ISSN 1001-0742
Journal of Environmental Sciences Vol. 18, No. 5, pp. 969—972, 2006 CN 11-2629/X

Article ID: 1001-0742(2006)05-0969-04 CLC number: X172 Document code: A

Isolation and characterization of gasoline-degrading bacteria from gas station leaking-contaminated soils

LU Si-jin, WANG Hong-qi*, YAO Zhi-hua
(College of Water Sciences, Beijing Normal University, Beijing 100875, China. E-mail: whongqi310@sohu.com; vopt976@yahoo.com.cn)

Abstract: The effects of culture conditions *in vitro* and biosurfactant detection were studied on bacterial strains capable of degrading gasoline from contaminated soils near gas station. The main results were summarized as follows. Three bacteria (strains Q10, Q14 and Q18) that were considered as efficiently degrading strains were isolated and identified as *Pseudomonas* sp., *Flavobacterium* sp. and *Rhodococcus* sp., respectively. The optimal growth conditions of three bacteria including pH, temperature and the concentration of gasoline were similar. The reduction in surface tension was observed with all the three bacteria, indicating the production of biosurfactant compounds. The value of surface tension reduced by the three strains Q10, Q14 and Q18 was 32.6 mN·m, 12.4 mN·m and 21.9 mN·m, respectively. Strain Q10 could be considered as a potential biosurfactant producer. Gasoline, diesel oil, benzene, toluene, ethylbenzene and xylene (BTEX) could easily be degraded by the three isolates. The consortium was more effective than the individual cultures in degrading added gasoline, diesel oil, and BTEX. These results indicate that these strains have great potential for in situ remediation of soils contaminated by gas station leaking.
Keywords: gasolene; BTEX; bacteria; biosurfactant; consortium

Introduction

Worldwide concern is increasing over the environmental pollution by petroleum leaked from underground storage tanks (USTs) of gas stations. There are approximately 3×10^6 of USTs storing petroleum products in the USA, and as many as 5×10^5 of USTs may be leaking petroleum into the ground (EPA, 2001; Rothenstein, 2003). This problem is especially acute in China, although there are not clear data about it (Xue, 2003).

Contamination of soil and groundwater by gasoline and other petroleum-derived hydrocarbons released from USTs is a serious and widespread environmental problem. Great attention has been paid to human and environmental safety, concerning the release of hydrocarbons to the environment. Gasoline contains benzene, toluene, ethylbenzene and xylene (BTEX) isomers, which are hazardous substances regulated by many nations (Alvarez and Vogel, 1991; Ribeiro *et al.*, 2005). In addition to BTEX, other gasoline constituents such as methyl-*t*-butyl ether (MTBE), 1, 3, 5-trimethylbenzene (1, 3, 5-TMB), and 1, 2, 4- trimethylbenzene (1, 2, 4-TMB) are also toxic to humans.

Microbial biodegradation is considered as a major process that accounts for both containment of the petroleum-hydrocarbon plume and the reduction of leaking contaminant concentrations. Many bacterial strains capable of degrading petroleum pollutants have been isolated. Sorkhoh *et al.* (1993) isolated two strains of *B. stearothermophilus* which was able to degraded 80%—89% of crude oil (5 g/L) within 5 d at 60℃. Ijah and Antai (2003) reported the isolation of a *Bacillus* strain from highly polluted soil samples which was able to rapidly degrade crude oil. Rather, there was a great deal of specialization and interspecies interaction and very few organisms could degrade a single compound completely. Degradation of petroleum compounds was often the result of community-interacting microbial populations, generally termed as a consortium (Alexander, 1980). However, few studies were conducted to isolate and characterize bacteria capable of degrading hydrocarbon leaked from gas stations. The main objectives of this study were to isolate and characterize gasoline-degrading bacteria in contaminated soils, to establish optimum conditions for the growth of isolated bacteria, to determine excreting-biosurfactants ability of bacteria, and to compare degradation ability of individual bacteria and consortium for gasoline, diesel oil, benzene, toluene, xylene and BTEX.

