



Isolation and Characterization of a Novel Toluene-Degrading *Bacterium* Exhibiting Potential Application in Bioremediation

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ABSTRACT

Background: Toluene is a cyclic aromatic hydrocarbon which is widely used as an industrial feedstock and as a solvent. It is one of the major parts of pollution in oil-contaminated environments.

Objectives: The main aim of this study was to isolate and characterize a *Bacterium* with high potential application in toluene bioremediation.

Materials and Methods: To isolate a toluene-degrading *Bacterium*, several seawater and wastewater samples were added to toluene-containing basal salt media (BSM). The isolate was identified by morphological features, biochemical tests, and molecular characterization. Also, physiological characteristics of the isolated strain were determined.

Results: The isolate represented the capability of growing on toluene under both aerobic and anaerobic conditions. Moreover, this *Bacterium* could also use different toxic compounds as the sole sources of carbon and energy. Sequence analysis of 16S rDNA showed that the isolated strain was closely related to Uncultured *Bacterium* clone A1-E3_M13R (98%) and was submitted as *Bacterium* Ex-DG74 in NCBI. *Bacterium* Ex-DG74 showed a tolerance to organic solvent and saline conditions as it could grow in the medium containing over 15% toluene (v/v) and NaCl (w/v), and degraded 79% and 45% of toluene (1% (v/v)) in aerobic and anaerobic conditions, respectively.

Conclusions: In this investigation we succeeded to isolate a novel toluene-degrading *Bacterium* from wastewater. This isolated strain could be considered as a biological material for the toluene bioremediation.

Keywords: *Bacterium* Ex-DG74; Bioremediation; Gas chromatography; Toluene

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▶ Implication for health policy/practice/research/medical education:

This research introduced a novel toluene degrading strain, *Bacterium* Ex-DG74, which can be potentially used in bioremediation of toluene in both aerobic and anaerobic environments.

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1. Background

Polycyclic aromatic hydrocarbons (PAHs) and BTEX (benzene, toluene, ethylbenzene and xylene) compounds are common environmental pollutants associated with petroleum product releases. BTEX compounds are the most water-soluble and most toxic petroleum contaminants in groundwater. Of these, toluene which widely exists in petroleum and related products is a serious cause for concern due to its adverse health effects and carcinogenic potential (1, 2). Because of the amount of toxic and hazardous petroleum hydrocarbons contaminating the environment, improved bioremediation technologies seem to be necessary. Since microorganisms are able to use petroleum hydrocarbon as a carbon and energy source, they can be used for bioremediation applications. Bioremediation is the process by which organisms can degrade or transform toxic organic compounds to a less toxic state (1-4).

Bioremediation is also considered as a cost-effective and environmentally friendly means for the treatment of hydrocarbons in comparison to the physical and chemical methods of cleaning up pollutants (5). Toluene is degraded by many species of aerobic bacteria such as *Burkholderiacepacia*, *Ralstoniapickettii* and *Pseudomonasmendocina* (6-8). The anaerobic degradation of toluene has also been observed in denitrifying strains, including *Thaueraaromatica* K172, *T. aromatica* T1, and *Azoarcussp.* strain T (9, 10). The strains described above are not able to degrade toluene under both aerobic and anaerobic conditions. Several studies indicated that only a few strains are capable of growing on toluene under both aerobic and anaerobic conditions (11-13). In this study, a toluene-degrading bacterial strain which degrades toluene under both aerobic and anaerobic conditions was isolated from wastewater.

2. Objectives

The objectives of this research were to isolate and characterize a toluene-degrading bacterium from water environments and to investigate its ability in toluene bioremediation.

3. Materials and Methods

3.1. Sampling

To isolate toluene-degrading bacteria, seawater samples were collected from three different sites in the Persian Gulf (the coasts of Bushehr, Bandar-Abbas and Qeshm) and two sites in the Caspian Sea (the coasts of Bandar-Anzali and Gisoum) and wastewater samples were collected from a wastewater treatment plant in Isfahan, Iran, and transported on ice to the laboratory.

3.2. Media and Growth Conditions

The best toluene-degrading strain, *Bacterium Ex-DG74* was isolated from wastewater by a standard culture enrichment technique using basal salt medium (BSM) supplemented with 1% (v/v) toluene as the sole carbon and energy source. BSM contained 4 g KH₂PO₄, 4 g Na₂HPO₄, 2 g NH₄Cl, 0.2 g MgCl₂, 0.001 g CaCl₂ and 0.001 g FeCl₃ in 1000 ml twice distilled water. The pH was adjusted to 6.8 before autoclaving (14). The isolated strain was cultivated aerobically on toluene-containing medium and anaerobically on toluene in growth medium containing 5 mM KNO₃ using anaerobic culture tubes at 28 °C.

