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Removal of MTBE by novel *Exiguobacterium* in seawater

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ABSTRACT

For over three decades, methyl-*tert*-butyl ether (MTBE) has been used as an additive to either increase the octane number in high and medium grade gasoline in substitution of tetraethyl lead or to raise its oxygen content. However, the fate of MTBE in the environment is a great concern. In this work, biomass of novel toluene degradator *Exiguobacterium*, isolated from toluene enriched seawater, could utilize MTBE by 72% and produced CO_2 and formate during degradation. Production of ketone and *tert*-butyl alchol (TBA) has not been seen by gas chromatography (GC). Degradation of MTBE has been seen in aerobic and anaerobic conditions from 4 to 30 °C. It is interesting that the highest degradation occurred during growth in aerobic and cold condition (4 °C). This suggested that in cold areas *Exiguobacterium* is a very good candidate to degrade MTBE. Since this novel isolate could tolerate high salt, it is useful for removal of MTBE from seawater.

Keywords: Exiguobacterium; MTBE; Pollution; Seawater

1. Introduction

Among fuel oxygenates, methyl-*tert*-butyl ether (MTBE) is the most commonly used agent because of its high-octane level, low production cost, ease of blending with gasoline, ease of transfer and distribution [1,2]. The addition of MTBE to gasoline began on a relatively small scale in the late 1970s with its use as an octane enhancer to replace tetraethyl lead [3]. Currently an average of 11% MTBE by volume is added to about 30% of the gasoline sold in the United States [4]. Like most other gasoline components, MTBE is introduced into various environmental compartments during the production, distribution, use and storage of oxygenate-blended fuels. 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 rates comparable to groundwater velocities. The mobility of MTBE in the subsurface is due to its high aqueous solubility, low water partition coefficient and chemical structure which are relatively resistant to microbial attack [3]. Scientific information on the assessment of the carcinogenicity of MTBE in humans comes from animal investigations. However, the potential carcinogenic effect of MTBE on humans remains a matter of debate [5]. Based on taste and odor concerns, the EPA's Office of Water has established a drinking water advisory level of 20–40 μg/l as guidance for state and local authorities [6]. MTBE is poorly adsorbed, chemically and biologically stable and very soluble in water, making it very persistent in the environment. Therefore, effective technologies are in an urgent demand to remove MTBE from contaminated water. Conventional treatment of MTBE-contaminated groundwater is inefficient and unsatisfactory. Air stripping is difficult and requires a high air-to-water ratio

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(200/1 l for 95% removal) because of its very low Henry's law constant [7]. Several techniques are mainly used for MTBE removal, including physicochemical attenuation mechanisms by UV/TiO_2, UV/H_2O_2 and O_3/H_2O_2 process [8] and biodegradation.

It can be treated biologically with special bacterial strains or natural isolates under aerobic condition, although to our best knowledge these strains grow slowly with low yields of biomass and are sometimes unstable. As a result, a reliable bioremediation process for MTBE has not been reported up to now.

The purpose of this research is to investigate biodegradation of MTBE by a novel extremophile *Exiguobacterium.*

2. Material and methods

2.1. Chemicals and reagents

Toluene and MTBE were purchased from Merck-Schuchard Hohenbrunn.

2.2. Culture media and isolation of bacterial strain

Exiguobacterium sp. strain was isolated from toluene-contaminated wastewater by a standard culture enrichment technique using basal salt medium (BSM) supplemented with 1% (v/v) toluene as the sole carbon source. BSM contained: 4 g KH_2PO_4, 4 g Na_2HPO_4, 2 g NH_4Cl, 0.2 g $MgCl_2$, 0.001 g $CaCl_2$ and 0.001 g $FeCl_3$ in 1000 ml twice distilled water [9]. The strain was stored in 50% (v/v) glycerol at –80 °C.

2.3. Identification of isolated strain

Identification of the isolated strain was based on colony morphology, Gram stain, acid-fast stain, catalase test, oxidase test, oxygen requirement, motility, the ability of growth on different carbon sources, and in the presence of some inhibitors according to standards for microbial identification in *Bergey's Manual of Systematic Bacteriology*. Sequences analysis of the 16S rRNA gene also was performed for identification of strain according to Lin Wang et al. method [10].

2.4. MTBE removal assay

Ten microliter of 0.5 McFarland bacterial suspensions was transferred to fresh BSM with 10 µl MTBE. Removal of MTBE was assayed by UV spectrum at 200–600 nm and reaction with COD Hach reagent (4.913 g $K_2Cr_2O_7$ was added to 500 ml water with 167 ml H_2SO_4 and 33.3 $HgSO_4$), after 24 h. This reagent dissolved and diluted to 1000 ml.

One milliliter of the supernatant from grown cells on MTBE were added to 1 ml digestion solution (COD Hach reagent), the obtained blue green colour was measured by a turbidity measurement as (O.D. at 600 nm) in a UV-visible spectrophotometer (Shimadzu UV-160, Japan) against blank. The reduction of blue green colour showed the removal of MTBE.

