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Isolation and Characterization of Polyaromatic Hydrocarbons Degrading Bacteria from Compost Leachate

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ABSTRACT

Polycyclic aromatic hydrocarbon (PAHs) degrading bacteria were isolated from compost leachate (CL) that collected from a composting site located in central Scotland, UK. Isolation was carried out by enrichment using phenanthrene (PHR), Pyrene (PYR) and Benzo(a)pyrene (BaP) as the sole source of carbon and energy. The isolates were characterized using a variety of phenotypic, morphologic and molecular properties. Six different isolates were collected based on the difference in morphological and biochemical tests by using API 20E and API NE, also for their efficiency in PAHs utilization. The 16S rDNA sequence analysis confirmed the results of biochemical identification, as both of biochemical and molecular identification of the isolates assigned them to *Bacillus licheniformis, Pseudomonas aeruginosa, Alcaligenes faecalis, Serratia marcescens, Enterobacter cloacae* and *Providenicia rettgeri* which were identified as the prominent PAHs-utilizers isolated from (CL). This study indicates that the (CL) samples contain a diverse population of PAHs-degrading bacteria and the use of (CL) may have a potential for bioremediation of PAHs contaminated sites.

Indexing terms/Keywords

Polycyclic aromatic hydrocarbon (PAHs), Compost leachate, PAHs degrading bacteria,

Academic Discipline And Sub-Disciplines

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SUBJECT CLASSIFICATION

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INTRODUCTION

For more than 30 years, awareness has been growing about the dangers posed on human health and the environment by ‘‘persistent toxic substances’’ PTS. Many of these substances of the greatest concern are organic compounds, such as polycyclic aromatic hydrocarbons (PAHs). PAHs are of great environmental and human health concerns due to their widespread occurrence, persistence in terrestrial ecosystems, and carcinogenic properties (1-2), as many are subject to atmospheric, aquatic or biological transport over long distances, and are thus globally distributed, detectable even in areas where they have never been used. Their occurrence in the environment is partly due to anthropogenic activities including the incomplete combustion of fossil fuels, accidental discharge during transport, use and disposal of petroleum products, and incineration of refuse and wastes (3-4-5). PAHs releases to soils and the wider environment have led to higher concentrations of these contaminants than would be expected from natural processes alone (6). All of the previous, may result in a wide range of environmental problems that can accumulate in agricultural environment, which threatened to become a negative impact on sustainable agricultural development. This indicates the need to identify and cleanup sites that have become heavily contaminated so that they do not pose unnecessary risks to health. Thus, the potential of using physical, chemical, or biological technologies (or hybrid combinations of these) to remediate PAH-contaminated sites has received much attention (2-7-8). Research on the biological degradation of PAHs has since 1970s demonstrated that bacteria, fungi, and algae possess catabolic abilities that may be used for the bioremediation of PAH-contaminated waste and water (9-10-11). Numerous studies have shown that composting has an enormous potential for bioremediation and clean-up of soils contaminated with hazardous materials as PAHs by sustaining microbial populations of wide range of microorganisms, which are able to degrade a variety of organic contaminants at laboratory scale and/or field scale. Composts are rich sources of xenobiotic-degrading microorganisms, including bacteria, actinomycetes and lignolytic fungi, and these can degrade pollutants such as PAHs. Although many surface soils contain native bacteria and fungi capable of degrading PAHs and other hydrocarbons, composted materials have been blended with PAH-contaminated soils to aid in the degradation of PAHs as an *ex-situ* remediation process (12-13-14). During composting approach mineralization is considered the more significant mechanism of PAHs- removal reported in most studies.

There are have to date few published studies on compost leachate treatment. Thus, this study focuses on developing a cost-effective bioremediation technology for polyaromatic hydrocarbons (PAHs) contaminated lands through studying physiochemical and microbial characteristics of compost leachate (CL) in attempt to identify those microorganisms spectra with high potential to degrade PAHs, in order to investigate the suitability of CL as a nutrients and microbial source for bioremediation process of PAHs in agricultural ecosystem.

MATERIALS AND METHODS

Chemicals

Phenanthrene (PHR), Pyrene (PYR) and Benzo(a)pyrene (BaP) with purity of 98% and acetonitrile, hexane, dichloromethane DCM and acetone in HPLC grade were purchased from Merck Company. Nutrient agar media and chemical materials for mineral salt medium (MSM) were purchased from Merck, Sigma and Aldrich Chemical Companies.

Source of composting leachate (CL)

Forty liters of compost leachate was collected from composting site located in central Scotland and transported in plastic containers to the University of Edinburgh greenhouse.

