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MICROBIAL DEGRADATION OF NAPHTHALENE THROUGH *BACILLUS CEREUS* RD6 ISOLATED FROM REFINERY OIL SLUDGE

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ABSTRACT

Oil contains a number of hydrocarbons. It is a complex mixture of linear, branched and cyclic alkanes, and mono- and polyaromatic compounds like benzene, toluene, xylene, ethylbenzene, naphthalene etc. These compounds are of utmost concern to human health because of their genotoxic, mutagenic and carcinogenic effects. Naphthalene is a common polycyclic aromatic hydrocarbon (PAH) and has been considered as a model for microbial degradation study. The aim of this study was to evaluate the naphthalene degradability by indigenous bacterial strain isolated from oil sludge. Twelve bacterial isolates were obtained from the oil sludge of Guwahati Refinary, Assam, India and after preliminary screening the isolate designated as RD6 was considered for degradation study of naphthalene. The isolate was further identified as *Bacillus cereus* through biochemical tests and molecular characterization by 16SrDNA gene sequencing. Phylogenetic tree was constructed with BLAST result of closely related sequences using MEGA 7 software. The sequence of *Bacillus cereus* RD6 (MH114968) was submitted to NCBI gene bank for global retrieval. Detection of eight metabolic biomarkers such as oxalic acid, dibutylphthalate, 1,2-benzene dicarboxylic acid, fumaric acid, 2-propenoic acid, phthalic acid, Benzoic acid, and Eicosane in GC-MS analysis reveals the degradation of naphthalene by *Bacillus cereus* RD6.

KEY WORDS : *Bacillus cereus* RD6, Naphthalene, bioremediation, GC-MS, biomarker.

INTRODUCTION

Naphthalene contamination from petroleum hydrocarbon is a major source of environmental pollution. Hydrocarbons are a group of organic compounds composed exclusively of carbon and hydrogen and are either monocyclic or polycyclic aromatic in structure. Monocyclic aromatic hydrocarbons like Benzene, Toluene Ethylbenzene, and Xylene (BTEX) having a single benzene ring, are commonly found in gasoline and are highly volatile substances (Coates *et al*., 2002). Polycyclic aromatic hydrocarbons (PAHs) contain two or more benzene rings and are relatively less aqueous solubility than monocyclic aromatic hydrocarbons. Aromatic hydrocarbons are considered as highly toxic, mutagenic and carcinogenic to human health. These pollutants are released from combustion of fossil fuels and petroleum hydrocarbons.Due to their lipophilic property it enters the ecosystem and pollutes the environment. The aromatic hydrocarbons like Naphthalene, Benzene, Toluene, Ethylbenzene, and Xylene are commonly found in crude petroleum and petroleum products and are considered as one of the major causes of environmental pollution (Farhadian *et al*., 2008). Among the polycyclic aromatic hydrocarbons (PAHs), naphthalene is the simplest one and has been extensively used as a model for biodegradation. Native bacteria species belong to *Styphylococcus*, *Corynebacterium*, *Pseudomonas*, *Bacillus* and *Micrococcus* isolated from oil contaminated soils in Iran were reported as efficient degradable agents of naphthalene (Kafilzadeh, 2011). Pawar *et al.* (2013) reported degradation of naphthalene by Gram positive and Gram negative

bacteria belonging to genus *Micrococcus*, *Bacillus*, *Styphylococcus* and *Pseudomonas* isolated from marine and petroleum soil sample. Study on degradation of polycyclic aromatic hydrocarbons, fraction of refinery effluent (Esedafe *et al*., 2015) indicates capability of microorganisms to degrade certain hydrocarbon pollutants both in pure and mixture forms.Fourteen different types of bacteria were isolated from oily soil of Tabriz which was able to degrade a mixture of PAHs (naphthalene, phenanthrene and anthracene) with low and high molecular weight (Sadighbayan *et al*., 2016). Microbial degradation of aromatic compounds has been reported by a number of authors (Heider *et al*. 1999; Mrozik *et al*., 2013; Su Seo *et al*., 2009; Doley and Barthakur, 2016; Doley*et al*, 2017).Aerobic degradation of naphthalene and other polycyclic aromatic hydrocarbon has been studied by a number of authors (Sullivan *et al*., 2001; Rocken and Strand, 2001; Rothermich *et al*., 2002). Biodegradation of naphthalene is either through salicylate or phthalate pathway. Abo-State *et al*. (2018) detected o-phthalic acid in strains of *Bordetellaavium*; MAM-P22 was a clear indication that these two bacteria were able to utilize the "phthalic pathway" to biodegrade Naphthalene. However, Annweiler *et al*. (2000) reported the existence of both the pathways in the biodegradation of Naphthalene by the Thermophilic *Bacillus thermoleovorans*.

