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Microbial Community from MTBE-Contaminated Soil for Aerobic Biodegradation of MTBE

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Abstract

This Methyl *tert*-butyl ether (MTBE) is one of the main additives in gasoline to increase octane rating and consequently reduce air pollution. The physico-chemical properties of this substance (high water solubility, low sorption in soil) result in high mobility and considerable concentrations in aquifers. In this survey, Isfehan Refinery that was encountered with MTBE contamination problem was selected as a case study and the MTBE degradation ability of this contaminated area by its indigenous microorganisms was investigated. In the first step of this survey, the influence of various factors on the aerobic degradation of MTBE such as mixed culture type, incubation time, microbial culture and optimal concentration of MTBE were investigated in shaking flasks and the most important factors were specified by means of fractional factorial design ½. In the second stage by using optimal values which obtained from the first stage, the effects of co-substare parameter and inoculum parameter were assayed by means of response surface method. The results of the experiments showed that the mixed culture type and initial concentration of MTBE were the most significant factors. The results of the experiments showed that the mixed indigenous culture acted better than activated sludge. The initial concentration of MTBE was also one of the most significant factors. At the best condition about 31 percent of MTBE was treated by co-substrating with n-hexane in a ratio of 0.2.

Keywords

Bioremediation, MTBE Contamination, Groundwater, Cometabolism, Indigenous Microorganisms

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1. Introduction

Methyl *tert*-butyl ether (MTBE), a synthetic compound produced from methanol and isobutylene, has been used as a gasoline additive to increase octane ratings instead of lead-based and aromatic compounds since the late 1970s. For the last 15 years, it has been added in higher concentrations (up to 15% (v/v)) to increase fuel combustion efficiency and to lower emissions of CO and other air pollutants [1]. The release of MTBE to the environment, mainly from damaged gasoline underground storage tanks or distribution systems spills, has provoked extended groundwater pollution [2]. Solubility of MTBE in water is approximately 50,000 mg/L at 25˚C and has a low organic carbon-based partitioning coefficient (K_{OC}) of 11 cm^3/g resulting in minimal sorption and retardation in natural aquifer [3]. MTBE has a higher vapor pressure than benzene, its Henry's Law constant (0.022 at 25˚C) is $1/10^{th}$ benzene. This results in minimal losses of MTBE from the aqueous phase to the atmosphere. Due to significant partitioning of MTBE into the aqueous phase and limited sorption to soil, MTBE plumes in polluted groundwater are generally longer and more stable than coexisting gasoline plumes [4].

There are different technologies that have been applied to remediate groundwater polluted by MTBE and other gasoline compounds such as pump and treat, air sparging, multi-phase extraction, and *in-situ* chemical oxidation [5]-[7]. Traditional pump and treat technologies, including air stripping, used to eliminate gasoline components from groundwater have not been as successful for MTBE removal [4]. Bioremediation is an alternative to traditional technologies because it is considered to be cost-effective and energy efficient. Its main advantage is that it can completely mineralize MTBE to harmless products such as carbon dioxide, biomass, and water. Initial studies show that MTBE is highly recalcitrant (because of its structure containing both an ether link and a branched moiety) but recently some authors have reported that certain bacteria can degrade MTBE as their sole carbon and energy source. Depending on the microorganisms involved in the process, biodegradation is more frequently observed under aerobic conditions by pure or mixed cultures [5]. To date, the only bacterial strains that have been reported to completely consume MTBE as their sole source of carbon and energy are *Methylibiumpetroleiphilum* PM1, *Hydrogenophagaflava* ENV735, *Mycobacterium austroafricanum* IFP2012 and *Aquincolatertiaricarbonis*. A diverse group of bacterial strains such as *Methylobacterium*, *Rhodococcus*, *Arthrobacter*, *Bacillus*, *Pseudomonas* or an entrapped microbial consortium have been reported to partially degrade MTBE [8]-[11].

