

Characterization of naphthalene-degrading bacteria isolated from the Persian Gulf and the Caspian Sea as potential agents for naphthalene removal from polluted environments

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Abstract

Over fifty bacterial strains were isolated from seawater samples in the presence of naphthalene as a sole source of carbon and energy. Among them, three isolates with higher growth rate and naphthalene degradation ability were selected for further studies. Biochemical and molecular analysis revealed that two Persian Gulf isolates, strain PG-10 and strain PG-48 belonged to the group of hydrocarbonoclastic bacteria (HCB). The other isolated strain (SA-58, from the Caspian Sea) was not related to this group. After 1 week incubation at 30 °C, the rates of naphthalene degradation by PG-10, PG-48 and SA-58 was 91.2, 78.5 and 87.3%, respectively. Furthermore, the effects of addition of salicylate on naphthalene degradation by the isolated bacterial strains were investigated. The naphthalene degradation rate of the strains PG-10 and PG-48 increased with addition of salicylate. In contrast, biodegradation of naphthalene by strain SA-58 was decreased approximately 30% in the presence of salicylate. These isolates were also able to grow on different contaminants, including crude oil, kerosene, toluene and hexane as the sole sources of carbon and energy. Hence, we suggest these bacterial strains as a potential tool in bioremediation of oil-polluted environments.

Key words: Bioremediation, Hydrocarbonoclastic bacteria, Marine environment, Polycyclic aromatic hydrocarbon

Highlights

- Isolation and characterization of naphthalene-degrading bacteria from the Persian Gulf and the Caspian Sea
- Assessment of the role of salicylate in naphthalene biodegradation
- Degradation of petroleum compounds by these naphthalene-degrading bacteria

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Introduction

The Persian Gulf is an important marine environment in south of Iran that supplies approximately 60% of the marine-transported oil in the world. This area was polluted with crude oil during the 1990 to 1991 Persian Gulf War. The Caspian Sea is another marine environment in Iran that its level of oil contamination has increased in recent years (1). Since the ecological impacts of oil pollution are truly serious in these areas, bioremediation strategy seems necessary to efficiently eliminate environmental pollutants.

Polycyclic aromatic hydrocarbons (PAHs), which are widely distributed crude oil-associated pollutants in diverse environments, are of most concern because of their toxicity, low volatility, and resistance to degradation (2). Naphthalene, the simplest PAH with two aromatic rings, is commonly used as a model for studying PAH metabolism due to its high abundance in PAH-contaminated environments (3). This hydrocarbon is one of the great causes to concern because it has toxic and carcinogenic effects, as well as high solubility in water (4). PAH bioremediation is considered as an effective and environmentally friendly cleanup technology. Many bacteria, which play important roles in the biodegradation of naphthalene, have been isolated from PAH-contaminated sea, wastewater and soil (5). *Pseudomonas* spp., *Vibrio* spp., *Acinetobacter* spp., *Marinobacter* spp., and *Sphingomonas* spp. are common naphthalene-degrading bacteria (6).

The aim of this study was to isolate naphthalene-degrading bacteria from the Persian Gulf and the Caspian Sea, and examine their ability in naphthalene degradation.

Materials and methods

Sampling sites and conditions

To isolate and characterize naphthalene-degrading bacteria, seawater samples were collected from the Persian Gulf and the Caspian Sea marine environments. The Persian Gulf sample collection sites were located in the coasts of Khark, Siri, and Hormoz islands, and the Caspian Sea sampling sites were located in the coasts of Bandar-Anzali, Tonkabon, and Babolsar. Seawater samples were collected from a depth of 15 cm in sterile 100-mL bottles and transferred on ice to the laboratory.

Isolation of naphthalene-degrading bacteria

To isolate naphthalene-degrading bacteria, ONR7a and Bushnell Hass Mineral Salts (BHMS) media were used (7 and 8). The media were supplemented with 1% (v/v) naphthalene as the sole carbon source. Aliquots of seawaters (5 mL) were added to Erlenmeyer flasks containing 100 mL of each medium, and the flasks were incubated for 1 week on a shaker (30 °C, 150 rpm). After four subcultures, 100 μ L of each sample was streaked out on ONR7a agar and BHMS agar, and phenotypically different colonies were purified. Autotrophic and agar-digesting bacteria were eliminated by transferring the colonies to fresh media with and without naphthalene. Finally, the isolates that were capable of growing only on ONR7a medium were defined as hydrocarbonoclastic bacteria (HCB), and the

strain that showed growth on both ONR7a and BHMS media was defined as hydrocarbon-degrading bacterium. Prior to biochemical and molecular analysis, these strains were stored at -20 °C (9).

