [m]Mean of three determinations.

Table 3. The amount of crude oil degraded in soil amended with pig dung.

Incubation time (days)	Weight loss (%)[m] of crude oil in soil	
	Pig dung	No pig dung
4	13.5 ± 0.5	10.4 ± 0.2
8	33.7 ± 1.8	24.3 ± 0.6
12	56.9 ± 2.2	38.6 ± 1.4
16	68.2 ± 1.9	50.7 ± 1.6

[m]Mean of three determinations.

shown that fifteen of the isolates were not able to degrade the crude oil.

The results of the weight loss of oil from the soil treated with crude oil and amended with pig dung are presented in Table 3. At the end of the incubation period, 68.2% of crude oil was degraded in soil amended with pig dung as against 50.7% of same oil in unamended soil after incubation period.

These were confirmed by the results obtained from gas chromatograph in Figure 1 (crude oil amended with pig dung) and Figure 2 (control; crude oil not amended with pig dung), where it is shown that the bacterial population contained in pig dung utilized almost all the components of the crude oil. The fractions of isoprenoids (pristane and phytane) were however, not attacked by the bacterial community in the pig dung after 16 days of incubation. The pattern nonetheless shows that the microorganisms first attacked the lower and higher hydrocarbon chains and those of middle length were attacked later in the course of incubation.

The pH values obtained in soils treated with crude oil and amended with pig dung reveal that the pH of control soil ranged between 5.6 and 5.8 (Table 4). While that of the unamended soil ranged between 5.7 and 6.0 after sixteen days of incubation. The pH of the amended soil increased from 6.2 at time zero of sampling to 7.2 over same period, which is from acidic to slightly alkaline pH (Table 4).

There were significant ($P < 0.05$) differences among the treatments and the period of incubation with respect to weight loss of the crude oil. A linear correlation was observed between the gas chromatographic rates of biodegradation and weight losses of crude oil in soils amended with chicken manure.

DISCUSSION

Many types of microorganisms have been reported to be present in pig dung, which include bacteria and fungi (Laukova, 2000; Mansour et al., 1999). This is in addition to the mineral nutrients, which include nitrogen and phosphorous in the sample for microbial growth and metabolism. In this present study, eleven genera of bacteria were identified from the pig dung sample used, which were mainly enterics. Though, *Bacillus* species was the most predominant isolated bacterial species, it prevalence could be attributed to the fact that it forms spores, which help microorganisms to withstand harsh conditions, such as sun drying employed in this work. Isolation of *Bacillus* species from animal dung could also be attributed to its ubiquitous distribution in nature. Mansour et al. (1999) reported the isolation of *Bacillus, Acinetobacter, Staphylococcus* and *Enterobacter* among other bacteria from pig dung.

There are volumes of literature on bacterial degradation of crude oil in the ecosystem. However, what is interesting is the source of the oil degrading bacteria in the natural ecosystem. It is interesting to find that pig dung, in addition to being rich in mineral elements nitrogen and phosphorous (Diez et al., 2001)] necessary for crude oil biodegradation, also contain bacteria with varying de-

Table 4. The pH reactions of soil treated with crude oil and amended with pig dung.

Incubation time (days)	pH values[m]		
	Non polluted, not amended soil	Crude oil polluted soil	Crude oil polluted soil amended with pig dung
0	5.6 ± 0.1	5.7 ± 0.1	6.2 ± 0.1
4	5.7 ± 0.0	5.8 ± 0.0	6.9 ± 0.0
8	5.8 ± 0.3	5.9 ± 0.1	6.9 ± 0.2
12	5.9 ± 0.1	6.0 ± 0.1	7.1 ± 0.4
16	5.8 ± 0.2	5.9 ± 0.2	7.2 ± 0.1

[m]Mean of three determinations.

