Indian Journal of Biotechnology
Vol 15, April 2016, pp 243-250

Characterisation of oil degrading bacteria from tailored compost containing crude oil sludge

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Received 26 October 2014; revised 10 April 2015; accepted 29 May 2015

In the present study, different bacteria with polycyclic aromatic hydrocarbon (PAH)-degrading capabilities were isolated from compost prepared from oil sludge and animal manures. These bacteria were isolated on a mineral base medium and mineral salt agar plates. A total of 31 morphologically distinct isolates were carefully selected from 5 different compost treatments for identification using polymerase chain reaction (PCR) of the 16S rRNA gene with specific primers (16S-P1 PCR (5′AGAGTTTGATCCTGGCTCAG3′) and 16S-P2 PCR (5′AAGGAGGTGATCCAGCCGCA3′). The amplicons were sequenced using 16S-PA SEQ (5/CTACGGGAGGCAGCAG3/) and sequences were compared with the known nucleotides from the GenBank. The phylogenetic analyses of the isolates showed that they belong to 3 different clades: *Firmicutes, Proteobacteria* and *Actinobacteria.* The bacteria identified were closely related to the genera *Bacillus*, *Arthrobacter*, *Staphylococcus*, *Brevibacterium*, *Variovorax*, *Paenibacillus*, *Ralstonia* and *Geobacillus.* The results showed that *Bacillu*s species were predominant in all composts. Based on the results of the degradation of the PAH in the composts and results of previous studies on bacterial degradation of hydrocarbons in oil, the characteristics of these bacterial isolates suggests that they may be responsible for the breakdown of PAH of different mol wt in the composts. Thus, they may be potentially useful for bioremediation of oil sludge during compost bioremediation.

Keywords: Animal manures, bioaugmentation, biodegradation, bioremediation, composting, oil sludge, PAHs

Introduction

Waste sludge encountered during crude oil refining and cleaning of oil storage vessels contains large amounts of hydrocarbons including aromatic hydrocarbons in the range of 1-40 carbon atoms[1]. Oil sludge has been classified by the United States Environmental Protection Agency (US EPA) as a hazardous organic complex[2]. Treatment of oil sludge can be achieved through physical, chemical and biological methods[3,4]. Of these, the biological method stands out as the most environmentally friendly option. A common biological approach is the use of microorganisms for the degradation oil sludge in the environment[5]. Microbial biodegradation is an effective and inexpensive approach to the degradation of petroleum hydrocarbons[6], it has minimal negative effect and may eliminate the waste permanently without long term liability, as such it has greater public acceptance.

Compost bioremediation, a controlled biological process used to breakdown organic contaminants is gaining importance as a bioremediation technology. The large amounts of organisms and nutrients present, and the high temperatures generated during the process have made the technology attractive to researchers in this field[7]. Compost bioremediation degrade oil into humus-like substances as well as inert products, such as, carbon dioxide, water and salts. In the present study, compost bioremediation was used to breakdown components of oil sludge. Results obtained from analysis showed that polycyclic aromatic hydrocarbons (PAHs; 2-6 rings) present in oil sludge were reduced by 66-80% in all co-composting experiments over a period of ten months, indicating that compost systems have the ability to degrade PAHs in the oil sludge. Microbial communities present in the compost are believed to be responsible for these degradative actions. Culture dependent and non-culture dependent methods are used to determine the composition of the metabolically active microbial community that plays a

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role in the degradation of pollutants[8]. These methods enable the identification of more than 90% microorganisms. Perhaps, the most widely used non-culture dependent method is the molecular method, which involves DNA extraction, PCR amplification and nucleotide sequence for identification of microbes[9,10]. It is a reliable method that can be used to detect diversity, quantity and sometimes viability of microbial contents of a sample[8,10].

The aim of the present study was to isolate and characterise bacteria present in a compost bioremediation system containing oil sludge.