2.5. GC analysis of MTBE removal by biomass

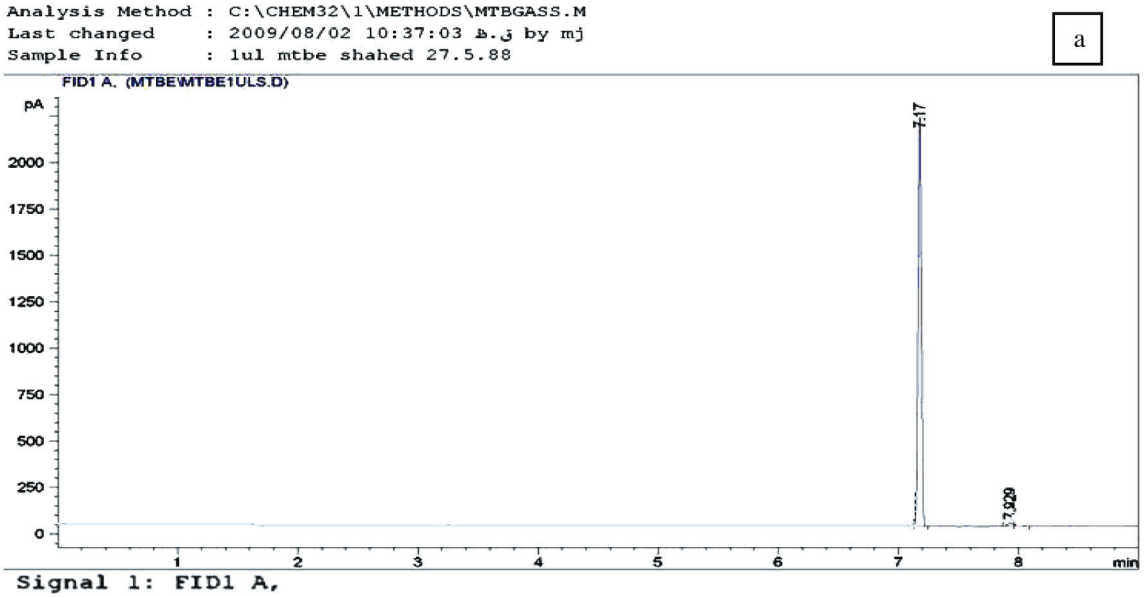
Removal of MTBE by cell biomass was detected by GC, after 12 h. GC measurements were performed on gas chromatograph Agilent Technologies 6890N (Avondale, USA) equipped with flame ionization detection (FID) and a split–splitless injector. The carrier gas was helium with a pressure of 34 psi in the injection port. The detector temperature was maintained at 240 °C. Oven temperature was programmed as follows: from 60 to 130 °C at 7 °C min^{-1}. One hundred percentage dimetyle polysiloxane HP1 (L: 60 m, I.D.: 0.25 mm) was employed for the GC separation.

2.6. Determination of formaldehyde production

The concentration of produced formaldehyde by isolated strain was measured by Hantzsch method [11]. Equal volumes of Hantzsch reagent (2 M ammonium acetate, 50 mm acetic acid, 20 mm acetyle acetone) was added to 2 ml of centrifuged cell biomass grown on nutrient broth and induced with MTBE (4000 ppm) for 2 h. This mixture was incubated at 60 °C for 10 min. The obtained yellow colour of 3,5-diacetyl–1, 4-dihydrolotidin which produced from reaction between formaldehyde and pentane 2,4-dion (acetyle acetone) in the present of ammonium acetate was centrifuged and measured at 412 nm against blank.

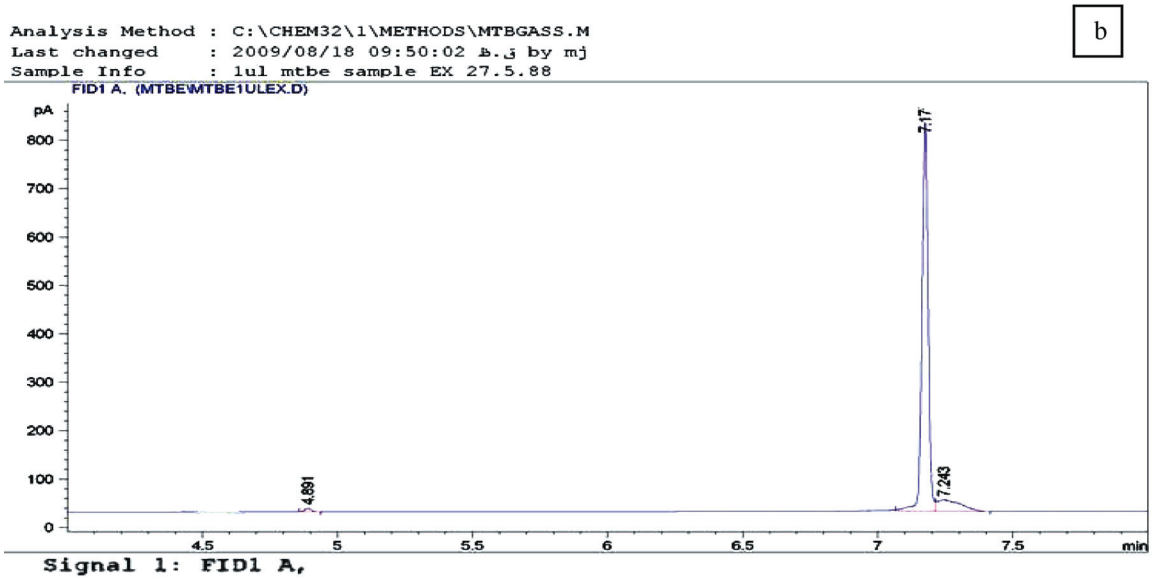
Table 1
Preliminary identification of isolated *Exiguobacterium*