Physiochemical analyses

CL samples were primarily characterized using standard analytical methods applied in the contaminated land assessment and remediation research centre (CLARRC), University of Edinburgh, UK: for pH, electrical conductivity (EC), total ∑18 (PAHs) of US-EPA, extraction method was applied by accelerated solvent extractor (ASE 300, DIONEX, Camberley, Surrey, UK) to recover residual PAHs in CL, extracts were then analyzed for total (PAHs) by (Thermo Trace GC-MS). Total organic carbon (TOC) was determined by loss on ignition (LOI) (15). The extract for determination of total N and P was prepared through Kjeldahl digestion (16). Kone supra chemcial analyser (Helsinki, Finland) was used for the colorimetric determination of both ammonia (for total N) and phosphate (for total P) in Kjeldahl digests. Extract for the determination of extractible (dissolved) N was prepared using potassium chloride and for extractible P was prepared using 2.5% glacial acetic acid (AcA) solution, then extracts were analysed using the Kone supra chemical analyzer. Anions were analyzed using a DX-500 ion chromatograph (DIONEX, Sunnyvale, CA, USA), metal concentrations were determined using Perkin Elmer Optima 5300DV; UK inductively coupled plasma-optical emission spectrometer (ICP-OES) instrument. COD for CL was determined with Palintest tubetests system and BOD was determined by respirometric (manometric) measurement using the OxiTopÆ IS 12-6 system, supplied by the Wissenschaftlich-Technische Werkstatten (WTW), Weilheim, Germany.

Total number of heterotrophic bacteria

The total number of culturable bacteria was estimated by determining the number of colony-forming units per g of dry soil (CFU g^{-1}soil). Serial dilution aliquots (0.1 ml) were spread, in duplicate, onto autoclaved nutrient agar (NA) medium (pH = 6.8) contained per liter: 5.0 g Peptone, 3.0 g Meat Extract, 5.0 g NaCl, 20.0 g Agar and cycloheximide (50 mg l^{-1}). Plates were incubated inverted at 25 °C and colonies counted after 5 days.

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Total number of culturable fungi

Potato dextrose agar (PDA) medium (39 g l^{-1}, pH = 5.6), contained per liter 4 g* potato extract, 20 g dextrose, and 15 g Agar. (*4.0 g of potato extract is equivalent to 200 g of infusion from potatoes) and streptomycin (30 mg l^{-1}), was used. The inoculation technique and incubation conditions were identical as for bacteria.

Enrichment of PAHs-degrading consortia and isolation of PAHs-degraders

About 5 ml of CL was added to 50 ml of mineral salt (MSM) medium, contained per litre: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 1 g KNO_3, 0.2 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$ $2H_2O$, 0.1 g NaCl, 0.01 g $FeCl_3.6H_2O$ and 1 mL trace element solution. The trace element solution contained per liter: 23 mg $MnCl_2.2H_2O$, 30 mg $MnCl_4$, 32 mg H_3BO_3, 39 mg $CoCl_2.2H_2O$, 50 mg $ZnCl_2$, 30 mg $NaMnO_4.2H_2O$ and 20 mg $NiCl_2$ (17). The media supplied with 0.5 ml of 3∑PAHs (PHR, PYR and BaP) mixture solution. The PAHs mixture was dissolved in acetone and filtered through a 0.22 µm pore film and added at final concentrations of 0.1, 0.1 and 0.035 g L^{-1}, respectively. The solvent was allowed to evaporate before adding the sample or inoculation. Enrichment was conducted at 25°C and 150 rpm on a rotary shaker in dark for about 1 month. The enrichment cultures were transferred every week with 1 ml inoculums to 50 ml fresh MSM medium with PAHs spiked, and the enrichment repeated for more three times. The isolates that caused visible turbidity were potentially able to use PAHs as a carbon and energy source. About 10^{-6} dilutions were spread on MSM agar plates and incubated at 25°C. At the end of incubation, individual colonies were picked out and streaked on solid media LB for conservation contained per liter: 10 g NaCl, 10 g tryptone, 5 g yeast extract. The first screening of bacterial strains was done after colony morphology and Gram staining to eliminate apparently similar isolates. This resulted in collection of thirty isolates. All bacterial isolates were submitted to a preliminary test for enumeration and utilization of hydrocarbons.

Preliminary test for enumeration and selection of promising PAHs-degrading communities

Spray plate method was used to estimate the inherent PAHs-degradation capacity of CL and isolates. It consisted of growing heterotrophic microorganisms on mineral medium (MSM) with PAHs as the sole source of carbon. PHR, PYR and B[a]P were first separately dissolved in acetone to a concentration of 4 mg ml^{-1} (18-19). Then, 1 ml of each PAHs solution was spread onto solidified MSM medium. The acetone was allowed to evaporate under sterile conditions and left an opaque white thin granular layer of hydrocarbon on the surface. CL or isolates suspension aliquots (0.1 ml) from 10^{-1} and 10^{-6} dilutions were then spread onto the PAHs-coated MSM plates in duplicate and incubated in polyethylene bags for 30 d at 25 °C. At the end of the incubation, PHR, PYR and BaP-degrading bacteria were distinguished as colonies surrounded by clear zones (halos) called zone- forming units (ZFU) due to PAHs uptake and utilization.