Materials and Methods

Material Characterisation

Garden soil was air-dried and analysed for soil type, organic carbon content, total nitrogen content, total phosphorus content (C, N & P), soil pH and water holding capacity (WHC). Soil sample were also characterized using concentrated acid digestion method (CADM) with Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA). Oil sludge from a refinery was characterized by gas chromatography/mass spectrometry (GC/MS) method. The PAHs in samples were recovered using automated soxhlet extraction with dichloromethane and the extracts were quantified by GC/MS using US EPA 8270. The stock standard Restek Cat. No 8270-1 of 1000 ppm, which contained semivolatile mix, was used to prepare calibration standards of 10, 30 and 50 ppm using dichloromethane. The GC/MS was Agilent 7860GC system and 5975C MSD, equipped with a 7683B autosampler. The sampler syringe was 5.0 µL and splitless injection was 1.0 µL. The carrier gas used was helium 30 cm/sec and at a constant flow rate of 1 mL/min. The inlet mode was splitless, maintained at 260°C, purge flow was 50 mL/min at 0.5 min and gas saver was at 80 mL/min at 3 min. Inlet liner was the deactivated dual taper direct connect. The column was Agilent HP-5 ms ultra-inert 30 m×0.25 mm×0.25 µm film thickness. The oven program was started at 40°C for 1 min to 100°C (15°C/min), 10°C/min to 210°C (1 min), 5°C/min to 310°C, and it was held for 8 min. The detection was MSD source at 300°C, quadrupole at 180°C, transfer line at 290°C, scan range 45 to 450 amu. PAHs were identified by retention times matching to standards concentration. The value of the chromatogram was quantified using peak area integration. Animal manures, such as, cow, pig, horse, and poultry manures were collected from University of Pretoria, Onderstepoort, Pretoria, South Africa, and were characterized for organic carbon content, total nitrogen content, total phosphorus content. Bulking agent, wood-chips of 8-10 cm×0.3-0.5 cm was used to enhance aeration. Carbon tetrachloride (CCl4, 99.55%), molar mass 153.8236 g/mol, density 1.594 g/mL) was purchased from Merck, South Africa and used to dissolve the oil sludge before mixing with soil.

Table 1—Concentration of selected PAHs present in oil sludge

Selected PAH	Initial conc. (mg kg^{-1})
Naphthalene	95.32± 0.03
1-methyl naphthalene	205.81±10.14
2-methyl naphthalene	195.70±0.01
Acenaphtylene	5.05±0.1
Acenaphthene	7.94±0.96
Fluorene	23.11±0.96
Anthracene	40.44±0.69
Phenanthrene	1.44±0.49
Fluoranthene	1.44±0.46
Pyrene	10.83±1.33
Chrysene	44.77±4.07
Benzo[a] anthracene	1.44±0.59
Benzo[b] fluoranthene	21.66±2.1
Benzo[k] fluoranthene	2.17±0.6
Benzo[a]pyrene	7.22±1.12
Indenol (1,2,3-cd) pyrene	5.78±1.38

Values are mean of three actual values ± standard error

Preparation of Compost Mixture

Crude oil sludge was mixed with 4 kg of soil and 100 g sample was analysed for selected PAHs (Table 1). The mixture was divided into five and four parts were separately mixed with about 1 kg of either pig, cow, horse or poultry manures in a ratio of 2:1 (w:w). The fifth portion was used as the control with no manure added and incubated in PVC troughs at room temperature for a period of 10 months. Temperature, moisture content, pH and respiration of compost organisms were measured monthly. Organisms present in the compost were isolated and characterised by molecular methods.

Isolation of Degrading Bacteria

Mineral Salt Medium (MSM)

Enrichment of compost organisms was done in mineral salts medium containing (mg L^{-1}): 500 KH_2PO_4, 500 $MgSO_4.7H_2O$, 500 $NaH_2PO_4.H_2O$, 500 NH_4Cl, 4000 NaCl, 500 $NaHCO_3$ and 500 Na_2CO_3, and 1 mL trace elements solution containing (mg L^{-1} DW): 1500 $FeCl_2.H_2O$, 9000 NaCl, 197 $MnCl_2.4H_2O$, 900 $CaCl_2$, 238 $CoCl_2.H_2O$, 17 $CuCl_2.H_2O$, 287 $ZnSO_4$, 50 $AlCl_3$, 62 H_3BO_3,

24 $NiCl_2.6H_2O$, and 10 mL 10.18 M HCl (32%), which were dispensed into 250 mL Erlenmeyer flasks. All solutions were dispensed under aseptic conditions.