Test	Reaction
Gram stain	+
Shape	Rod, Coryneform
Spore	–
Acid-fast	–
OF	+/+
Motility	+
Oxidase	+
Catalase	+
Growth aerobically	+
Growth anaerobically	+
Growth in NaCl	2–15%
Growth range	4–50 °C
Fumarate and H production	+



Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	7.17	VB	0.0306	4268.00732	2203.19824	39.27553
2	7.929	BV	0.0480	52.87696	18.15537	0.48659



Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	4.525	BB	0.0283	759.94739	416.72913	34.54876
2	4.891	BB	0.0271	12.31330	7.15873	0.55979
3	7.17	BV	0.0257	1284.57141	799.98822	58.39924

Fig. 1. Degradation of MTBE by *Exiguobacterium* studied by GC. (a) Blank (MTBE, 10 µl), (b) MTBE with *Exiguobacterium*.

2.7. Formate determination by potassium permanganate

Production of formate was tested with significant reduction of purple colour of potassium permanganate (1 mm) [11].

2.8. Production of CO_2 from MTBE

Filter paper Watman number one (2 × 1 cm^2) was cut, soaked with 0.1 ml KOH 40% and dried. The weight of this paper was measured and insert to small minifuge tube in sealed cap universal tube with MTBE (0.02 ml/ml). The MTBE oxidation to CO_2 was measured by CO_2 reaction with KOH.

2.9. Growth of strain and MTBE removal in extreme conditions

The growth rate of *Exiguobacterium* in anaerobic condition and refrigerator temperature was assessed indirectly by a turbidity measurement (O.D. at 600 nm) in a UV-visible spectrophotometer (Shimadzu UV-160, Japan). The MTBE removal assay was carried out by Hantzsch reagent at 412 nm against blank.

3. Results and discussion

The *Exiguobacterium* was isolated from toluene enriched seawater in media with toluene as the sole carbon source. Preliminary test showed that the isolate was Gram-positive, coryneform, motile and capable to grow at 4–50 °C and identified as *Exiguobacterium* (Table 1). As it is shown in Table1, the isolate is a salt tolerant strain.

Cell biomass of *Exiguobacterium* (OD = 0.7) was added to Teflon cap tube with 1 µl (740 ppm) MTBE. The remaining MTBE was detected by gas chromatography fitted with a flame ionization detector (FID) and equipped with a capillary column. The toluene grown cell biomass could remove MTBE by 72%. The results of GC are shown in Fig. 1.

MTBE monooxygenase was induced in *Exigoubacterium* when it was grown on toluene. This isolate could produce formate as a main intermediate of MTBE degradation.

Table 2
Removal of MTBE by *Exiguobacterium* (the data are average of three replications)

Conditions	Growth in MTBE broth (OD600)	Removal of MTBE (%)
Aerobic, 28 °C	0.16	42.12
Anaerobic, 28 °C	0.08	26.31
Aerobic, 4 °C	0.2	61.53
Anaerobic, 4 °C	0.13	38

Although, production of formaldehyde, TBA and acetone were negative, the isolate could utilize formaldehyde. On the other hand, some bacteria like *Gordona* and *Pseudomonas* species could produce formaldehyde as result of TBA fermentation [12].

The MTBE consumption by the isolate was tested on aerobic and anaerobic conditions at 28 °C and 4 °C. The results are shown in Table 2. As it is shown, this isolate could degrade MTBE by 61.53% at 4 °C in aerobic condition which make this bacterium a good candidate for MTBE removal from the seawater.

4. Conclusion

The main sources of MTBE in the environment are leaking underground storage tanks and other activities related to its production and transport [13]. Because of the low affinity of MTBE for soil particles, matrix and reaches the underground water contaminating aquifers not only by itself, but also with other MTBE-related organic ethers [14]. There have been some reports on the partial biodegradation of MTBE by pure bacterial cultures, including *Rhodococcus*, *Flavobacterium*, *Pseudomonas*, *Burkholderia and Methylobacter* [15]. Here is the first report for degradation of MTBE by novel *Exiguobacterium* isolated from toluene enriched seawater. This microorganism is Gram-positive, rod-shaped, facultative aerobe, motile with peritrichous flagella and its growth temperature range is from –2.5 to 40 °C [10].

Therefore, this new coryneform extremophile isolate is a good candidate to degrade MTBE and toluene in aerobic, anaerobic, salted and cold conditions.

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