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Asian Journal of Animal and Veterinary Advances

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Asian Journal of Animal and Veterinary Advances 8 (7): 841-851, 2013
ISSN 1683-9919 / DOI: 10.3923/ajava.2013.841.851

Isolation and Characterization of Marine Beneficial Bacteria from Petroleum Contaminated Sites for Better Environment of Animal Life

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are persistent hydrophobic organic pollutants ubiquitously found in the environment. PAHs consist of cytotoxic, mutagenic and carcinogenic properties and causes serious hazard to human health and environment. Hence, in this investigation eight phenanthrene (250 mg L^{-1}) degrading bacterial strains were isolated by enrichment method from petroleum contaminated sludge samples. Among these isolates, one efficient degrading strain was identified and characterized by using morphological, biochemical, chemotaxonomic (FAMEs analysis) and molecular (16S rDNA sequencing) methods. Based on these studies the strain was identified as gram positive, motile, spore forming, pink pigmented cocci with oxidase negative reaction and utilized sugars such as arabinose, xylose and rhamnose as carbon source. It is susceptible to kanamycin, nalidixic acid and novobiocin type of antibiotics. The dominant fatty acids found in this strain are 15:0 anteiso (44.16%), 15:0 iso (26.36%). Phylogenetic analysis of this strain showed 99% sequence similarity with *Kocuria rosea* and the strain name as PDM-7 was adopted. The degradation study illustrates that this strain utilizing about 82% of phenanthrene in six days. Therefore, this strain can be efficiently used for the bioremediation of PAHs contaminated marine environments and soils.

Key words: Biodegradation, phenanthrene, *Kocuria rosea,* animal life, phylogenetic analysis

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are important hydrophobic organic pollutants widely found in the environment and they are the first atmospheric pollutants to be identified as suspected carcinogens (IARC, 1983; Wang *et al*., 2008). Polyaromatc hydrocarbons are composed of fused benzene and/or pentacyclic rings arranged in angular or cluster positions (Muckian *et al*., 2007). They are thermodynamically stable due to their negative resonance energy and possess high melting and boiling points together with low water solubility and vapour pressures (WHO, 1998; Johnsen *et al*., 2005). The chemical properties and environmental fate of PAHs depends on the number of aromatic rings present and the nature of the linkage between these rings. Many PAHs

contain A, B, Bay, K and L-regions, which can be metabolized to highly reactive epoxides. Phenanthrene is the simplest aromatic hydrocarbon which contains these regions. Polyaromatic hydrocarbons are mainly produced from incomplete combustion of organic materials, fossil fuels, and partly also from natural processes such as forest fires and volcanic eruptions (Duke, 2008; Chen *et al*., 2012). More than 160 PAHs found in nature have been characterized. Among these only 16 were selected as priority pollutants by the United States Environmental Protection Agency (USEPA) and the European Union due to their toxic, mutagenic, or carcinogenic properties (ATSDR, 2005). The International Agency for Research on Cancer (IARC) has identified 15 PAHs including six of the 16 USEPA regulated PAHs as potential carcinogens. Most of the PAH environmental burden is found in the soil (95%) as opposed to air (0.2%) (ENDS, 1994). The highest PAHs concentration is generally found in the urban environment. The main reasons for this high PAHs concentration are rapid industrialization, increased vehicular traffic and domestic waste (Moja *et al*., 2013). Distribution of PAHs in marine environment is mainly due to the oil spillage and transport of toxic substances from the urban environment. The presence of increased concentration of PAHs in the marine water and sediment are toxic to both benthic and marine animals. It causes threat to the ecosystem through the transfer of PAHs into the food chain (Vagi *et al*., 2005; Sei and Fathepure, 2009; Lors *et al*., 2009).

Consequently, in view of the above problem, many strategies have been advanced such as biological, physical, chemical, thermal and solidification approaches. Among these the biological treatment is the most efficient and economical compared to others (Doyle *et al*., 2008). Bioremediation is a technology that utilizes the metabolic potential of microorganisms to clean up contaminated environment. It shows particular promise as a safe and cost-effective option and it is the most significant and influential in removal of PAHs (Haritash and Kaushik, 2009; Nwachukwu, 2010; Lu *et al*., 2011). Therefore, in recent years considerable interest has been focussed in the isolation and characterization of microorganisms with PAHs degrading potential. The studies on the fate and accumulation of PAHs in natural environments have also been increased (Igwo-Ezikpe *et al*., 2009).

