CHARACTERIZATION OF PYRENE UTILIZING *BACILLUS* SPP. FROM CRUDE OIL CONTAMINATED SOIL

Purnima Khanna[1], Dinesh Goyal[1*], Sunil Khanna[2]

[1] Department of Biotechnology & Environmental Sciences, Thapar University, Bhadson Road, Patiala – 147 004, Punjab, India; [2] Professor, Biotechnology & Bioinformatics, NIIT University, Neemrana, Rajasthan, India.

ABSTRACT

Pyrene, a high molecular weight polycyclic aromatic hydrocarbon (PAH), is a priority pollutant present in soil contaminated with crude oil, coal-tar and complex PAHs. Bacterial consortium CON-3 developed from crude oil contaminated soil of Patiala, Punjab (India) cometabolized 50 µg ml^{-1} pyrene in the presence of glucose (0.5 %; w/v) at 30 °C, as determined by reverse-phase high performance liquid chromatography (HPLC). *Bacillus* sp. PK-12, *Bacillus* sp. PK-13 and *Bacillus* sp. PK-14 from CON-3, identified by 16S rRNA gene sequence analysis, were able to cometabolize 64 %, 55 % and 53 % of pyrene in 35 days, respectively. With the increase in glucose concentration to 1.0 % (w/v) in growth medium isolates PK-12, PK-13 and PK-14 showed 19 - 46 % uptake of 50 µg ml^{-1} pyrene in 4 days, respectively. Uptake of pyrene was correlated with growth and biosurfactant activity, which is suggestive of the potential role of members of *Bacillus* genera in pyrene mobilization and its uptake.

Key words: Pyrene, *Bacillus*; crude oil contaminated soil, high performance liquid chromatography (HPLC)

INTRODUCTION

A variety of polycyclic aromatic hydrocarbons (PAHs) are formed as a result of anthropogenic activities such as incomplete combustion of coal, oil, gas and wood (13), use of creosote as wood preservatives (34), generation of wastes from petrochemical industries (14), oil refining (43) and coal gasification plants (17). PAHs e.g. anthracene, phenanthrene and pyrene have been identified as biohazardous chemicals by different State and Central Pollution Control Boards due to their toxic, carcinogenic and tetragenic effects on living systems (23). Physico-chemical properties of PAHs such as low water solubility, high adsorption coefficient and high stability of the complex aromatic ring structure limit the application of conventional remediation techniques (16, 26). *In situ* bioremediation is an attractive process due to its cost effectiveness, versatility and the benefit of pollutant mineralization to carbon dioxide and water (12). Bacterial degradation of petroleum and xenobiotic aromatic hydrocarbon contaminants in natural ecosystems may result from catabolism by individual strains such as *Mycobacterium* (9; 13), *Bacillus*, *Pseudomonas* (10), *Aeromonas*, *Beijerinckia*, *Flavobacterium*, *Nocardia*, *Corynebacterium*, *Burkholderia* (7), *Paracoccus* (43), *Stenotrophomonas* (4) and *Sphingomonas* (29, 37) or from combined metabolism by mixed communities called 'consortia' (4, 16, 24).

*Corresponding Author. Mailing address: Department of Biotechnology & Environmental Sciences, Thapar University, Bhadson Road, Patiala – 147 004, Punjab, India.; Tel/Fax.: +91-175-2393011.; E-mail: d_goyal_2000@yahoo.com

The metabolic pathways, enzymatic reactions, and genetic control of the catabolism of lower-molecular-weight (LMW) PAHs (naphthalene, phenanthrene, and anthracene) have been well documented (9, 12, 13). However a low rate of bacterial degradation of higher-molecular-weight (HMW) PAHs (pyrene, and benzo[*a*]pyrene) has been reported which is attributed to their inability to degrade chemicals that are insoluble in water. The aqueous solubility of PAHs decreases almost logarithmically with increasing molecular mass, therefore HMW PAHs ranging in size from four to seven rings are of special environmental concern (17). Some hydrocarbon-degrading bacteria respond to these non soluble carbon sources by producing surface-active compounds (10, 22) which help to pseudo-solubilize the hydrocarbons (32) and promote their bioavailability in the environment (6, 35).

Pyrene, a model compound for HMW PAH degradation studies, is commonly found in Indian soils contaminated with crude oil, coal tar and other complex mixtures of PAHs (10, 27). It has a chemical structure found in several carcinogenic PAHs and is included in the list of 129 'Priority Pollutants' compiled by the U.S. Environmental Protection Agency (12, 14). Pyrene degradation at the metabolic, genomic and proteomic level by actinomycetes group of bacteria has been documented (18; 19 and references therein). However, information about the prevalence of PAH-catabolic nonactinomycetes bacterial genotypes (3, 23, 33) in PAH-contaminated Indian soils is meagre and according to Habe and Omori (12) there may still be many unidentified PAH-degrading bacteria. The aim of the present study was to isolate an aerobic, mesophilic bacterial consortium and its monoculture bacterial isolates from crude oil-contaminated soil with ability to grow in aqueous medium and utilize pyrene.

MATERIALS AND METHODS

Development of bacterial consortium

Crude oil contaminated soil samples were collected from a refinery located in Patiala (Punjab), India. Ten gram of soil was added to 100 ml Bushnell-Haas (BH) liquid medium containing 1 % (v/v) crude oil (Bombay High, India) and shaken on a rotary shaker (120 rpm) at 30 ^{o}C. After 3 weeks of incubation the mixed culture (CON-3) of crude oil utilizing microorganisms was obtained. For selective enrichment 10 ml of the consortium was grown in 100 ml BH medium supplemented with 0.5 % (v/v) crude oil and 10 µg ml^{-1} pyrene (Merck - Schuchardt, Germany) as carbon sources with orbital shaking (120 rpm) at 30 ^{o}C. BH medium (Himedia labs, India) was composed of (g L^{-1} of deionized water): MgSO$_4$.7H$_2$O, 0.2; CaCl$_2$.2H$_2$O, 0.02; KH$_2$PO$_4$, 1; K$_2$HPO$_4$, 1; NH$_4$NO$_3$.6H$_2$O, 1 and FeCl$_3$, 0.05; pH 7.0 $\pm$ 0.2 (39). The medium was sterilized by autoclaving at 121 ^{o}C for 20 min in all tests *in vitro* and pyrene dissolved in acetone solvent (5 %; w/v stock solution) was aseptically added to the media flasks followed by shaking on a rotary shaker (120 rpm) at 30 ^{o}C for 12 hrs before inoculation to allow acetone evaporation as described by Vila *et al.* (41). After 4 weeks of incubation, 10 ml of enriched CON-3 culture was transferred into another flask containing 100 ml fresh sterile BH medium with 0.4 % (v/v) crude oil, 0.2 % (w/v) glucose and 20 µg ml^{-1} pyrene and incubated. Glucose was added to increase the biomass level of enriched culture (38). Every 4 weeks, in fresh 100 ml BH medium, the concentration of crude oil was decreased in steps of 0.1 % (v/v) upto 0.2 % while the concentration of glucose was increased in steps of 0.1 % (w/v) upto 0.5 %. A total of five gradual enrichments of pyrene in steps of 10 µg ml^{-1} upto 50 µg ml^{-1} were carried out to develop the HMW-aromatic hydrocarbon degradation phenotype and to selectively enrich the pyrene-utilizing bacterial isolates in consortium.