Identification of selected bacterial strains by API

A morphological screening and Gram staining; KOH test, growth on MacConkey agar, and on Difco-Cetrimide Agar Base (selective media for *Pseudomonas sp.*), oxidase test were performed. Biochemical tests were carried out using API 20E which is a standardized identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative rods, and API 20NE identification system for non-fastidious, non-enteric Gram-negative rods, (bioMèrieux, Marcy- L'Etoile, France).

Molecular identification of selected bacterial strains using16S rDNA sequence analysis

16S rDNA sequence analysis of isolates was performed by 16S rDNA PCR as reported previously by Obuekwe *et al*. (20) involving an initial DNA extraction and purification using a Wizard Genomic Purification Kit (Promega Corporation, Madison, Wisconsin). Amplification the 16S rRNA gene was done by the use of the primers 27f (AGAGTTTGATCCTGGCTCAG), and 1492r (L) (GGYTACCTTGTTACGACTT) primers was the most successful pair. Program of the thermal cycler was as follows: 95 °C for 10 min, 30 cycle (95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 45 sec) finally 72 °C for 10 min. Store the PCR products at 15 to 25 °C until you are ready to use them. The DNA sequencing of the purified PCR product was carried out using an API 310 automated DNA sequencer. The DNA sequence homology was analyzed in Gene bank by means of the BLAST program.

RESULTS

Physiochemical and microbial characterization of the compost leachate

The physiochemical characteristics of compost leachate (CL), including PAHs content, and the population of microbes, mainly bacteria and fungi, were determined and presented in Tables 1 and 2. Results of physico-chemical characteristics of compost leachate generally showed that the 18∑PAHs concentrations which characterized in leachate were ≤ 0.02 that is to say less than the detection limit (< dl) of GC/MS (Table 1). Results also showed that most of metals concentrations in the leachate were below the Dutch list* (soil and ground water criteria used in the Netherlands for contaminated land) values for contaminated land or less than the detection limits of ICP-OES.

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Table 1 . 18 ∑ PAHs initial concentrations in composting leachate (CL.)

Compounds	CL. (mg/L)
Naphthalene	0.03
1-Methylnaphthalene	< dl
2-Methylnaphthalene	< dl
Acenaphthylene	0.00
Acenaphthene	0.01
Fluorene	0.01
Phenanthrene	0.02
Anthracene	0.02
Fluoranthene	0.02
Pyrene	0.03
Benzo(a)anthracene	0.03
Chrysene	0.04
Benzo(b)fluoranthene	0.03
Benzo(k)fluoranthene	0.05
Benzo(a)pyrene	< dl
Indeno(1,2,3-cd)pyrene	< dl
Dibenzo(a,h)anthracene	< dl
Benzo(g,h,i)perylene	< dl

< dl : less than the detection limit of GC/MS

Table 2. Some physico-chemical and biological properties of compost leachate (CL)

Characteristics	Compost leachate(CL)
pH (1:2.5), H_2O	6.8
EC ($\mu S\ cm^{-1}$)	2313
TOC (%)	41
C:N	21.8
N- content ($mg\ l^{-1}$) Total Kj N Extracted/Dissolved NH_4-N Extracted/ Dissolved TON (NO_2-N+NO_3-N)	 1200±80 1105±369 6.6
P- content ($mg\ l^{-1}$) Total P Extracted/Dissolved PO_4-P	 19±2 10.3±2
COD (mgO2/l)	25333±3.215
$BOD_{5(mgO2/l)}$	8240±223
PAHs ($mg\ l^{-1}$) ∑18 EPA PAHs	 <0.02 (<dl)
Microbial content ($CFUml^{-1}$) Total number of heterotrophic bacteria Total number of culturable fungi	 $2.9\times10^7 \pm 14.5\times10^6$ $3.5x10^5 \pm 1\times10^4$

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Isolation of promising PAHs degrading bacteria

Fifteen different isolates were collected based on the difference in colony morphology (Table 3). Out of those fifteen isolates, eight isolates were more efficient in PAHs degradation compared to the rest of isolates. The confirmation of PAHs utilization was further judged by the growth of isolates in liquid MSM medium amended with 3∑ PAHs under this investigation. The turbidity of transparent media indicates the possible growth of these isolates on PAHs as a carbon and energy source.

Table 3. Growth efficiency of the bacterial isolates on MSM medium amended with hydrocarbons (PHR, PYR and BaP) as a sole of carbon and energy.

Isolate ID	Phenathrene	Pyrene	Benzo(a)Pyrene
2a	+++	+++	++
2b	+++	++	+
3a	++	+++	++
3b	+++	+++	+
4a	+++	++	+
5b	+++	++	++
6a	++	+	+
6b	++	+	+
B2	+++	++	+
7b	++	++	++
8b	+++	+++	+++
9a	+++	+++	+++
10	++	+	+
11	+++	++	+
12	+	++	+

Identification of bacterial isolates potent in PAHs bioremediation

The oxidase activity test showed that 4 of the potent isolates were oxidase positive and 11 were oxidase negative. The efficient eight isolates were subjected to various morphological, Gram stain and biochemical identification (Table 4). The long rod gram positive spore forming isolate was among the oxidase positive isolates and this isolate belongs to genus *Bacillus*. The identification of the rest isolates by biochemical tests using API 20E and API 20NE identification systems were found belonging to *Pseudomonas aeruginosa, Alcaligenes faecalis*, *Providenicia rettgeri, Serratia marcescens*, and *Enterobacter cloacae*. The distribution of identified isolates with the species is illustrated in (Fig. 1).