Enrichment Culture (EC)

Five samples (100 g) each were collected from different parts of compost at the end of 10 months incubation and pooled to make a composite sample. In each 250 mL Erlenmeyer flasks containing 100 mL sterile MSM spiked with 10 mL oil sludge, 15 g of compost from the pooled sample was added. Control experiments similar to the one described above but without oil sludge were set up. The flasks were closed with aluminium foil cotton wool bungs and incubated in the dark at 28°C on a rotary shaker at 150 rpm for 21 d. All the experiments were set up in triplicates. From each flask, 1 mL content was aseptically transferred into another set of 250 mL flasks containing 100 mL sterile MSM spiked with 10 mL of oil sludge and incubated for another 21 d at 28°C in a rotary shaker in the dark. At the end of each incubation period, samples were withdrawn from each flask for determination of concentration of selected PAHs by GC/MS with same conditions stated above. The final culture was used for the isolation and identification of the oil sludge degraders.

Mineral Salts Agar (MSA)

In 900 mL of mineral salts medium, 20 g of bacteriological agar was added and the mixture was sterilised in an autoclaved at 121°C for 15 min. The medium was allowed to cool to about 50°C before adding 1 mL of trace element solution, which was previously filter through 0.2 µm membrane filter (Millipore) before dispensing in Petri dishes under aseptic conditions. Each plate was overlaid with 50 µL of filter sterilized oil sludge.

Isolation of Oil Degrading Bacteria from Enrichment Cultures

Oil degrading bacteria were isolated from the EC by serial dilution (10^{-8}). A 0.1 mL aliquot of each diluent from the 10^{-6} to 10^{-8} dilutions was inoculated on the MSA plates. The plates were incubated for 21-28 d at 28°C and checked daily for bacteria growth. Distinct colonies from the MSA plates were transferred onto nutrient agar plates and incubated for 3 d at 28°C. Pure cultures were made from distinct colonies from nutrient agar plates by serial dilution (10^{-3}), plated on nutrient agar plate and incubated for 3 d at 28°C. Distinct colonies were further purified by streaking onto nutrient agar plates. Identification of the isolates was done by molecular techniques.

Nutrient Broth

Nutrient broth powder (16 g) was mixed in 1 L bottle containing 1000 mL distilled water, which was sterilised at 121°C for 15 min and allowed to cool at room temperature before 10 mL each was dispensed into autoclaved test tubes with cap. Then single colonies obtained on the nutrient agar plates were transferred into the 10 mL nutrient broth mixture and incubated for 3 d at 28°C.

Molecular Characterisation of Isolates

Genomic DNA Extraction of Bacteria Isolates

Genomic DNA extraction was conducted using cetyltrimethyl ammonium bromide (CTAB) method. About 1.5 mL of the pure cultures was transferred into 2 mL eppendorf tubes and centrifuged with a microcentrifuge (Eppendorf MiniSpin plus, Westbury, NY, USA) at 14000 rpm for 5 min. The pellets was recovered, and then resuspended in a solution containing 567 µL of Tris ethylene diamine tetraactic acid buffer (Tris EDTA or TE buffer), 30 µL of 10% sodium dodecyl sulphate (SDS), 3 µL of proteinase K (20 mg/mL) and incubated at 65°C for 1 h. Then 180 µL of 5 M NaCl and 80 µL of 10% CTAB solutions were added to the mixture and incubated for 10 min at 65°C. Equal volumes (400 mL) of phenol and chloroform were added to each tube and centrifuged at 14000 rpm for 15 min and then DNA was precipitated by adding 0.6 mL cold isopropanol to 300 µL of the supernatant in each tube. The precipitate was centrifuge at 14000 rpm for 15 min and DNA pellets was washed with 200 µL of 70% ethanol by spinning at 14000 rpm for 10 min. The supernatant was discarded and the DNA pellets were air-dried. About 100 µL of TE buffer was added to the dried DNA pellets, followed by 1 µL of RNAase and then incubated at 37°C for 60 min.

Polymerease Chain Reaction (PCR) and Sequencing

The master mix aliquot for the PCR was dispensed into individual PCR tubes and the different DNA samples were added to each tube. The negative control was used to check for contamination in the master mix. The PCR reagents in each tube amounted to 50 µL containing: buffer (5 µL), $MgCl_2$ (1.5 µL), primer1 (2 µL forward), primer2 (2 µL reverse), dNTP mix (1 µL), Dream Taq (0.25 µL), sterile sabax water (35.25 µL) and DNA samples (3 µL). The PCR reactions were performed using MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR products (20 µL each) were later cleaned up using 160 µL of 13% polyethylene glycol (PEG) 8000, 20 µL of 5 M NaCl solution and 200 µL of 70% ethanol. Cleaned PCR

products were sequenced. The sequences of the 16S rRNA region obtained were edited using BioEdit software and the edited sequences were copied in a fasta format. Blasting of sequences was done on National Centre for Biotechnology Information (NCBI) website.