Isolation of pyrene utilizing bacterial isolates

The finally developed bacterial consortium CON-3 was serially diluted in 0.85 % (w/v) saline (NaCl) solution and plated on pyrene coated BH agar plates containing 0.25 % (w/v) glucose, in duplicate and incubated for 48-72 hr at 30 ^{o}C.

Pyrene coating of BH agar plates was done according to Kiyohara *et al.* (20) by uniformly spreading 0.1 ml of pyrene stock solution over the surface of the media plate. The acetone immediately vaporized at ambient temperature and a white, thin layer of pyrene remained on the entire surface. Morphologically different, discrete bacterial colonies were picked, purified by repetitive streaking on the same medium (16) and Luria-Bertani (LB) agar medium (24) and evaluated for growth on 25, 50 and 75 µg ml^{-1} pyrene in 100 ml BH medium containing glucose in 250-ml capacity flasks with orbital shaking (120 rpm) at 30 ^{o}C for 30 days. Glucose was provided at 0.25 %, 0.5 % and 0.75 % (w/v), respectively. Growth was measured spectrophotometrically (Hitachi model U-2900, Japan) at 600 nm. Pyrene utilization efficiency was determined by high performance liquid chromatography (HPLC) analysis.

Molecular characterization by 16S ribosomal RNA gene analysis

The phylogenetic affiliation of bacterial isolates with maximum pyrene utilization efficiency greater than 50 % was determined. Genomic DNA was extracted by modified boiling lysis method of Krivobok *et al.* (21). Briefly, bacterial pellet from 2 ml monoculture (cells harvested in eppendorf tube by centrifugation at 10,000 x *g*, 15 min) was suspended in 0.1 ml of 0.22 µm filter-sterilized milli-Q water (Millipore, Germany) by vigorous vortexing. The bacterial cells were lysed by incubating the cell suspension in water bath set at 95 ^{o}C for 10 minutes followed by immediate chilling to 5 ^{o}C. Cell debris was pelleted down by centrifugation at 10,000 x *g* for 15 min. The supernatant (2 µl) containing genomic DNA (~ 10 ng) was used along with *Taq* DNA polymerase (1 unit reaction^{-1}), 4 deoxyribonucleoside triphosphates (200 µM each), MgCl$_2$ (1.5 mM final concentration; MBI Fermentas Life sciences, USA) and universal bacterial primers E8F (*E. coli* position 8-27, 5'-AGA GTT TGA TCC TGG CTC AG-3'; 25) and E1492R (*E. coli* position 1492-1513, 5'-GGT TAC CTT GTT ACG ACT T-3'; 42) (0.5 µM each; Qiagen Operon GmBH, Germany) to amplify the 16S rRNA gene in a PCR thermal cycler (GeneAmp® 9700, Applied Biosystems). The template DNA in the reaction mixture underwent initial denaturation at 92 ^{o}C for 2 min and 10 sec followed by 36 cyclic episodes of denaturation (92 ^{o}C for 1 min and 10 sec), renaturation (48 ^{o}C for 30 sec) and extension (72 ^{o}C for 2 min and 10 sec). Final extension occurred at 72 ^{o}C for 6 min and 10 sec. The PCR product was visualized on 0.8 % agarose gel, ligated with pGEM-T Easy vector (Promega, Wisconsin, USA) and sequenced by Bangalore Genei Pvt Ltd, Bangalore, India. Sequence analysis and nucleotide identity (similarity) search was performed with BLAST program (1) using the nucleic acid sequences deposited in multiple databases like National Centre for Biotechnology Information (NCBI) GenBank database and Ribosomal Database Project release 10 (RDP X).

The 16S rRNA gene sequences obtained in present study have been deposited with the NCBI GenBank database under accession numbers EU685814 to EU685816. Bacterial isolates *Bacillus* sp. PK-12 and *Bacillus* sp. PK-14 have been deposited at Microbial Type Culture Collection library at IMTECH, Chandigarh (India) with MTCC number 1002 and 1003 respectively.

Pyrene uptake studies

Pyrene uptake by bacterial consortium: The capacity of the developed consortium CON-3 to uptake pyrene was evaluated in BH medium (100 ml) containing 50 µg ml^{-1} of pyrene with and without 0.5 % (w/v) glucose in triplicate flasks with orbital shaking (120 rpm) at 30 ^{o}C for 30 days. In another experiment the effect of media optimization on rate of pyrene uptake and absolute / optional requirement of glucose for pyrene metabolism by consortium CON-3 was studied in BH medium (100 ml) supplemented with 50 µg ml^{-1} pyrene at interval of 10 days for 30 consecutive days at 30 ^{o}C in presence of either 0.5 % glucose, 2 ml of trace elements stock solution and 0.1 ml of trace vitamins stock solution (TEV), or 1.0 % glucose. Trace element 50 X stock solution (40) contained (mg ml^{-1}): Nitrilotriacetic acid, 15; MgSO$_4$, 5; FeSO$_4$.7H$_2$O, 1;

$CoCl_2$, 1; $CaCl_2.2H_2O$, 1; $ZnSO_4$, 0.1; $CuSO_4.5H_2O$, 0.1; $AlK(SO_4)$, 0.1; H_3BO_3, 0.1   and Na_2MoO_4, 0.1 and was autoclaved. Trace vitamins 1000 X stock solution (28) contained (mg ml^{-1}): Pyridoxine HCl, 10; Thiamine HCl, 5; Riboflavin, 5; Nicotinic acid, 5; Calcium pentothenate, 5; DL-α-Lipoic acid, 5; Biotin, 2 and Folic acid, 1 (Himedia labs, India), was filter-sterilized and stored at 4 $^{\circ}$C. Batch cultures of CON-3 consortium and uninoculated media (control) flasks were withdrawn at 10 day interval, solvent extracted and quantified by HPLC for pyrene utilization.