Table 4. General properties of potent PAHs-Degrading bacteria

Characteristics	Potent PAHs-Degradering bacterial isolates					
	9a, 12	8b,10	5b	B2	11	3A
Cell Morphology	SR	SR	SR	LR	SR	SR
Gram Stain	- n	-n	-n	+p	-n	-n
Motility	M	M	M	M	M	M
Spore formation	-	-	-	+	-	-
KOH test	+	+	+	-	+	+
lactose fermenting bacteria (Growth on Maconkey Agar)	Lac^+ Red/pink	Lac^+ Red/pink	Lac^- White/colorless	ND	Lac^+ Red/pink	Lac^- White/colorless
Oxidase test	-	-	+	+	-	+

ND: not detected; SR: short rod; LR: long rod; +: positive; - : negative; - n: Gram –ve; +p: Gram +ve; M: motile

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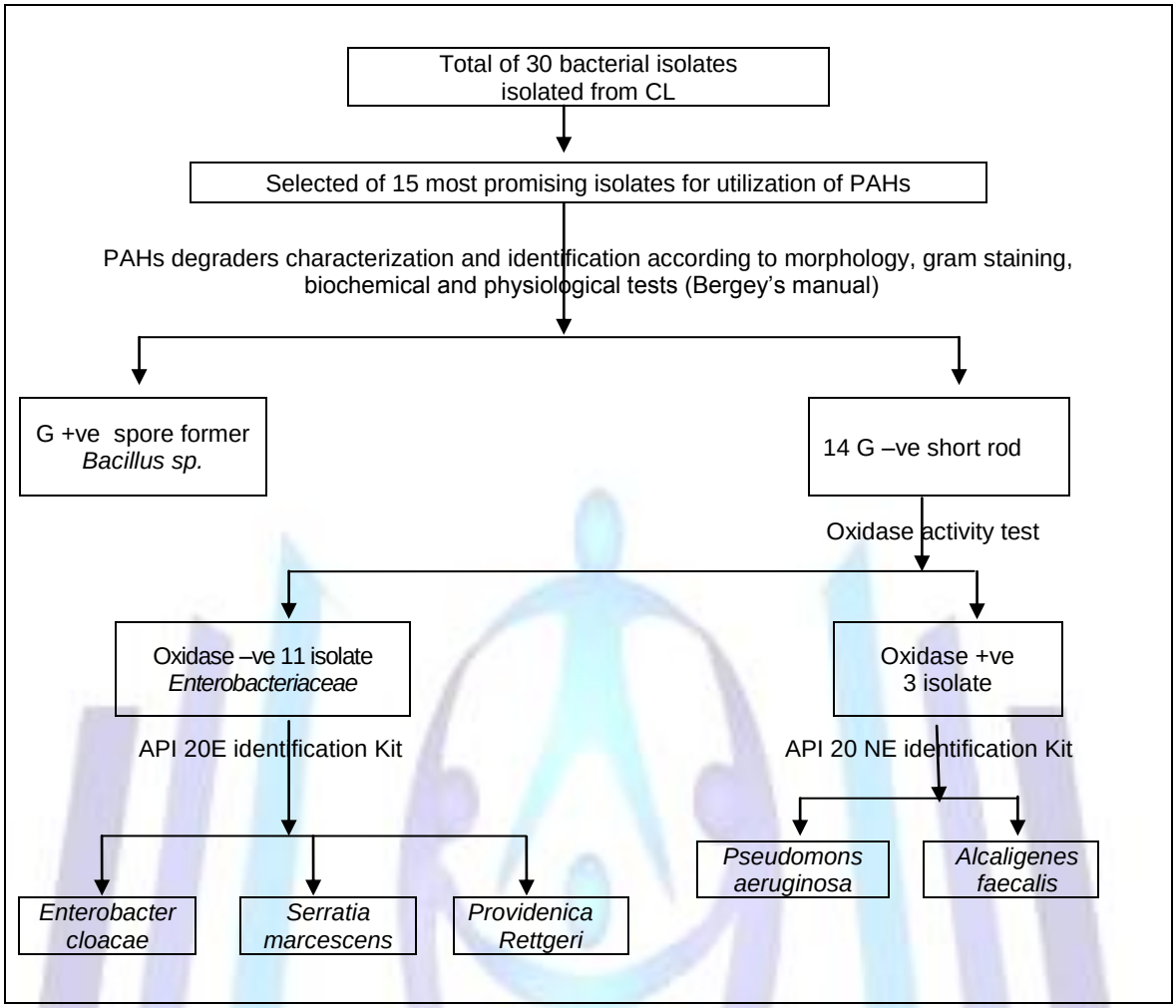

Figure 1. Flowchart for identification of most potent (promising) PAHs-utilizing bacteria.