Cometabolic MTBE biodegradation has received significant research attention. Cometabolism involves the use of an additional carbon source for growth and may have a role in natural attenuation. Some compounds present in gasoline have been reported for their ability to induce enzymes able to degrade MTBE [12]. Cometabolic MTBE degradation has been reported by C3-C7 n-alkanes [12]-[16] and generally, aromatic compounds do not support MTBE degradation [12]. Bacteria reported to degrade MTBE by cometabolism include *Nocardia* [14], *Mycobacterium* [17], *Rhodococcus* [18] and *Pseudomonas* [12].

In this study, the aerobic MTBE degrading capacity of the indigenous microbial community in soil from gasoline contaminated sites in Isfehan Refinery, is examined in batch experiments simulating different *in-situ* conditions.

2. Material and Methods

2.1. Soil Sample and Enrichment Culture

Soil samples were collected at 5 - 20 cm depth from MTBE contaminated ground surface of Isfehan Refinery. These samples were used for reaching the indigenous microorganisms. Ten grams of soil sample were dissolved in 100 ml sterilized water and shaken at 150 rpm and 30˚C for 3 hrs and then left standing for 30 mins. The supernatant 10 ml was then incubated into 200 ml modified M9 as growth medium with the following formulation: Na_2HPO_4, 6.0 g/l; KH_2PO_4, 3.0 g/l; NaCl, 0.5 g/l; NH_4Cl, 1.0 g/l; $MgSO_4.7H_2O$ 1.0 M, 2.0 ml/l; $CaCl_2$ 1.0 M, 0.1 ml/l (all chemicals were purchased from Merck). MTBE as the sole carbon and energy source was added to a final concentration of 200 ppm and the mixture was shaken in a 500 ml flask for 24 h at 150 rpm and 30˚C. Subsequently, 20 ml of the inocula (supernatants of the above cultures) was transferred to another 500 ml flask containing 200 ml of modified fresh M9 medium supplemented with MTBE (300 ppm) and was incubated for another 24 hrs under the same conditions. This procedure was repeated over 10 days until the concentration of MTBE reached to 1000 ppm in the last fresh modified M9 medium. After the final transfer, 100 µl of the culture was spread on Nutrient agar medium plates and incubated for 24 hrs at 30˚C. Nutrient agar medium plates were

maintained at 4˚C.

2.2. Batch Scale Study

In this part of study, feasibility of MTBE bioremediation was assessed by a combined microbial culture that was separated from MTBE contaminated soil. To evaluate this method's applicability, a series of experiments based on design of experiments by Minitab software were done in which superficial MTBE contaminated water samples were used for preparing the mixed bacterial culture. Experiments were conducted to examine the feasibility of MTBE biodegradation under aerobic and aerobic-cometabolic condition. Experiments were conducted in two stages. At first stage, the effects of mixed culture type, initial concentration of MTBE, incubation time and culture medium type were investigated. All experiments were carried out in sealed 250-mL Erlenmeyer flasks. The flasks contained 90 mL of a culture medium type (M9 or nutrient broth) and MTBE. Based on design table (see **Table 1**) 10 ml of isolated microorganisms from contaminated soil with the optical density adjusted to 1 at 600 nm by sterile normal saline (0.89% NaCl) and municipal activated sludge with MLSS of 12 g/l with the optical density adjusted to 1 at 600 nm by sterile normal saline, were added to the flasks as inoculums. For each flask, one flask was prepared as abiotic control which every control contains 250 mg/l $HgCl_2$ and 500 mg/l NaN_3 [19]. Abiotic controls for each flask were prepared based on **Table 1**. Flasks were incubated at 25˚C and 150 rpm for 24 and 120 hours based on **Table 1**. The volume of the flask headspace was sufficient to prevent any limitation to O_2 [20]. The pH of media was adjusted to 7.2 using 0.1 N NaOH.

At the second stage (after analyzing the results of stage 1) the effects of inoculum size and the presence of co substrate factors were investigated based on **Table 2**. Indigenous microorganisms of the region were used at this stage. Initial concentration of MTBE was 200 ppm at M9 salt broth. All the conditions of experiments were the same as stage 1. Incubation time in shaking flask was considered 48 hours.