A variety of genera of gram-positive and gram-negative bacteria, fungi, algae have been isolated and characterized for their ability to utilize PAHs as their sole carbon and energy source. During the last few decades a variety of microorganisms capable of degrading PAHs have been discovered and which include *Pseudomonas, Aeromonas, Beijerinckia, Flavobacterium, Nocardia, Corynebacterium, Sphingomonas, Agmenellum, Alcaligenes, Acinetobactor, Burkholderia, Flavobacterium, Micrococcus, Paenibacillus, Corynebacterium, Nocardioids, Moraxella, Lutibacterium, Rhodococcus, Streptomyces, Cycloclasticcus Mycobacterium, Stenotrophomonas, Paracoccus, Burkholderia, Brevibacillus, Martelella, Nitratireductor, Diaphorobacter, Pseudoxanthomonas, Oceanicola, Thalassospira* (Cerniglia, 1984; Juhasz and Naidu, 2000; Daane *et al*., 2002; Samanta *et al*., 2002; Van Hamme *et al*., 2003; Zhang *et al*., 2004; Kodama *et al*., 2008; Yuan *et al*., 2009; Klankeo *et al*., 2009; Reddy *et al*., 2010; Lai *et al*., 2011; Cui *et al*., 2012). Therefore, the objective of this present study was to isolate and characterize the PAHs degrading bacteria from petroleum contaminated sludge samples and also to study their degradation potential.

MATERIALS AND METHODS

Sampling: Petroleum contaminated sludge samples were collected from Hindustan Petrochemical Limited (HPCL) refinery located in Visakhapatnam, Andhra Pradesh, India. The samples were

collected from areas where the waste crude oil remnants from the clean-up of oil storage containers were dumped in separate locations for more than 15 years. Samples were collected from three different sites from these locations in aseptic conditions in plastic containers and stored in refrigerator until further process for bacterial isolation.

Culture medium and chemicals: Mineral salts medium (MSM) (composition: KH_2PO_4 1.0 g, Na_2HPO_4. $2H_2O$ 1.25 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2$ 0.05 g and $FeSO_4$ 0.05 g L^{-1} with the trace element solution comprising $FeSO_4$ 40 μg, $MnSO_4$ 40 μg, $ZnSO_4$ 20 μg, $CuSO_4$ 5 μg, $CoCl_2$ 4 μg, Na_2MoO_4 5 μg, $CaCl_2$ 0.5 μg, KH_2PO_4 136 μg and NaCl 1 mg) was used for this study. The chemicals required for preparation of various culture media were purchased from Sigma-Aldrich, Himedia, Merck, Qualigens, SD-Fine, FINAR and Loba-Chem. Phenanthrene with greater than 98% purity were purchased from Sigma-Aldrich chemicals. Biochemical (KB003 Hi25™) bacterial identification kit and nine different types of antibiotic discs such as Polymyxin-B (pb^{300}), Ampicillin (A^{10}), Kanamycin (K^{30}), Nitrofurazone (Nr^{100}), Novobiocin (NV^{30}), Neomycin (N^{30}), Oxytetracycline (O^{30}), Nalidixic acid (Na^{30}) and Penicillin-G (P^{10}) were purchased from Himedia laboratories.

Enrichment culture for isolation of phenanthrene degrading bacterial strains: The petrochemical sludge samples were cultured on PAH enriched culture medium according to Kiyohara *et al.* (1982). Phenanthrene (250 mg L^{-1}), a three ring PAH with aqueous solubility of 1.29 mg L^{-1} was used as the sole carbon and energy source to enrich the MSM medium. One gram of sludge sample was inoculated in 50 mL of sterilized culture medium taken in a conical flask and incubated at 30°C on orbital shaker at 150 rpm for seven days. A 2 mL of aliquot was transferred every week to fresh sterile medium and incubated under above conditions. One milliliter of the culture (after six transfers) was diluted with saline solution (0.9% NaCl) and plated on MSM agar plates. An ethereal solution of phenanthrene (250 mg L^{-1}) was uniformly sprayed over the plates wherein, the ether vaporizes leaving a thin-white layer of phenanthrene. The plates were incubated at 30°C and after 3-7 days, colonies of candidate phenanthrene-degrading strains were picked up on the basis of clear zones around them. The colonies were further purified by repetitive streaking on fresh plates. The pure cultures of the final bacterial isolates were preserved under refrigeration on nutrient agar slants or as glycerol stocks at -20°C.