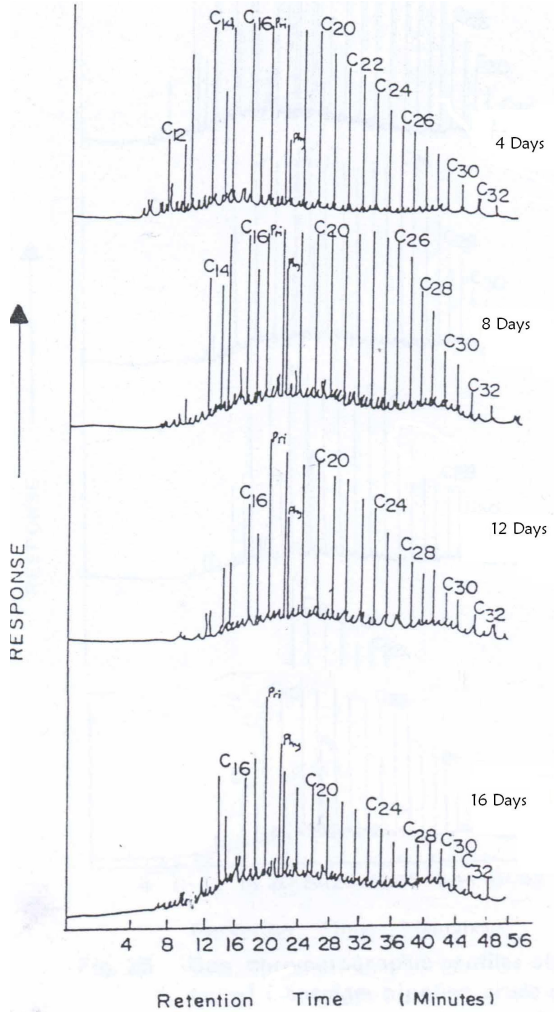

Figure 1. Gas chromatograph of lagoma light crude oil amended with pig dung after, 4, 8, 12 and 16 days of incubation at 28°C.

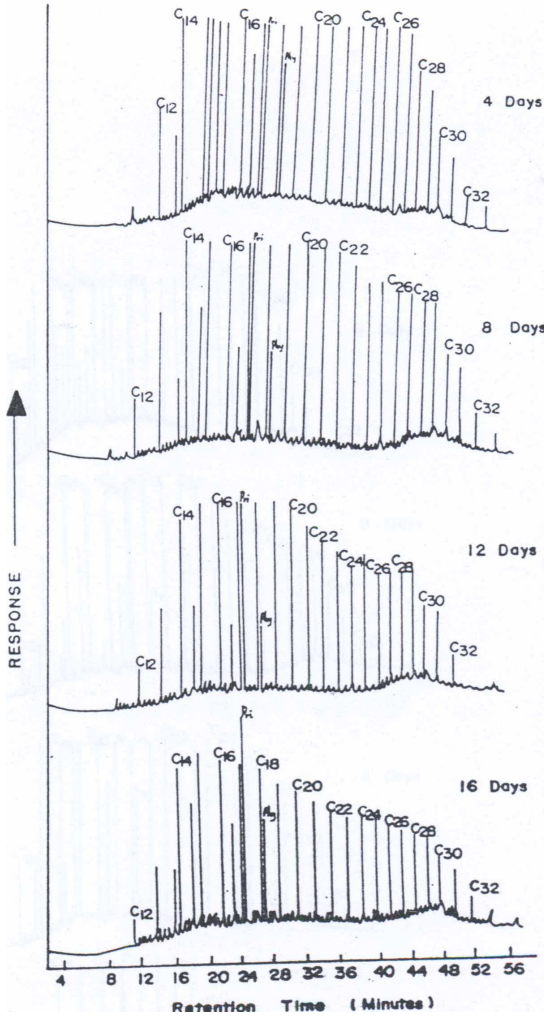

Figure 2. Gas chromatograph of lagoma light crude oil (control) not amended with pig dung after, 4, 8, 12 and 16 days of incubation at 28°C.

grees of crude oil degrading capabilities. This capability is shown here to be further widespread among the bacteria present in the pig dung. The oil-utilizing bacteria isolated from the pig dung in the present work have previously been implicated in crude oil biodegradation, though from different sources (Ijah and Antai, 2003; Ijah, 1998).

Stimulated biodegradation of crude oil is at present being encouraged because it ensures rapid remediation of oil-polluted ecosystems (Ijah and Antai, 2003). Most

often, chemical fertilizers are used to enhance microbial degradation of crude oil in the natural environments. Mineral elements have been reported to be essential for crude oil biodegradation (Mitchell, 1999; Atlas, 1995). Apart from the oil utilizing bacteria in animal dung, if manure is spread back on the land, not only will it add much needed nutrients, but also organic matter, which is not found in chemicals. Therefore, the addition of pig dung to the soil enhanced the rates of crude oil biodegradation.