Biochemical and molecular identification

Preliminary identification of the isolates was based on macroscopic and microscopic observations, biochemical tests such as Gram staining, catalase and oxidase, oxygen requirement, and motility according to Bergey's Manual of Systematic Bacteriology (10).

To identify the naphthalene-degrading isolates, the bacterial *16S rRNA* loci were amplified using the forward primer DG74_F (5'-AGGAGGTGATCCAACCGCA-3') and the reverse primer RW01_R (5'-AACTGGAGGAAGGTGGGGAT-3') (11). The DNA amplification program was set as follows: initial denaturation at 94 °C for 2 min (1 cycle), denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 45 sec (32 cycles), and final elongation step at 72 °C for 5 min (12). Sequence analysis was performed by Eurofins MWG Operon's sequencing service, Germany.

Growth rate and naphthalene removal assay

Bacterial suspensions (5.0 mL of 0.5 MacFarland) were inoculated to naphthalene-containing media and incubated for 1 week (30 °C, 150 rpm). To determine the growth rate of the isolates, optical density of the media at 600 nm were measured using a UV-visible spectrophotometer (Shimadzu UV-160, Japan). To measure naphthalene removal rate by the isolates, residual naphthalene of medium was dissolved in n-hexane, and then

its optical density was defined against a blank at a wavelength of 276 nm (13).

Gas chromatography analysis

The naphthalene removal was also monitored using gas chromatograph Agilent Technologies 6890N (Avondale, USA) equipped with flame ionization detector (FID) and a split-splitless injector. One milliliter of each culture growing in the presence of naphthalene was collected and residual naphthalene was extracted by n-hexane (1:1, v/v). The extracts were analyzed by gas chromatograph using HP-5 for the GC separation. Control sample was the sterile medium without bacterial inoculation. The injector and detector temperature were maintained at 110 °C and 150 °C, respectively. Oven temperature was programmed as follows: from 50 to 250 °C at 10 °C min⁻¹. The retention time for naphthalene was 5.9 min.

Determination of salicylate effect on naphthalene biodegradation

To examine the effect of salicylate on biodegradation of naphthalene, 5.0 mL of bacterial cultures growing exponentially on naphthalene were added to 50.0 mL of ONR7a and BHMS media supplemented with 1 g/L sodium succinate. Then, 0.5 mL of 3.5 mM sodium salicylate solution was added to 4.5 mL of bacterial cultures during the early exponential phase of growth. After 1 h, the bacterial cells were collected by centrifugation at 6000 rpm for 10 min. The pellets were resuspended in sodium phosphate buffer (0.05 M, pH 7), and centrifuged again. The washing step was repeated, and the cells were suspended in the buffer at a maximum absorbance of 0.3. Finally, 0.02 mL of 10 mM naphthalene in

ethanol was added to 3.0 mL of suspension, and the decrease in absorbance of 276 nm was followed using the UV-visible spectrophotometer (13).

Determination of growth rate of the isolates on other toxic compounds

To study the ability of the isolates to grow on other pollutants as the sole sources of carbon and energy, bacterial suspensions were added to ONR7a and BHMS media supplemented with 1% (v/v) crude oil, kerosene, n-hexane, and toluene. The growth rate was assessed by measuring the turbidity ($OD_{600\text{ nm}}$) after 1 week incubation at 30 °C, then was ranged from '+' to '++++' corresponding from low to high growth rate.

Results

Isolation and identification

Over fifty bacterial strains that could grow on naphthalene were isolated from enrichment cultures. Based on high growth rate and naphthalene degradation ability, three strains were selected by spectrophotometric method for further studies. Two strains (PG-10 and PG-48) were from the Persian Gulf, and the other strain (SA-58) that had been previously described by Emtiazi *et al.*, was from the Caspian Sea (14). These strains were identified using biochemical tests (Table 1).