Time course uptake of pyrene by bacterial isolates: Time course studies were conducted for determining utilization percentage of pyrene by monoculture isolates. For each bacterial isolate, a batch of sixteen flasks containing BH medium (100 ml) with 50 μg ml^{-1} pyrene, 0.5 % (w/v) glucose and 5 % culture inoculum was incubated at 30 $^{\circ}$C with orbital shaking (120 rpm). One culture flask was withdrawn at zero time (control) to determine initial pyrene concentration. Another flask containing the same amount of pyrene but uninoculated was used as control to determine abiotic losses. Culture flasks (in triplicate) from each batch were withdrawn every 7th day upto 35 days, solvent-extracted and quantified by HPLC.

Effect of glucose concentration on pyrene uptake by bacterial isolates: Glucose concentration in 100 ml BH medium was increased from 0.5 to 1 % (w/v) and the pyrene metabolized (utilized) by the selected bacteria was determined after every 24 hr interval. Total protein content in culture (mg ml^{-1}) was estimated using biuret method of Itzhaki and Gil (15) and glucose utilization (%) was determined by 3, 5-Dinitrosalicylic acid (DNS) assay as given by Plummer (30).

Biosurfactant activity: Standard emulsification assay / index of Barkay *et al.* (2) and Jacques *et al.* (16) was followed to monitor biosurfactant activity of bacterial isolates with maximum pyrene utilization efficiency greater than 50 %. The isolates were grown in duplicate flasks containing BH medium (100 ml) with 50 μg ml^{-1} pyrene plus 1.0 % (w/v) glucose and

incubated at 30 $^{\circ}$C for 4 days with orbital shaking (120 rpm). From one flask withdrew a 5 ml aliquot of the culture supernatant (cells were removed by centrifugation at 10,000 *x g* for 30 min, at 4 $^{\circ}$C) and mixed with 2 % (v/v) Mobil oil (Racer 2T, HP Corporation Ltd., Mumbai) in a glass tube (150 × 18 mm) by vortexing for 1 min (16). The tubes were rested for 10 min and then the degree of dispersion of mobil oil and stability of the emulsion were measured spectrophotometrically at 550 nm (2) against a blank of uninoculated medium with 2 % mobil oil. Contents of the duplicate flasks were estimated for pyrene uptake by HPLC.

Estimation of pyrene by HPLC: Residual concentration of pyrene was determined by liquid-liquid extraction of residual pyrene from BH - glucose medium (inoculated with culture or noninoculated control) followed by spectrophotometric and reverse phase HPLC (PerkinElmer) analysis. The growing culture was acidified to pH 2.0 with 6 N HCl (13; 16) and extracted thrice with 50 ml hexane solvent (Merck; purity > 99.8 %). The extracted material was dried in fume-hood, resuspended in 5 ml acetonitrile and measured spectrophotometrically at 254 nm for pyrene (5). Extraction efficiency was found to be 87 % $\pm$ 3 %. Spectrophotometric results were confirmed by quantifying the amount of pyrene in a reversed phase high-performance liquid chromatograph using C18 column (33 x 4.6 mm). A linear gradient of 50 - 95 % methanol in MQ-water was developed over 20 min at a flow rate of 1 ml min^{-1}. A 0.02 ml aliquot of acetonitrile extract was injected. Pyrene was identified by comparing characteristic absorption spectra (at 254 nm) and retention times to authentic pyrene, using a PerkinElmer diode array detector with data display, and analyzed using PerkinElmer Total Chrome Ver 6.0 software.

RESULTS AND DISCUSSION

Boonchan *et al.* (4), Johnsen *et al.* (17), Jacques *et al.* (16) and Lin and Cai (24) have reported microbial consortia to

possess multiple metabolic capacities that increase the efficiency of the bioremediation process. In present study bacterial consortium CON-3 was developed from refinery waste contaminated soil by selective enrichment technique, by providing pyrene as co-carbon source in the concentration range 10 - 50 μg ml⁻¹ in five successive transfers over a period of five months. The finally developed CON-3 consortium showed 49 % removal of 50 μg ml⁻¹ pyrene from BH mineral salt medium containing 0.5 % (w/v) glucose during 30 days of growth (Fig 1). Utilization of pyrene by bacterial consortium CON-3 could be due to the synergistic effect of various bacterial isolates (4) or the consortium might contain predominantly certain class of bacteria that have wide range of substrate specificity (34). The capability of CON-3 consortium to remove pyrene was quite different from those of PAH-degrading consortia enriched by Jacques *et al.* (16) and Lin and Cai (24) from petrochemical sludge landfarming site and mangrove sediment samples which degraded 22 and 92 % of pyrene in mineral medium, after 30 and 21 days of incubation, respectively.