The major goal of this study was to isolate and identified an indigenous bacterialstrain from oil sludge and its applicability to degrade naphthalene, a common pollutant of petroleum industry.

MATERIALS AND METHODS

Chemicals: All chemicals are of analytical grade, Purchased from Sisco Research Laboratory Pvt. Ltd., Mumbai.

Sample collection: Samples of oilsludge were collected in a sterilized polythene bagfrom Guwahati oil refinery, Assam, India. The soil sample was allowed to dry for two days.

Isolation of bacterials

Stock solution was prepared by mixing 100 mg of the oil sludge in 100 mL of sterilized distilled water and then subjected to serial dilution from 10^{-2} to 10^{-9}. Plating was done in Minimal Salt medium (MS), supplemented with naphthalene (20 mg/L) as carbon and energy source. The plates were incubated at 37 °C for 48 hours. Well growth colonies were picked up and stored in slant of MS medium for further investigation.

Screening for most promising naphthalene degrading isolate

The well growth bacterial isolates were selected and inoculated in each 250 mL Erlenmeyer flask, containing 100 mL MS medium amended with 20, 40, 60, 80 and 100 mg/L naphthalene. The flasks were incubated at 37 °C for 48 hours. Bacterial growth was monitored through uv-vis spectrophotometer (Agilent Carry-60) at 600 nm. The best growth isolate was selected for further studies. Concentration of naphthalene where optimum growth was recorded was considered for further naphthalene degradation study.

Biochemical characterization of bacterial isolate

The isolates were characterized based on morphological and biochemical attributes (Cappuccino and Sherman 2005; Holt 1984-89). The biochemical traits studied included Indole test, Methyl Red test, Voges-Proskauertest, Catalase test, Oxidase test, Starch hydrolysis, Gelatin liquefiction, Citrate utilization and carbohydrate fermentation test.

Identification of bacterial strain through 16SrDNA sequence analysis

For identification of the isolated strain, DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel. A single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1484 base pairs was observed when resolved on Agarose gel. The PCRamplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDTv3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16SrDNA gene sequence was used to carry out BLAST with the database of NCBI Genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs. Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7. Sequencing of the 16S

rDNA was carried out by Eurofin Genomic, Bangalore, India.

Naphthalene biodegradation study

Evaluation of biodegradation was done by inoculating the best growth bacterial strain (CFU3x10^6) in 5 Erlenmeyer flasks (250 mL volume) containing 100 mL MSmedium,amended with 60 mg/L naphthalene as a sole carbon source. It was then incubated at 37 ºC for 9 days. Naphthalene utilization by the bacterial strain in the medium was monitored periodically by measuring the optical density at 310 nm. Culture media inoculated with bacterial suspension without supplementation of naphthalene was treated as control. After incubation the culture of all the five flasks were pooled together and centrifuged at 10285.6 G. The supernatant were then extracted twice in in 200 mL of n-hexane.

Analytical methods

GC-MS study

The n-hexane extracts of naphthalene broth was concentrated in rotary evaporator to about 5 mL. Concentrated extract was analyzed through GC-MS, using carrier gas: He; EB-5MS column (30 m x 0.25 mm x 0.25 mm); injector temperature 250 ºC; initial oven temperature 40 ºC with 1 min hold time, 120 ºC with 1 min hold time and then 280 ºC with 5 min hold time; sample amount 1µL in splitless mode with column flow of 1.11 mL/min.