Table 1- Biochemical characteristics of the isolates

Biochemical tests	Strain PG-10	Strain PG-48	Strain SA-58
Morphology	Bacillus	Coccobacillus	Bacillus
Gram staining	Negative	Negative	Negative
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Aerobic/ anaerobic growth	Aerobic	Aerobic	Aerobic

Strain SA-58 was capable of growing on both ONR7a and BHMS media, while the Persian Gulf isolates represented growth only on ONR7a medium. Thus, these two strains, through their obligate requirement for NaCl, were characterized as the hydrocarbonoclastic bacteria (HCB).

Molecular analysis confirmed that the strain PG-10 and the strain PG-48 belonged to this group of bacteria. The *16S rDNA* sequences of the isolated HCBs have been deposited to the GenBank database at the National Center for Biotechnical Information (NCBI) (Table 2).

Table 2- Closest relatives of the *16S rRNA* gene sequences of the isolates

Isolate	Accession No.	Closest relative (Accession No.)	Maximum identity
PG-10	HM055367	<i>Marinobacter hydrocarbonoclasticus</i> (JQ799059)	99%
PG-48	HM055368	<i>Halomonas cupida</i> (AB681322)	98%

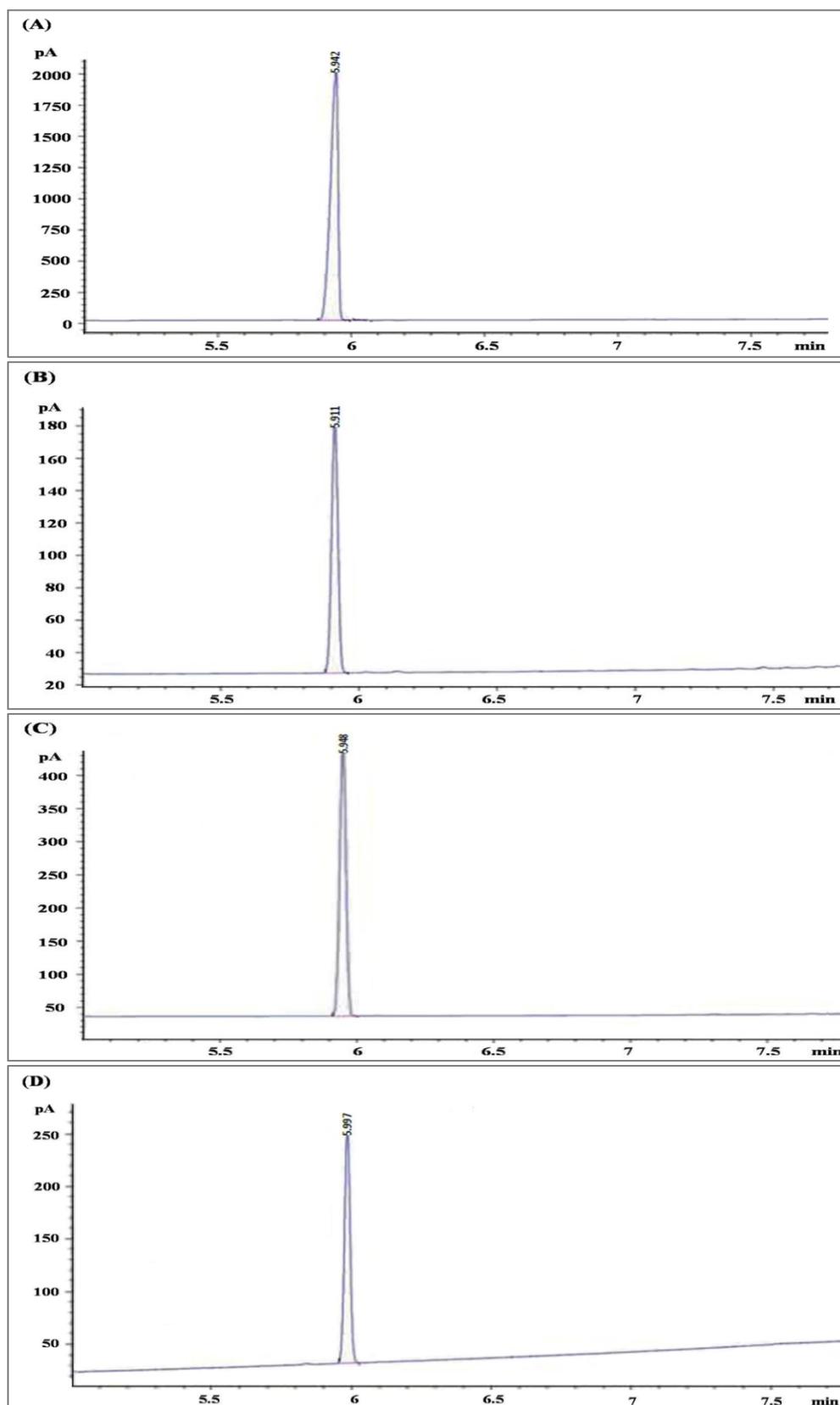


Fig. 1- GC analysis of naphthalene degradation by PG-10 (B), PG-48 (C), and SA-58 (D). Control was the sterile medium supplemented with naphthalene (A)

Growth rate and naphthalene removal

Table 3 represents the maximum growth rate and GC analysis of naphthalene removal by the isolated strains after 1 week incubation at 30 °C. As shown, despite the lower growth rate, strain PG-10 exhibited the highest level of biodegradation, degrading 91.2% of the naphthalene (Fig. 1).

Table 3- Maximum growth rate and GC analysis of naphthalene removal by the isolates