Minerals and vitamins are required for the enhanced growth and activity of bacteria (26). Addition of trace elements and vitamins (TEV) in basal medium stimulated pyrene uptake by CON-3 in 30 days to 58 %, both in the absence and presence of 0.5 % (w/v) glucose (Fig. 1). When glucose concentration was doubled to 1.0 % (w/v) it stimulated the pyrene uptake to 63 %. This result favoured the requirement of glucose for maximum uptake of pyrene from basal BH medium. According to Cerniglia, (8) pyrene cannot be utilized as a sole carbon and energy source, so a growth substrate must be supplied to initiate growth of the microorganisms and to induce the production of catabolic enzymes. Therefore the growth of CON-3 consortium in BH medium in presence of pyrene is cometabolic in nature. Churchill *et al.* (9) and Boonchan *et al.* (4) also reported cometabolic reaction in *Pseudomonas, Acinetobacter, Nocardia, Bacillus, Mycococcus, Methylosinus* and *Arthrobacter* bacterial species. De-Sisto *et al.* (11) grew bacteria on waste electrical transformer oil in basal medium supplemented with 1 % yeast peptone glucose. Das and Mukherjee (10) observed enhancement of pyrene utilization and bacterial growth in medium containing 0.01 % glucose.
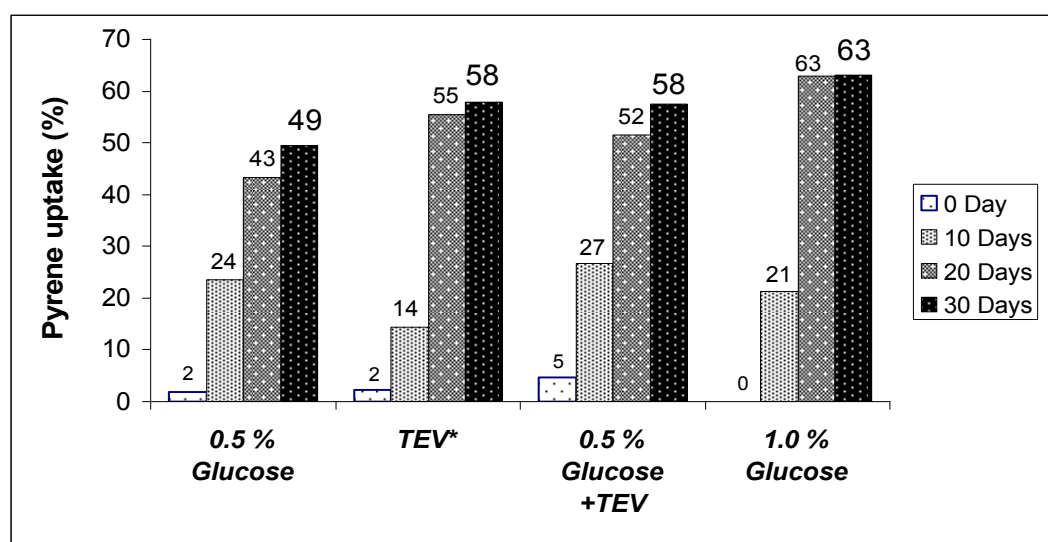

Figure 1. Pyrene uptake by bacterial consortium CON-3 in BH medium supplemented with 50 μg ml⁻¹ pyrene at 10, 20 and 30 days interval at 30 °C.

* TEV indicates trace elements and trace vitamins.

Ten bacterial isolates (PK-12 to PK-21) were obtained from the selectively enriched bacterial consortium CON-3. In the initial screening it was observed that no isolate was capable of utilizing pyrene as sole carbon source. All isolates utilized pyrene as a co metabolite and removed 6 – 98 % of 25 μg ml^{-1} pyrene in presence of 0.25 % (w/v) glucose after 30 days of incubation (Table 1). Five isolates PK-12, PK-13, PK-14, PK-15 and PK-16 showing more than 50 % uptake of pyrene as 98 %, 61 %, 55 %, 54 % and 51 %, respectively, were further exposed to higher concentrations of pyrene in BH plus glucose medium. It was found that 75 μg ml^{-1} pyrene concentration inhibited the growth of all five isolates; however 50 μg ml^{-1} pyrene could support good growth in presence of 0.5 % and 1 % (w/v) glucose respectively. Therefore extent of pyrene uptake by bacterial isolates decreased with increasing concentrations of pyrene. In a 35 day time-course study it was observed that bacterial isolates PK-12, PK-13, PK-14, PK-15 and PK-16 cometabolized 50 μg ml^{-1} pyrene (in presence of 0.5 %; w/v glucose) by 18, 13, 18, 11 and 17 %, respectively, after 14 days of incubation which increased to 59, 53, 50, 50 and 47 %, respectively, as compared to negligible change in abiotic control after 28 days (Fig 2). Thereafter pyrene uptake for two

bacterial isolates PK-15 and PK-16 remained stable till 35 days, while for three isolates PK-12, PK-13 and PK-14 it (uptake) increased upto 64, 55 and 53 %, respectively (Fig 2). Results demonstrate that maximum uptake of pyrene by isolates occurred between 14 and 28 days of growth. Lin and Cai (24) also observed 66 % and 34 % utilization of 50 μg ml^{-1} pyrene by *Bacillus cereus* and *B. megaterium,* respectively, in three weeks.

Table 1. Pyrene (25 mg l^{-1}) uptake efficiency of bacterial isolates from CON-3 consortium, in presence of 0.25 % (w/v) glucose at 30 °C in 30 days.

Bacterial isolates	Pyrene uptake (%)
PK12	98
PK13	61
PK14	55
PK15	54
PK16	51
PK17	8
PK18	16
PK19	6
PK20	17
PK21	10

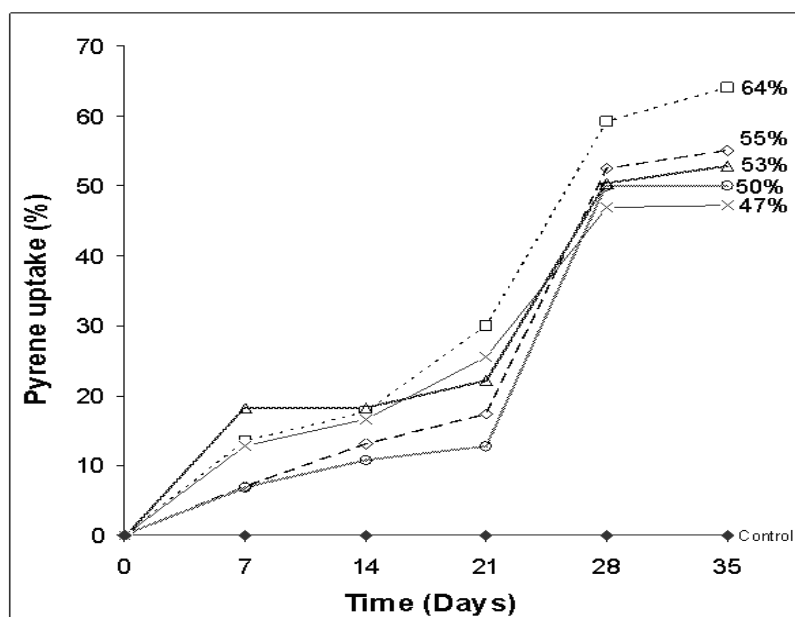**Figure 2.** Pyrene (50 μg ml^{-1}) uptake by bacterial isolates PK-12 (- - □ - -), PK-13 (- - ◊ - -), PK-14 (- -Δ- -), PK-15 (…o…) and PK-16 (—×—), from CON-3 consortium, in presence of 0.5 % (w/v) glucose at 30 °C over a time course of 35 days.