1 Materials and methods

1.1 Sampling site and enrichment cultures

The bacterial strains used in this study were isolated from soils contaminated by a 4-year-old gas station in Beijing, China. Soil samples were collected near the underground storage tanks (about 2 m depth) and stored in closed containers at 4℃ prior to use.

All microbial enrichment and isolation were performed in the media prepared from the following composition (g/L): NH_4Cl 0.1, $MgCl_2 \cdot 6H_2O$ 0.08, K_2HPO_4 0.25, $CaCl_2 \cdot 2H_2O$ 0.1. Gasoline was added as the sole carbon source to autoclaved basal medium (at the rate of 500, 1000, and 2000 mg/L, respectively). The pH of the inorganic culture media was adjusted to 7.0 with either HCl or NaOH.

1.2 Isolation and characterization of bacteria

An enrichment culture technique was used to isolate gasoline-degrading bacteria. The medium containing soil and gasoline was incubated at 35℃ with orbital shaking (120 r/min). An aliquot of 1 ml of the medium was transferred every day to the same type of sterile medium and incubated under the same

Foundation item: The National Natural Science Foundation of China (No.40472129302000030); *Corresponding author

conditions. After four transfers, 1 ml was diluted and placed on agar plates of basal medium containing 500 mg/L gasoline and incubated for 48 h at 30℃ in darkness. The well-growth bacterial colonies were purified on the same medium.

Well-separated colonies from each medium were assayed for their degrading ability in universal bottles (25 ml) containing basal medium plus gasoline, as appropriate, which were incubated on a shaking platform at 120 r/min at 30℃. The concentrations of gasoline were determinated by infrared spectrophotometer. The most efficient bacteria were selected and streaked again onto fresh gasoline-containing agar plates and nutrient agar plates to ensure purity.

Cell morphology, mortality, Gram-reaction and physiological characteristics of the purified cultures were examined under a light microscope.

1.3 Determination of optimum temperature, pH and substrate concentration for the growth of isolated bacteria

The influence of pH, temperature, substrate concentration and some hydrocarbons on the growth of selected isolates was assessed using basal medium, each with three replications. The autoclaved medium was adjusted to pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using predetermined amounts of 1 mol/L HCl or 1 mol/L NaOH and incubated at 30℃. The incubation temperatures were 20, 25, 30, 35 and 40℃ and the gasoline concentrations were 0, 125, 250, 500, 1000 and 2000 mg/L.

The inocula, containing 5% of the total volume, were sampled in the logarithmic phase cultures in basal medium broth and incubated for 24 h with orbital shaking (120 r/min). After incubation for 24 h, 1 ml of medium was diluted and 0.1 ml of the 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on nutrient agar and incubated for 24 h at 30℃ in darkness and colonies were directly counted and expressed as CFU/ml.

1.4 Surfactant detection

The surfactant produced by three isolates were tested by the reduction of surface tension. The surface tension was measured by model ZHY-180 tensiometer. Evaluation was performed by the presence and absence of cells which were removed by centrifugation at 10000 r/min for 30 min at 4℃. In this experiment the surface tension of basal medium was 68.7 mN·m.

1.5 Microbial consortia preparation and degradation

The microbial consortia was formulated by mixing equal proportions of all pure bacterial cultures that were isolated from hydrocarbon-contaminated soils.

In order to obtain a standard inoculum, individual bacteria were grown for 24 h on Tryptone Soy Broth at 30℃ on an orbital shaker at 120 r/min. The cells were then harvested by centrifugation, rinsed three times in sterile saline water before being resuspended in sterile basal medium to yield an absorbance reading of 0.5 at 600 nm. When used as an inoculum at 10% (v/w), the resulting colony forming unit (CFU)/g soil was around 1.6×10^{7} CFU/g.

1.6 Substrate degradation by isolates and consortium

In order to test the degradation ability, isolates and consortium were added into several glass flask containing 500 mg/L gasoline, diesel oil, and BTEX, respectively. The incubation were carried out at 30℃ with an orbital shaker at 120 r/min for 48 h. Then the concentration of gasoline were determinated.

1.7 Control experiment

Considering the volatility of gasoline, in all experiment sterile controls containing gasoline or BTEX compounds, but no cells, were used to monitor abiotic losses of organic compounds.