2.3. Design of Experiments

Minitab 16 was used for designing of experiments and all of statistical analysis. The method for the first stage designing was fractional factorial ½ with resolution of IV and for second stage was response surface method (Central Composite Design). ANOVA was used to determine the significance of regression and factors. ANOVA was also used to assess differences between appropriates measured parameters. The first stage design table and the second stage design table are given in **Table 1** and **Table 2** respectively.

2.4. Analysis of MTBE

MTBE in groundwater are measured using headspace solvent micro extraction (HSME) method, with gas chromatography-flame ionization (GC-FID). HSME is a novel method of sample preparation for chromatography

Table 1. Design of experiments based on fractional factorial ½ method.

No. of Exp.	Factors				MTBE Biological Removal Percent
	Mixed Culture Type	Initial Concentration of MTBE (ppm)	Incubation Time (h)	Culture Medium Type	
1	Indigenous microorganisms	200	24	M9	22.79
2	Indigenous microorganisms	1000	24	NB	2.69
3	Indigenous microorganisms	200	120	NB	17.80
4	Indigenous microorganisms	1000	120	M9	5.00
5	Activated sludge	1000	24	M9	1.31
6	Activated sludge	200	24	NB	2.66
7	Activated sludge	200	120	M9	1.07
8	Activated sludge	1000	120	NB	0.30

Table 2. Design of experiments based on response surface method.

No. of Exp.	MTBE/n-Hexane (w/w)	Inoculum Size (OD)	MTBE Biological Removal Percent
1	0.2	0.2	30.38
2	0.2	0.4	29.76
3	0.2	0.6	30.98
4	0.6	0.2	25.43
5	0.6	0.4	24.95
6	0.6	0.6	25.59
7	1	0.2	22.38
8	1	0.4	22.80
9	1	0.6	21.92

analysis. This system involves a microdrop of a high boiling point organic solvent (n-decane in this study) extruded from the needle tip of gas chromatographic syringe, which is exposed to the headspace above a sample. Volatile organic compounds (MTBE in this study), are extracted and concentrated in the microdrop. Then, the microdrop is retracted in the microsyringe and injected directly into a chromatograph. The high volatility and low polarity of MTBE lead the compound to have a fast diffusion to headspace and an enhanced distribution into the microdrop, respectively. Theses characteristic leads to study the determination of MTBE in water samples by a HSME method using a GC-FID equipment [21]. The GC-FID analysis was performed using a Varian CP 300 gas chromatography equipped with a flame ionization detector. The injector and detector temperatures were 210˚C and 230˚C, respectively. A constant flow (1 ml/min) of Helium was used as carrier gas. The analysis was performed with an initial column temperature 35˚C held for 5 min followed by heating to 150˚C at 25˚C/min. MTBE eluted in 3.7 mins. All quantifications made in this study were based on the relative peak area of MTBE to the internal standard (1000 ppm chloroform in methanol) from the average of three replicate measurements.

2.5. MTBE Biodegradation Determination

The control samples were prepared for considering MTBE removal by abiotic processes. The percentages of biotic and abiotic removal of MTBE (R%) was obtained by the following equations:

$$R_{\text{Biotic}}(\%) = \frac{\text{MTBE}_{\text{Control}} - \text{MTBE}_{\text{Treatment}}}{\text{MTBE}_{\text{Initial}}} \times 100 \quad (1)$$

$$R_{\text{Abiotic}}(\%) = \frac{\text{MTBE}_{\text{Initial}} - \text{MTBE}_{\text{Control}}}{\text{MTBE}_{\text{Initial}}} \times 100 \quad (2)$$

3. Result and Discussion

Influences of Factors and Analysis of Variance

The results of stage one showed that indigenous microorganisms of the region could biodegrade MTBE effectively comparing to activated sludge. Based on the analysis of Minitab software, it was found that out of four main factors, two of them including culture type and initial concentration of MTBE had significant effects on biodegradation of MTBE with p-value < 0.05. In contrast, incubation time and culture medium factors had insignificant effect on MTBE degradation with p-value > 0.05. MTBE disappearance in the all controls was negligible.