Isolate	Growth rate (OD _{600 nm})	Naphthalene removal
PG-10	0.41	91.2%
PG-48	1.2	78.5%
SA-58	0.87	87.3%

Salicylate effect on naphthalene biodegradation

Fig. 2 illustrates the effects of salicylate on naphthalene biodegradation. As shown, biodegradation rates of naphthalene by the Persian Gulf bacterial strains were increased in the presence of salicylate, while an opposite effect was observed in the strain SA-58.

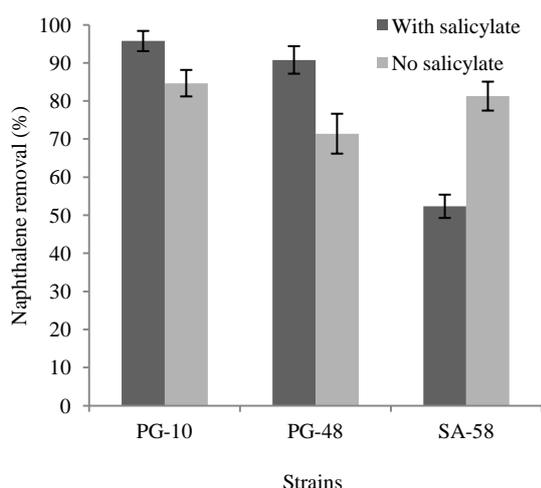


Fig. 2- Effects of salicylate on naphthalene degradation by the isolates

Growth on other toxic compounds

The results in Table 4 indicate that all of the tested bacterial isolates were also able to degrade a wide range of different environmental pollutants including crude oil, kerosene, toluene, and hexane as well as naphthalene.

Table 4- Comparison of growth of the isolates on other toxic compounds

Isolate/Carbon sources	Crude oil	Kerosene	Toluene	Hexane
PG-10	+++	++	+	++
PG-48	++	++	+++	++
SA-58	++++	+++	+++	+

Discussion and conclusion

Recently, hydrocarbonoclastic bacteria (HCB) have been described as Gram-negative, obligate marine halophilic bacteria capable of utilizing only petroleum hydrocarbons as carbon and energy sources. HCBs were first characterized by Yakimov *et al.* who isolated *Alcanivorax borkumensis* from the North Sea. Marquez *et al.* and Hassanshahian *et al.* described other genera belonging to this group of bacteria (15-17). In this study, one hydrocarbon-degrading bacterial strain (isolate SA-58) and two HCBs including one strain of *Marinobacter* (isolate PG-10) and one strain of *Halomonas* (isolate PG-48) capable of utilizing naphthalene, as the sole source of carbon were isolated from marine environments of the Persian Gulf and the Caspian Sea.

