Research Article

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Potential microbial drivers of biodegradation of polycyclic aromatic hydrocarbons in crude oil sludge using a composting technique

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Abstract

BACKGROUND: Improper disposal of crude oil sludge poses a public health risk due to the toxic nature of components of the waste, especially polycyclic aromatic hydrocarbons (PAHs). Such toxic wastes can be treated by bioremediation of the polluted sites with composting, an eco-friendly technique that is gaining acceptance. Crude oil sludge was composted for four months during which surfactants were introduced. Fungi and bacteria were isolated from the compost using basic culture techniques. Residual concentrations of PAH components of the sludge during the composting period was monitored with gas chromatography – mass spectrometry (GC-MS).

RESULTS: Molecular characterization of the internal transcribed spacer (ITS) genes of fungal isolates classified fungi in the compost into different genera including *Aspergillus, Bionectria, Doratomyces, Exophiala, Fusarium, Galactomyces, Geotrichum, Mucor, Penicillium, Trichoderma* and *Trichurus*. The bacterial isolates were classified into *Stenotrophomonas, Pseudomonas, Bordetella, Brucella, Bacillus, Achromobacter, Advenella, Klebsiella, Mesorhizobium, Mycobacterium, Ochrobactrum, Pusillimonas* and *Raoultella*. Results from analysis of residual PAH concentrations of composted samples showed higher degradation efficiency of the higher molecular weight (HMW) PAHs resulting in their complete degradation, whereas the lower molecular weight PAHs were not degraded completely.

CONCLUSIONS: Compost bioremediation is a sustainable waste management technique for hazardous wastes such as crude oil sludge. Inclusion of surfactants at different concentrations did not constantly improve the bioavailability of PAH compounds in the crude oil sludge but it promoted fungal growth in the treatments.

Supporting information may be found in the online version of this article.

Keywords: composting; fungi; bacteria; PAHs; bioremediation; crude oil sludge

INTRODUCTION

Fossil fuels are major energy sources for most countries globally[1] and crude oil sludge is a waste product generated during the refining of crude oil.[2] Inappropriate disposal of crude oil sludge presents a major environmental hazard due to the toxic nature of the polycyclic aromatic hydrocarbons (PAHs).[3,4] These substances are toxic, mutagenic, known environmental carcinogens that are recalcitrant due to their high molecular weights (HMW) and strong molecular bonds.[5] Some conventional methods of remediation have been used to degrade PAHs in crude oil sludge, but most of these have the limitation of either being cost-intensive and laborious, or environmentally unfriendly and tending to generate toxic by-products.[6] Biological methods of remediation, which include bioremediation, have proven to be efficient, cost effective and eco-friendly.[7,8] Bioremediation, an increasingly popular and beneficial biological approach to remediation, uses naturally occurring microorganisms that possess the ability to utilize contaminants as their nutrient and energy sources to drive the degradation process.[9]

Compost bioremediation is the use of microorganisms in compost to metabolize contaminants. It is an emerging bioremediation technology and has several advantages including simplicity of design and operation, high treatment efficiency, presence of a diverse microbial population, and, most importantly, being environmentally friendly.[10,11] Composting involves the addition of organic amendments and bulking agents such as wood chips, rice hulls or manure to improve not only microbial activities, aeration and water

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retention capacity, but also nutrient composition of the compost.[12] The inclusion of organic amendments also could serve as bioactivators as different fungal and bacterial species have the potential to metabolize/co-metabolize PAHs.[13,14] For example, some species of fungi such as *Amorphoteca, Aspergillus, Fusarium, Talaromyces, Paecilomyces* and *Penicillium* have been reported to degrade hydrocarbons.[15,16] In certain situations, complete metabolism of HMW and low molecular weight (LMW) PAHs has been achieved by using a combination of the lignolytic and non-lignolytic fungi.[17] Specifically, the lignolytic fungi are involved in the degradation of recalcitrant PAHs due to their ability to secrete extracellular lignolytic enzymes. These enzymes are vital to the biodegradation of PAHs as they catalyze the opening up of the ring structure of complex aromatic compounds.[17,18] The enzyme cytochrome P450 monooxygenase commonly catalyzes the metabolism of PAHs by non-lignolytic fungi. This enzyme catalyzes the initial degradation of PAHs to epoxide and to dihydrodiols by epoxide hydrolase. The dihydrodiols are further degraded to PAH catechols by dihydrodiols dehydrogenase.[19] It has been advocated that the metabolism of the HMW PAHs often is initiated by fungal exoenzymes, thereby creating a diffusion gradient that facilitates further PAH biodegradation by other types of microorganisms, especially bacteria.[9] Bacteria usually degrade LMW PAHs and a crucial phase in the aerobic bacterial metabolism of PAHs is the oxidation of the fused-benzene ring of the PAHs through the integration of molecular oxygen by the dioxygenase enzyme to form cis-dihydrodiols. Dehydrogenation of the dihydrodiols results in the generation of dihydroxylated intermediates such as catechol, which are further broken down to CO_2 and water by catechol dioxygenases.[20] Catechol-2,-3-dioxygenase (C23O) is an essential enzyme in the aerobic biodegradation of aromatic hydrocarbons. It acts as a marker for the detection of aromatic hydrocarbon degradability in a wide range of bacteria.[21]

Certainly, production of enzymes by both bacteria and fungi with complementary biodegradation attributes provides a platform for complete biodegradation of PAHs in natural environments. However, this is not always the case as some other important factors may influence the biodegradation process directly or indirectly.[22,23] These factors are the substrate uptake capability of the microbes, as well as sorption and transmembrane processes. Furthermore, the ability of microbial isolates to produce suitable enzymes for the metabolism of HMW PAHs could be hindered by low bioavailability of PAHs.[24] Bioavailability of HMW PAHs could be improved by the addition of surfactants impacting positively on biodegradation of hydrocarbons.[25–28]

