Original article

Removal of toxic methyl tert-butyl ether by Naphthalene degradating bacterium isolated from freshwater

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How to cite this article:
Rahbari M, Emtiazi G, Kamali S. Removal of toxic methyl tert-butyl ether by Naphthalene degradating bacterium isolated from freshwater. Jundishapur J Microbiol. 2011; 4(3): 195-201.

Received: May 2010
Accepted: February 2011

Abstract

Introduction and objective: Naphthalene as a cyclic aromatic hydrocarbon is one of the major part of pollution in oil-contaminated environments. Methyl tert-butyl ether (MTBE), an additive to gasoline, is widely used. MTBE is highly water soluble, toxic and causes cancer. Naphthalene degraders have gene for co-oxidation of other toxic compounds therefore Naphthalene degrader could utilize and oxidize other toxic contaminant. The aim of this study is to isolate and identify bacteria that could degrade Naphthalene and MTBE as a circular and liner component respectively.

Material and methods: Freshwater was contaminated with Naphthalene and after several subcultures, strain SA86 was isolated and identified as *Pseudomonas* by biochemical test. Then bacterium was grown in culture with determined amount (1000ppm) of Naphthalene and the disappearance of Naphthalene was monitored with Gas Chromatography (GC) after six days. Then inoculate of the above bacteria was transferred to Teflon cape tube containing 500ppm MTBE and incubated for 24h. The removal of MTBE was measured by GC.

Results: The results showed that strain *Pseudomonas* (SA86) can degrade 99% of Naphthalene during six days incubation period. In addition this strain can remove 70% of MTBE. Meanwhile by products resulted from oxidation of MTBE were not observed.

Conclusion: The results emphasise that Naphthalene degrading bacterium isolated in this study can remove MTBE as well as Naphthalene, while there are different in their chemical structures. Obviously Naphthalene induced the MTBE oxidation enzymes.

Significance and impact of the study: Usually co-metabolism reaction is necessary for MTBE oxidation. However, here it is shown that Naphthalene grown cells are useful for MTBE removal with no co-metabolism reaction.

Keywords: Naphthalene; MTBE; Biodegradation; Freshwater; *Pseudomonas*

Introduction

Naphthalene is an important industrial chemical compound, which is of great concern because of its toxic and carcinogenic effects in the environment [1] and its high solubility in water [2]. The environmental protection agency (EPA) has reported that Naphthalene is one of the major pollutants in the environment [3]. Many bacteria, which are capable of degrading Naphthalene, have been isolated from contaminated sea, wastewater and soil. Some of them belong to genus *Pseudomonas* [2]. Naphthalene has two aromatic rings that are the important components of the coal and crude-oil.

Methyl tertyl-butyl ether (MTBE) is used as oxygenated for petroleum oil, an additive to gasoline to reduce atmospheric emissions of carbon monoxide and volatile organics [4]. The result of widespread use of this component leads to contamination of underground water. The great concern is that, MTBE become the second most common contaminant of urban ground waters because of its high aqueous solubility [5]. MTBE has been detected in urban air, surface water, storm water and groundwater. In fact, MTBE has been shown to persist in aquifers, and MTBE plumes have been shown to migrate at the rates comparable to groundwater velocities.

The mobility of MTBE in the subsurface is due in part to its high aqueous solubility, low octanol water partition coefficient and its molecular structure which is relatively resistant to microbial attack [6]. Because of the possible toxic effect of MTBE, the US Environmental Protection Agency (1999) has classified this compound as a potential carcinogen that is allowed to be with the maximum level of 20-40µg/l in drinking water [7].

Several techniques are mainly used for MTBE removal, including physicochemical attenuation mechanisms and biodegradation. It can be treated biologically with natural bacterial biomass or pure special bacterium. However, the inductions of enzymes are necessary for biodegradation and thus biomass inoculation with no enzyme induction might inhibit the degradation. Some reports showed the mechanism of MTBE biodegradation [8]. The biodegradation of MTBE has been investigated in some strain such as, *Mycobacterium*, *Rubrivivax*, *Rhodococcus* and *Pseudomonas* [9,10]. In this survey, the ability of Naphthalene degradation and its induction for MTBE oxidation, by strain isolated from fresh water were investigated.