Phylogenetic Analyses

The bacterial sequences from the five treatments, control, cow, horse, pig and poultry were aligned using online version of MAFFT software. The phylogenetic analyses were done using Mega 4 software and evolutionary distance of the isolates was computed using neighbour-joining (NJ) methods. The bootstrap consensus tree was inferred from 1000 replicates and all positions containing gaps as well as missing nucleotide data were eliminated from the data-set. *Vibrio cholerae* was used as the out-group.

Results and Discussion

The soil used in the present study was a sandy-loam soil. The initial pH of the soil was 5.56 and carbon, nitrogen and phosphate content was 102, 20 and 4.4 mg L^{-1}, respectively. The water holding capacity of the soil was 32.62%. Selected PAHs present in the oil sludge are shown in Table 1. The characteristics of the manures (pH of pig, 5.3; horse, 5.3; cow, 5.5; & poultry, 5.5), including carbon, nitrogen phosphorus content are shown in Table 2. A total of 31 morphologically distinct isolates were selected from the different compost treatments for molecular characterisation. They included 5 isolates from the control experiments, 2 from horse manure compost, 10 from cow manure compost, 5 isolates from poultry manure compost and 9 isolates from pig manure compost. The oligonucleotide primers used amplified 1500 base pairs of 16S rRNA gene fragments using 3 µL DNA sample. The homology sequence and phylogenetical analyses of the 16S rRNA of the isolates showed that they belong to 3 different clades, namely, *Firmicutes, Proteobacteria* and *Actinobacteria* (Figs 1-5). In the analyses, *Vibrio cholerae* was used as the out-group.

Table 2—Characteristics of animal manures for carbon, nitrogen and phosphorus

Animal manures	Total organic C (%)	Total N (mg L^{-1})	Total P (mg L^{-1})
Poultry	49.2 ± 14.2	277 ± 63	254 ± 14
Cow	54.9 ± 5.9	109 ± 8	46 ± 8
Horse	52.7 ± 2.7	81 ± 3	50 ± 2
Pig	50.6 ± 5.9	904 ± 84	252 ± 29

Values are mean of three actual values ± standard error
The analysis was conducted by Waterlab (Pty) Ltd
The procedures followed were standardised method for C:N:P analysis (US EPA, 1984)

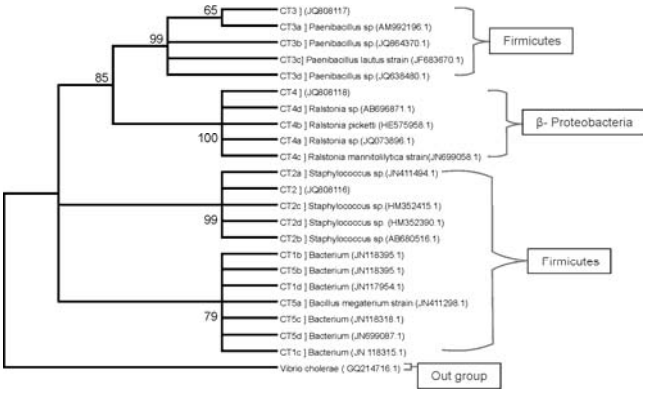

Fig. 1— Neighbour joining tree showing partial sequences of 16S rRNA of bacteria isolates from group 1 (control) and their related species obtained from the GeneBank as computed by boostrap NJ method.

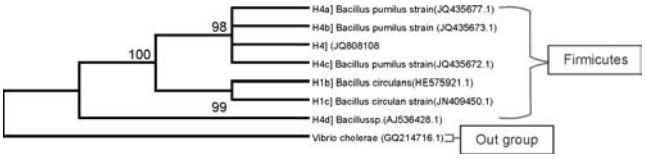

Fig. 2—Neighbour-joining tree showing partial sequences of 16S rRNA of bacterial isolates from group 2 (cow) and their related species obtained from the GenBank as computed by boostrap NJ method.