Also, the bacterial consortium in the pig dung attacked and degraded almost all the components of lagoma light crude oil, except the fractions of isoprenoids (pristane and phytane) that were resistant to bacterial attack. Not a single bacterium can degrade all the component fractions of any crude oil, and significant oil biodegradation is normally achieved by microbial synergy. Pig dung could, therefore, harbour both primary and secondary crude oil biodegraders. As such, the use of pig dung may be a promising potential in integrated oil spill abatement.

Furthermore, the pig dung raised the pH of the soil to a range between 6.2 and 7.2, which is ideal for most crude oil utilizing bacteria (Vidali, 2001). This indicates that the pig dung had a buffering effect on the soil, and since strong acidity is a limitation in biodegradation, it would have contributed to the enhanced crude oil degradation in the soil. The use of pig dung to stimulate crude oil bio-degradation in the soil could be one of the severally sought environmentally friendly ways of eliminating petroleum hydrocarbon in the natural ecosystem.

REFERENCES

Atlas MR (1995). Bioremediation of petroleum pollutants. Inter. Biodeterior. Biodegrad. 35: 317-327.

Bushnell LD, Haas HF (1941). The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41: 653-673.

Diez JA, de la Torre AI, Cartagena MC (2001). Waste management evaluation of the application of pig slurry to an experimental crop using agronomic and ecotoxicological approaches. J. Environ. Qual. 30: 2165-2172.

Grangemard I, Wallach J, Marget-Dana R, Peypoux F (2001). Licenysin: a more efficient cation chelator than surfactin. Appl. Biochem. Biotechnol. 90:199-210.

Ijah UJJ (1998). Studies on relative capabilities of bacterial and yeasts isolates from tropical soil in degrading crude oil. Waste Manage. 18: 293-299.

Ijah UJJ, Antai SP (2003). The potential use of Chicken-drop microorganisms for oil spill remediation. The Environmentalist. 23: 89-95

Laukova A (2000). In vitro treatment of different isolates from cattle dung and pig slurry by nisin. Acta Vet Brno. 69: 147-151.

Mansour MA, Bottefroy D Linder A (1999). Inhibition of Bacillus lincheniformis spore growth in milk by nisin, monolaurin, and pH combinations. J. Appl. Microbiol, 86: 311-324.

Margesin R, Schinner F (2001). Bioremediation (natural attenuation and biostimulation) of Diesel-Oil-Contaminated soil in an Alpine Glacier Skiing area. Appl. Environ. Microbiol. 67(7): 3127-3133.

Margesin R, Schinner F (1999). A feasibility study for the in situ remediation of a former tank farm. World J. Microbiol. Biotechnol. 15: 615-622.

Mitchell JG (1999). In the wake of the spill: ten years after Exxon Valdez. Natl. Geogr. 195(3): 96-117.

Okpokwasili GC, Okorie BB (1988). Biodeterioration potentials of microorganisms isolated from engine lubricating oil. Tribol. Inter. 21: 215-217.

Olesnicky EC, Miller L, Fukunaga J, Laskin AI (2002). Biodegradation of brominated biphenyls by members of the genus Sphingomonas (C). In: American Society for Microbiology. 102nd General meeting, Salt lake city, Utah. pp. 19-23.

Plohl K, Leskovšek H, Bricelj M (2002). Biological degradation of motor oil in water. Acta Chim. Slov. 49: 279-289.

Roman R, Caballero R, Bustos A, Diez JA, Cartagena MC, Vallejo A. Caballero A (1996). Water and solutes movement under conventional corn in central Spain: I. Water balance. Soil. Sci. Soc. Am. J. 60: 1530-1536.

Solano-Serena F, Marchal R, Casarégola S, Vasnier C, Lebeault JM, Vandecasteele JP (2000). A Mycobacterium strain with extended capacities for degradation of gasoline hydrocarbons. Appl. Environ. Microbiol. 66(6): 2392-2399.

Vidali M (2001). Bioremediation. An overview. Pure Appl. Chem. 73(7): 1163-1172.