RESULTS

Isolation of the bacteria

A total of 12 different bacterial colonies were isolated from refinery oil sludge of which three colonies were well growth in MS medium supplemented with 20 mg/L naphthalene. Furthermore, the isolate labeled as RD6 showed best growth in enrichment culture. However, the growth of RD6 was optimum in culture broth amended with 60 mg/L naphthalene. Thus among the three well growth isolates RD6 was selected for further studies. As the growth of RD6 was optimum in culture broth amended with 60g/L naphthalene, therefore this concentration was further considered for naphthalene degradation studies.

Characterization of Bacterial strain

The biochemical and morphological study revealed that RD6 was Gram positive, rod-shaped and motile bacteria. It showed positive test for Catalase, Oxidase, Vogues-Proskeurand was able to ferment carbohydrates such as Glucose and Fructose. It was able to hydrolysis starch and gelatin. However the bacterial strain was variable for Sucrose and TSI test. It showed negative test for Methyl Red, Indole, Citrate, Galactose, Lactose, hydrogen sulphide and Urease (Table 1).

Bacterial identification by 16S rDNAsequence analysis

Amplification of the fragment of 16S rDNA was done by 27F and 1429R primers which resulted in the production of 1500 base pairs (Fig. 1). From forward and reverse sequence data consensus sequence of 16S rDNA gene was generated which was used to carry out BLAST with the NCBI genebank database. First ten sequences were selected based on maximum identity score and aligned using multiple alignment software programs and the phylogenetic tree was constructed using MEGA 7. From the analysis, the isolated strain has been identified as *Bacillus cereus* with 100% identity (Fig. 2).

Table 1. Biochemical characteristics of RD6 strain

Test Parameter	Result	Test Parameter	Result
Gram staining	+	Citrate utilization	-
Motility	+	Fructose	+
Shape	Rod	Galactose	-
Indole test	-	Glucose	+
MR test	-	Lactose	-
VP test	+	Sucrose	Variable
Catalase test	+	TSI	Variable
Oxidase test	+	Hydrogen Sulphide production	-
Starch hydrolysis	+	Urease test	-
Gelatin liquefication	+		

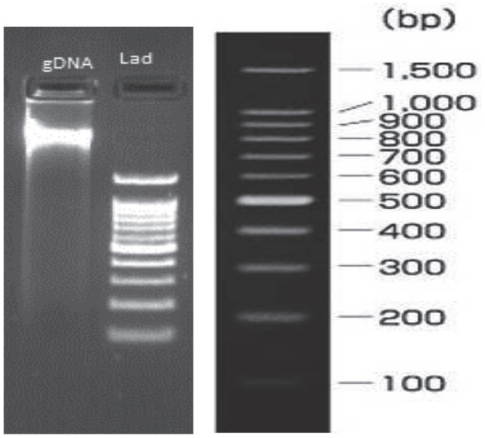

Fig. 1. gDNA and ladder specification of RD6

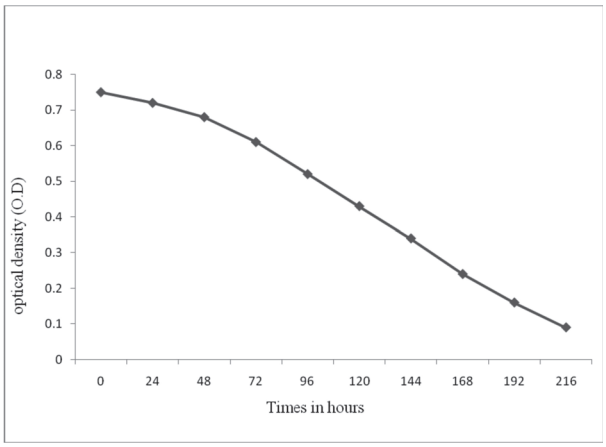

Fig. 3. Naphthalene utilization by *Bacilluscereus*

Biodegradation of Naphthalene by *Bacillus cereus* RD6 strain

The bacterial strain *Bacillus cereus* RD6 was able to grow on the culture media by utilizing naphthalene as carbon and energy source. Optical density of the culture media examined periodically in UV-visible spectroscopy at 310 nm exhibited gradual decrease of absorption of naphthalene from 48 hours onward indicated the utilization of naphthalene by the bacterial strain as carbon and energy source (Fig. 3).