Further analysis was performed to define taxonomical status and phylogenic relation and similarity between the most potent PAHs-utilizing bacterial strains obtained and other standard strains by using partial 16S rDNA gene sequence analysis . 16S rDNA of 8 representative isolates from CL were partially sequenced and identified. In general, this 16S rDNA sequence analysis confirmed the results of biochemical identification by the API 20E and API NE (Table 7). Six genera, *Bacillus, Pseudomonas, Alcaligenes, Serratia, Enterobacter* and *Providenicia* were identified as the prominent PAHs -utilizers isolated from Compost Leachate (CL). Strain 5b (*Pseudomonas aeruginosa*) showed best matches with *Pseudomonas aeruginosa* strain HSD1 (*Genebank accession no.* HQ457014.1 with 99 % similarity); strains 9a and 12 (*Serratia marcescens)* matched best with *Serratia sp.* zl_3 and *Serratia marcescens* strainN4-5 (*Genebank accession no.*FJ715491.1 and EF035134.1 with 99% and 96% similarity, respectively). Strains 10, 8b (*Enterobacter cloacae*) matched with *Enterobacter sp*. RP24.2 and *Enterobacter cloacae* strain S125R (*Genebank accession no.*GU056352.1 and JF513137.1 with 99% and 93%, similarity, respectively). Strain 3A (*Alcaligenes faecalis*) showed best matches with *Alcaligenes faecalis* (*Genebank accession no.* FJ449841.1 with 98% similarity).Strain11 (*Providenicia rettgeri*) showed best matches to *Providenicia rettgeri* (*Genebank accession no* FJ687602.1 with 97% similarity). Strain B2 (*Bacillus sp.*) matched with *Bacillus licheniformis* (*Genebank accession no.*JF819667.1 with 96% similarity). In the present study, 16S rDNA sequence analysis appeared to be more sensitive than biochemical tests using API kits.

DISCUSSION

In developing countries, where agriculture is the main economical activity, the conventional sources of organic matter are restricted. The compost amendment and its leachate may represent a cost-effective solution promoting both soil fertility and microbial activity. The incorporation of compost leachate (CL) into agricultural soils lacking organic matter may be proposed as a feasible, inexpensive and environmentally sound disposal practice. In addition, CL may enhance the bioremediation of hydrocarbon-polluted soils by introducing microorganisms and nutrients that compensate scant indigenous microbial activity. Biological remedial technology combining chemical analysis with microbiological assessment were undertaken to evaluate the suitability and efficiency of using CL in bioremediation of polyaromatic hydrocarbons (PAHs) of contaminated soils, as chemical analysis alone cannot provide a full picture of the bioremediation process and its effectiveness (21).

Table7. Combined identification biochemical and molecular of potent PAHs-Degrading bacteria

Isolate ID	Gram stain	Biochemical identification	Molecular identification (the closest organism in Genbank database)	Accession no.	Identity (%)
5b	-ve	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* strain HSD1	HQ457014.1	99
9a, 12	-ve	*Serratia marcescens*	*Serratia sp.* zl_3; *Serratia marcescens* strainN4-5	FJ715491.1 EF035134.1	99 96
10, 8b	-ve	*Enterobacter cloacae*	*Enterobacter sp.* RP24.2; *Enterobacter cloacae* strain S125R	GU056352.1 JF513137.1	99 93
B2	+ve	*Bacillus sp.*	*Bacillus licheniformis*	JF819667.1	96
3A	-ve	*Alcaligenes faecalis*	*Alcaligenes faecalis*	FJ449841.1	98
11	-ve	*Providenicia rettgeri*	*Providenicia rettgeri strain BPK-15*	FJ687602.1	97

Results of physico-chemical and microbiological characteristics of CL generally indicated that the content of soluble or suspended material in the leachate mainly dependent on the composition of the feed stocks that being processes there. That is to say, "leachate" is only as good as the compost from which it comes. This is strongly supported by many investigations which emphasized that the variability in CL quality from composting facilities varied depending on the type of compost, composting process implemented, and environmental conditions (22).On the other hand multiple studies reported various levels of metals such as copper, lead and zinc to be present in CL and run-off (23-24). Sellars (22) reported other parameters found in food waste/mixed food waste leachate included Cl^- (700 – 10000) mg l^{-1} and Pb (10 – 200) micrograms per liter (ug l^{-1}). While the runoff included Cl (30 -500) mg l^{-1}; Cd non-detect to 200 µg l^{-1} and Pb non-detect to 50 µg l^{-1}.