Selection of efficient phenanthrene degrading isolates: The purified and selected phenanthrene degrading bacterial isolates were again screened to select high efficient strains. To study their degradation potential, the bacterial isolates were grown on liquid MSM medium to which 250 mg L^{-1} of phenanthrene was added and incubated for 48 h at 30°C and 150 rpm. Their growth was estimated by using turbidometric method and protein content was estimated by using Bradford method.

Identification, morphological, biochemical and chemotaxonomic characterization of the isolates: The bacteria were identified by bright field and electron microscopy and the KB003 Hi25™-Himedia bacterial identification kit was used for biochemical characterization through colorimetric identification. Antibiotic susceptibility was studied by nine types of antibiotic discs (Himedia) according to manufacturer instructions. For chemotaxonomic characterization, FAMEs analysis was performed by Gas-Liquid-Chromatography (Agilent Technologies 5890, 6890 or 6850 GC) with the Sherlock MIS (Microbial Identification System, MIDI, Inc., Newwark, Del) software.

Molecular characterization of the bacterial isolates: Genomic DNA of the selected bacterial isolate was amplified by using the primers: 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3'). These primers are complementary to the conserved regions at the 5' and 3' ends of the 16S rDNA corresponding to positions 9-27 and 1477-1498 of the *Escherichia coli* 16S rRNA gene. Amplified DNA fragments were separated by 1% (w/v) agarose gel electrophoresis. The separated bands were visualized under UV transillumination and then digitalized by Electrophoresis Documentation and Analysis system (Kodak DS, USA). The DNA fragment identified as 16S rDNA was eluted and purified using the clean Genei kit (Bangalore Genei) and sequenced with Gene Amp PCR machine (9600, Perkin-Elmer). The purified sequencing reaction mixtures were electrophoresed using ABI Prism Model 377 version 2.1.1 automatic DNA sequencer. The results were subjected to Bioinformatics analysis for identification of the isolates.

Biodegradation studies: Biodegradation experiments were performed by inoculating exponential phase culture of the bacterial strain in MSM medium containing 250 mg L^{-1} phenanthrene. Incubation was carried out under standardized conditions of temperature 30°C, pH 7.0, 150 rpm and inoculums size of 5 vol. The amount of phenanthrene degraded was estimated every 12 h for 6 days with High Performance Liquid Chromatography (HPLC) (Story *et al*., 2004). For the HPLC analysis, the culture medium (containing the sample) in which the bacteria were growing was separated by centrifugation and bacterial pellet was removed. The supernatant was extracted three times with equal volumes of ethyl acetate, concentrated to 1 mL by rotatory evaporator and analyzed by using HPLC.

The reverse phase HPLC of Schimadzu model LC-10AT chromatograph was used in this study. It is equipped with SPD-M20A prominence Photo Diode Array UV-vis detector and a CMB-20A model prominence Communication Bus Module (CBM). Separation was achieved on Octyldecyl Silane Hypersil column (250 by 4.6 mm and 5 μm particle size) with a injection volume of 20 μL. The mobile phase used was a linear gradient of acetonitrile in 10 mM KH_2PO_4 buffer (pH 3.5) (0 min, 50:50, 0-30 min ramp to 0:100 and 30-50 min isocratic at 0:100). The experiment was repeated twice and the peak area values obtained by the samples were compared with those of calibration curve with R^2 value of 0.9907 (Fig. 1). The rate of degradation was calculated by using Class-VP software provided with the HPLC system.