Surfactants are amphiphilic surface-active agents that reduce the surface and interfacial tensions of the medium in which they are dissolved. Surfactants possess a hydrophilic head and a hydrophobic tail which often is a hydrocarbon chain.[29] These surface-active agents are classified as anionic, nonionic, cationic or zwitterionic surfactants based on the ionic charge of the hydrophilic head of the molecule.[26] Addition of surfactants is believed to enhance solubilization and dissolution of hydrocarbons resulting in improved bioremediation process,[26,30] by changing the binding effects of PAHs thereby increasing mass transfer of hydrocarbons into the aqueous phase.[28,30] However, the addition of commercially available surfactants during a bioremediation process could have some drawbacks which include the possibility of preferential utilization of surfactants as the principal substrate by microorganisms; and toxicity of some surfactants to the microorganisms involved in the degradation process. This could sometimes limit or possibly hinder the desorption and/or biodegradation of the PAHs in the presence of some surfactants.[25] This study explores the potential of bacteria and fungi to drive the process of biodegradation of PAHs in crude oil sludge during composting; and the effect of addition of commercially available anionic surfactant, Sodium dodecyl sulphate (SDS), and nonionic surfactant, Tomadol 900 (TD) during the degradation process.

MATERIALS AND METHODS

Materials for composting

Soil, bark chips, soybean meal and horse manure

Agricultural topsoil was purchased from Plantland in Crowthorne, Midrand, South Africa; it was air-dried, homogenized and kept at room temperature preceding the composting process. The characteristics of the topsoil are pH 6.1, total organic carbon 5.7 g kg^{-1}, total nitrogen 0.3 g kg^{-1}, total phosphorus 0.1 g kg^{-1} and dry matter content 84.28%. Bark chips ≈2–4 cm × 1–2 cm in dimensions were used as bulking agents. Soybean meal and horse manure (organic amendments) were obtained (respectively) from Willowton Group, Isando, Kempton Park, Johannesburg and a horse farm in Midrand, Johannesburg, South Africa.

Crude oil sludge, surfactants and petroleum ether

Crude oil sludge was obtained from a crude oil refinery in Durban, KwaZulu-Natal, South Africa. It was characterized for selected PAHs by solvent extraction with a Soxhlet extractor and quantified by GC-MS analysis.[31] The anionic surfactant SDS was purchased from Rochelle chemicals (Johannesburg, South Africa), whereas the nonionic surfactant TD was purchased from Fine Organics Chemicals (PTY) (Alberton, Johannesburg, South Africa). The nonionic and anionic surfactants were used as purchased without any further treatment. Petroleum ether was purchased from Rochelle Chemicals.

Compost experimental set-up

Fifteen transparent composting bins with dimensions 20 cm × 40 cm × 20 cm were used as composters to set up 15 treatments including controls. Crude oil sludge, a highly viscous compound was dissolved in petroleum ether before mixing with agricultural soil to enhance its fluidity. The composters each held 300 g crude oil sludge, 1.5 kg agricultural topsoil and 180 g bark chips. Soybean meal and horse manure were introduced into the composters in various quantities, as indicated in the ratios presented in Table 1, to give three distinctive sets of experiments.

Furthermore, anionic (SDS) and nonionic surfactants (TD) were added to different compost mixtures (treatments) at concentrations of either 0.5% or 1.0% (Table 1). Control mixtures also were set up and experiments were conducted in triplicates. The treatments were turned at three day intervals for the first six weeks and then at weekly intervals from Week 7 to Week 16. Analysis of microbial community and residual PAH concentrations in compost samples were carried out after 16 weeks of composting.

Isolation and PCR of fungal isolates

In order to isolate the fungi from the compost samples, 1 g homogenized compost sample was serially diluted and plated on potato dextrose agar (PDA) medium that had been supplemented with 0.05% (w/v) Tetracycline.[32] The plates were incubated at 30 °C for seven days. Grown cultures were aseptically subcultured onto PDA + 0.05% (w/v) tetracycline plates to obtain pure cultures and subsequently stored at 4 °C on PDA slants for future use. Genomic DNA was isolated from the fungal hyphae

Table 1. Experimental design for compost treatments

Treatments	SETS	Soy bean meal (kg)	Horse manure (kg)	Surfactants (w/v)	
				Anionic (SDS) (%)	Nonionic (TD) (%)
1	SET A	1	0	0.5	0
2		1	0	1	0
3		1	0	0	0.5
4		1	0	0	1
5 (control)		1	0	0	0
6	SET B	0.5	0.5	0.5	0
7		0.5	0.5	1.0	0
8		0.5	0.5	0	0.5
9		0.5	0.5	0	1
10 (control)		0.5	0.5	0	0
11	SET C	0	1	0.5	0
12		0	1	1	0
13		0	1	0	0.5
14		0	1	0	1
15 (control)		0	1	0	0

that were scraped from the PDA slants. Zymo Research Soil Microbe DNA Miniprep isolation kit was employed for the isolation of DNA following the manufacturer's protocol (Zymo Research Corp., Irvine, CA, USA). Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Randburg, South Africa) was used to quantify filtered DNA extracts and extracts were stored at −20 °C for further use.[33] The universal fungal primers *ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC-3′ were used to amplify the ITS region of the fungal DNA using PCR.[34] A total volume of 20 µL amplification reaction mix was prepared which comprised 1 µL (10 µmol L^{-1}) of each primer, 10 µL 2× Thermo Scientific Phusion Flash High-Fidelity PCR Master mix, 2 µL DNA templates and 6 µL of sterile distilled water. Controls were set up. The amplification reaction was preheated to 98 °C for 10 s in a BIORAD T100™ Thermal Cycler and the cycle was run at 98 °C, 1 s; 55 °C, 1 min; 72 °C, 15 s in 34 cycles and elongated at 72 °C, 1 min. The amplification reaction was held at 4 °C until the amplicons were removed from the thermal cycler. One percent agarose gel electrophoresis was used to confirm the size of amplicons, and stored at −20 °C for subsequent use.