3.3. Biochemical and Molecular Identification

The isolated strain was identified using microbiological and biochemical procedures according to the microbial identification standards (15). 16S rDNA gene was amplified with DG74-AGGAGGTGATCCAACCGCA as a forward primer and RW01-AACTGGAGGAAGGTGGGGAT as a backward primer (16). Polymerase chain reaction products were separated by agarose gel electrophoresis. Purification and sequencing were performed by Eurofins MWG Operon's sequencing service, Germany.

3.4. Growth Rate and Toluene Removal Assay

The growth rate of the isolate was indirectly assessed by a turbidity measurement as optical density (OD) at 600 nm in a UV-visible spectrophotometer (Shimadzu UV-160, Japan). The toluene removal assay was performed by dissolving residual toluene of medium in 3 ml n-hexane and reading the optical density of the toluene against a blank at 200-400 nm wavelengths (17).

3.5. Gas Chromatography

Toluene removal rate was also detected using Gas Chromatography (GC) by the bacterial cells grown in aerobic condition. GC measurements were performed on gas chromatograph Agilent Technologies 6890N (Avondale, USA) equipped with flame ionization detection (FID) and a split-splitless injector. The carrier gas was helium with a pressure of 34 psi in the injection port. The detector temperature was maintained at 240 °C. Oven temperature was programmed as follows: from 60 to 130 °C at 7 °Cmin⁻¹. 100% dimethyl polysiloxane HP1 (L: 60 m, I.D.:0.25 mm) was employed for the GC separation.

3.6. Effect of Toluene Concentration on Growth

To determine the effect of NaCl concentrations on growth rate, the isolate was incubated in BSM supplemented with 1, 5, 10, and 15% (v/v) toluene. Cell growth was monitored by measuring OD (600 nm) of overnight culture (17).

3.7. Effect of pH, Temperature, and Salinity on Growth

The effect of pH on growth rate of the isolate was measured in 1% (v/v) toluene-containing medium adjusted to the pH ranging from 5 to 11. The optimum temperature for growth was determined by conducting the assay at various temperatures ranging from 4 to 50 °C. To determine the effect of NaCl concentrations on growth rate, the isolate was cultivated in the presence of 1 to 20% (w/v) NaCl. The growth rate of *Bacterium Ex-DG74* grown on toluene was recorded by a turbidity measurement (OD at 600 nm).

3.8. Biodegradation of Other Petroleum Contaminants

To study the potential of *Bacterium Ex-DG74* for the degradation of other pollutants, 1 mL bacterial suspension of 0.5 MacFarland was added to 1000 mL BSM supplemented with 1% (v/v) crude oil, kerosine, xylene, toluene, and 1 mg naphthalene as the only sources of carbon and energy. The growth rates of the isolate were assessed by a turbidity measurement as OD at 600 nm.

4. Results

4.1. Isolation and Identification of *Bacterium Ex-DG74*

After sampling from different water environments and enrichment procedures in toluene-containing basal medium, about twenty toluene-degrading bacterial strains were isolated. Among them, a newly isolated bacterium from wastewater showing great ability of toluene degradation was selected for more studies.

Preliminary tests showed that the bacterial cells were Gram-positive, coryneform, motile, and capable of growing at 4–50 °C and are able to tolerate high salt concentrations. Biochemical characteristics of this strain are shown in Table 1.

The 16S rDNA sequence analysis for wastewater strain exhibited 98% homology with the 16S rDNA of *Uncultured bacterium clone AI-E3_M13R* and it was submitted as *Bacterium Ex-DG74* with the accession number of HQ414235.1 in the NCBI (Table 2).

4.2. Growth Rate and Toluene Biodegradation

Bacterium Ex-DG74 was capable of growing on toluene as the sole carbon and energy source under both aerobic and anaerobic conditions. Figure 1 and 2 illustrate that *Bacterium Ex-DG74* was efficiently capable of removing toluene from medium. The maximum toluene removal rate was observed when it was cultivated under aerobic condition at 28 °C.

As shown, the maximum toluene degradation was obtained after 24 hours for oxic (79%) and 48 hours for anoxic (45%) conditions in which the highest growth rate was also observed. According to these results, it can be concluded that the consumption of toluene by this strain is directly related to its growth rate.

According to the GC results, the aerobically grown cell biomass could remove toluene by 77.5%, whereas in the abiotic control the toluene content remained unchanged (Figure 3).