On the microbial level, Tetra Tech. Inc. (25) measured bacterial densities in leachate derived from food waste compost processed in passively aerated windrows (PAW) as well as limited turned windrows (LAW), for both types of windrows *Salmonella* densities were reported < 0.3 MPN/100 ml. However, fecal coliform densities ranged from ND-90 MPN/100 ml for PAW and ND-50,000 for LAW systems. Bartlett (23) reported that *E. coli* densities at 30 MPN/100 ml for leachate generated from food residuals and yard trimmings compost. At the position of groundwater quality, there is very limited information on groundwater quality underneath or in proximity to composting facilities. Savage and Tyrrel (26) suggested that liquor was highly variable in composition and very strong in nature (e.g. BOD_5 10,000–50,000 mg/l; NH_3=NH_4 300–1200 mg/l). It is also known that the composition of compost liquor is likely to vary according to the duration of composting (27) as they found that liquors leached from aerobically digested green waste contained higher levels of BOD_5, ammoniacal nitrogen and electrical conductivity in the first 6 weeks of composting than in the second 6 weeks of the trial. From all the previous, a conclusion can be built on that by controlling of materials that entering the composting process, can produce a good quality of compost byproducts (as leachate or run off) which could have beneficial role in soil quality.

Recent advances in microbial ecology make it possible to combine molecular and culture-dependent approaches in order to describe bacterial diversity at environmental sites. Molecular methods based on 16S rRNA gene analyses are useful for describing microbial community structure, while bacterial isolation *via* culture-dependent approaches is needed to characterize the degradation pathways. The combination of both culture-independent and culture-dependent techniques might provide useful and complementary information on the structure of microbial communities. The application of molecular-biology-based techniques has improved our understanding of the composition, phylogeny and physiology of metabolically active members of the microbial community in the environment (28-29-30). It can be concluded that there is a great diversity of microorganisms which are capable of degrading low-molecular-weight (LMW) PAHs, such as naphthalene and phenanthrene (31-32). However, relatively few organisms have been observed to degrade the high-molecular-weight (HMW) PAHs, and there is only limited information regarding the bacterial biodegradation of PAHs with five or more rings in both environmental samples and pure or mixed cultures (2-32-33).

In present study, a series of experiments was conducted to isolate and identify the most promising strains efficient in PAHs degradation. Selective enrichment of compost leachate samples led to isolation of microbial consortia adapted to grow on 3∑PAH namely phenantheren (PHR), pyrene (PYR) and benzo(a)pyrene (BaP) as a sole carbon and energy source for growth. Finally, six isolates were selected as the best concomitant PAHs utilizing bacteria compared to others. The results obtained from the morphological, Gram stain and biochemical identification using API kits identified the isolates as, *Pseudomonas aeruginosa, Serratia marcescens*, *Enterobacter cloacae*, *Bacillus sp., Alcaligenes faecalis, and Providenicia rettgeri*. Further conformation was performed to define taxonomical status of the most potent PAHs- utilizing bacteria by using of molecular approach by partial 16S rDNA gene sequence analysis. Similar approach was conducted

by (34) they used selective cultures for enrichment of PAHs-degrading strains. Culturable PAHs-degrading strains were obtained from these enrichments showed that 7 isolates were novel, using the criterion that they were 97% or less similar to known isolates based on the 16S rRNA gene sequence. The study emphasized the hypothesis that a high diversity of bacterial strains with the ability to degrade multiple PAHs can be confirmed by the combined use of classical selective enrichment and molecular analyses.

Results of phenotypic and molecular identification were indicating that all isolates were identifiable at the genus and species level, by biochemical identification with identity percent ranging from 95.2 % to 98.9%. Furthermore 16S rDNA sequencing technique was conducted to confirm identification of environmental isolates obtained from enrichment CL samples. Partial 16S rDNA gene sequencing and database homology search for the eubacterial isolates, also, revealed their tentative close relationship to members of families*: Alcaligenaceae (Alcaligenes faecalis)*, *Pseudomonadaceae (Pseudomonas aeruginosa)* , *Enterobacteriaceae* (*Serratia marcescens,* and *Enterobacter cloacae*) and *Bacillaceae (Bacillus licheniformis).* The relationship obtained among the isolates under study was the same with biochemical and molecular assays. Similar results achieved in molecular identification of environmental isolates that showed a similarity greater than 99% to *Alcaligenes Sp., Bacillus megaterium, Bacillus firmus, Paenibacillus lautus,* by Riccardi *et al.* (35) in a study of improving the biodegradation of pyrene. On the other hand, Weissenfels *et al.* (36) and Kanaly and Harayama, (37) documented that the isolation of the soil microorganism *Alcaligenes denitrificans* strain WW1, which biodegraded fluoranthene at a rate of 0.3 mg ml^{-1} per day and which also co-metabolized other PAHs, including pyrene and benz(a)anthracene. Also, the presence of *Alcaligenes* sp. in soils contaminated from naphthalene and phenanthrene was detected (38). Furthermore, Abd-Elsalam *et al.* (39) isolate anthracene and phenanthrene degrading bacteria from different contaminated sites in middle delta, Egypt. The isolates were identified as *E. coli*, *Alcaligenes* sp. and *Thiobacter subterraneus* after subjecting to 16S rDNA sequence analysis. Mohsen *et al.* (40) found highest removal (more than 85 %) of phenanthrene in their biotreatment study of phenanthrene in solid phase reactor with two bacterial mixed cultures for a period of 20 weeks, and the microbial analysis using confirmative series tests and analytical profile index (API) kit tests revealed that the *Pseudomonas fluorescence*, *Serratia liquefaciens*, and *Bacillus* strains were of dominant bacteria in the mixed cultures.