Molecular characterization of three maximum pyrene utilizing bacterial isolates by analysis of approx. 1500 bp long 16S rRNA gene sequence and similarity searches revealed isolates PK-12, PK-13 and PK-14 belonged to the genus *Bacillus* (Table 2). The *Bacillus* strain PK-12 was genetically very close to type strain *B. pumilus* (T) FO-036b (99 % identity). Isolate PK-13 was most homologous to the type specie *B. flexus* (T) IFO-15715 and isolate PK-14 clustered nearest with the type strain *B. firmus* (T) IAM-12464 (both 98 % identity). Members of the genus *Bacillus* have been used in past studies for PAH biodegradation (10; 16; 24, 39). Toledo *et al.* (39) have majorly attributed *Bacillus* strains with the property to colonize environments contaminated with PAHs. They isolated eight *B. pumilus* strains from waste crude oil

capable of growth on naphthalene, phenanthrene or pyrene as sole carbon source. *B. flexus* is not reported to be involved in the degradation of any polycyclic aromatic hydrocarbons till date. Mohamed *et al.* (27) have isolated *B. firmus* as bacterial degraders of petroleum hydrocarbons from contaminated soils in Kuwait. All the low molecular weight (LMW) PAH dioxygenase genes were in gram-negative bacteria, while the high molecular weight (HMW) PAH dioxygenase genes were in gram-positive strains was suggested by Habe and Omori (12). Our findings are in agreement as the three pyrene utilizing isolates, *Bacillus* spp. PK-12, PK-13 and PK-14, isolated from CON-3 consortium belong to gram-positive category.

Table 2. Nucleotide identity (BLAST search output) of bacterial isolates 16S rRNA gene sequence to sequences deposited in the GenBank database.

Bacterial isolate (GenBank Accession No.)	NCBI match (Accession no)	Nucleotide identity (%)
PK-12 (EU685814)	*Bacillus pumilus* (T) FO-036b (AF234854)	99 %
PK-13 (EU685815)	*Bacillus flexus* (T) IFO-15715 (AB021185)	98 %
PK-14 (EU685816)	*Bacillus firmus* (T) IAM-12464 (D16268)	98 %

Pyrene uptake (PU) by the selected bacteria *Bacillus* spp. PK-12, PK-13 and PK-14 in presence of glucose and its correlation with total protein in basal growth medium was studied (Fig. 3). In time course study (Fig. 2) *Bacillus* spp. PK-12, PK-13 and PK-14 showed 14, 7 and 18 % uptake of 50 µg ml^{-1} pyrene respectively, from BH medium containing 0.5 % (w/v) glucose in 7 days. When glucose concentration in BH medium was doubled to 1.0 % (w/v) *Bacillus* spp. PK-12, PK-13 and PK-14 showed increased and rapid cometabolic uptake of pyrene which was 46, 19 and 37 % respectively, in 4 days (Fig 3). Tao *et al.* (37) also observed enhanced cell growth of *Sphingomonas* sp. GY2B on 100 µg ml^{-1} phenanthrene upon addition of glucose at concentration 0.001 to 0.02 % in minimal medium. However glucose concentration greater than

0.05 % inhibited the bacterial growth. In contrast we have found that glucose concentration, as high as 1.0 % (w/v) exerted a positive and stimulatory effect on pyrene utilization, by CON-3 consortium and its three member isolates. A corresponding increase in total culture protein was also observed. Trend in total protein produced in four days of growth was similar to percent pyrene uptake (Fig. 3). Total protein content estimated was maximum for *Bacillus* sp. PK-12 and minimum for *Bacillus* sp. PK-13.

Bacillus sp. are capable of using numerous carbohydrates, but glucose is the most preferred carbon source according to Stulke and Hillen (36) and it often represses the expression and activity of catabolic systems that enable the utilization of secondary substrates (3). Our results however support that

glucose supplementation stimulates pyrene metabolism. Glucose is believed to act as an inducer and co-source of carbon, energy and reducing power for microbes that leads to substantial increase in bacterial biomass with a corresponding increase in metabolic activities (10). Das and Mukherjee (10) have shown 48 % utilization of 2500 µg ml^{-1} pyrene as sole carbon source by *B. subtilis* DM-04 in 4 days of growth at 55 ^{o}C incubation temperature. In present study *Bacillus* sp. PK-12 was able to cometabolize 46 % of 50 µg ml^{-1} pyrene as a co-carbon source in same time interval of 4 days but at 30 ^{o}C, which is optimum for lab-scale and *in situ* bioremediation applications.

It is known that microorganisms growing on hydrocarbons frequently produce biopolymers with emulsifying (6) or surfactant activity (39) so as to improve their ability to utilize these compounds (31). In this study, emulsification assays were carried out to know the capacity of the genetically identified, pyrene utilizing bacteria to produce biosurfactant activity (BA). The supernatants of exponentially growing cultures of *Bacillus* spp. PK-12, PK-13 and PK-14 were evaluated for residual glucose concentration and biosurfactant activity by their ability to emulsify mobil oil at 24 hour interval (Fig. 3). Almost half of the 1.0 % (w/v) glucose provided in the growth medium was utilized by *Bacillus* sp. PK-14 (Fig. 3C) at the end of 4 days, while *Bacillus* spp. PK-12 (Fig. 3A) and PK-13 (Fig. 3B) consumed less glucose. Interestingly the trend for biosurfactant activity was also similar with maximum biosurfactant activity (1.5 units) for *Bacillus* sp. PK-14 (Fig. 3C) followed by *Bacillus* sp. PK-12 (1.1 units; Fig. 3A) and least for *Bacillus* sp. PK-13 (0.7 units; Fig. 3B) after 4 days of incubation. It was further observed that in *Bacillus* sp. PK-14 pyrene uptake occurred during exponential phase of growth and biosurfactant activity (Fig 3 C), while in *Bacillus* spp. PK-12 and PK-13 (Fig. 3A and B, respectively) major uptake of pyrene was observed during the stationary phase in the culture medium.