Isolation of bacteria and PCR of bacterial isolates

Isolation of bacteria from compost samples was done through enrichment culture technique using mineral salts medium (MSM).[35] Mineral salts medium contained 5.0 g NaCl, 5.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4{\cdot}7H_2O$, 2.0 g $NaNO_3$, 0.02 g $FeCl_2{\cdot}4H_2O$, 0.02 g $CaCl_2$ per litre of distilled water, pH 7.2; whereas mineral salts agar (MSA) consisted of MSM and 12 g of bacteriological agar per litre of distilled water. About 1.5% of compost samples and 1% of crude oil sludge were inoculated into 100 mL of sterile MSM and incubated at 28 °C under rotatory conditions 140RPM. Aliquots (5 mL) were periodically subcultured into a sterile MSM for the nine-week duration of enrichment. Enriched culture was aseptically plated out on MSA and incubated at 28 °C for seven days. Several subculturing exercises resulted in the generation of pure colonies, which were stored in nutrient agar slants for further classification. Colony PCR was used to amplify the 16S rRNA region of the DNA of bacterial cells.[36] This is the use of colonies from agar plates as DNA template for PCR. A small amount of bacterial colony was picked from the nutrient agar slants using a sterile pipette tip and placed in 50 µL sterile distilled water in a 2-mL microcentrifuge tube. The tube was incubated for 10 min at 95 °C. This method lyses the bacterial cell thereby releasing the DNA from the cell into the sterile distilled water in the 2 mL tube. The method described in section 2.3 was used to prepare the amplification reaction as well as cycling conditions but the annealing temperature was set at 53 °C using the universal 16S rRNA bacterial primers, 27F (5′-AGA GTT TGA TCC TGG CTC AG -3′) and 1492R (5′- CGG CTA CCT TGT TAC GAC TT -3′) (synthesized by Inqaba biotechnological Industries PTY Ltd, Pretoria, South Africa).[36]

Sequencing and phylogenetic analysis of fungal ITS and bacterial 16S rRNA genes

The amplified products of PCR from the fungal and bacterial isolates were sequenced at Inqaba Biotechnological Industries Pretoria, South Africa. Bioedit and ClustalW software were used to align the gene sequences of the amplicons and closest taxonomic species were identified with the aid of BLAST (Basic Local Alignment Search Tool) program from NCBI (National Centre for Biotechnology Information).

Based on the partial ITS and 16S rRNA gene sequences, the software MEGA v6.0 (Molecular Evolutionary Genetics Analysis) was used to construct phylogenetic trees. Bootstrap analysis with 100 repetitions was carried out and the sequences were arranged for the assignment of accession numbers using the software Sequin. Similar ITS and 16S rRNA gene sequences were respectively clustered into operational taxonomic units (OTUs) using Mothur 1.25.1.

Catechol-2,3-dioxygenase (C23O) gene detection in bacteria isolates

The presence of C23O genes in bacterial isolates was assessed using the method outlined in the previous section with the degenerate primers C230F (5′- AAG AGG CAT GGG GGC GCA CCG GTT CGA -3′) and C230R (5′-TCA CCA GCA AAC ACC TCG TTG CGG TTG CC -3′) (Inqaba biotechnological Industries PTY