Table 1. Preliminary Identification of *Bacterium Ex-DG74*

Tests	Characters
Gram Staining	Positive
Morphology	Rod, Coryneform
Spore Formation	–
Acid-fast	–
OF test	F/O
Motility	+
Oxidase	+
Catalase	+
Growth Aerobically	+
Growth Anaerobically	+
Growth in NaCl	1 to 20%
Growth Temperature Range	4 to 50°C
Fumarate production	+
PHA Production	+
Chemotaxisto Toluene	+

Table 2. Molecular Identification of *Bacterium Ex-DG74*.

Strain	Sequence of 16S rDNA	Maximum Identity (98%)
<i>Bacterium Ex-DG74</i>	TCATGAATCACACCGTGGTAACCGTCCCCCGAAGGTTAGACTAGC-TACTTCTGGTGCAACCCACTCCCATGGTGTGACGGCGGTGTGTGTA-CAAGGCCGGGAACGTATTCACCGCGACATTCTGATTCGCGATTACTAGC-GATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCG-GTTTTGTGAGATTAGCTCCACCTCGCGGCTTGGAACCCTCTGTACCGAC-CATTGTAGCACGTGTGTAGCCAGGCCGTAAGGGCCATGATGACTTGACGT-CATCCCCACCTTCTCCAGTT	<i>Uncultured bacterium clone AI-E3_M13R</i>

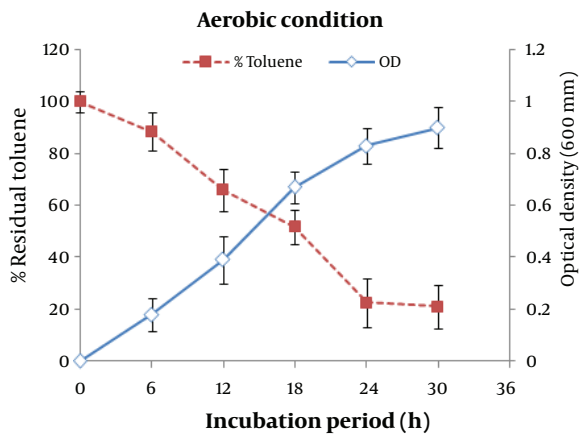


Figure 1. Growth Curve and Toluene Removal by Bacterium Ex-DG74 in Aerobic Condition.

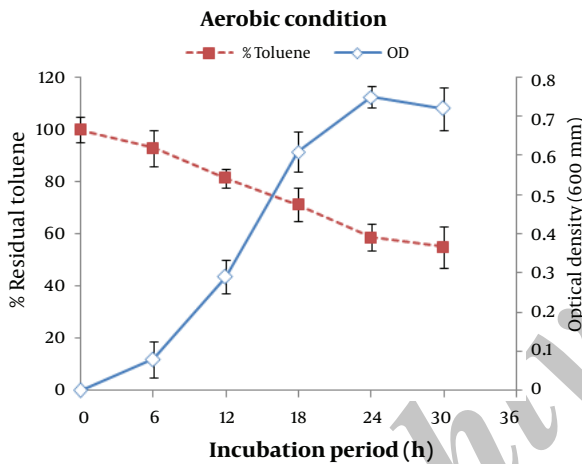


Figure 2. Growth Curve and Toluene Removal by Bacterium Ex-DG74 in Anaerobic Condition. The toluene removal assay was also performed using gas chromatography after 24 hours.

4.3. Effect of Toluene Concentration on Growth

Figure 4 shows the effect of different concentrations of toluene on growth of *Bacterium Ex-DG74*. As shown, the desired toluene concentration for obtaining the maximum growth of the strain was between 1% and 5% (v/v). The results indicated that this strain is able to survive in higher toluene concentrations.

4.4. Effect of NaCl, pH, and Temperature on Growth

Figure 5 shows the effect of different concentrations of NaCl on growth of the strain in the presence of toluene. As shown, 1% (v/v) NaCl was the best concentration for the growth of *Bacterium Ex-DG74*. As shown, it could also tolerate above 15% (v/v) of NaCl.