Many researches have been performed on naphthalene biodegradation so far, and scientists have succeeded to isolate and identify various bacterial species with this capability. The rate of naphthalene

biodegradation by these bacteria was found to be varied. Based on the results, all of the isolated strains were able to degrade more than 75% of the naphthalene provided. However, *Polaromonas naphthalenivorans*, a Gram negative naphthalene-degrading coccus, could metabolize 100% of the naphthalene (18). In addition, maximum naphthalene degradation rate of 13% and 97% was detected in *Paenibacillus* sp. ORNaP1 and *Pseudomonas* sp. ORNaP2 (19).

One of the strategies to enhance the degradation rate of PAHs is to offer bacteria inducers, such as salicylate, to stimulate both selective growth of PAH degraders and induce PAH metabolism (20). Naphthalene metabolism has been known for a long time to be induced by salicylate (21). Salicylate also efficiently enhances initial removal rates of high-molecular weight PAHs such as fluoranthene, pyrene, anthracene, chrysene, and pyrene (22). According to the results of the present study, addition of salicylate induced naphthalene degradation by PG-10 and PG-48, but it had an inhibitory effect on naphthalene removal by strain SA-58. Salicylate, an inducer of naphthalene oxygenase at certain concentrations, is an intermediate in the naphthalene degradation pathway (23). Thus, higher amount of salicylate may have negative effect on enzyme induction of some strains like what was observed in the strain SA-58.

As illustrated in this study, all of the tested isolates were able of degrading a wide range of different environmental pollutants. As previously described, strain

SA-58 was also able to utilize 75% of MTBE as the sole source of carbon and energy (14).

Besides the positive impact of salicylate on naphthalene degradation, in this study, it was found that the presence of salicylate can have inhibitory effect on degradation of naphthalene by some bacterial strains. Since hydrocarbon-degrading bacteria and HCBs play an important role in the marine microbial communities as primary destructors of organic compounds, the isolated bacterial strains might have potentials in bioremediation of toxic compounds as well as naphthalene in marine environments.

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شناسایی باکتری‌های تجزیه کننده نفتالین جدا شده از خلیج فارس و دریای خزر به عنوان عوامل حذف کننده نفتالین از محیط‌های آلوده

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چکیده

بیش از ۵۰ سویه باکتریایی از نمونه‌های آب دریا و در حضور نفتالین به عنوان تنها منبع کربن و انرژی جداسازی شدند. از میان این سویه‌ها، سه باکتری که دارای توانایی بالا در رشد و مصرف نفتالین بودند به منظور مطالعه بیشتر انتخاب شدند. بررسی‌های بیوشیمیایی و مولکولی بر روی آن‌ها مشخص کرد که دو باکتری جداسازی شده از خلیج فارس (سویه‌های PG-10 و PG-48) به گروه باکتری‌های هیدروکربنو کلاستیک تعلق دارند. در حالی که سویه SA-58 که از دریای خزر به دست آمده بود، به این گروه متعلق نیست. پژوهش‌ها نشان می‌دهد که میزان تجزیه نفتالین پس از یک هفته انکوباسیون در دمای ۳۰ درجه سانتی‌گراد توسط سویه‌های PG-10، PG-48 و SA-58 به ترتیب ۹۱/۲، ۷۸/۵ و ۸۷/۳ درصد است. همچنین، اثر افزودن سالیسیلات بر تجزیه زیستی نفتالین بررسی شد. میزان تجزیه نفتالین توسط سویه‌های PG-10 و PG-48 در حضور سالیسیلات افزایش می‌یابد. حال آن‌که تجزیه زیستی نفتالین توسط سویه SA-58 در حضور سالیسیلات حدود ۳۰ درصد کاهش یافت. این سویه‌ها قادر به رشد بر روی سایر ترکیبات نفتی از قبیل نفت خام، نفت سفید، تولوئن و هگزان به عنوان تنها منبع کربن و انرژی بودند. بنابراین، از این باکتری‌ها می‌توان به عنوان ابزارهایی بالقوه برای پاکسازی محیط‌های آلوده به ترکیبات نفتی استفاده کرد.

واژه‌های کلیدی: تصفیه زیستی، باکتری‌های هیدروکربنو کلاستیک، محیط دریایی، هیدروکربن آروماتیک چند حلقه‌ای

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