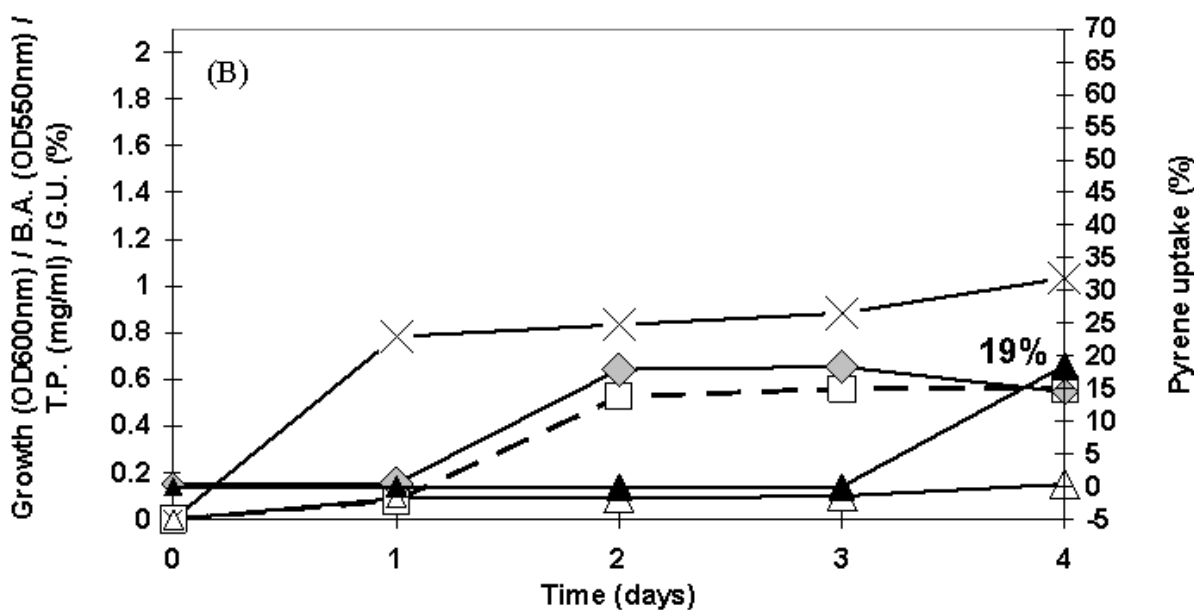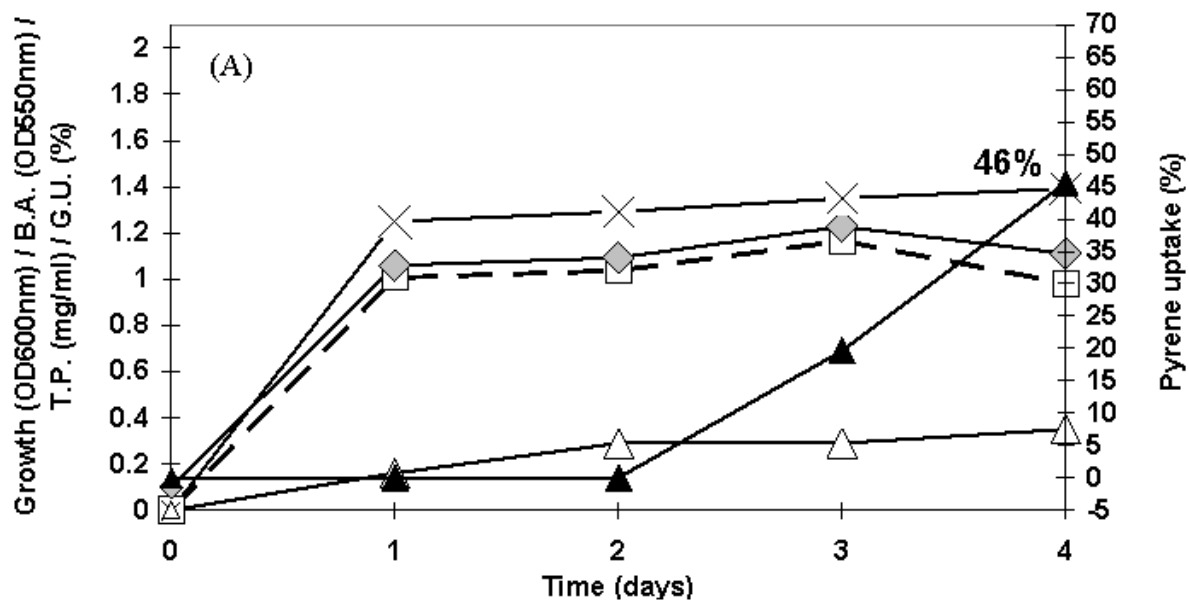At the end of the biosurfactant assay, emulsification of mobil oil resulted from the presence of biosurfactant activity in the culture medium. And this may be the reason that biosurfactant activity corresponded to the change in pyrene concentration in four days. Our proposed assay for biosurfactant activity is quite practical and convenient to use, since it permits preliminary prediction of production of extracellular biopolymers with biosurfactant or bioemulsifier activities by bacteria on the basis of a simple and rapid test. Jacques *et al.* (16) have used a similar emulsification index for diesel oil to estimate surfactant activity produced by bacterial and fungal isolates capable of utilizing a variety of PAHs. In another report Barkey *et al.* (2) tested for emulsifying activity of alasan preparations of *Acinetobacter radioresistens* KA53 by measuring emulsion formation spectrophotometrically at 600 nm.

The dynamics of glucose utilization and biosurfactant activity in the cultures were consistent with that of pyrene concentration change, indicating that the pyrene uptake by the bacterial isolates PK-12, PK-13 and PK-14 may be correlated with biosurfactant activity. In present study pyrene uptake by *Bacillus* sp. PK-14 occurred during the log phase of growth and biosurfactant activity. Glucose utilization and biosurfactant activity of *Bacillus* sp. PK-14 were highest among the three isolates. Therefore, it may be said that the uptake of pyrene is logarithmically associated with biosurfactant activity in *Bacillus* sp. PK-14. In contrast *Bacillus* sp. PK-12 displayed 1.4 times less biosurfactant activity and 1.2 times more pyrene uptake during stationary phase of growth. Correspondingly glucose utilization was also 1.6 times less. This result suggests that biosurfactant activity of *Bacillus* sp. PK-12 was more efficient than *Bacillus* sp. PK-14 in mobilizing pyrene uptake from growth medium. It has also been found by Das and Mukherjee (10) that the biosurfactant secreted by *B. subtilis* DM-04 was more efficient than *P. aeruginosa* strains in enhancing the solubility of pyrene in aqueous media resulting in a higher uptake and utilization of pyrene by the former bacteria. They also formulated the hypothesis that a minor variation in biosurfactant isoforms between *P. aeruginosa* NM

and M strains may result in a large variation of the emulsification property and specificity.

