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Bioremediation of methyl tertiary-butyl ether (MTBE) by three pure bacterial cultures

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Abstract

Background: Bioremediation of groundwater and soil contamination is more economical than physicochemical remediation. The present study focused on the bioremediation capability of two bacterial species (*Klebsiella planticola* and *Enterobacter cloacae*) from the family Enterobacteriaceae. These bacteria have been identified as new species with capability of degrading methyl tertiary-butyl ether (MTBE). In order to enhance their degradation capability, selected concentrations and retention time were investigated.

Methods: The bacteria were cultured on the nutrient agar (NA) medium at room temperature. pH of the medium was adjusted to 7. The medium was autoclaved at 121°C for 15 minutes and incubated for 24 hours at 35°C. After 24 hours, the mixture was inoculated into 50 mL of Luria Bertani (LB) liquid medium containing 50 and 150 ppm MTBE. The cultures were incubated for 2 and 5 days at 35°C and shacked on a shaker at 150 rpm. Cell concentrations of the bacteria in pure culture were determined from the optical density at 600 nm using a UV–VIS spectrophotometer. Then, the culture was centrifuged at 3800 rpm for 20 minutes. In the next step, the MTBE concentration in the supernatant was measured by gas chromatography/mass spectrometry (GC/MS, Agilent Technologies, 5975, US10304411, 5.02.07).

Results: The results showed that both strains are able to grow in the presence of 50 and 150 ppm MTBE. In the best conditions, when cell density was 3×108 CFU/mL during 5 days, the highest rate of MTBE degradation for *K. planticola* and *E. cloacae*, was 43% and 40%, respectively. It was also revealed that *Escherichia coli* can degrade 50 and 150 ppm MTBE about 19.8% and 13.65%, respectively.

Conclusion: It seems that *E. coli* can be a good candidate for MTBE degradation at high concentrations for a time longer than that in the present study. It was also found that the species have high performance at 50 ppm than 150 ppm. So, these bacteria can remove MTBE from the environment.

Keywords: Biodegradation, *Klebsiella planticola, Enterobacter cloacae, Escherichia coli*, methyl tertiary-butyl ether

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Introduction

Unleaded gasoline consists hydrocarbons and different chemical compounds such as methyl tertiary-butyl ether (MTBE) (1,2). MTBE ($C_5H_{12}O$) is an oxygenate organic compound, that has been used as additive in gasoline since the late 1970s, to replace tetraethyl lead (TEL) and other toxic chemicals (3-5). MTBE is a persistent compound in the environment because it is highly soluble in water, poorly adsorbed by soil and is biologically and chemically stable against degradation (6). Accidental fuel leakage during storage or transportation is the main source of environmental contamination with MTBE (7). Therefore, the presence of MTBE in water is responsible for taste and odor related issues, genotoxicity and skin and eye irritation. Taste and odor thresholds for MTBE are 20-40 ppb (8,9). Generally, MTBE is the most common oxygenate compound, because it is cost-effective and easy-to-use (7). Due to its economic benefits, bioremediation with 99% efficiency, is a more attractive option than physicochemical remediation technologies such as ozone utilization, activated carbon, vaporization extraction and other methods (3,7,10-12). All microorganisms are not able to degrade MTBE easily (13). Some bacteria such as *Pseudomonas, Rhodococcus, Mycobacterium, Enterobacter,*

and *Achromobacter* are capable of degrading MTBE co-metabolically but not tert-Butyl Alcohol (TBA) (1). A few bacterial strains such as *Methylibium petroleiphilum* PM1, *Hydrogenophaga flava* ENV735, *Achromobacter xylosoxidans* MCM 1/11, *Pseudomonas* sp., *Bacillus* sp., and *Streptococcus* sp. can utilize MTBE as the sole source of carbon and energy (14-18). Two bacterial isolates (IsoSL1 and Iso2A) degraded MTBE in both nutrient-rich and nutrient-limited media. The highest rate of MTBE degradation was reported 29.6% and 27.8%, respectively, in 28 days (19). Researchers reported that bacteria such as *A. xylosoxidans* MCM 1/11 can use MTBE in 7 days (17). In this study, bioremediation of MTBE by *Klebsiella planticola* and *Enterobacter cloacae* at laboratory conditions was investigated. On the other hand, according to the abundance of *Escherichia coli* and its capability to utilize a wide range of hydrocarbons while the engineered E. coli was used for bioremediation, therefore, MTBE-degrading capacity of this bacterium was also compared. MTBE is one of the gasoline components that, nowadays, is spreading in the environment and can pollute soil, water, and groundwater. The influence of microbial degradation of organic substances and MTBE is well known (17-19). Many studies have been conducted on the MTBE biodegradation by pure bacterial cultures such as *Bacillus cereus* and *Klebsiella terrigena*, *Enterobacter* sp. NKNUO2, and other microorganisms (7,14-17,20). The first step in bioremediation is selecting the best bacteria because only some bacteria can use MTBE as a source of carbon and energy. A few pure or mixed bacterial cultures can grow on MTBE and use it as a carbon and energy source and some strains grow slowly on this oxygenated compound with low cell yields (21). Due to the complex molecular structure of MTBE, this compound is resistant to biodegradation because its ether bond and tertiary carbon atom are relatively unreactive (21-23). The toxic effects of products produced during metabolism can cause it. The intermediate products of MTBE biodegradation are tert-butoxy methanol (TBM), formaldehyde and TBA, respectively (23-25). Steffan et al demonstrated that the growth rates of propane-oxidizing bacteria on MTBE is very slow. Numerous microorganisms including EVN 735, *Variovorax paradoxus* CL-8, *Chryseobacterium* sp. A-3, *B. cereus, K. terrigena, Enterobacter* sp., and NKNUO2, have also the capability to remove MTBE from the environment (12,20,26-30). The biological degradation of MTBE and most of the organic matter is nowadays known in science (31,32). In a study by Salanitro et al, biomass yields (gram of dry weight cells per gram of MTBE) were 0.21 to 0.28 (32). Some studies have also investigated MTBE biodegradation by Mycobacterium (33,34). But so far, no study has been conducted to investigate the role of K. planticola in bioremediation of MTBE, while some species of Klebsiella have been found to be capable of utilizing MTBE, n-hexadecane, and other hydrocarbons contaminating soil (20,35,36).

