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RESEARCH ARTICLE

Isolation and characterization of diesel degrading bacteria, *Sphingomonas* sp. and *Acinetobacter junii* from petroleum contaminated soil

Qiuzhuo ZHANG, Duanchao WANG, Mengmeng LI, Wei-Ning XIANG, Varenyam ACHAL (✉)

Shanghai Key Laboratory for Urban Ecological Processes and Eco-Restoration, College of Resources and Environmental Science, East China Normal University, Shanghai 200241, China

Abstract Two indigenous bacteria of petroleum contaminated soil were characterized to utilize diesel fuel as the sole carbon and energy sources in this work. 16S rRNA gene sequence analysis identified these bacteria as *Sphingomonas* sp. and *Acinetobacter junii*. The ability to degrade diesel fuel has been demonstrated for the first time by these isolates. The results of IR analyses showed that *Sphingomonas* sp. VA1 and *A. junii* VA2 degraded up to 82.6% and 75.8% of applied diesel over 15 days, respectively. In addition, *Sphingomonas* sp. VA1 possessed the higher cellular hydrophobicities of 94% for diesel compared to 81% by *A. junii* VA2. The isolates *Sphingomonas* sp. VA1 and *A. junii* VA2 exhibited 24% and 18%, respectively emulsification activity. This study reports two new diesel degrading bacterial species, which can be effectively used for bioremediation of petroleum contaminated sites.

Keywords diesel degrading bacteria, 16S rRNA, cell surface hydrophobicity, emulsification, bioremediation

1 Introduction

Pollution by diesel frequently occurs in environment throughout the world and affects natural habitat. Diesel contains many highly concentrated toxic materials (Dillard et al., 1997), and diesel contamination can negatively influence soil microbes and plants, as well as quality of groundwater, which may be used for drinking or agriculture. Consequently a variety of methods have been developed to treat diesel contamination. Conventional physico-chemical treatments to the remediation of hydrocarbons (i.e., diesel) are usually expensive, laborious, and generally require the use of hazardous solvents.

Bioremediation is recognized as an economically feasible, in site and ex site application and effective method for the treatment of oil contaminants in soil. Several studies have reported bacteria such as *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Sphingomonas* sp., with enhanced oil degrading abilities (Hong et al., 2005; Lin et al., 2005; Aislabie et al., 2006; Kebria et al., 2009). Biodegradation of hydrocarbons by natural population of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment inexpensively (Leahy and Colwell, 1990; Lidderdale, 1993). To make this process more meaningful and viable, we need to explore some indigenous bacterial strains of diesel contaminated environment that can stimulate the uptake of diesel as nutrient for better biodegradation performance.

In this work, two indigenous bacterial strains were isolated from petroleum contaminated soil. These strains were identified on the basis of biochemical and molecular characteristics. Growth and diesel degradation profile of the isolates on the medium containing diesel as the sole

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E-mail: varenyam@re.ecnu.edu.cn

carbon source was demonstrated. Further, cellular hydrophobicity and emulsion tests were performed to access potential of bacterial isolates in diesel bioremediation applications.

2 Materials and methods

2.1 Isolation of diesel-degrading microorganisms

Petroleum contaminated soil samples were collected from sites near an oil station (31.00°N, 121.23°E) in Shanghai, China in radiation sterilized polypropylene bottles. For culture enrichment, 100 mL of Bushnell Haas (BH) medium ($MgSO_4$, 0.20 g/L; $CaCl_2$, 0.02 g/L; K_2HPO_4, 1 g/L; NH_4NO_3, 1 g/L; $FeCl_3$, 0.05 g/L; KH_2PO_4, 1 g/L; pH 7.0), was supplemented with 1 g contaminated soil and 1% diesel (v/v) as the sole carbon source. Samples were incubated at 30°C with shaking condition (130 rpm) for 7 days. Bacteria from this culture were enumerated using serial dilution technique by total plate count method on diesel supplemented BH agar medium. The plates were incubated at 30°C overnight. Two bacterial strains, designated as VA1 and VA2 were finally selected as most active strains and used for further studies.