2 Results and discussion

2.1 Isolation and identification of bacterium

Nineteen preselected bacteria were isolated from gas station leaking contaminated soils, which were able to grow well using gasoline as the sole source of carbon. Among them, three strains (Q10, Q14 and Q18) were the most efficient in degading gassoline (data not shown).

Physiological characteristics of the three isolates were examined. The bacterial colonies of strain Q10 were circular, low-convex, gray, smooth and shining. The bacterial colonies of strain Q14 were typically circular, smooth, yellow with regular and complete margins. The bacterial colonies of strain Q18 were low-convex, dryness with uncomplete margins. The details of other morphological and physiological characteristics are summarized in Table 1.

According to the morphological and biochemical properties, strains Q10, Q14 and Q18 were tentatively classified as *Pseudomonas* sp., *Flavobacterium* sp. and *Rhodococcus* sp., respectively.

2.2 Optimum temperature, pH and substrate

Table 1 Biochemical and physiological characteristics of strains Q10, Q14 and Q18

Characteristics	Q10	Q14	Q18
Color of bacterial olonies	Gray	Straw yellow	Salmon pink
Morphology	Short-rod	Rod	Short-rod
Diameter, μm	0.8	1.4	0.6
Gram staining	-	-	+
Acrobic growth	+	+	+
Hydrolysis of Gelatin	+	+	+
Starch	-	-	-
Substrate utilization Glucose	+	+	-
Lactose	-	+	-
Saccharose	-	+	-
Indole production	+	+	-
Methyl red test	-	+	-
Voges-Proskauer test	+	-	-

concentration for growth

In order to determine the optimal growth conditions for the selected isolates, including temperature, pH and gasoline concentration were evaluated.

Fig.1 shows the growth response of three strains Q10, Q14 and Q18 to different temperatures. Three strains showed similar response to temperature. At the beginning, the numbers of bacteria increased but decreased later with temperature rising. The largest biomass of bacteria of strains Q10 and Q14 were found at 30℃, but was at 35℃ for strain Q18. Although temperature could increase substrate bioavailability, but higher temperature would also had adverse effects on the development of cell.

The growth of strains Q10, Q14 and Q18 with

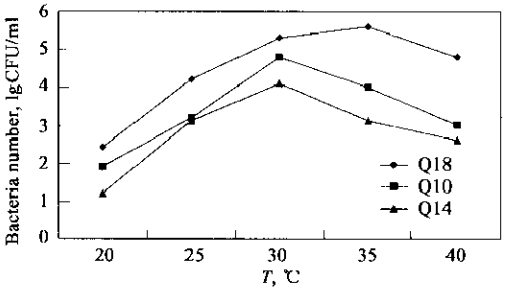

Fig.1 Effect of temperature on the growth of three isolates

different initial pH value is shown in Fig.2. The highest cell number of the three strains were found at pH 6.0. When initial pH value was above 6, the numbers of strains Q10 and Q14 decreased quickly. But Q18 decrease slowly until initial pH value above 7. Therefore, the optimum pH value for 3 strains growth was around 6.0 showing that a neutral to slightly acidity pH may be required for the growth of cells. But for strain Q18 slightly alkalescence pH was also tolerable.

The effect of gasoline concentration on cell growth was revealed in Fig.3. The three isolated strains (Q10, Q14 and Q18) showed similar response to gasoline concentration. Their cell numbers increased with increasing concentrations of gasoline up to 1000 mg/L. When the concentrations were above 1000 mg/L, the numbers of bacteria decreased, but the decreasing trend was slow. These results suggest that 1000 mg/L was the optimum