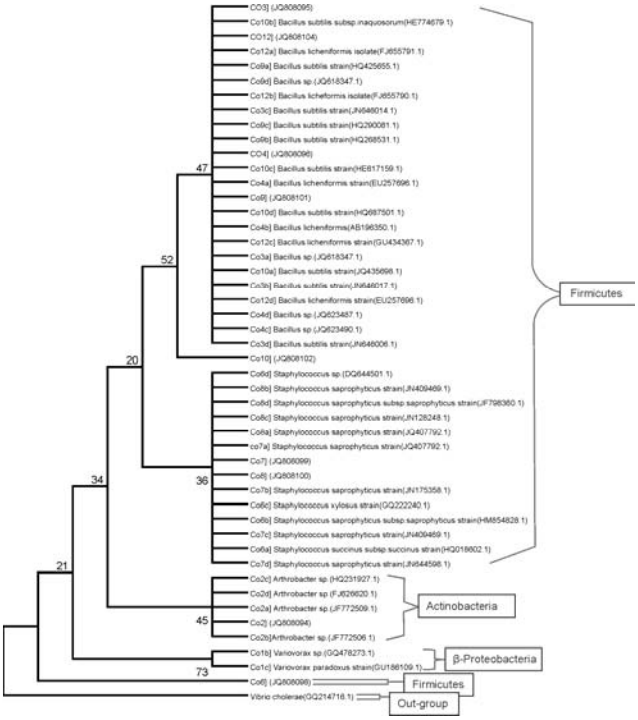

Fig. 3—Neighbour-joining tree showing partial sequences of 16S rRNA of bacterial isolates from group 3 (horse) and their related species obtained from the GenBank as computed by boostrap NJ method.

The molecular and phylogenetic analyses of the nucleotide sequences showed that they are closely related to the following 8 genera: *Staphylococcus*, *Brevibacterium*, *Variovorax*, *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Ralstonia* and *Geobacillus* (Figs 1-5). These isolates have been allocated the accession numbers (JQ808080-JQ808120) and their closely related genera from the GenBank are shown in Figs 1-5. The results from the samples obtained from enrichment cultures, which were analysed using GC/MS, showed that these bacteria were able to utilise the hydrocarbons in 21 d (Table. 3), as indicated by the reduction (61.81% to 80%) in concentrations of the PAHs. This is similar to those reported for the comparison of bioaugmentation and composting for remediation of oil sludge by Ouyang *et al*[11], suggesting the existence of active bacterial community in the culture media capable of utilizing oil sludge hydrocarbons[12]. This also implies that the animal manures were rich in nutrients, such as,

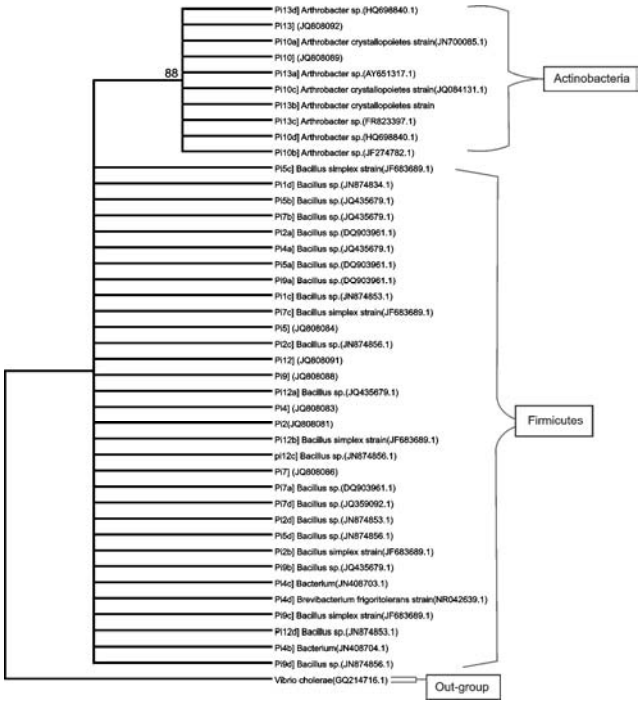

Fig. 4—Neighbour-joining tree showing partial sequences of 16S rRNA of bacterial isolates from group 4 (pig) and their related species obtained from the GenBank as computed by boostrap NJ method.