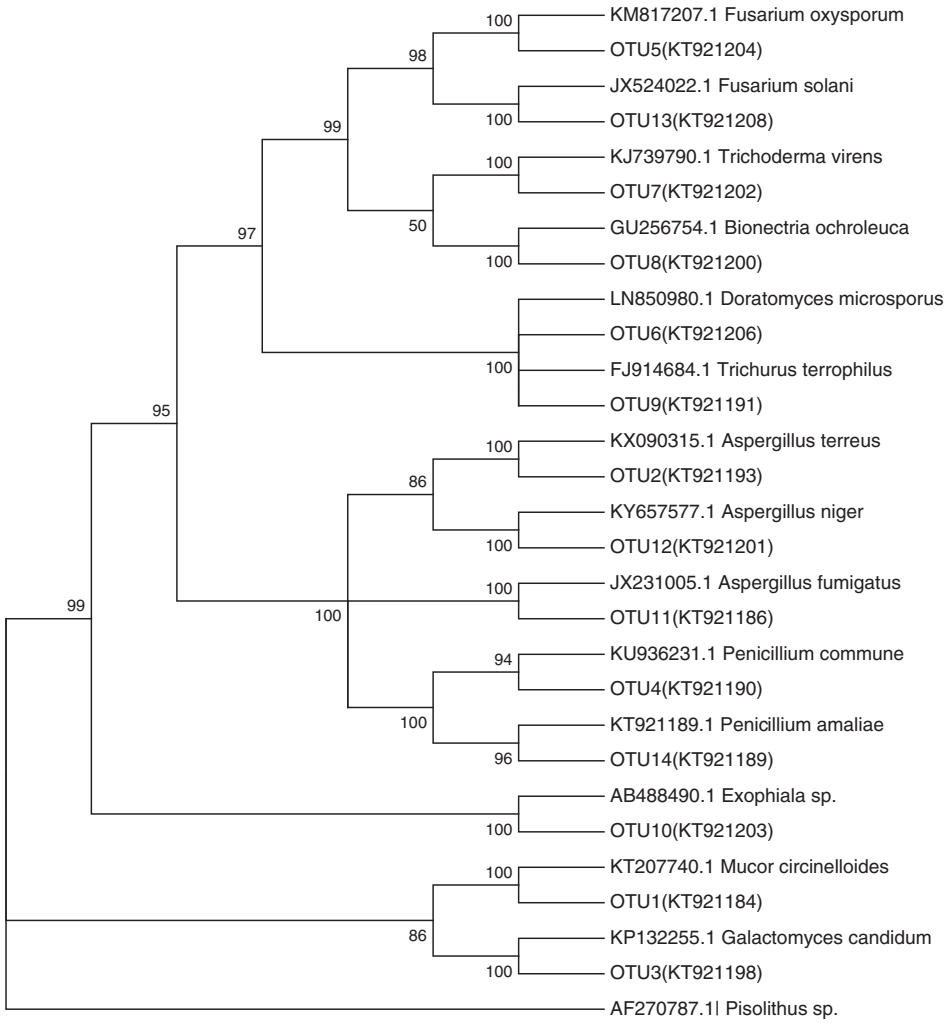

Figure 1. Percentage removal of selected PAH components of crude oil sludge present in treatments 1, 2, 3, 4 and 5 after four months of composting. Error bars represent SD ($n = 3$).

Ltd), which annealed at 55 °C for 1 min.[37] Agarose gel electrophoresis (1%) was used to assess the amplified products. The ITS gene sequences obtained in this study were placed in the GenBank database with accession numbers KT921184 to KT921209 and 16S rRNA gene sequences with accession numbers KT337506 to KT337538, and KT445946 to KT445948. Examination of the partial sequences of ITS and 16S rRNA genes were used to create an OTU to facilitate clear-cut classifications.

Screening of microbial isolates for PAH biodegradation potential

Bushnell Haas agar (BHA) containing 0.02 g $CaCl_2$, 0.2 g $MgSO_4$, 1.0 g K_2HPO_4, 1.0 g $KHPO_4$, 0.05 g $FeCl_3$, 1.0 g NH_4NO_3 and 12 g bacteriological agar in 1 L distilled water was prepared at pH 7.0 and dispensed into plates. A filter membrane with 0.2-µm pore size was used to sterilize the crude oil sludge of which 1.5% was spread-plated on the solidified BHA.[35] Then 1 cm^2 agar plugs of pure fungal isolates and a loop of bacterial isolates were transferred aseptically to separate crude oil sludge laced BHA plates and incubated at 30 °C for three to seven days.

Aliquots (10 mL) of Bushnell Haas broth (BHB) medium were distributed into sterile glass test tubes. The glass tubes were spiked with a mixture of 0.5% (w/v) 2,6-dichlorophenol indophenol (2,6-DCPIP), 3% (v/v) sterile crude oil sludge, and 0.1% Tween 80. Agar plugs (≈2 cm^2) of fungal isolates and bacteria isolates were transferred aseptically to separate tubes containing the spiked BHB medium. The medium was incubated in a rotary incubator at 30 °C and 170 rpm for seven days. The experiment was set up in duplicate with controls and observed regularly for discoloration of the deep blue medium.[38] At the end of the incubation period, the broth was filtered and the filtrate was centrifuged at 8000 rpm for 15 min to separate the biomass. Analysis of the supernatant was carried out at an absorbance of 609 nm using a UV-visible spectrophotometer (Hach spectrophotometer DR 5000). The percentage biodegradation of PAHs in crude oil sludge was evaluated with Eqn (1):

$$\%\,of\ degradation = 1 - \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Efficiency of treatments for PAH degradation

The compost matrix was analyzed for residual PAH content to evaluate the efficiency of microbial degradation of PAH constituents of the crude oil sludge. Residual PAH concentrations were analyzed in single ion monitoring mode by GC-MS after soxhlet extraction.[31] The efficiency of different treatment conditions for