Materials and methods

Sampling and isolation

Fresh water including; Zayanderood river, Karon river as well as Isfahan's waste water and activated sludge from Isfahan was sampled. All samples were stored in sterile bottle at 4°C before analysis. 5ml of samples was added to Bushnell-Haas (BH), Brown and Braddock [11] and shaked in 150rpm for one week at room temperature. After several subcultures, 100µl from culture medium was spread on BH agar plate (1.5%) [11]. The plates were inverted and Naphthalene crystals, as a sole source of carbon and energy, placed in each lid followed by parafilm wrapping of the plates and keeping them at 25°C for 48h. The resulting colonies were picked and inoculated into BH containing Naphthalene crystals with concentration of 1000ppm. Purity was verified by restreaking colonies onto BH agar Naphthalene plates

Phenotypic test

Preliminary identification of the isolates was based on colony morphology, Gram stain, catalase and oxidase, oxygen requirement, motility, acid produced from carbohydrate and Oxidative/Fermentative (O/F) test. All biochemical tests were

performed according to standards for microbial identification written in Bergey's manual of systematic bacteriology [12].

Growth of bacteria in Naphthalen
The isolate was grown in BH medium with 1000ppm Naphthalene as the only source of carbon, then the growth rate was determined by measuring optical density at 600nm using spectrophotometer.

Naphthalene degradation experiment
UV spectrophotometry
Approximately $1/5\times10^7$ per ml bacteria was add to 5ml of BH medium, containing 1000ppm Naphthalene in different tube. Then extract Naphthalene with hexan each 6h, and determined Naphthalene that remained with UV spectrophotometry at 277nm.

GC analysis
Bacterium (*Pseudomonas*) was grown in culture with determined amount of Naphthalene and the disappearance of Naphthalene was monitored with a gas chromatograph (model 6890 USA). 5ml of BH bacterial culture (approximately $1/5\times10^7$ per ml bacteria) containing 1000ppm of Naphthalene was added to each Teflon cape tube and were shaken in the darkness with 120rpm, at room temperature for six days. Control tube contained no bacteria. Then 1ml of each culture was removed and extracted for 60s with hexanes (1:1, v/v).

The extracts were analyzed by gas chromatograph using HP-5 column. The initial oven temperature was 50°C and the oven temperature was increased at a rate of 10°C/min until it reached 250°C. The injector and detector temperature were maintained at 110°C and 150°C, respectively.

Growth of bacteria in MTBE
Three ml of growth bacteria on BH media ($1/5\times10^7$per ml) incubated with 60µl of MTBE teflon cap tubes.

MTBE removal assay
MTBE removal was assayed in cultures containing determined amount of MTBE and the disappearance of MTBE was measured with gas chromatography (GC). 2ml of BH culture with Naphthalene grown bacteria (OD:0.4) with 2µl of MTBE added to each Teflon cape tube and were shaken in the darkness with 150rpm, at room temperature for 24h.

Control tube contained no bacteria, also other set of experiment have done with heat shock (100°C). Then 0.1ml of evaporated MTBE in Teflon cap tube injected to gas chromatograph with HP-1 column. The initial oven temperature was 40°C and the oven temperature was increased at a rate of 15°C/5mins until it reached 100°C. The hitter and detector temperature were maintained at 100°C and 220°C, respectively. Carrier gas was helium [13].

Result
A Gram-negative bacterium which had high ability to degrade Naphthalene was isolated from freshwater. Phenotypic test showed that isolated strain belongs to *Pseudomonas* genus (Table 1). Results of growth rate of this bacterium in Naphthalene are shown in figure 1. As it is shown, complete degradation of Naphthalene, (determined with UV specterophotometry at 277nm), has occurred in 90h. This figure also shows bacterial growth on Naphthalene (Fig. 1). Results of Naphthalene degradation measured by using GC and GC analysis are shown in figures 2a and 2b for strain *Pseudomonas* SA86 and control, respectively.

This result indicated that strain SA86 could remove more than 99% of Naphthalene available in the culture during six days. Although the above isolate could grow on Naphthalene however no growth was observed on MTBE as the only source of carbon and energy (even with the addition of 20µl substrate by injection into Teflon cap tube daily). Result of MTBE removal was shown by GC analysis in figures 3a and 3b. As it is shown, *Pseudomonas* SA86 could remove MTBE that by 70%. Also our results showed heat shocked bacteria could not remove MTBE. However, MTBE removal is not surface absorbance and its oxidation occurrs.