2.2 Phenotypic characterization

Morphological characterization (i.e., colony, cell and spore morphology, Gram-reaction, motility, catalase, oxidase, citrate and malonate utilization, nitrate reduction, phenylalanine deamination, lecithinase, starch, casein, gelatin and esculin hydrolysis) were identified (Achal and Pan, 2011). To determine the growth of the bacterial isolates at different temperatures and pHs, the isolates were incubated at intervals 5°C from 5°C to 55°C and pH values from 5 to 11.

2.3 Molecular characterization

Genomic DNA was extracted from overnight grown bacterial cells by alkaline lysis. The 16S rRNA gene from the genomic DNA was amplified by PCR using the following primers; 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions: 8–27) and 5′-AAGGAGGTGATCCAGCCGCA-3′ (*E. coli* positions: 1521–1540) corresponding to the forward and reverse primers of 16S rDNA, respectively. The amplification was done by initial denaturation at 95°C for 5 min followed by 10 cycles of 93°C for 1 min, 63°C for 1 min, 71°C for 1.5 min; 20 cycles of 93°C for 1 min, 67°C for 1 min, 71°C for 2 min and final extension at 71°C for 5 min. The purified PCR product was cloned in Ins TA PCR cloning kit (Fermentas, USA) as per the manufacturer's instructions and sequenced in both directions using an Applied Biosystems 3730xl DNA Analyzer. The 16S rRNA gene sequence was compared with Ribosomal Database Project-II and with those from GenBank using the BLASTN program (Altschul et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.0 software (Tamura et al., 2007). The 16S rRNA gene sequences for VA1 and VA2 determined in this study was deposited in GenBank of NCBI under the accession numbers KF270891 and KF270892, respectively.

2.4 Diesel fuel biodegradation

Both bacterial isolates (*Sphingomonas* sp. VA1 and *A. junii* VA2) were tested for the ability to degrade diesel fuel. One percent of overnight grown bacterial isolates (equivalent to 5×10^8 cells/mL) was inoculated into 50 mL of BH medium supplemented with 0.5 mg/mL (500 ppm) concentration of commercial diesel fuel as the sole carbon and energy source. Samples were incubated at 30°C with shaking condition (130 rpm) for 15 days and collected periodically for analysis. The samples were extracted using perchloroethylene as an extraction solvent (Kebria et al., 2009). The concentration of the petroleum hydrocarbons in the liquid was measured by IR radiation with TPH (Total Petroleum Hydrocarbon) meter (EPA, 1997) and bacterial growth was monitored by viable counts on nutrient agar plates. Control experiment was also prepared in similar manner without addition of bacterial cells.

2.5 Cell surface hydrophobicity

The cellular hydrophobicity of both isolates was analyzed mainly according to the methods of Rosenberg et al. (1980). Briefly, overnight grown bacterial cells were collected and rinsed with saline solution followed by suspension in phosphate-urea-magnesium buffer and absorbance was measured by taking OD at 400 nm. Different volumes (0–0.5 mL) of diesel were added into cell suspensions and incubated at 30°C for 10 min under shaking condition (120 rpm). The mixtures were allowed to stand for 15 min to separate the diesel and aqueous phase. Samples were taken carefully from the aqueous phase and absorbance was measured at 400 nm. Hydrophobicity (%) was calculated by: (1-A_{400} of aqueous phase/A_{400} of initial cell suspension) × 100%.

2.6 Emulsification activity

The emulsification activity of both bacterial isolates was determined by measuring the emulsion index (E_{24}) as described by Cooper and Goldenberg (1987). The cell pellet collected from overnight grown bacterial culture was subjected to sonication at 50 Watt for 5 min using an ultrasonic processor. Five mL of the cell residue suspension was mixed vigorously (5000 rpm for 5 min) with eight mL of diesel in a test tube and allowed to stand overnight at room temperature and the emulsion index (E_{24}) was

determined by: (height of emulsified zone/height of total liquid) × 100%.