There is little literature on uptake of pyrene by *Bacillus* species and its link to biosurfactant activity and glucose utilization. Different members of same genera showing different pyrene uptake capabilities in this study may be related to the biosurfactant activities and glucose utilization ability of respective bacterial species. The present study showing increased pyrene uptake and enhanced emulsification capacities of the bacterial isolates *Bacillus* sp. PK-12 and *Bacillus* sp. PK-14 indicates that these bacteria can be used for biotreatment and bioaugmentation of soils contaminated with PAHs.
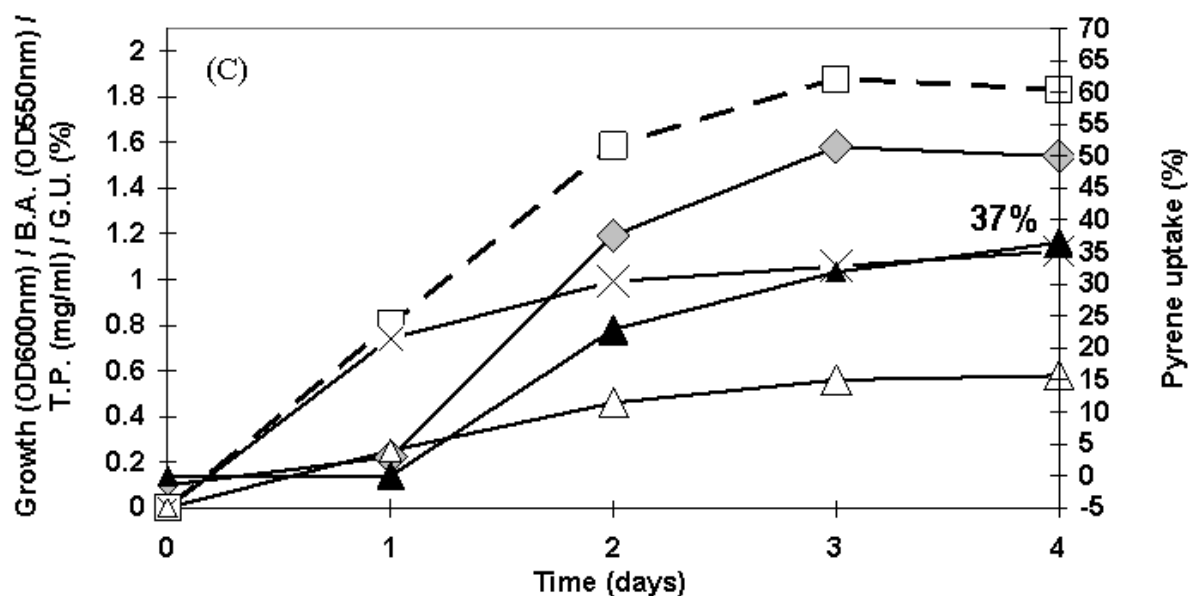
Figure 3. Pyrene (50 µg ml^{-1}) uptake, (—▲—), Biosurfactant activity (B.A; —♦—), Total protein (T.P.; —x—), Glucose utilization (G.U.; –Δ–) and growth (- - □ - -) profile of bacteria *Bacillus* spp. (A) PK-12, (B) PK-13 and (C) PK-14 in presence of 1.0 % (w/v) glucose at 30 °C.

CONCLUSION

PAHs occur at relatively high concentrations in crude oil-contaminated soils and sediments (14), so there is an increasing need in the use of PAH-acclimatized microbial consortia and monoculture isolates for the bioremediation of PAHs in the environment. The present study shows 3 different bacteria isolated from the same bacterial consortium (CON-3) from crude oil-contaminated soil and belonging to the same *Bacillus* genera, but they show different pyrene uptake, biosurfactant activity and glucose utilization profiles. *Bacillus* spp. PK-12, PK-13 and PK-14 exhibited 46 %, 19 % and 37 % cometabolic uptake of 50 µg ml^{-1} pyrene in presence of 1.0 % (w/v) glucose in 4 days. High glucose utilization (0.4 – 0.6 %) and enhanced biosurfactant activities (OD$_{550nm}$ > 1.0) of bacterial isolates *Bacillus* spp. PK-12 and PK-14 may be related to their enhanced pyrene uptake and subsequent utilization abilities. Therefore, soils contaminated with crude oil from refinery wastes, serve as an abode for pyrene metabolizing bacterial microorganisms. Increased pyrene uptake and enhanced emulsification capacities of the soil bacteria *Bacillus* spp. PK-12 and PK-14 in the present study indicates that these bacteria can be used for biotreatment and bioaugmentation of soils contaminated with PAHs.

REFERENCES

1. Altschul, S.F.; Gish, W.; Miller, W.; Myersttt, E.W.; Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.
2. Barkay, T.; Navon-Venezia, S.; Ron, E.Z.; Rosenberg, E. (1999).

Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. *Appl. Environ. Microbiol.* 65, 2697–2702.

3. Basu, A.; Apte, S.K.; Phale, P.S. (2006). Preferential utilization of aromatic compounds over glucose by *Pseudomonas putida* CSV86. *Appl. Environ. Microbiol.* 72, 2226-30.

4. Boonchan, S.; Britz, M.L.; Stanley, G.A. (2000). Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal bacterial cocultures. *Appl. Environ. Microbiol.* 66, 1017-1019.

5. Bugg, T.; Foght, J.M.; Pickard, M.A.; Gray, M.R. (2000). Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. *Appl. Environ. Microbiol.* 66, 5387–5392.

6. Cameotra, S.; Bollag, J.M. (2003). Biosurfactant-enhanced bioremediation of polycyclic aromatic hydrocarbons. *Crit. Rev. Env. Sci. Technol.* 33(2), 111-126.

7. Cerniglia, C.E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. *Adv. Appl. Microbiol.* 30, 31–71.

8. Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation.* 3, 351–368.

9. Churchill, S.A.; Harper, J.P.; Churchill, P.F. (1999). Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Appl. Environ. Microbiol.* 65, 549–552.

10. Das, K.; Mukherjee, A.K. (2007). Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability. *J Appl. Microbiol.* 102, 195-203.

11. De-Sisto, A.; Fusella, E.; Urbina, H.; Leyn, V.; Naranjo, L. (2008). Molecular Characterization of Bacteria Isolated from Waste Electrical Transformer Oil. *Moscow University Chemistry Bulletin.* 63, 120–125.

12. Habe, H.; Omori, T. (2003). Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem.* 67, 225–243.

13. Heitkamp, M.A.; Franklin, W.; Cerniglia, C.E. (1988). Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.* 54, 2549–2555.