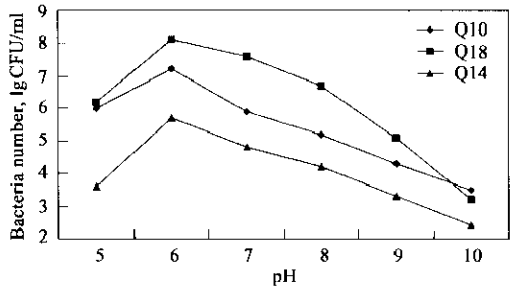

Fig.2 Effect of pH on the growth of three isolates

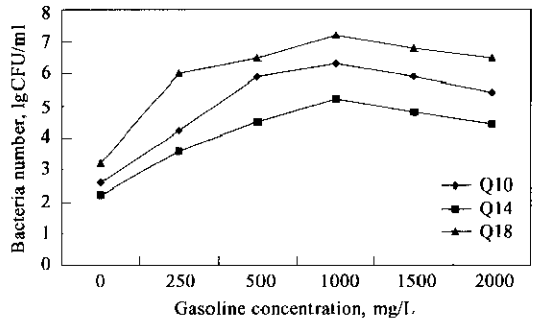

Fig.3 Effect of gasoline concentration on the growth of three isolates

Table 2 Reduction in surface tension of the medium by three strains

Strain	Surface tension, mN·m	Surface tension reduced, mN·m
Q10	36.1	32.6
Q14	43.6	12.4
Q18	40.7	21.9

concentration of gasoline for strains Q10, Q14 and Q18, and these isolates had a great utilization ability of gasoline.

2.3 Biosurfactant production

Oil compounds have a low solubility in water, which baffled microbial degradation. However, many microorganisms have some mechanisms to produce biosurfactant, which could increase the dissolution flux of the substrate and reduced the difficulty of the substrate coming into bacteria cells (Cameotra and Bollag, 2003). In general, biosurfactant production was estimated by the reduction in surface tension of the medium free of cells after growing in the substrate. Results are listed in Table 2.

The reduction of surface tension was obsereved in all the three strains. The value of surface tension reduced by strains Q10, Q14 and Q18 was 32.6, 12.4, and 21.9 mN·m, respectively. Strain Q10 caused the greatest surface tension reduction and it could be considered as a potential biosurfactant producer (Desai and Banat, 1997). The emulsification phenomenon was not found in this study and these three isolates did not produce high molecular weight iosurfactants.

This biosurfactant-excreting ability of strains Q10, Q14 and Q18 may have greatly enhanced the degradation of gasoline, especially *in situ* remediation because the bioavailability and biodegradation of gasoline would be increased by biosurfactant and in future biosurfactant would play more role in oil compound degradation.

2.4 Other substrate degradation ability

These isolates grew well on basal medium containing diesel oil, and BTEX. The degradation efficencies of strains Q10, Q14 and Q18 on gasoline, diesel oil and BTEX after 48 h inoculation were listed in Table 3.

Strain Q10 had the greatest degradation ability for gasoline, toluene and *o*-xylene, with an efficiency of 62.6%, 72.8% and 66.7%, respectively, but it had less

degradation ability for benzene, with an efficiency of 28.3% (Table 3). Strain Q18 was the most active against diesel oil, benzene, ethylbenzene, with an degradation efficiency of 51.2%, 81.6% and 83.5%, respectively. But it was the least against *o*-xylene and the degradation efficiency was 4.8%. The degradation ability of strain Q14 was between strain Q10 and strain Q18.

In general, these three strains had a great ability

Table 3 Degradation efficiency of gasoline, diesel oil and BTEX

Substrate	Efficiency, %			
	Q10	Q14	Q18	Consortium
Gasoline	62.6	41.3	54.5	82.2
Diesel oil	35.4	22.7	51.2	62.4
Benzene	28.3	67.1	81.6	91.6
Toluene	72.8	42.3	52.3	84.5
o-Xylene	66.7	31.5	4.8	70.1
Ethylbenzene	76.3	47.2	83.5	91.5

to degrade gasoline and other oil compounds. Therefore, in future these strains (Q10, Q14 and Q18) maybe have great applying prospect in spot remediation of gas-station-leaking contaminated soils.

2.5 Substrate degradation by consortium

Because microorganism had different degradation ability to different compounds and gasoline or other oil product contained complex hydrocarbon, biodegradation of those hydrocarbon usually requires the cooperation of more than a single species. In our experiments, assemblages of mixed populations were conducted. The degradation efficiency of the consortium, containing equal proportions of the three isolates, on gasoline, diesel oil and BTEX are listed in Table 3.