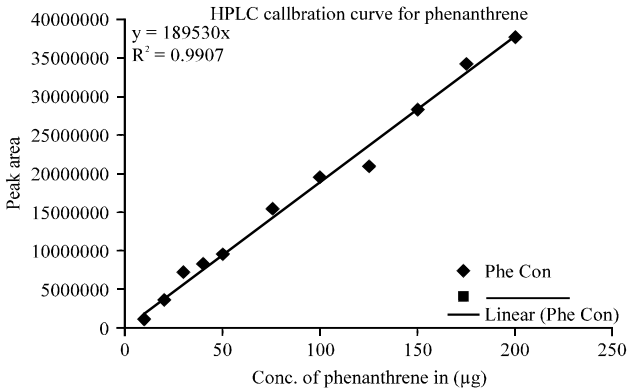

Fig. 1: HPLC standard calibration curve constructed by using Class-VP software for quantification of phenanthrene degradation

RESULTS

Morphological and biochemical characterization: Microscopic studies showed that the strain-7 is a Gram positive, motile, spore forming, aerobic, cocci, occurring singly and in pairs. When grown on nutrient agar plates, colonies of the strain-7 were circular, smooth with pink pigmentation and had round margins. The optimum growth pH and temperature are 6.8-7.0 and 30°C, respectively.

The enzyme activity studies of strain-7 showed that the oxidase and urease activities were negative. Hydrogen sulfide was not produced. Indole and citrate utilization showed negative reaction. The activity of lysine decarboxylase, ornithine decarboxylase and ONPG hydrolysis was positive. The nitrate reduction and phenylalanine deamination reactions were positive. The reactions for methyl red, VogesProskauer's test and esculin hydrolysis were negative and malonate test was also negative. Among the sugars tested, arabinose, xylose and rhamnose were utilized with acid production and utilization of adonitol, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose were not detected with acid production (Table 1).

Antibiotic susceptibility test: Antibiotic susceptibility test for the strain-7 showed that it was susceptible to kanamycin, nalidixic acid and novobiocin; resistant to ampicillin, polymyxin-B, nitrofurazone, oxytetracycline, penicillin-G and neomycin (Fig. 2, Table 2).

Table 1: Biochemical activity tests for the strain PDM-7: It includes 13 conventional biochemical tests and 12 carbohydrate utilization tests

Biochemical tests	Activity
ONPG hydrolysis	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Urease	_
Phenylalanine deamination	+
Nitrate reduction	+
H_2S production	_
Citrate utilization	_
VogesProskaur's test	_
Methyl red	_
Tryptophan deamination (Indole)	_
Malonate	_
Esculin hydrolysis	_
Carbohydrate utilizatiou tests	
Arabinose	+
Xylose	+
Adonitol	_
Rhamnose	+
Cellobiose	_
Melibiose	_
Saccharose	_
Raffinose	_
Trehalose	_
Glucose	_
Lactose	_
Oxidase	_

+: Positive reaction, -: Negative reaction

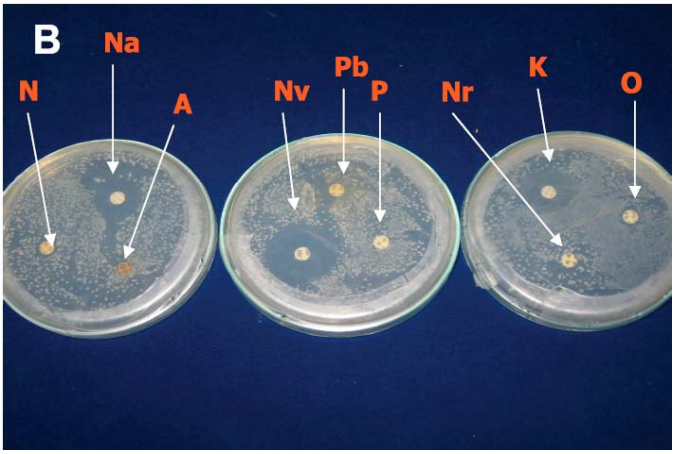

Fig. 2: Agar plates with nine different types of antibiotics (discs) showing zone of inhibition for antibiotic susceptibility test of the strain PDM-7

Table 2: Antibiotic susceptibility tests of the strain PDM-7 for different antibiotics

Types antibiotics	Activity
Polymyxin-B	+
Ampicillin	+
Kanamycin	-
Nitrofurazone	+
Novobiocin	-
Neomycin	+
Oxytetracycline	+
Nalidixic acid	-
Penicillin-G	+