Table 1: The biochemical characteristic of isolated bacterium (SA86)

Strain name	**SA86**
Aerobic/ anaerobic growth	Aerobic
Acid production from glucose	+
Motility	+
Oxidase	+
Catalase	+
O/F	O^+
Colony colour	Yellow
Gram reaction	Gram-negative (bacillus)
Site of isolation	Isfahan waste water
Production soluble brown pigment	+

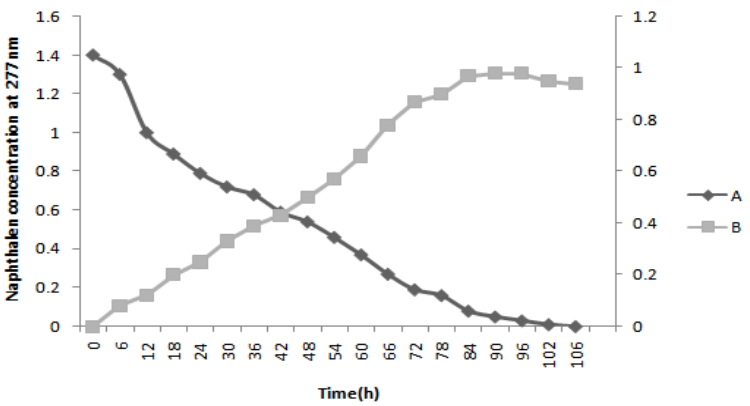

Fig. 1: Growth of bacteria, during incubation of strain SA86 in BH medium using Naphthalene as the sole carbon and energy source. A-Naphthalene concentration. B-Bacterial growth

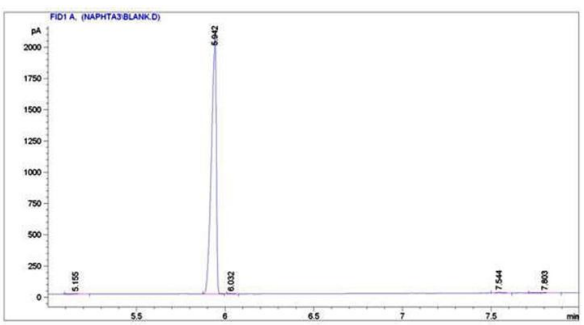

Fig. 2a: Analysis of Naphthalene (1000ppm) with GC after six days incubation, as blank

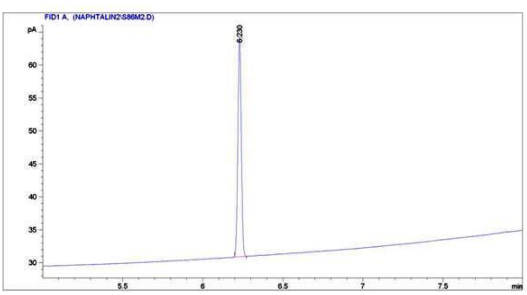

Fig. 2b: Analysis of Naphthalene (1000ppm) with GC by strain SA86, after six days