The comparison of the degradation potentials of the mixed and pure cultures revealed that the mixed culture was more effective than the pure cultures in degrading gasoline, diesel oil and BTEX. The degradation efficencies of gasoline, diesel oil, benzene, toluene, *o*-xylene and ethylbenzene were 82.2%, 62.4%, 91.6%, 84.5%, 70.1% and 91.5%, respectively.

The consortium, consisting three strains isolated from gasoline contaminated soil could effectively degrade hydrocarbons and was more effective than pure cultures in biotreatment systems. Because the three isolates exhibited a biodegradation pattern that was a subset of that exhibited by the mixed culture and interspecies interactions may be necessary for the complete biodegradation of multicomponent hydrocarbon mixtures such as gasoline. However, the types of microorganism and hydrocarbon mixtures may determine the rate and extent of hydrocarbon remediation. And these all needed to explore in further studies.

3 Conclusions

Three efficiently degrading strains (strains Q10, Q14 and Q18) were isolated and identified as *Pseudomonas* sp., *Flavobacterium* sp. and *Rhodococcus* sp., respectively. The optimal growth conditions of three bacteria including pH, temperature and the concentration of gasoline were similar. All the three could excrete biosurfactant during degrading gasoline. And strain Q10 could be considered as a potential biosurfactant producer. Gasoline, diesel oil and BTEX could easily be degraded by the three isolates. The consortium was more effective than the individual cultures in degrading added gasoline, diesel oil, and BTEX. These strains may have great potential for *in situ* remediation of soils contaminated by gas station leaking.

References:

Alexander M, 1980. Biodegradation of chemicals of environmental concern[J]. Science, 211: 132—133.

Alvarez P J, Vogel T M, 1991. Substrate interactions of benzene, toluene, and *para*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries [J]. Appl Environ Microbiol, 57(10): 2981—2985.

Cameotra S S, Bollag J M, 2003. Biosurfactant-enhanced bioremediation of polycyclic aromatic hydrocarbons [J]. Critical Reviews in Environmental Science and Technology, 30: 111—126.

Rothenstein C, 2003. FY 2003 semi-annual end-of-year activity report [Z]. Office of Underground Storage Tanks.

Desai J D, Banat I M, 1997. Microbial production of surfactants and their commercial potential [J]. Microbiology and Molecular Biology Reviews, 61: 47—64.

Fayolle F, Hernandez G, Le Roux F *et al.*, 1998. Isolation of two aerobic bacterial strains that degrade efficiently ethyl *t*-butyl ether (ETBE)[J]. Biotechnol Letts, 20: 283—286.

Garcia-Junco M, Gomez-Lahoz C, Niqui-Arroyo J L *et al.*, 2003. Biosurfactant and biodegradation enhanced partitioning of polycyclic aromatic hydrocarbons from nonaqueous phase liquids [J]. Environmental Science and Technology, 37: 2988—2996.

Ijah U J J, Antai S P, 2003. Removal of nigerian light crude oil in soil over a 12-month period [J]. International Biodeterioration & Biodegradation, 51: 93—99.

Mulligan C N, 2005. Environmental applications for biosurfactants [J]. Environmental Pollution, 133: 183—198.

Ribeiro N I, Ribeiro R, Zaiat M *et al.*, 2005. Anaerobic packed-bed reactor for bioremediation of gasoline-contaminated aquifers [J]. Process Biochemistry, 40: 587—592.

Samanta S K, Singh O V, Jain R K, 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation [J]. Trends in Biotechnology, 20: 243—248.

Sorkhoh N A, Ibrahim A S, Ghannoum M A *et al.*, 1993. High temperature hydrocarbon degradation by *Bacillus stearothermophilus* from oil-polluted Kuwait desert [J]. Applied Microbiology and Biotechnology, 39: 123—126.

USEPA, 2001. Cleaning up underground storage tank system releases [EB/OL]. http: //www. epa. gov/swerustl/cat/.

Wong J W C, Fang M, Zhao Z *et al.*, 2004. Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions [J]. Journal of Environmental Quality, 33: 2015—2025.

Xue J, 2003. Pollution of gas station and its control [J]. Heilongjiang Environmental Journal, 27: 27—28.

(Received for review November 18, 2005. Accepted February 28, 2006)