+: Resistant, -: Susceptible, v: Moderately susceptible

Chemotaxonomic characterization: The major fatty acids found in the strain are 15:0 anteiso (44.16%), 15:0 iso (26.36%). The other minor fatty acids found in this isolate were 16:1 w 7c/16:1 w6c (3.82%), 18:1 w7c (2.00%), 16:0 (1.91%), 17:1 iso w9c (1.81%), 14:0 iso (1.78%), 14:0 (1.64%), 17:1 anteiso w9c (1.56%), 16:0 iso (1.38%), 17:0 cyclo (1.29%), 17:0 anteiso (1.05%) and 17:0 (1.03%), respectively (Table 3).

Molecular characterization and phylogenetic analysis: Partial sequence of the 1250 nucleotides length of 16S rDNA of the bacterial strain was obtained. Based on phylogenetic analysis it was closely related to *Kocuria rosea* with bootstrap value of 86. It also showed close relationship with different strains of their respective species groups with bootstrap values of more than 50. Distance matrix calculated for the strain with some of the most related strains indicates that the strain has 99% sequence similarity with *K. rosea*. Bar, 0.005 nucleotide substitutions per nucleotide position. This similarity clearly indicated that the strain-7 belonged to *K. rosea* (Fig. 3). Hence, the strain was identified as *K. rosea* and given the strain name of PDM-7.

Table 3: Fatty Acid Methyl Esters (FAMEs) analysis of different types of fatty acids present in PDM-7

Fatty acids	Composition (%)
15:0 iso	26.36
15:0 anteiso	44.16
14:0 iso	1.78
14:0	1.64
16:0 iso	1.38
16:1 w7c alcohol	-
17:0 anteiso	1.05
17:0 anteiso B/iso I	-
17:1 iso w10c	-
17:0 iso	-
17:0 iso w9c	1.81
17:1 iso w10c	-
17:1 anteiso w9c	1.56
17:0 cyclo	1.29
17:0	1.03
16:0	1.91
16:1 w1 1c	-
16:1 w7c/16:1w6c	3.82
16:1 w6c/16:1w7c	3.82
18:1 w6c	2.00
18:1 w7c	2.00

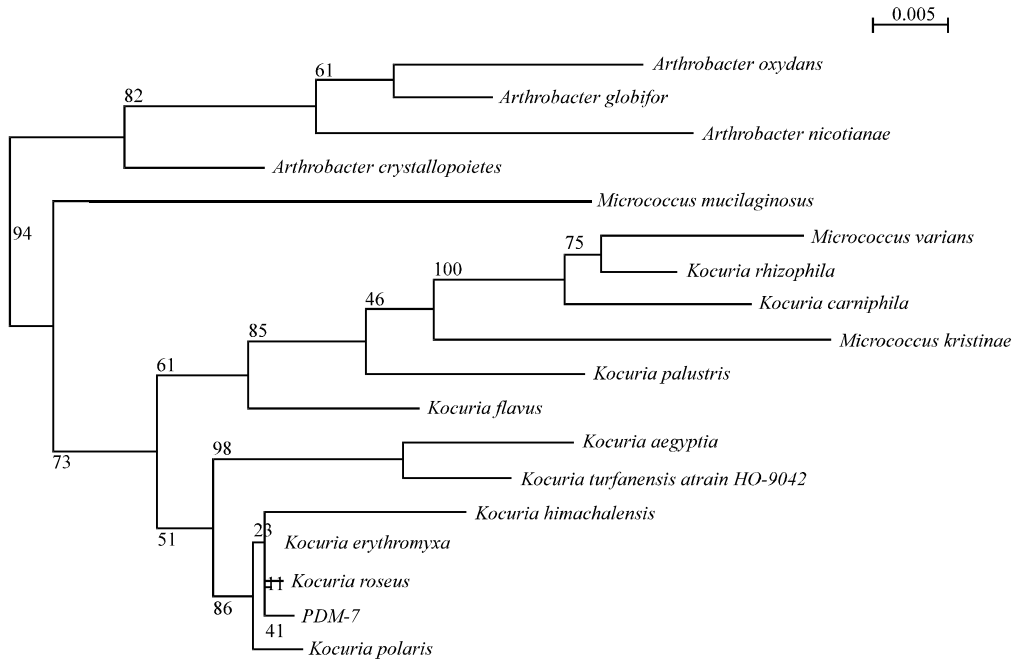

Fig. 3: Phylogenetic position of PDM-7 among related bacteria

Biodegradation studies: The HPLC analysis showed that the strain *Kocuria rosea* degrading 81.92% of phenanthrene in six days (Fig. 4). Based on these results, the bacterial strain