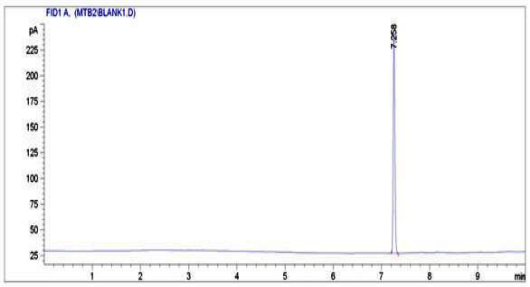

Fig. 3a: Analysis of MTBE (500ppm) with GC after 24h incubation, as blank

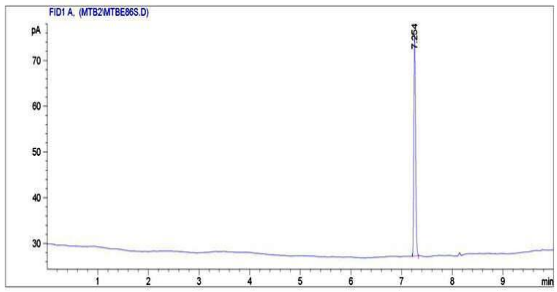

Fig. 3b: Analysis of MTBE (500ppm) with GC by strain SA86, after 24h incubation

Discussion

Naphthalene is one of the compounds that belongs to the Polycyclic Aromatic Hydrocarbons (PAHs) that is widespread in the environment. Different genera have previously been reported with the ability to degradate Naphthalene such as, *Pseudomonas*, *Rodococous*, *Marinobacter*, *Vibrio*, *Flavobacterium*, *Mycobacterium*, etc [14]. Most of research indicated that *Pseudomonas* has a great ability to degrade the toxic material such as Naphthalene [15]. MTBE is an additive to gasoline, introduced into various environments during the production, distribution and use and storage of oxygenate fuels. MTBE has been detected in surface water [16], and ground water [17,18]. So far, only a few microorganisms able to degrade MTBE have been reported. [9,10,19].

Some MTBE biodegradations co-metabolise with other organic material for example propan, n-Botan Benzene and pentane in *Pseudomonas aeruginosa* [20]. On the other hand, MTBE biodegradation may be inhibited in the presence of more easily biodegradable compounds [21]. This inhibition can occur when MTBE degrading cultures preferentially utilize easily degradable hydrocarbons instead of MTBE. For example, when BTEXs is mixed with MTBE, this leads to negative effect in MTBE biodegradation [22]. But in the other case in *Methylibium petroleiphilum* pm1 there is no negative effect in the presence of this xenobiotic [23]. There are some reports that presence of short chain alkenes has positive effect in MTBE Biodegradation in *Pseudomonas* strain [24].

In this study, we isolated and identified a *Pseudomonas* SA86 from fresh water that showed ability to use Naphthalene as the only carbon and energy source. This strain (SA86) could also remove MTBE when grown on Naphthalene. In fact Naphthalene biodegradation is catalyzed by dioxygenase and monooxygenase. There are reports that some *Pseudomonas* with ability to degrade Naphthalene has the cytochrome p450 dependent monooxygenase [25]. Cytochrome p450 is responsible for xenobiotic Biodegration like ETBE and MTBE that have been reported in *R. aethrivorans* IFP 2017 [26].

Conclusion

In our research, the isolated strain, SA86 could not remove MTBE in the absence of Naphthalene. Presence of cytochrome p450 in *Pseudomonas* and the ability of these genera to degrade Naphthalene indicate that this enzyme may be involved in induction of MTBE degradation. Because of the current pollution of fresh water with aromatic and oxygenated material from different source, it is very important to use this strain for bioremediation, due to its

ability to remove of both Naphthalene and MTBE.

Conflict of interest statement: All authors declare that they have no conflict of interest.

Sources of funding: None.