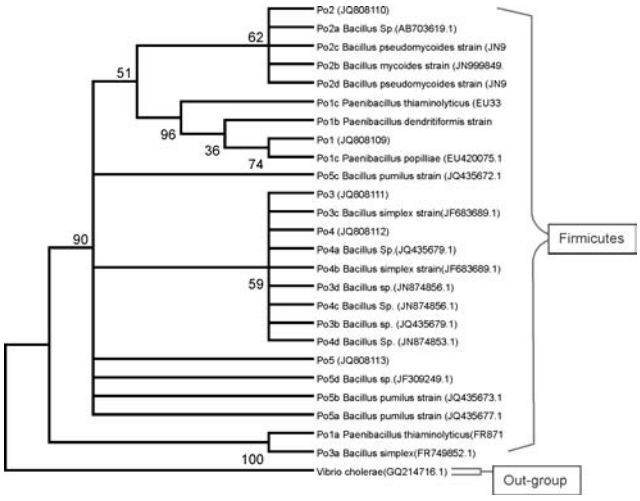

Fig. 5—Neighbour-joining tree showing partial sequences of 16S rRNA of bacterial isolates from group 5 (poultry) and their related species obtained from the GeneBank as computed by boostrap NJ method.

Table 3—Reduction in concentrations of selected PAHs in enrichment culture.

Selected PAH	Final conc. in enrichment culture (mg kg^{-1})	% reduction	Final conc. in subculture (mg kg^{-1})	% reduction
Naphthalene	19.06±0.1	80.00	31.77±2.14	66.67
1-methyl naphthalene	41.16±1.2	80.00	51.38±1.92	75.04
2-methyl naphthalene	39.14±0.99	80.00	48.93±3.96	74.99
Acenaphtylene	1.01±0.09	80.00	1.68±0.76	66.73
Acenaphthene	1.59±0.64	79.98	2.65±1.67	66.63
Fluorene	4.62±.69	80.01	5.78±1.81	74.99
Anthracene	9.09±0.18	77.52	13.48±2.53	66.67
Phenanthrene	0.29±0.14	79.86	0.48±0.16	66.67
Fluoranthene	0.36±0.13	75.00	0.38±0.21	73.61
Pyrene	2.71±1.06	74.98	3.61±1.4	66.67
Chrysene	8.95±1.7	80.00	11.19±2.26	75.01
Benzo[a] anthracene	0.26±0.13	81.94	0.55±0.21	61.81
Benzo[b] fluoranthene	4.33±1.28	80.00	7.20±1.94	66.67
Benzo[k] fluoranthene	0.43±0.14	80.18	0.72±0.29	66.82
Benzo[a]pyrene	1.44±0.46	80.06	1.81±0.9	74.93
Indenol (1,2,3-cd) pyrene	1.16±0.23	79.93	1.45±0.58	74.91

Values are mean of three actual values ± standard error

nitrogen, carbon and phosphorus, necessary for basic microbial activities and stimulated microbial growth and activities in the compost pile. The distinct grouping of the organisms was strongly supportive and phylogenetically coherent, showing their synergistic relationship to degrade their target pollutants (Figs 1-5). Bacteria identified in this study were also reported in various studies of soils contaminated with petroleum hydrocarbons. They were also shown to be involved in degrading oil and utilizing hydrocarbons as their sole source of carbon and energy[13-15].

Some of the bacterial strains identified in the present study were those that are efficient biosurfactant producers on petroleum hydrocarbon impacted soil according to[14,16]. The biosurfactants they produce are capable of emulsifying oil sludge, thus increasing the surface area of the substrates and enhancing solubility, and allowing the constituent hydrocarbons to become available for bacterial biodegradation as their sole source of carbon and energy[14]. The advantage of biosurfactants produced by bacteria is that they are natural, non-toxic, biodegradable and cost-effective approach in solubilisation of oil sludge during biodegradation[16]. They are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons[14].

Apart from biosurfactants produced by bacteria, other factors that may be responsible for the uptake and high metabolism of PAHs by these bacteria include growth conditions of bacteria. These conditions are pH and temperature, presence of a specific and high amount of inducible enzymes secreted by bacteria, substrate specificity of PAHs degrading enzyme and bacterial cell surface hydrophobicity[17]. However, neutral pH or near neutral are more favourable to bacteria. In the present study, pH values in the composting piles were observed to be increased from 5.6 to 7.9, which were within the recommended pH range for composting organic compounds. As such, pH enhanced the bacterial growth and activities that would have inhibited degradation of the contaminant hydrocarbon in the composting pile.