Thus, present results indicated that the CL may have a great potential for application in cost-effective bioremediation processes of PAHs contaminated lands, which attributed to the microbial consortium contained in it. Also, CL could increasingly used to promote the fertility and the microbial activity of agricultural soils, it is an unlimited source of organic matter and minerals, however possible toxic effect to soil biota should be considered and case-specific studies including appropriate test-organisms should be carried out. Thus, CL may consider as suitable source of nutrients and microbes for bioremediation of PAHs contaminated lands.

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REFERENCE

[1] Brandt, C. A., Becker, J. M, and Porta, A. 2002. Distribution of polycyclic aromatic hydrocarbons in soils and terrestrial biota after a spill of crude oil in Trecate, Italy. Environmental Toxicology and Chemistry 21: 1638–1643.

[2] Ukiwe, L. N., Egereonu, U. U., Njoku, P. C, Nwoko, C. I. A. and Allinor, J. I. 2013. Polycyclic Aromatic Hydrocarbons Degradation Techniques: A Review. International Journal of Chemistry 5, No. 4; 43- 53

[3] Beck, A. J., Johnson, D. L. and, Jones, K. C. 1996. The form and bioavailability of non-ionic organic chemicals in sewage sludge-amended agricultural soils. The Science of the Total Environment 185:125–149.

[4] Fernandez, P., Grimalt, J. O, and Vilanova, R. M. 2002. Atmospheric gas-particle partitioning of polycyclic aromatic hydrocarbons in high mountain regions of Europe. Environmental Science and Technology 36: 1162–1168.

[5] Dyke, P. H., Foan, C. and Fiedler, H. 2003. PCB and PAH releases from power stations and waste incineration processes in the UK. Chemosphere 50: 469–480.

[6] Kanaly, R. A., Bartha, R., Watanabe, K, and Harayama, S. 2000. Rapid mineralization of benzo[a]pyrene by a microbial consortium growing on diesel fuel. Applied and Environmental Microbiology 2000; 66: 4205–4211.

[7] Maini, G., Sharman, A,, Knowles, C., Sunderland, G, and Jackman, S. 2000. Electrokinetic remediation of metals and organics from historically contaminated soil. J. Chem. Technol. Biot., Journal of chemical technology and biotechnology 75: 657-664.

[8] Zeng, V., Hong, P. K. A, and Wavrek, D. A. 2000. Integrated chemical- biological treatment of benzo[a]pyrene. Environmental Science and Technology 34: 854-862.

[9] Dean-Ross, D., Moody, J. and Cerniglia, C. E. 2002. Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS Microbiology Ecology 2002; 41: 1-7.

[10] Wang, Q., Zhang, S., Li, Y. and Klassen, W. 2011. Potential Approaches to Improving Biodegradation of Hydrocarbons for Bioremediation of Crude Oil Pollution. Journal of Environmental Protection 2: 47-55

[11] Maiti, A., Das, S. and Bhattacharyya, N. 2012. Isolation and Characterization of a New Bacterial strain from Petroleum Oil Contaminated Soil, India. Journal of Science (JOS). 2, No. 2: 103-108

[12] Antizar-Ladisla, B., Lopez-Real, J. and Beck, A. J. 2006. Degradation of polycyclic aromatic hydrocarbons (PAHs) in an aged coal tar contaminated soil under in-vessel composting conditions, Environmental Pollution 141: 459–468.

[13] Cai, Q. Y., Mo, C. H., Wu, Q. T., Zeng, Q. Y., Katsoyiannis, A., and Ferard, J. F. 2007. Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated sewage sludge by different composting processes. Journal of Hazardous Materials 142: 535–542.

[14] Sayara, T., Borras, E., Caminal, G., Sarra, M. and Sanchez, A. 2011. Bioremediation of PAHs-contaminated soil through composting: Influence of bioaugmentation and biostimulation on contaminant biodegradation. International Biodeterioration & Biodegradation 65: 859-865.

[15] Heiri, O., Lotter, A. F, and Lemck, G. 2001. Loss on ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results. Journal of Paleolimnology 25: 101–110.

[16] Schumacher, B, A., Neary, A. J., Palmer, C. J., Maynard, D. G., Pastorek, L., Morrison, I. K. and Marsh, M. 1995. Laboratory methods for soil and foliar analysis in long-term environmental monitoring programs EPA/600/R-95/077, 1995.

[17] Ressler, B., Kneifel, H. and Winter, J. 1999. Bioavailability of polycyclic aromatic hydrocarbons and formation of humic acid-like residues during bacterial PAH degradation. Applied Microbiology and Biotechnology 53: 85-91.

[18] Bengtsson, G, and Zerhouni, P. 2003. Effects of carbon substrate enrichment and DOC concentration on biodegradation of PAHs in soil. Journal of Applied Microbiology 94: 608–617.