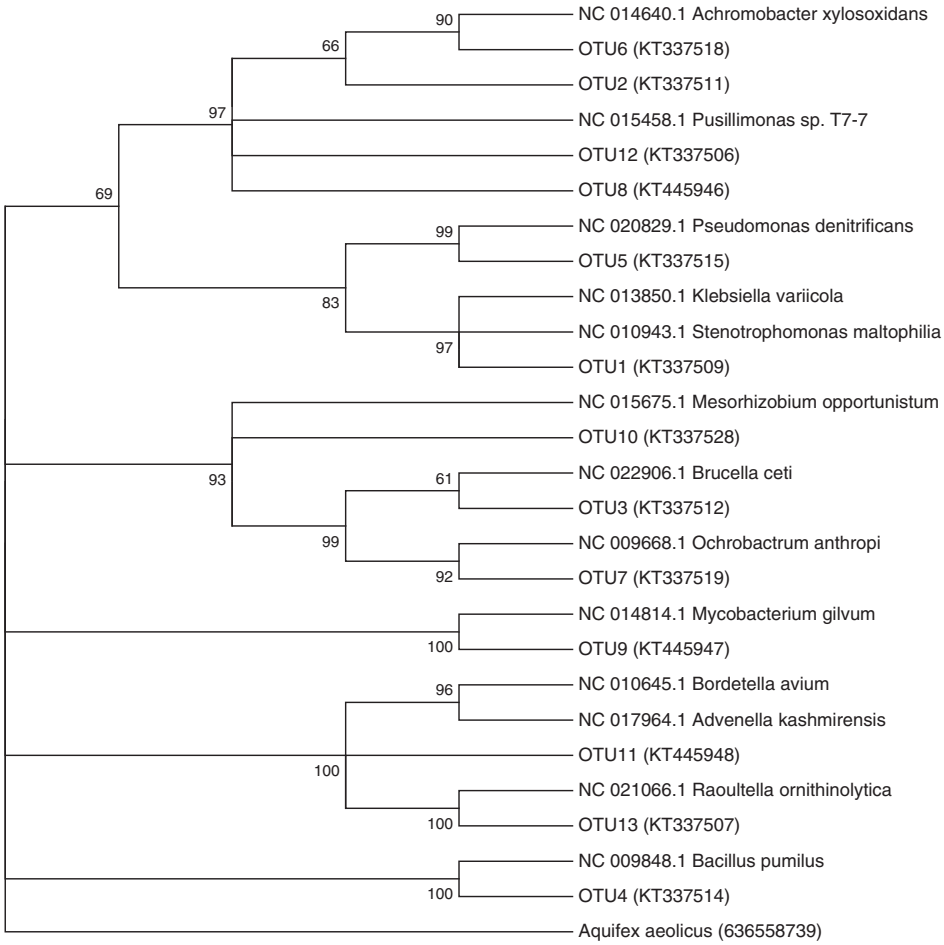

Figure 2. Percentage removal of selected PAH components of crude oil sludge by treatments 6, 7, 8, 9 and 10 after four months of composting. Error bars represent SD ($n = 3$).

degrading individual PAHs was determined by evaluating the percentage (%) degradated expressed as percentage PAH removed. Degradation percentages for individual PAHs after treatment were calculated with Eqn (2) and the results are presented in Figs 1, 2 and 3.

$$\%\,PAH\,removed = \frac{C_{0-}C_{16}}{C_0} \times 100 \qquad (2)$$

where C_0 is the initial concentration of PAHs and C_{16} is the concentration after 16 weeks.

Statistical analysis

Two-way ANOVA was used to examine the percentage degradation of PAHs. This was to determine the presence of significant differences among the different treatments if any. Mean difference among the treatments was calculated using the Student's *t*-test LSD (least significant difference) at a 5% significance level ($P \leq 0.05$). The standardized residuals were tested for deviations from normality using the Shapiro–Wilk test.[39,40] SAS v9.4 statistical software was used for data analysis.[41]

RESULTS

A total of 55 pure fungal isolates were obtained from the compost samples after subculturing several times on PDA. These fungi resisted the toxic nature of crude oil sludge and proliferated. The enrichment culture technique also produced 36 bacterial isolates that possibly utilized crude oil sludge for their energy and carbon requirements, as crude oil sludge was the sole carbon source during the enrichment. The results of the identification and characterization of bacterial isolates have been published previously.[36] A total of 17 PAHs were quantitatively identified in the crude oil sludge used in this study, 15 of which are in the US EPA list of priority pollutants (Table 2).

Molecular characterization and phylogenetic of microbial isolates

The products of the amplified ITS regions of fungal isolates ranged between 400 and 600 bp in size and isolates were identified in the following genera: *Aspergillus, Bionectria, Doratomyces, Exophiala, Galactomyces, Fusarium, Mucor, Penicillum, Trichurus* and *Trichoderma*. DNA sequence analysis of the 16S rRNA genes of isolated bacteria showed that they belong to the following genera: *Stenotrophmonas, Pseudomonas, Bordetella, Brucella, Bacillus, Achromobacter, Advenella, Klebsiella, Mesorhizobium, Mycobacterium, Ochrobactrum, Pusillimonas* and *Raoultella*.

The ITS gene sequences were classified into 14 OTUs (Table 3) and *Pisolithus* sp. served as the outgroup (Fig. 4). Subsequent characterization of the 16S rRNA genes sequences (Table 3) resulted in bacterial sequences being grouped into 13 OTUs with

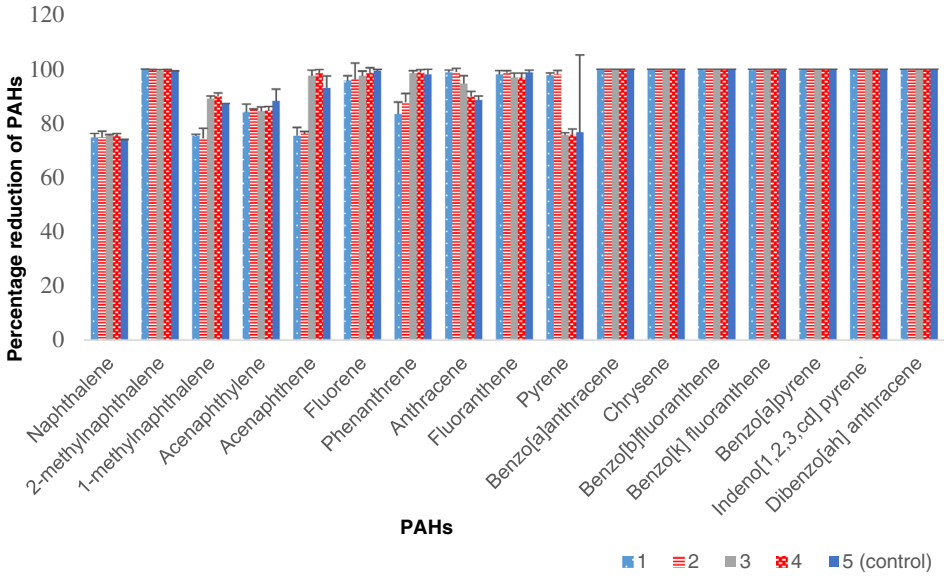

Figure 3. Percentage removal of selected PAH components of crude oil sludge present in treatments 11, 12, 13, 14 and 15 after four months of composting. Error bars represent SD ($n = 3$).