2.7 Statistical analysis

All the experiments were performed in triplicate. The data were analyzed by Analysis of Variance (ANOVA) and the means were compared using Tukey's test. All the analyses were performed using GraphPad Prism (4.1) software.

3 Results and discussion

3.1 Isolation, characterization and identification of diesel-degrading bacteria

Among the different bacteria isolated, two bacterial strains, designated as VA1 and VA2 were finally selected as the most active strains based on vigorous growth on diesel supplemented BH agar medium. Both isolates were Gram negative and rod shaped, non-motile, catalase positive and oxidase negative. No pigments were produced by these isolates. VA1 was able to hydrolyze starch, casein, gelatin and esculin whereas VA2 hydrolyzed gelatin and starch only. Acid production was noted with lactose, xylose, fructose, dextrose, raffinose, trehalose, arabinose, glycerol, sorbitol, mannitol, sucrose and ONPG in case of VA1 while only with sucrose, lactose, mannitol and ONPG in case of VA2. Citrate was utilized by VA1 only. The VA1 and VA2 isolates showed growth between a pH range of 5–10 and 6–9 respectively. When subjected to salinity and temperature tests, VA1 and VA2 were able to survive NaCl concentration of 0–8% and 0–9% and a temperature range of 20°C–45°C and 20°C–40°C respectively (Table 1). These physiological features show significant differences between these two isolates.

Lecithinase test was used to confirm non-pathogenic behavior of isolates in this work. Lecithinase is an enzyme known to be involved during invasion in pathogenesis (Nandy et al., 2007). Both the strains did not show any lecithinase activity and were confirmed as non-pathogenic strains.

Phylogenetic analysis revealed that VA1 was close to some strains of *Sphingomonas* sp. and *Sphingomonas xenophaga*, while VA2 showed 100% similarity with *Acinetobacter junii* (Fig. 1). *Legionella pneumophila* was used as out group taxa. Based on molecular characterization bacterial isolates VA1 and VA2 were identified as *Sphingomonas* sp. and *A. junii* respectively.

3.2 Diesel fuel biodegradation

The results of IR analyses of TPH show that the addition of both bacterial isolates enhances degradation of diesel fuel compared to the control (without bacteria). The diesel was degraded significantly by both the isolates. *Sphingomonas* sp. VA1 reduced diesel from 0.5 mg/mL to 0.087 mg/mL, while *A. junii* VA2 degraded up to 0.12 mg/mL (Fig. 2(a)). The diesel reduction by isolates *Sphingomonas* sp. VA1 and *A. junii* VA2 was 82.6% and 75.8%, respectively. The biodegradation of diesel oil was very efficient during first five days of inoculation; however efficiency was low until the fifteenth day. Further, *Sphingomonas* sp. VA1 reached the peak value after five days of inoculation and then stayed stable until the end of the experiment (Day 15), as a result, the final biodegradation efficiency of 82.6% until the fifteenth day. Isolates found in this study are not known as common hydrocarbon degraders. Mostly species of *Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhodococcus*, *Microbacterium* and *Arthrobacter* are reported as diesel degrading bacteria (Milcic-Terzic et al., 2001; Menezes Bento et al., 2005; Yousaf et al., 2010; Zhang et al., 2010). Zhang et al. (2010) reported 11 bacterial strains, mostly firmicutes, which degraded more than 70% of TPHs in diesel oil.