The results in Table 3 show that microorganisms were able to utilise and break down the hydrocarbons at 28°C because of increased solubility of the target contaminants. The principal mechanism for the aerobic bacterial metabolism of PAHs according to[18] is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form *cis*-dihydrodiol, which is dehydrogenated to form dihydroxylated intermediates that can be metabolised further *via* catechols to CO_2 and H_2O. *Cis*-dihydrodiols of PAHs are potentially soluble and more bioavailable[18].

Most microorganisms have been reported as being capable of either completely assimilate a defined range of compounds or carry out their transformation as well as mineralisation to different extent. *Bacillus* species has been reported to have the potentials to degrade and transform different PAHs but may not mineralise them[19]. However, *B. subtilis*, which is one the dominant bacteria species identified in the present study, showed the potentials to utilise several PAHs including benzo(a)pyrene, anthracene, naphthalene and dibenzothiophene as sole source of carbon energy[20].

Staphylococcus spp. identified in this study has shown the potentials to biotransform PAHs, such as, phenanthrene, using the monooxygenase by initial attack at C1 and C2 position giving 1,2,dihydroxy phenanthrene to catechol. *Arthrobacter* species, extremely tolerant and resistant to most metals and other toxic substances, are also capable of degrading some hydrocarbons to their less toxic forms[21]. *Arthrobacter* species in conjunction with several other strains use their vast metabolic activities to degrade pollutants in a synergistic relationship, using their target contaminant as their sole source of carbon and energy. This happens as a result of their specific adhesion mechanisms and production of extracellular emulsifying agents for contact with water-insoluble hydrocarbons[22]. Hence, their presence in pig and cow compost treatment of the present study (Figs 2 & 4), suggest that they are useful in bioremediation[15,23]. *Paenibacillus* species can withstand environmentally stressful conditions, which allow them long-term survival[17], hence they were present in poultry and control compost treatments of the present study (Figs 1 & 5). *Paenibacillus* species are capable of fixing nitrogen, phosphate solubilisation and degradation of environmental pollutions [17]. They also produce extracellular enzymes, such as, polysaccharides-degrading enzymes and protease, which can catalyse a wide variety of synthetic reactions and utilise aromatic substrates (PAH-degradation)[17]. Hence, similar was their role in the present study. *Variovorax* species identified in this

study have been found to degrade a broad range of different compounds, such as, pesticides and herbicides intermediates, to CO_2[24]. Sørensen *et al*[24] used a combination of *Variovorax* sp. strain SRS16 and *A. globiformis* strain D47 to mineralise phenylurea herbicide diuron and its intermediates by hydrolysis of amide bond to CO_2. *Variovorax* sp. strain SRS16 and *A. globiformis* strain D47 have close relationship based on the similarity in their degradation pathways, hence their presence and phylogenetic relationship in Fig. 2 of the present study.

The bacteria mentioned above have been isolated from the liquid enrichment cultures and are adapted to growing on and utilising hydrocarbon compounds as sole source of carbon[17,23], and are responsible for the degradation of the oil sludge components in the compost pile. In the present study, the oil amended medium was a growth control medium meant to exclude heterotrophic bacteria leaving the active bacterial community responsible for oil sludge degradation during compost bioremediation of oil sludge.

The results obtained in the present study indicate that *Bacillus* species were predominant community and as such they might had higher potential and were major contributors among other isolated bacterial strains to degrade oil. Strains of *Bacillus* species have shown potential to grow on a large number of hydrocarbon compounds as a source of carbon and energy demonstrating that they might be efficient hydrocarbon degraders[17]. *Bacillus* species are more tolerant to high levels of oil due to their resistant endospores. They possess more competent and active oil degrading enzymes than other biodegraders[17]. Degradation of oil by these microorganisms shows that they adapt in oil polluted environments and also have specialized co-metabolic capacities. These bacteria are the most predominant microorganism among other microorganisms in either *in situ* or *ex situ* bioremediation processes, indicating that they are the main agents responsible for the degradation of oil. The use of these native bacterial consortiums with oil utilising capabilities as seed onto oil-contaminated environment could prove a more environmentally-friendly approach to bioremediation.

Acknowledgement

The authors wish to acknowledge the financial support given by the South African National Research Foundation for this project.

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