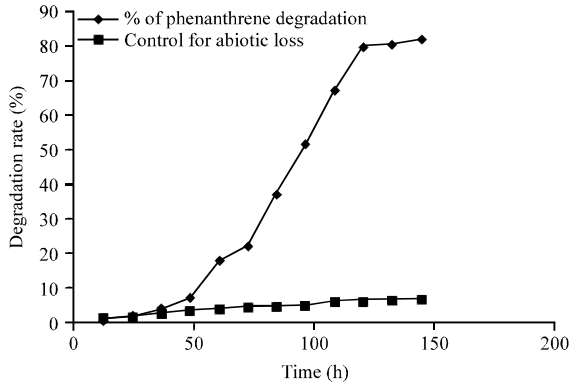

Fig. 4: Rate of phenantherene degradation at different time intervals by *Kocuria rosea* PDM-7

Kocuris rosea PDM-7 could be used to clean petrochemical contaminated marine environments and soils for the benefit of marine animals.

DISCUSSION

Microbial bioremediation of a toxic chemical depends mainly on the capacity of a microorganism to survive in that site and its capacity in utilizing that compound as a substrate for growth. Hence, selection of appropriate organisms becomes first and foremost criteria for this aspect. In this study eight phenanthrene degrading bacterial strains were isolated by enrichment culture, of which two highest degrading strains, strain-3 and strain-7 were selected for identification and characterization. In previous studies one of the highest phenanthrene degrading strains with biosurfactant production was reported as *Brevibacillus parabrevis* PDM-3 (Reddy *et al.*, 2010). In the present study, the other PAHs degrading bacterial strain-7 was identified and characterized as *Kocuria rosea* PDM-7. The bacterial strain PDM-7 was deposited in China Type Culture Collection Centre (CTCC) with deposit number CCTCC AB 209088^T and its 16S rDNA sequence was deposited in the GenBank database with the accession number FN 185994. The genus *Kocuria* was first proposed by Stackebrandt *et al.* (1995) based on the genus *Micrococcus* (Stackebrandt *et al.*, 1995). At present there are 17 species are available in the genus *Kocuria* (Yun *et al.*, 2011).

Kocuria sp. were also isolated previously from a variety of environmental samples such as narrow-leaved cattail (Kovacs *et al.*, 1999), an Antarctic cyanobacterial mat samples (Reddy *et al.*, 2003), marine sediments (Kim *et al.*, 2004), saline desert soil (Li *et al.*, 2006), fermented food (Park *et al.*, 2010a, b), seawater (Seo *et al.*, 2009) and salt- fermented sea food (Yun *et al.*, 2011). Recently Ahmed *et al.* (2010) isolated and characterized PAHs degrading bacterial strain *Kocuria rosea* CMG2042 from crude oil contaminated beach. Other than PAHs *Kocuria rosea* also exhibits the keratinolytic activity for feather degradation (Vidal *et al.*, 2000). However, till to date there are very limited reports are available on *Kocuria rosea* regarding their activity of degrading PAHs as sole carbon and energy source. The strain obtained in this study are novel with unique characteristics of degrading phenanthrene efficiently.

CONCLUSION

Biodegradation of polyaromatic hydrocarbons is essential because of their ubiquitous nature and carcinogenic properties. For this reason phenanthrene degrading bacterial isolate *Kocuria rosea* PDM-7 was isolated and characterized by using morphological, biochemical, chemotaxonomic and

molecular methods. Based on this study PDM-7 has the potential to utilize phenanthrene as the sole source of carbon and energy and degrading about 82% of phenanthrene in six days. Therefore, this strain can be applicable widely as beneficial bacteria in the field of environmental biotechnology to bioremediate the PAHs contaminated marine environments and soils for better environment of animal life.

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