References

1) Preuss R, Angerer J, Drexler H. Naphthalene an environmental and occupational toxicant. *Int Arc Occup Environ Health*. 2003; 76: 556-76.
2) Lee HJ, Villaume J, Cullen DC, Kim BC, Gu MB. Monitoring and classification of PAH toxicity using an immobilized bioluminescent bacteria. *Biosens Bioelectron*. 2003; 18: 571-7.
3) Environmental Protection Agency (EPA), USA. Toxicological review of Naphthalene (CAS No. 91-20-3). In support of Summary Information on the Integrated Risk Information System (IRIS). 1998; 7-34.
4) Lopes Ferreira N, Labbe D, Monot F, Fayolle-Guichard F, Greer CW. Genes involved in the methyl tert-butyl ether (MTBE) metabolic pathway of *Mycobacterium austroafricanum* IFP 2012. *Microbiology*. 2006; 152: 1361-74.
5) Steffan RJ, McClay K, Vainberg S, *et al*. *Appl Environ Microbiol*. 1997; 63: 4216-22.
6) Deeb R, Scow K, Alvarez-Cohen L. Aerobic MTBE biodegradation: an examination of past studies, current challenges and future research directions. *Biodegradation*. 2000; 11: 171-86.
7) Agency USEP. Achieving clean air and clean water: the report of the blue ribbon panel on oxygenates in gasoline. US Government Printing Office, Washington DC. 1999.
8) Fayolle F, Francois A, Garnier L, *et al*. Limitation in MTBE Biodegradation. *Oil Gas Sci Technol*. 2003; 58: 497-504.
9) Lopes Ferreira N, Francois A, Mathis H, *et al*. biodegradation of Methyl tert-Butyl Ether, and other oxygenates by a new strain, *Mycobacterium austroafricanum* IF 2012. *Appl Environ Microbiol*. 2002; 86: 2754-62.
10) Lopes Ferreira N, Maciel H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW. Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTBE. *Appl Environl Microbiol*. 2006; 70: 358-65.
11) Brown EJ, Braddock JF. Sheen screen, a miniaturized most-probable number method for enumeration of oil -degrading microorganisms. *Appl Environ Microbiol*. 1990; 56: 3895-6.
12) Holt JG, Krieg NR, Moulder JW, *et al*. Bergeys manual of determinative bacteriology. 9th ed, USA, Williams & Wilkins. 1994; 151-68.
13) Baek K, Yang SJ, Sneath PHA, *et al*. Immoblization behavior of methyl tert-butyl ether by cyclodextrins. *J Hazard Mate*r. 2003; 105: 169-77.
14) Leahy JG, Colwel RR. Microbial degradation of hydrocarbons in the environment. *Microbiol Rev*. 1990; 54: 305-15.
15) Hedlund BP, Geiselbrecht AD, Bair TJ, Staley JT. Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans*. *Appl Environ Microbiol*. 1999; 65: 251-9.
16) Reuter JE, Allen BC, Richards RC, *et al*. Concentrations, sources, and fate of the gasoline oxygenate methyl *tert*-butyl ether (MTBE) in a multiple use lake. *Environ Sci Technol*. 1998; 32: 3666-72.
17) Landmeyer J, Pankow J, Church C. Occurrence of MTBE and *tert*-butyl alcohol in a gasoline-contaminated aquifer. In: Proceedings of the 213th ACS National Meeting, San Francisco, CA, Vol 37, 1997; 4113-5.
18) Landmeyer JE, Chapelle FH, Bradley PM, *et al*. Fate of MTBE relative to benzene in a gasoline-contaminated aquifer (1993-98). *Ground Water Monit Remediat*. 1998; 18: 93-102.
19) Steffan RJ, McClay K Vainberg S, *et al*. Biodegradation of the gasoline oxygenates Methyl tert-butyl ether, ethel tert-butyl ether, and tert-amyl methyl ether by propane-oxidizing bacteria. *Appl Environ Microbiol*. 1997; 63: 4216-22.

20) Garnier P, Auria R, Auger C, *et al*. Cometabolic biodegradation of methyl t-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Appl Microbial Biotechnol*. 1999; 51: 498-503.
21) Mo K, Lora CO, Wanken AE, *et al*. Biodegradation of methyl t-butyl ether by pure bacterial cultures. *Appl Microbial Biotechnol*. 1997; 47: 69-72.
22) Lee EH, Cho KS. Effect of substrate interaction on the degradation of methyl *tert*-butyl ether, benzene, toluene, ethylbenzene, and xylene by *Rhodococcus* sp. *J Hazard Mater*. 2009; 167: 669-74.
23) Pruden A, Suidan M. Effect of benzene, toluene, ethylbenzene, and *p*-xylene (BTEX) mixture on biodegradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) by pure culture UC1. *Biodegradation*. 2004; 15: 213-27.
24) Morales MV, Nava E, Velasquez E, *et al*. Mineralization of methyl *tert*-butyl ether and other gasoline oxygenates by *Pseudomonads* using short n-alkanes as growth source. *Biodegradation*. 2009; 20: 271-80.
25) Bubinas A, Giedraityte G, kalediene L, *et al*. Degradation of Naphthalene by thermophilic bacteria via a pathway, through protocatechuic acid. *Cen Eur J Biol*. 2008; 3: 61-8.
26) Goodfellow M, Jones AL, Maldonado LA, *et al*. *Rhodococcus aetherivorans* sp. nov., a new species that contains methyl t-butyl ether-degrading *Actinomycetes*. *Syst Appl Microbiol*. 2004; 27: 61-5.