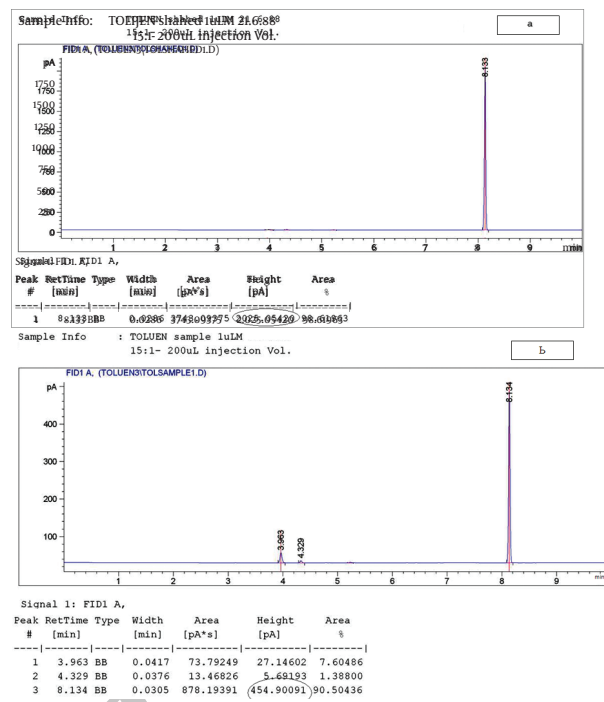


Figure 3. GC Analysis of Toluene Degradation by the Aerobically Grown Cells of Bacterium Ex-DG74. (a) Control (toluene), and (b) Toluene with Bacterium Ex-DG74.

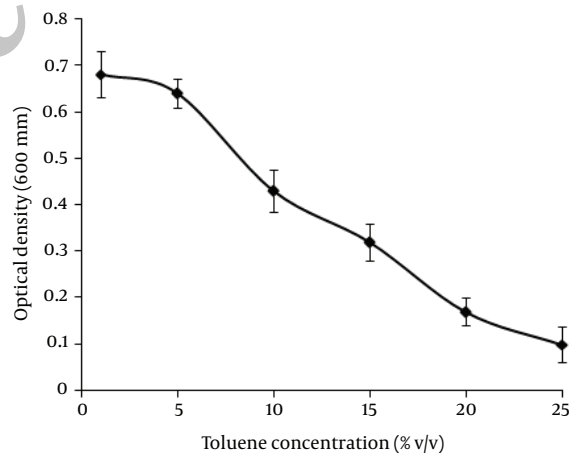


Figure 4. Effect of Toluene Concentration on the Growth of Bacterium Ex-DG74.

Figure 6 illustrates the optimal pH for the growth of the strain on toluene. This figure revealed that the neutral pH of 7 is the best for the growth of this strain.

Figure 7 shows the effect of different temperatures on the growth of the isolate on toluene. As shown, 28 °C was the best temperature for the growth of *Bacterium Ex-DG74*. This strain was also capable of growing and degrading toluene at 4 °C and 50 °C.

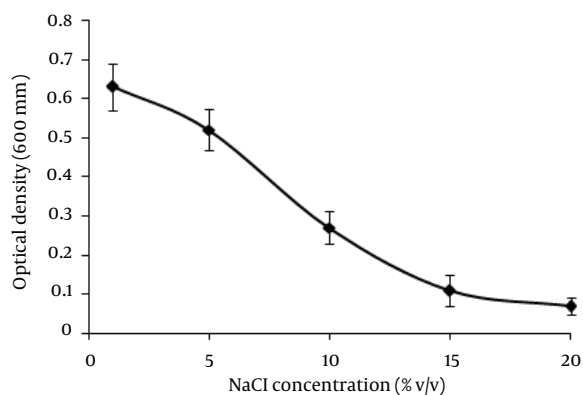


Figure 5. Effect of Medium Salinity on the Growth of Bacterium Ex-DG74.

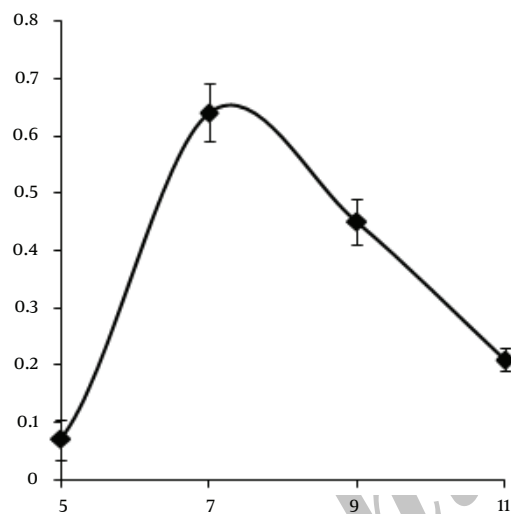


Figure 6. Optimal pH for the Growth of Bacterium Ex-DG74.