14. Irwin, R.J.; VanMouwerik, M.; Stevens, L.; Seese, M.D.; Basham, W. (1997). *Environmental contaminants encyclopedia,* National Park Service; Water Resources Division, Fort Collins, Colorado.

15. Itzhaki, R.F.; Gil, D.M. (1964). A micro-biuret method for estimating proteins. *Anal. Biochem.* 9, 401–410.

16. Jacques, R.J.S.; Okeke; B.C.; Bento, F.M.; Peralba, M.C.R.; Camargo, F.A.O. (2007). Characterization of a polycyclic aromatic hydrocarbon-degrading microbial consortium from a petrochemical sludge landfarming site. *Biorem. J.* 11, 1-11.

17. Johnsen, A.R.; Wick, L.Y.; Harms, H. (2005). Principles of microbial PAH-degradation in soil. *Environ. Pollut.* 133, 71–84.

18. Kanaly, R.A.; Harayama, S. (2000). Minireview: Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.* 182, 2059-2067.

19. Kim, S.J.; Kweon, O.; Jones, R.C.; Freeman, J.P.; Edmondson, R.D.; Cerniglia, C.E. (2007). Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. *J. Bacteriol.* 189, 464–472.

20. Kiyohara, H.; Nagao, K.; Yana, K. (1982). Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl. Environ. Microbiol.* 43, 454–457.

21. Krivobok, S.; Kuony, S.; Meyer, C.; Louwagie, M.; Willison, J.C.; Jouanneau, Y. (2003). Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases. *J. Bacteriol.* 185, 3828–3841.

22. Kumar, G.; Singla, R.; Kumar, R. (2010). Plasmid Associated Anthracene Degradation by *Pseudomonas* sp. Isolated from Filling Station Site. *Nature and Science.* 8, 89-94.

23. Kumar M.; Leon V.; Materano A. D. S.; Ilzins O. A.; Galindo-Castro, I.; Fuenmayor S. L. (2006). Polycyclic Aromatic Hydrocarbon Degradation by Biosurfactant-Producing *Pseudomonas* sp. IR1. *Z. Naturforsch.* 61, 203-212.

24. Lin, Y.; Cai, L. (2008). PAH-degrading microbial consortium and its pyrene-degrading plasmids from mangrove sediment samples in Huian, China. *Mar. Pollut. Bull.* 57, 703–706.

25. Martinez-Murcia, A.J.; Acinas, S.G.; Rodriguez-Valera, F. (1995). Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. *FEMS Microbiol. Ecol.* 17, 247– 255.

26. Mellor, E.; Landin, P.; O'Donovan, C.; Connor, D. (1996). The Microbiology of In Situ Bioremediation. Groundwater Pollution Primer CE 4594: Soil and Groundwater Pollution Civil Engineering Dept, Virginia Tech. Available at: http://www.cee.vt.edu/ewr/environmental/teach/gwprimer/biorem/index.html. Accessed 25 April 2010

27. Mohamed, M.E.; Al-Dousary, M.; Hamzaha, R.Y.; Fuchs, G. (2006). Isolation and characterization of indigenous thermophilic bacteria active in natural attenuation of bio-hazardous petrochemical pollutants. *Intl. Biodet. Biodeg.* 58, 213-223.

28. Pfennig, N. (1978). *Rhodocyclus purpureus* gen. nov. and a ring-shaped, vitamin B$_{12}$-requiring member of the family *Rhodospirillaceae. Intl. Syst. Bacteriol.* 28, 283–288.

29. Pinyakong, O.; Habe, H.; Omori, T. (2003). The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs).' *J. Gen. Appl. Microbiol.* 49, 1–19.

30. Plummer, D.T. (1988). *An introduction to practical Biochemistry*, 3rd Edn. Tata Mc Graw Hill Publishing Co., Ltd. New Delhi.

31. Ron, E.Z.; Rosenberg, E. (2002). Biosurfactants and bioremediation. *Curr. Opin. Biotechnol*. 13, 249–252.

32. Rosenberg, E.; Ron, E.Z. (1999). High and low-molecular-mass microbial surfactants. *Applied Microbial and Biotechnol*. 52, 154–162.

33. Sarma, P.M.; Bhattacharya, D.; Krishnan, S.; Lal, B. (2004). Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium *Leclercia adecarboxylata*. *Appl. Environ. Microbiol*. 70, 3163-3166.

34. Stringfellow, W.T.; Aitken, M.D., (1995). Competitive metabolism of naphthalene, methylnaphthalenes and fluorene by phenanthrene degrading *Pseudomonas*. *Appl. Environ. Microbiol*. 61, 357–362.

35. Stucki, G.; Alexander, M. (1987). Role of dissolution rate and solubility in Biodegradation of aromatic compounds. *Appl. Environ. Microbiol*. 53, No. 2, 292-297.

36. Stulke, J.; Hillen, W. (2000). Regulation of carbon catabolism in *Bacillus* species. *Annu. Rev. Microbiol*. 54, 849-80.

37. Tao, X.Q.; Lu, G.N.; Dang, Z.; Yang, C.; Yi, X.Y. (2007). A phenanthrene-degrading strain *Sphingomonas* sp. GY2B isolated from contaminated soils. *Process Biochem*. 42, 401–408.

38. Tian, L.; Ma, P.; Zhong, J.J. (2003). Impact of the presence of salicylate or glucose on enzyme activity and phenanthrene degradation by *Pseudomonas mendocina*. *Process Biochem*. 38, 1125–32.

39. Toledo, F.L.; Calvo, C.; Rodelas, B.; lez-Lopez, G.J. (2006). Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Syst. Appl. Microbiol*. 29, 244–252.

40. Van Hamme, J.D.V.; Odumeru, J.A.; Ward, O.P. (2000). Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture. *Can. J. Microbiol*. 46, 441–450.

41. Vila, J.; Lopez, Z.; Sabate, J.; Minguillon, C.; Solanas, A.M.; Grifoll, M. (2001). Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol*. 67, 5497–5505.

42. Widada, J.; Nojiri, H.; Kasuga, K.; Yoshida, T.; Habe, H.; Omori, T. (2002). Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. *Appl. Environ. Microbiol*. 58, 202–209.

43. Zhang, H.J.; Kallimanis, A.J.; Koukkou, A.I.J.; Drainas, C.L. (2004). Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. *Appl. Microbial Biotechnol*. 65, 124–131.