The total viable microbial population of *Sphingomonas* sp. VA1 and *A. junii* VA2 during biodegradation of commercial diesel fuel is presented in Fig. 2(b). After adding these isolates, the microbial population of *Sphingomonas* sp. VA1 immediately stimulated degradation of diesel fuel accompanied by significant TPH degradation, indicating that *Sphingomonas* sp. VA1 utilized a portion of the C supplied by the diesel fuel as a potential nutrient source. The utilization of diesel fuel as a carbon source stimulates the degradative capabilities of the indigenous

Table 1 Physiological characteristics of bacterial isolates VA1 and VA2 isolated from petroleum contaminated soil

Characteristics	VA1	VA2
Shape	Rod	Rod
Catalase	+	+
Oxidase	−	−
Lecithinase	−	−
Citrate utilization	+	+
Nitrate reduction	−	−
Temperature tolerance/°C	20–45	20–40
Alkalinity/pH tolerance	5–10	6–9
NaCl tolerance/%	0–8	0–9
Phenylalanine deamination	−	+
Starch hydrolysis	+	+
Casein hydrolysis	+	−
Gelatin hydrolysis	+	+
Esculin hydrolysis	+	−
Acid production Xylose, Fructose, Dextrose, Raffinose, Trehalose, Arabinose, Glycerol, Sorbitol	+	−
Mannitol, Sucrose, Lactose, ONPG	+	+

Note: +, Positive; −, Negative.

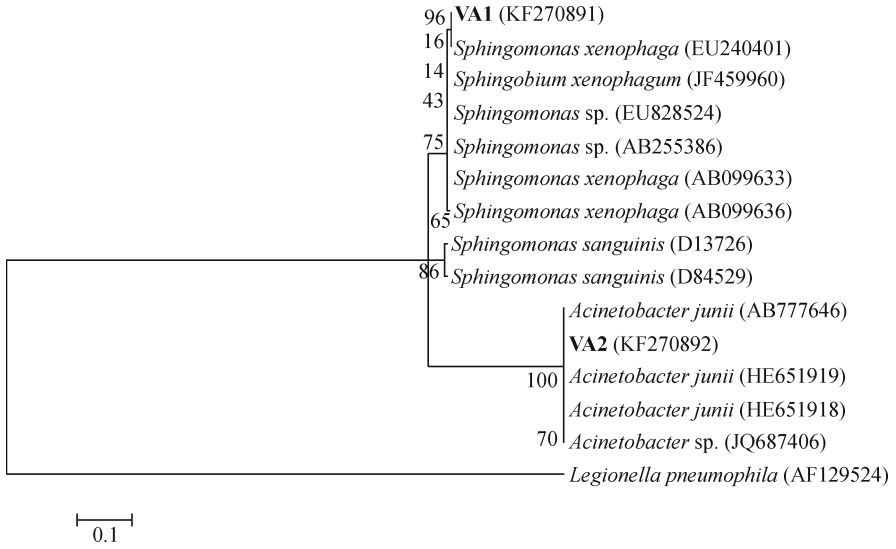

Fig. 1 16S rRNA gene sequence-based dendrogram showing the phylogenetic position of the isolates VA1 and VA2 along with sequences available in GenBank database. The tree was constructed using the neighbor joining method and rooted using *Legionella pneumophila* as a outgroup taxa. Numerical values indicate bootstrap percentile from 1,500 replicates. The scale bar shows the branch length corresponding to 0.1 nucleotide substitutions per position.