Table 2. PAHs identified in crude oil sludge

PAHs in sludge	Initial concentration of PAHs (mg kg^{-1})
Naphthalene	0.52
2-methylnaphthalene	0.83
1-methylnaphthalene	0.40
Acenaphthylene	0.40
Acenaphthene	0.54
Fluorene	0.54
Phenanthrene	0.53
Anthracene	0.76
Fluoranthene	0.52
Pyrene	0.68
Benzo[a]anthracene	0.56
Chrysene	0.09
Benzo[b]fluoranthene	0.26
Benzo[k]fluoranthene	0.10
Benzo[a]pyrene	0.22
Indenol[1,2,3,cd]pyrene	0.31
Dibenzo[ah]anthracene	0.57

>95% similarity (Fig. 5). *Aquifex aeolicus* served as the outgroup. Values >50% were presented after bootstrap analysis with 100 repetitions. Amplification of the C23O genes in the bacterial isolates indicated the presence of the catechol-degrading genes.

Microbial diversity of isolates

The diversity of isolated microorganisms was analyzed based on their phyla and class. The phylum Ascomycota representing ≈90% of the fungal populations, whereas Zygomycota represented ≈10%. The Sordariomycetes were the dominant class of the Ascomycetes representing ≈56% of the Ascomycetes populations, whereas Chaetothyriomycetes, Eurotiomycetes and Saccharomycetes represented 11%, 22% and 11%, respectively. Proteobacteria was the dominant bacterial phylum, representing ≈78% of the isolated bacteria population, whereas Firmicutes and actinobacteria represented 19% and 3% of the population, respectively. Amongst the Proteobacteria, γ- proteobacteria (39%) and β-proteobacteria (35%) dominated, whereas α-proteobacteria were the least populated with around 25%.

Crude oil sludge degradability

All of the fungal isolates and most of the bacterial isolates exhibited the potential to degrade PAH in crude oil sludge within the stipulated time of incubation. Speed of decolorization of redox indicator 2,6-DCPIP was used to assess PAH degradation potential. Isolates that decolourized the redox indicator 2,6-DCPIP in the shortest time were considered the top degraders of PAH in crude oil sludge. *Fusarium* sp. and *Pseudomonas* sp. were classified as the top degraders of PAH in crude oil sludge due to their ability to decolorize the 2,6-DCPIP within the shortest incubation time of three days. *Exophiala* sp. was identified as the least performing degrader due to its inability to decolorize the indicator within the experimental period. Isolates that belong to the genera *Galactomyces, Mucor, Aspergillus*, *Bionectria, Bacillus*, *Pusillimonas* and *Achromobacter* also exhibited high potential for PAH degradation as they decolorized the redox indicator within five days of incubation.

Mass balance and stoichiometry

Oxygen is incorporated by microbial cells during aerobic biodegradation of hydrocarbons as demonstrated by stoichiometric equations [Eqn (3)] for the mineralization of hydrocarbons:[42]

$$C_XH_Y + \left[X+(Y/4)\right]O_2 \rightarrow X\,CO_2 + \left(Y/2\right)H_2O \qquad (3)$$

Most of the HMW PAHs, especially Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k] fluoranthene, Benzo[a]pyrene, Indeno[1,2,3,cd]pyrene and Dibenzo[ah] anthracene, were completely degraded as they were not detected by GC-MS analysis of the composted samples after 16 weeks of composting. Significant differences exist amongst the treatments ($P < 0.05$).

Table 3. Microbial sequences and their OTU representatives (Obi *et al.* 2016)

Fungi			Bacteria		
OTUs	Number of sequences	OTU representative	OTUs	Number of sequences	OTU representative
OTU1	14	*Mucor circinelloides*	OTU1	4	*Stenotrophomonas* sp.
OTU2	6	*Aspergillus terrus*	OTU2	2	*Bordetella* sp.
OTU3	4	*Galactomyces cadium*	OTU3	1	*Brucella* sp.
OTU4	2	*Penicillum commune*	OTU4	7	*Bacillus* sp.
OTU5	5	*Fusarium oxysporum*	OTU5	5	*Pseudomonas* sp.
OTU6	3	*Doratomyces microsporus*	OTU6	4	*Achromobacter* sp.
OTU7	1	*Trichoderma virens*	OTU7	6	*Ochrobactrum* sp.
OTU8	2	*Bionectria ochroleuca*	OTU8	1	*Advenella* sp.
OTU9	1	*Trichurus terrophilus*	OTU9	1	*Mycobacterium* sp.
OTU10	1	*Exophiala sp.*	OTU10	1	*Mesorhizobium* sp.
OTU11	6	*Aspergillus fumigatus*	OTU11	1	*Klebsiella* sp.
OTU12	2	*Aspergillus niger*	OTU12	2	*Pusillimonas* sp.
OTU13	2	*Fusarium solani*	OTU13	1	*Raoultella* sp.
OTU14	2	*Penicillum amaliae*			

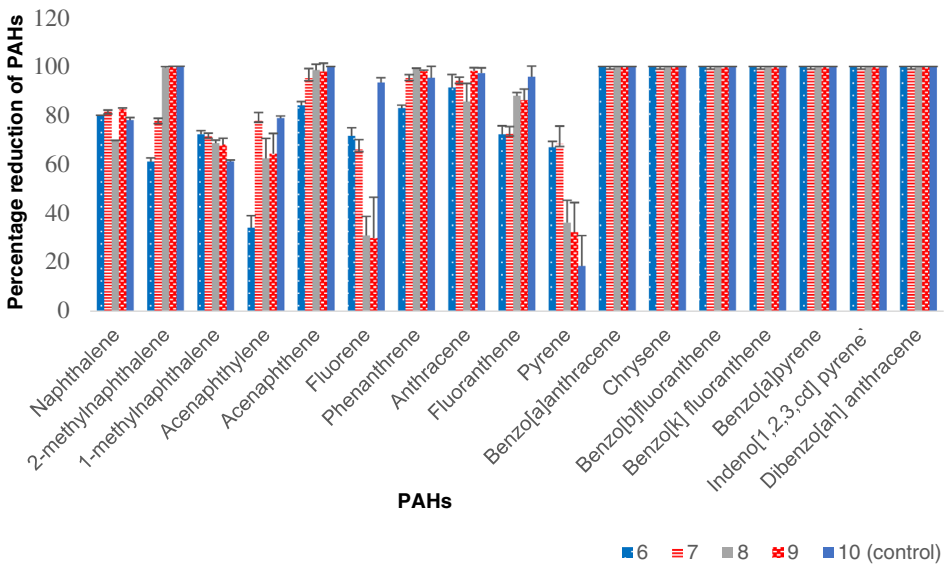

Figure 4. Phylogenetic tree of the ITS gene sequences of fungal isolates obtained from compost samples and their relatives found by BLASTn search.