Result showed that the indigenous microorganisms capability in MTBE degradation, as predicted, were higher compared to activated sludge. This phenomenon was probably interpreted in order of indigenous microorganisms acclimation with MTBE. According to data from experiments number 1 and 2 and also experiments number 3 and 4 and considering culture medium factor as an insignificant factor (p-value of media culture > 0.05),

MTBE degradation percent is decreased when MTBE initial concentration is increased. Similar results are reported in [20] when the MTBE initial concentration is increased above 50 - 100 ppm, the MTBE degradation rate decreased.

Incubation time factor has no significant effect on MTBE degradation (p-value > 0.05). According to data from experiments number 1 and 3 and also 2 and 4 and considering culture medium factor as an insignificant factor, it is found that although MTBE degradation percent is increased with spreading time, this factor has insignificant effect on MTBE degradation percent practically. This could be ascribed to the presence of *tert*-butyl alcohol (TBA) as an intermediate in the most of MTBE-degrading culture and its inhibitory effect on MTBE degradation so that is suggested that a competition between two components occurred. It can be presumed that the same enzyme possibly responsible for MTBE and TBA metabolism and they may be competing for the same active site [20]. The TBA accumulation had a negative effect on the MTBE degradation rate in the beginning hour of experiment, the similar result was also reported about butane-oxidizing *Arthrobacter* (ATCC 27778) bacteria [15].

According to the Minitab software, the medium culture has no significant effect on MTBE degradation. Although both M9 and NB can prepare Nitrogen and Phosphorous supply for microorganisms, MTBE degradation percent in M9, was higher comparison to NB. This can be intrepid by considering MTBE as the sole carbon source in M9.

Microorganism, MTBE initial concentration, incubation time and MTBE biological removal percent for similar studies are presented in **Table 3**.

According to results of stage two, the presence of cometabolite has significant effect on MTBE degradation (p-value < 0.05) and MTBE biological removal percent is increased. One potential reason is that some oxidizing enzymes during consumption of n-hexane are produced which promote MTBE removal efficiency. The result indicated that the highest MTBE degradation percent is achieved in MTBE to n-Hexane ratio (w/w) equal to 0.6.

The effect of inoculum size on MTBE biodegradation was tested. According to result of stage two, inoculum size factor had no significant effect on MTBE biodegradation (p-value > 0.05) and no difference was observed with the increasing of inoculum size. Although, inoculum size could play a role in MTBE removal soon after the inoculation, the degradation rate reaches a constant. This interpretation is consistent with findings of Zhang *et al*. [20]. Therefore, biological removal of MTBE could enhance with the decrease of MTBE to n-hexane ratio, while inoculum size had negligible impact on MTBE degradation.

4. Conclusion

The results of this study provide evidence of MTBE degradation by indigenous microorganisms. Aerobic microbial consortia is taken from the groundwater and soil of a contaminated site and enriched, to test whether they are capable of degrading MTBE as sole source of carbon. Current study shows that indigenous microbial populations are able to remove MTBE from aqueous samples. Furthermore, it emerges from the results of this study that when the initial concentration of MTBE is 200 ppm, biological removal of MTBE is enhanced. The results of design of experiments by response surface method show that using of n-hexane as the co metabolite provides a solution to overcome low biodegradation of MTBE.

Table 3. Aerobic MTBE degradation observed with different pure and mixed cultures of bacteria.

Microorganisms	Initial MTBE Concentration	Incubation Time	MTBE Removal Percent	Reference
*Achromobacter xylosoxidanns MCM*1/1	100 ppm	5 d	78%	[1]
Bacillus *Rhodococcus* *Micrococcus* *Aureobacterium* *Proteus*	100 ppm	5d	22% - 37%	[22]
Microbial consortium from MTBE contaminated groundwater	950 ppm	3d	25%	[5]
Indigenous microorganisms	200 ppm	1d	23%	Current study

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