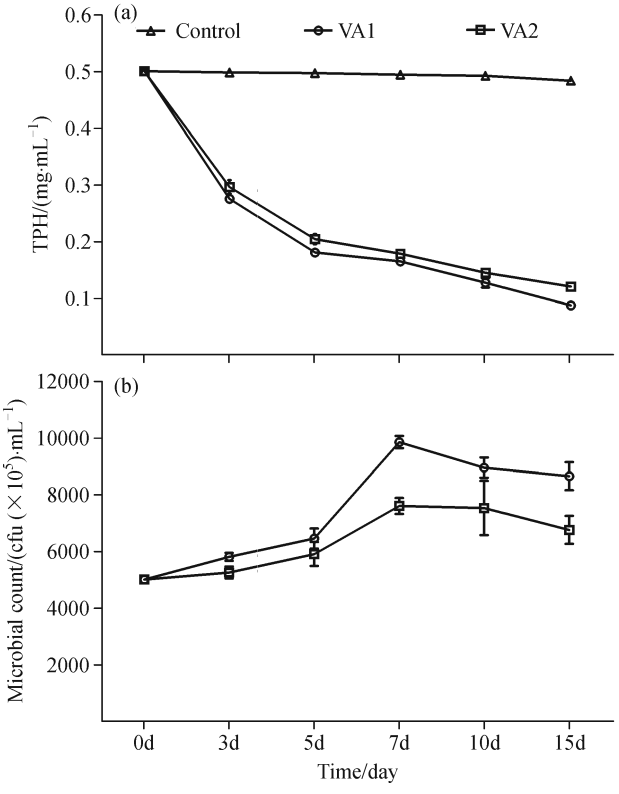

Fig. 2 (a) Degradation curve of diesel by *Sphingomonas* sp. VA1 and *A. junii* VA2, and (b) total viable microbial count during degradation of commercial diesel by *Sphingomonas* sp. VA1 and *A. junii* VA2. Values are mean±SD ($n = 3$).

microorganisms thus allowing the microorganisms to break down the organic pollutants at a faster rate (Ausma et al., 2002). Increases in microbial population corresponded to decreases in TPH concentration during the same time period. There was significant reduction in the microbial count in case of *A. junii* VA2 compared to *Sphingomonas* sp. VA1, might be the reason of lesser efficiency of this isolate in terms of diesel degradation.

3.3 Cell surface hydrophobicity

Sphingomonas sp. VA1 possessed the higher cellular hydrophobicities of 94% for diesel compared to *A. junii* VA2 (81%). These results suggest that the isolate with higher cell surface hydrophobicities float easily. Therefore it is reasonable that the isolate with higher cell surface hydrophobicities tended to approach the hydrophobic carbon source (diesel) located on the upper surface of the medium, contributing to the floating behaviour (Lin et al., 2005). The possession of cell-surface hydrophobicity by microorganisms creates chemical and physical compatibility between the organisms and the hydrophobic substrates, thus resulting in enhanced interaction between them (Stelmack et al., 1999). Many bacteria are known to degrade liquid hydrocarbons only after adherence to the substrate (Marcoux et al., 2000; MacLeod and Daugulis, 2005).

3.4 Emulsification activity

Both isolates displayed poor emulsification activities of their supernatants compared to cell residues. Also, emulsification activity of the cell residues exhibited opposite trend as compared with those for supernatants. The high emulsifying activities of both bacterial cell

residues also indicate their ability for intimate contact with diesel oil, most likely due to the hydrophobic characteristics of the cell surface. The isolates *Sphingomonas* sp. VA1 and *A. junii* VA2 exhibited 24% and 18% emulsification activity, respectively, in cell residues. It has been reported that the hydrocarbon utilizing bacteria often produce emulsifiers to assist the uptake of hydrophobic substrates (Navon-Venezia et al., 1995; Barriga et al., 1999). However, the supernatants of *Sphingomonas* sp. VA1 and *A. junii* VA2 possessed limited emulsification, as their E_{24} values were 10% and 7% respectively; implying that the emulsifying activity by these isolates might be cell (membrane)-bound, not extracellular (Ganesh and Lin, 2009).

4 Conclusions

In conclusion, this work demonstrated the diesel oil degrading potential of *Sphingomonas* sp. and *A. junii* isolated from petroleum contaminated soil. The results indicated that indigenous bacterial isolates have great potential for in situ bioremediation of petroleum-contaminated soils. A number of limiting factors such as temperature, pH and nutrients have been recognized to affect the biodegradation of petroleum hydrocarbons. Thus, it is necessary to characterize bacterial strains to know their bioremediating ability to soils contaminated with diesel. The strains shown in this work demonstrated their growth properties in the wide pH, temperature and saline ranges with potential to use diesel as C source. Future work requires identification of genes involved in diesel degradation by these bacteria and their over-expression to enhance degradation rate. The use of indigenous bacteria with diesel utilizing capabilities onto oil contaminated environment could prove a more environmentally-friendly approach to bioremediation, which would enhance sustainable development rather than the use of exotic bacterial strains and chemicals.

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