Results from treatments 1–5 (Fig. 1), indicate a higher capacity of PAH degradation when compared with those of treatments 6–10 (Figs 2 and 3). The incorporation of both anionic and nonionic surfactants to the treatments at different concentrations did not consistently improve the biodegradation efficiency.

DISCUSSIONS

Composting is emerging as an efficient technique for bioremediation of PAH-contaminated wastes, as it is not plagued by the challenges associated with bioremediation especially the problem of insufficient microbial diversity; and lack of pertinent nutrients to support the growth and activities of innate microbes.[43] Compost bioremediation of PAH-contaminated waste is a complex process with pathways yet to be fully understood. However, results from this study suggest that composting can be employed as an efficient degradation technology for the biodegradation of HMW PAHs. These results are supported by the findings of Wu and coworkers,[44] in which improved degradation of HMW PAHs was achieved by compost addition. A contrasting report found that increasing molecular weight of an aromatic compound reduced biodegradation efficiency of PAHs.[5] In the present study, composting of crude oil sludge resulted in highly efficient degradation of HMW PAHs with carbon atoms C_{18} – C_{22} including Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo [k] fluoranthene, Benzo[a]pyrene, Indeno[1,2,3,cd] pyrene and Dibenzo[ah]anthracene. However, composting of LMW PAHs with carbon atoms C_{10} – C_{16} resulted in lower degradation efficiencies achieving between 30% and 99% degradation. This finding corresponds to the reports of Cajthaml *et al.* [45] and Juhasz *et al.*[46] in which composting of PAH-contaminated wastes resulted in the more efficient degradation of HMW hydrocarbons than their

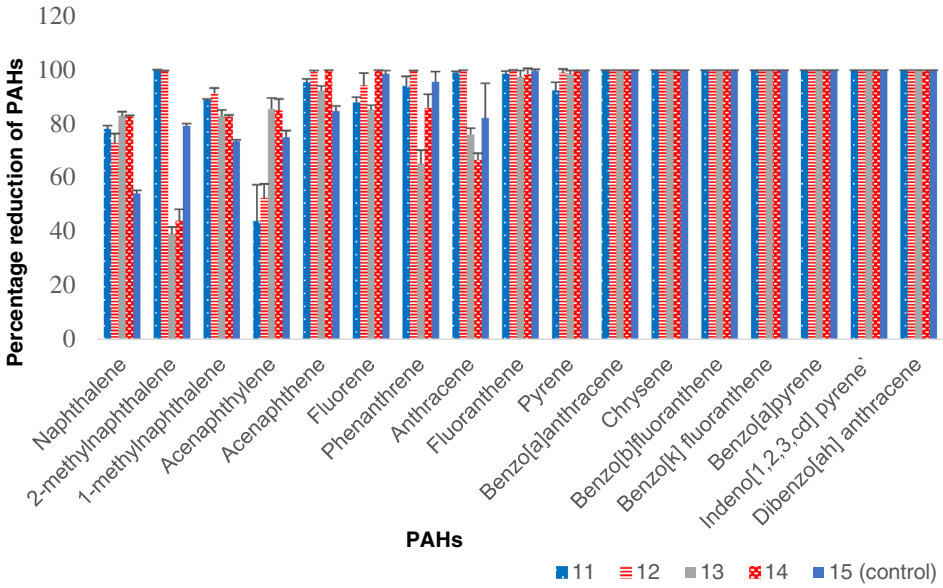

Figure 5. Analysis of phylogeny of the 16S rRNA gene sequences of bacteria isolates obtained from compost samples and their relatives found by BLASTn search.[40]

LMW counterparts. Higher degradation efficiencies of HMW PAHs could be attributed to the presence of bay regions in their molecular structure. However, these angular skeletal carbon orientations, although thermodynamically more stable, are susceptible to enzymatic attack thus making the PAHs with angular structure more biodegradable.[47] Furthermore, it has been reported that addition of compost to remediate a PAH-contaminated environment possibly increased selective sorption within the groups of PAH compounds resident in the contaminated environment. This selective sorption process that is activated by the addition of compost tends to decrease the sorption of the HMW PAHs onto particles instead of degrading the easily desorbed portions (LMW PAHs) of the contaminants. Decreased sorption of HMW PAHs signifies increased bioavailability for possible biodegradation of the PAH-contaminated waste.[44,48]

Compost bioremediation of crude oil sludge had many role players, it enthused the growth and activities of indigenous fungi and bacteria for the subsequent degradation of PAH-contaminated waste. A number of studies confirm the metabolism of petroleum hydrocarbons by fungi and bacteria that were isolated in the course of this study.[49–52] The presence of fungi and bacteria in the compost depicts their co-existence in a PAH-contaminated environment and possibly co-metabolism. A combination of fungi and bacteria synergistically enhanced the rate of degradation of HMW PAHs. The degradation process probably was initiated by the pre-oxidation of PAHs by fungi and the subsequent degradation of polar metabolites by bacteria.[49,53] The pre-oxidation process was made possible by the low substrate specificity of the extracellular enzymes of fungi which enabled their easy diffusion into the compost for subsequent oxidation of the HMW PAHs.[54,55] An investigation demonstrated the ability of fungal-bacterial culture to mineralize pentacyclic PAHs while reducing their mutagenicity. Boochan *et al*. and Kyser *et al*. also indicated the efficiency of bacterial-eukaryotic consortium in degrading HMW PAHs.[56,57] The hyphae of fungi have been reported to serve as a means of transporting specific bacteria in a contaminated environment, thereby improving the activities of the microorganisms as well as the bioremediation process.[58]