GC-MS analysis (Fig. 4) of the n-hexane extract of naphthalene amended MS broth detected metabolites such as oxalic acid (RT 23.44 min.), dibutylphthalate (RT 37.13), 1,2-benzenedicarboxylic acid (RT34.14), fumaric acid (RT22.49), 2-propenoic acid (RT16.06), phthalic acid (RT32.66), Benzoic acid (RT27.84), and Eicosane (RT10.29).

DISCUSSION

A total of 12 bacterial isolates were obtained from oil sludge of which RD6 was selected for the further study due to its best growth in naphthalene enrichment culture medium. The strain was identified as *Bacillus cereus* RD6 through biochemical tests, 16SrDNA sequence analysis and molecular phylogeny. The sequence with 1500bp (PL. 2) has been submitted to the Gene Bank of NCBI(accession no. MH114968) for global retrieval. Screening Electron Microscopic analysis suggested the short rodnature of cells.

Gradual decrease of optical density of naphthalene enrichment culture at OD310 nmis the indication of utilization of naphthalene by the bacterial strain as its carbon and energy source. *Pseudomonas putida* CSV86 showed preferential

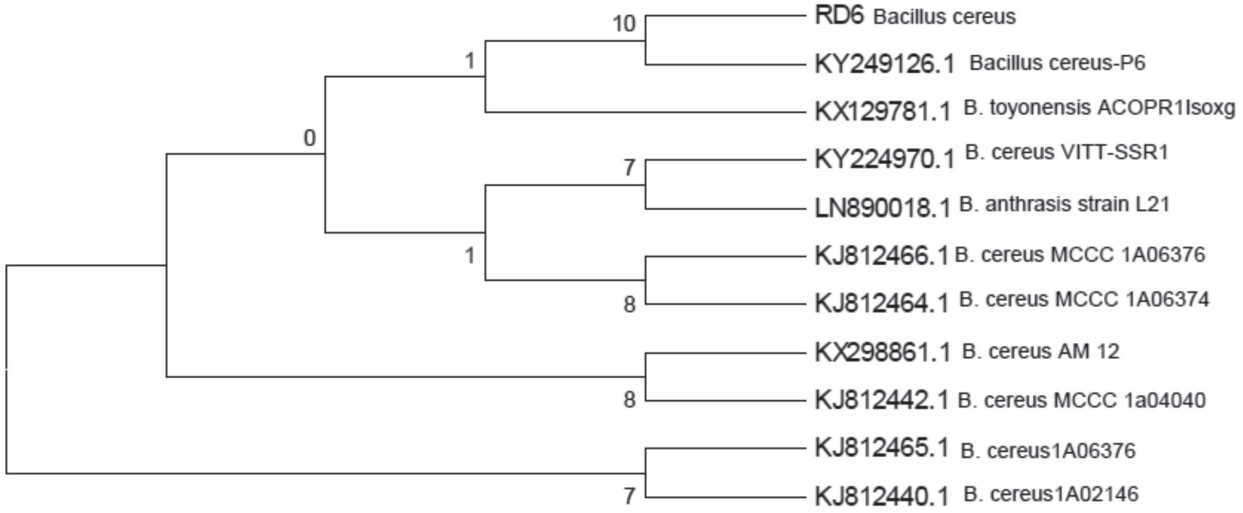

Fig. 2. Phylogenetic tree of bacterial isolate RD6

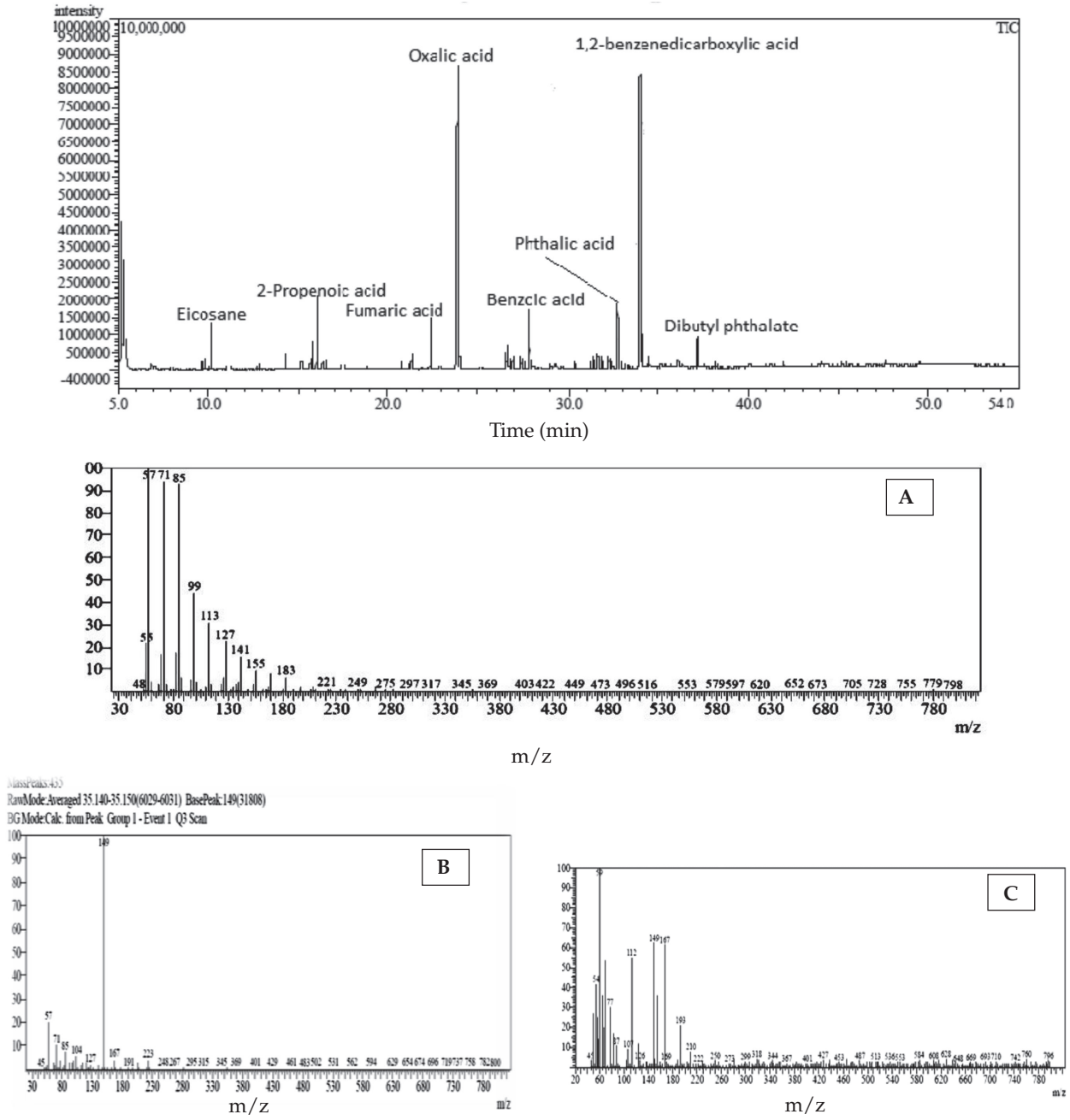

Fig. 4. GC-MS analysis and mass spectral profile of metabolites by strain RD 6 (A:Fumaric acid, B:1,2-Benzene dicarboxylic acid and C:Dibutylphthalate)

utilization of aromatic compounds over glucose (Basu *et al*., 2007). *Bacillus fusiformis* (BFS) degraded naphthalene into intermediate metabolites which were identified as o-phthalic acid and benzoic acid through GC-MS suggesting possible metabolic pathways (Lin and Chen, 2010). Biodegradation of petroleum and aromatic hydrocarbons by bacteria isolated from petroleum-contaminated soil (Mirdamadian *et al*., 2010) and also biodegradation potential of oily sludge by pure and mixed bacterial cultures (Cerqueira *et al*., 2018) was also reported. *Burkholderia* sp. VITR isolated from marine sediments degraded PAHs and the degraded end products of the PAHs were determined using FTIR (Revathy *et al*., 2015).