[19] Hamdi, H., Benzarti, S., Manusadzianas, L., Aoyama, I. and Jedidi, N. 2007. Solid-phase bioassays and soil microbial activities to evaluate PAH-spiked soil ecotoxicity after a long-term bioremediation process simulating land farming. Chemosphere 70: 135–143.

[20] Obuekwe, C. O., Al-Jadi, Z. K. and Al-Saleh, E. 2007. Insight into heterogeneity in cell-surface hydrophobicity and ability to degrade hydrocarbons among cells of two hydrocarbon-degrading bacterial populations. Canadian Journal of Microbiology 53: 252–260.

[21] Goi, A., Kulik, N, and Trapido, M. 2006. Combined chemical and biological treatment of oil contaminated soil. Chemosphere 63: 1754-1763.

[22] Sellars, B. 2006. Groundwater concern at composting sites. Oregon Department of Environmental Quality Inter-office Memorandum. December, 2006.

[23] Krogmann, U. and Woyczechowski, H. 2000. Selected characteristics of leachate, condensate and runoff released during composting of biogenic waste. Waste Management and Research 18: 235–248.

[24] Bartlett, J. 2006. Storm water treatment options at composting facilities. BioCycle. February 47, No. 2, p.23.

[25] Tetra Tech, Inc., Seattle, W. A. 2002. Commercial food waste composting study. Final project report. Oregon Department of Environmental Quality. October, 2002.

[26] Savage, A. J. and Tyrrel, S. F. 2005. Compost liquor bioremediation using waste materials as biofiltration media. Bioresource Technology 96: 557–564.

[27] Frederickson, J. 1997. Compost leachate; effect of composting duration on its composition and on its use as a fertilizer. In: Orbit 97.International conference, Harrogate, 3–5th September, pp. 107–112.

[28] Widada, J., Nojiri, H., Kasuga, K., Yoshida, T., Habe, H. and Omori, T. 2002. Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Applied Microbiology and Biotechnology 58: 202-209.

[29] Demnerova, K., Mackova, M,, Spevakova, V,, Beranova, K,, Kochankova, L., Lovecka, P, et al. 2005. Two approaches to biological decontamination of ground-water and soil polluted by aromatics—characterization of microbial populations. International Microbiology 8: 205–211.

[30] Al-Thukair, A. A., Abed, R. M., and Mohamed, L. 2007. Microbial community of cyanobacteria mats in the intertidal zone of oil-polluted coast of Saudi Arabia. Marine Pollution Bulletin 54: 173–179.

[31] Yun, T., Tianling, Z. and Xinhong, W. 2002. PAHs contamination and PAH-degrading bacteria in Xiamen Western Sea. Chemical Speciation and Bioavailability 14: 25–33.

[32] Zhou, H. W., Luan, T. G., Zou, F. and Tam, N. F. 2008. Different bacterial groups for biodegradation of three- and four-ring PAHs isolated from a Hong Kong mangrove sediment Journal of Hazardous Materials 152: 1179–1185.

[33] Luo, Y. R., Tian, Y,, Huang, X., Yan, C. L., Hong, H. S., Lin, G. H, and Zheng, T. L. 2009. Analysis of community structure of a microbial consortium capable of degrading benzo(a)pyrene by DGGE. Marine Pollution Bulletin 58: 1159–1163.

[34] Hilyard, E. J., Jones-Meehan, J. M., Spargo, B. J. and Hill, R. T. 2008. Enrichment isolation and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth river sediments. Applied and Environmental Microbiology 74: 1176.-1184.

[35] Riccardi, C., Papacchini, M., Mansi, A., Ciervo, A., Petrucca, A., La Rosa, G. et al. 2005. Characterization of bacterial population coming from a soil contaminated by polycyclic aromatic hydrocarbons (PAHs) able to degrade pyrene in slurry phase. Annals of microbiology 55:85-90.

[36] Weissenfels, W. D., Beyer, M., Klein, J. and Rehm, H. J. 1991. Microbial metabolism of fluoranthene: isolation and identification of ring fission products. Applied Microbiology and Biotechnology 34:528–535.

[37] Kanaly, R. A. and Harayama, S. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria Journal of Bacteriology 182: 2059–2067.

[38] Colores, G. M., Macur, R. E., Ward, D. M, and Inskeep, W. P. 2000. Molecular analysis of surfactant- driven microbial population shifts in hydrocarbon- contaminated soil. Applied and Environmental Microbiology 66: 2959-2964.

[39] Abd-Elsalam., H. E., Hafez, E. E., Hussain, A. A., Ali A. G. and El-Hanafy, A. A. 2009. Isolation and identification of three-rings polyaromatic hydrocarbons (anthracene and phenanthrene) degrading bacteria. American-Eurasian Journal of Agricultural and Environmental Science 5: 31-38.

[40] Mohsen, A., Simin, N, and Chimezie, A. 2009. Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in Petroleum Contaminated Soils. Iranian Journal of Chemistry & Chemical Engineering 28: 53-59.

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