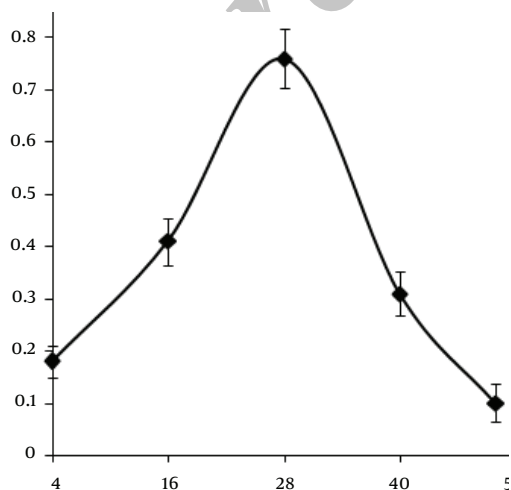


Figure 7. Optimal Temperature for the Growth of Bacterium Ex-DG74.

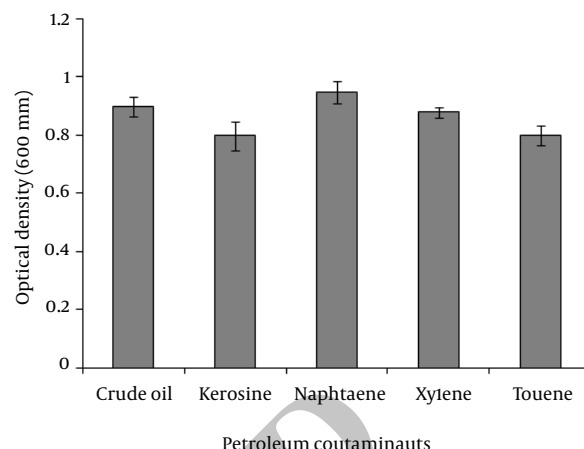


Figure 8. Biodegradation of Petroleum Contaminants by Bacterium Ex-DG74.

4.5. Biodegradation of Other Petroleum Contaminants

As shown in Figure 8, *Bacterium Ex-DG74* could use several pollutants as the sole carbon and energy sources. This strain was capable of growing on crude oil, kerosine, xylene, and naphthalene as well as toluene after 24 hours of incubation at 28 °C.

5. Discussion

Aromatic hydrocarbons are common pollutants associated with petroleum products release (2). Among these, toluene is one of the most important concerns due to its toxic and carcinogenic potential. Although anaerobic degradation of petroleum hydrocarbons by microorganisms has been occurred at negligible rates (18, 19), efficient degradation of toluene by a wide variety of microbes has been reported in aerobic environment (20). In this study, *Bacterium Ex-DG74*, a novel bacterium which degrades toluene under both aerobic and anaerobic conditions was isolated from wastewater.

According to our previous study, this bacterium was capable of producing significant amounts of peroxidase enzymes such as laccase and catalase which can help it in biodegradation of aromatic hydrocarbons (21). Interestingly, *Bacterium Ex-DG74* presented a good ability to remove toluene with the maximum removal efficiency of 79% and 45% in aerobic and anaerobic conditions, respectively. In aerobic condition, the maximum absorbance at 600 nm was over 0.8, which such a biomass growth on toluene has been reported for the first time. As we concluded, the consumption of toluene by this strain is directly related to its growth rate. Thus, less bacterial growth can generally lead to a decrease in toluene bio-

degradation.

As illustrated in this study, the temperature of 28 °C was better than other temperatures for the both microbial growth and toluene degradation. The temperature ranging from 30 to 40°C increases the rate of hydrocarbon metabolism to a maximum, above which the membrane toxicity of hydrocarbons is increased (22). Besides the presence of oxygen and temperature, toluene concentration, salinity and pH are the other factors influencing microbial growth and biodegradation rate. According to the results, low toluene concentrations (1 to 5% v/v) presented greater growth rates than the higher initial toluene concentrations. Moreover, the isolated strain could grow in higher toluene concentrations. The results of the present study agreed with Wang *et al.* experiments that showed the rates of hydrocarbon biodegradation decreased with increasing salinity (17). Most microorganisms prefer neutral pH to survive (23). Also, the neutral pH of 7 was the best for the growth of this strain.

Interestingly, *Bacterium Ex-DG74* could also degrade a wide range of different pollutants (Figure 8). As we previously described, this strain could use MTBE and produced CO₂ and formate during degradation (24). Undoubtedly, such properties make this newly isolated toluene-degrading bacterium unique in bioremediation of toluene and other toxic compounds under both aerobic and anaerobic conditions.

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Authors' Contribution

None declared.

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