Certain specific metabolic biomarkers are the indicators of biologically mediated degradation process of hydrocarbons (Beller, 2000; Pelz, *et al*.,

2001; Mancini *et al*., 2003). Detection of metabolites such as Benzoic acid, oxalic acid, dibutyl phthalate, fumaric acid, phthalicacid, 2-propenoic acid, Eicosaneand 1,2-benzenecarboxylic acidin GC-MS analysis of naphthalene enrichment culture broth suggested the degradation of naphthalene. Jia *et al*. (2008) reported two possible pathway of naphthalene biodegradation; the phthalic acid pathway and the other where the naphthalene was first degraded in to 1,2-dihydroxynaphthalene, and followed by ring cleavage caused the formation of salicyclic acid, catechol and 2-hydroxymuconic acid and finally these metabolites in to tricarboxylic acid (TCA) cycle.Phthalic acid, the transformation product of naphthalene may arise from degradation of 2-carboxylcinnamic acid (Annweiler *et al.*, 2000). However, 2-carboxylcinnamic acid was not detected in our study. The aromatic derivative dibutyl phthalate is a phthalate ester, obtained by the formal condensation of the carboxyl groups of phthalic acid with two molecule of butane-1-ol. Nzila *et al*. (2016) also reported dibutylphthalatein the culture broth of *Methylobacterium radiotolerans* (N7A0) *and Pseudomonas aeruginos* N7B1 strains in naphthalene enriched culture broth. Fumaric acid was the other derivative of naphthalene detected in our study (at retention time 45.66 min) is a dicarboxylic acid and is a precursor to L-malate in the tricarboxylic acid (TCA) cycle was similar to naphthalene degradation study by a number of authors (Annweiler *et al*., 2002; Meckenstock *et al*., 2000). Benzoic acid derived through ring fission of phthalic acid, whereas the later derived through ring fission and followed by oxidation of naphthalene. Nonane the other metabolic product of naphthalene (not detected in our study) was derived from reduction and polymerization of 1-Nonane-3-ol. *Bordetella avium* MAM-P22 strain degraded naphthalene through oxidation and followed by ring fission to give rise 1,2-Benzene dicarboxylic acid and 4-methyl-dimethyl ester (Abo-State *et al.,* 2018*)*. Furthermore, oxidation and followed by ring fission produced Butyl-2,4-dimethyl-2-nitro-4-pentenoate. Through reduction followed by demethylation to give 1-Nonen-3-ol and more reduction and polymerization followed by methylation to give rise Eicosane. Detection of Eicosane with retention time 10.29 min. in our study was similar to the finding of Abo-State *et al.* (2018), however, 4-methyl-dimethyl ester, Butyl-2, 4-dimethyl-2-nitro-4-pentenoate and 1-Nonen-3-ol were not detected.

CONCLUSION

The bacterial strain *Bacillus cereus* RD6, isolated from oil sludge and identified through biochemical and molecular characterization, degrades naphthalene, the polycyclic aromatic hydrocarbon in to its less toxic metabolites. The detection of certain metabolitesin naphthalene enriched culture media is a clear indication of degradation of the naphthalene.The intermediate metabolites detected through GC-MS such as 2-propanoi acid, oxalic acid, phthalic acids, dibutylphthalate, fumaric acid, eicosaneand1,2-benzenedicarboxylic acid strongly suggest the ability of *Bacillus cereus* RD6 to degrade naphthalene in to less toxic substances.

Conflict of Interests

The authors declare that there is no conflict of interests.

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