Complete degradation of benzo[a]pyrene and benzo[a]anthracene components of crude oil sludge was observed during the course of composting. Pure isolates of fungi obtained from this study have been associated with the degradation of such HMW PAHs, these fungi include *Fusarium, Aspergillus, Penicillium* and *Trichoderma* sp.[59–61] In addition, the identification of *Fusarium* sp. as the top degrader of crude oil sludge corroborates the studies that classified *Fusarium* sp. as a possible degrader of hydrocarbons.[38,62] Studies by Ijoma & Tekere, and Shahsavari also linked fungi (*Doratomyces, Galactomyces* and *Trichurus* sp.) isolated in the present study with hydrocarbon utilization in degradation experiments, thus supporting findings herein concerning the competency of these fungal isolates in degrading PAHs.[60,63] Similarly, some fungi obtained were identified to belong to both lignolytic and non-lignolytic classes of fungi; a combination of both classes was found to be beneficial to the bioremediation of PAHs through oxidation of the hydrocarbons.[64]

Furthermore, a selection of bacteria identified in this study has been identified to play a functional role in the degradation of aliphatic and aromatic hydrocarbons due to their involvement in the catabolic pathways of such hydrocarbons. Such organisms belong to the genera *Advenella, Achromobacter*, *Bacillus, Mycobacterium, Ochrobactrum, Pseudomonas*, and *Stenotrophomonas*.[14,65,66] *Mycobacterium*, as identified in the present study, has been linked with the capacity to degrade recalcitrant pollutants due to their multipurpose metabolic potentials.[67] Potential PAH-degrading bacteria possess the catechol genes that code for C23O enzymes of aerobic bacterial degradation pathways of aromatic compounds.[21] Amplification of the catechol genes in the bacterial isolates suggested their potential participation in biodegradation of PAH components of crude oil sludge. During the biodegradation, most aromatic compounds such as PAHs are degraded into central intermediates such as catechol and protocatechuate. The metabolism of catechol usually occurs through two alternative

pathways for aromatic compounds, the *ortho*-cleavage and the *meta*-cleavage pathways.[68,69] These metabolic pathways initially are catalyzed by the enzymes catechol-1,2-dioxygenase and C23O, respectively.[20,21]

Desorption of sorbed PAHs leading to inaccessibility of the substrates by potential degraders is a limiting aspect of PAH degradation.[70] During the course of this study, this challenge was supposedly mitigated through the inclusion of anionic and nonionic surfactants. Results obtained during this study did not show a significant consistent improvement of PAH degradation from incorporation of anionic (SDS) and nonionic (TD) surfactants at the concentrations of 0.5% and 1.0%. This could be because of complex interactions between PAHs that sometimes inhibit degradation.[71] Allen *et al*. obtained contrasting results in the application of nonionic surfactants to the biodegradation of PAHs, with growth inhibition of some strains observed leading to reduced degradation efficiency of the bacterial strains.[72] There also is insufficient information to indicate the contribution of the surfactants to improved bioavailability, which, as reported by Bezza and Chirwa, does not necessarily enhance the process of biodegradation of aromatic PAHs.[73] Although the addition of surfactants had no apparent effect on degradation efficiency, their presence enhanced fungal growth.[74] Enhanced fungal growth upon addition of surfactants signifies that the added surfactants were not toxic to indigenous microbial entities in the various treatments. This gives credence to previous findings that the toxicity of anionic surfactant SDS to microorganisms is not significant considering the biodegradability of SDS.[75] Biodegradation of surfactants possibly resulted in the complete removal of surfactants from the contaminated system, the microbial entities could have utilized the surfactants as preferred substrate while degrading the pollutant (PAHs) cometabolically.[76] This corresponds to the report of Bautista *et al*. which illustrated the ability of some identified bacterial isolates related to the present study to metabolize surfactants and PAHs.[77] The nonionic surfactant TD did not present any significant effect on the biodegradation of PAHs. Low cytotoxicity of nonionic surfactants in comparison to their ionic counterparts is due to a weaker interaction between a charged microbial cell and neutral surfactant molecules.[78] The surfactants (anionic and nonionic) involved in this study could not perhaps perform their vital role in bioremediation, which is reducing the binding capacity of PAHs by enhancing the mass transfer of PAHs from a solid/nonaqueous liquid phase to aqueous phase in order to improve bioavailability.[26]

CONCLUSIONS

Compost bioremediation is a sustainable waste management technique for hazardous wastes such as crude oil sludge and can serve as a carrier agent for the abundant indigenous microorganisms that are potential PAH degraders. Addition of surfactants did not enhance biodegradation of PAHs but it encouraged the growth of fungi. Further studies using metagenomics could enhance the identification of uncultivable potential degraders of hydrocarbons. Future studies also could focus on identification of PAH metabolites during compost bioremediation.

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CONFLICT OF INTEREST

None of the authors of this paper has a personal or financial relationship with other people or organizations that could unacceptably affect the content of the paper.

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DATA AVAILABILITY STATEMENTS

The data generated or analyzed during this study are included in this paper and its Supplementary Information. Any additional information concerning the data generated in this study are available from the corresponding author on reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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