Materials and Methods

Materials

MTBE (GC purity ≥98%) was purchased from Persian Type Culture Collection (PTCC). Other chemicals were analytical grade and purchased from Merck (Darmstadt, Germany).

After sterilization and passing through a 2-mm mesh sieve, specific concentrations of MTBE were added to 10 g of soil, and the growth rate of microorganisms and the concentration of MTBE were measured.

Microorganisms and incubation conditions

In order to determine a strain capable of growing on MTBE, two concentrations and two retention time were examined. *K. planticola* and *E. cloacae* were purchased from the PTCC. Then, these bacteria were cultured on the nutrient agar (NA) medium at room temperature. The composition of NA was as follows (gr^{-1}): 0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% sodium chloride, and distilled water. pH of the medium was adjusted to 7. The medium was autoclaved at 121°C for 15 minutes. In the next step, the cultures were incubated for 24 hours at 35°C. After 24 hours, the inoculum density at the beginning of the test were 1.5×10^8 CFU/mL and 3×10^8 CFU/mL, the mixture were then inoculated into 50 mL of Luria Bertani (LB) liquid medium containing 50 and 150 ppm MTBE (based on pretest) (12). LB medium was composed of (g/L): 10 g/L Trypton, 5 g/L yeast extraction, and 10 g/L NaCl. Then, the medium was autoclaved at 121°C for 15 minutes at pH 7. The cultures were incubated 2-5 days at 35°C in a shaker incubator at 150 rpm. Cell concentrations of *K. planticola* and *E. cloacae* in pure culture were determined from the optical density at 600 nm using a UV–VIS spectrophotometer (Hach model). Then, the culture was centrifuged in sealed tubes and cells were harvested from the medium by centrifugation at 3800 rpm for 20 minutes. Afterwards, the MTBE concentration in the supernatant was measured by gas chromatography/mass spectrometry (GC/MS, Agilent Technologies, 5975, US10304411, 5.02.07). The conditions for the GC analysis were as follows: 45°C held for 4 minutes, temperature ramped from 16°C/min to 70°C for 4.37 minutes, from 22°C/min to 100°C for 1.36 minutes, and 28°C/min to 220°C for 4.28 minutes (1,18,22). Helium was used as the carrier gas with an approximate flow rate of 1.10 mL/min. The sample without microorganisms was applied as blank in all tests. All specimens were tested two times. In this study, 38 samples were evaluated. Data were analyzed using statistical tests such as ANOVA, correlation, regression, and etc.

Results

During the incubation periods (2 and 5 days), *K. planticola* and *E. cloacae* were capable of growing on MTBE, as the source of carbon and energy, while initial concentrations of MTBE were studied. The removal rate of MTBE by two

Table 1. Biodegradation of MTBE by *Klebsiella planticola* and *Enterobacter cloacae*

Bacterial species	Initial concentration of MTBE (ppm)	Initial amount of bacteria (CFU/mL)	Time: 2 days		Time: 5 days	
			Removal percentage (%)	Residual value of concentration (ppm)	Removal percentage (%)	Residual value of concentration (ppm)
K. planticola	50	1.5×10^8	11.8	44.1	19	40.5
		3×10^8	29	35.5	43	21.5
	150	1.5×10^8	6.95	139.5	12.96	130.5
		3×10^8	24.3	12.5	33.5	99.5
E. cloacae	50	1.5×10^8	9.1	45.45	16.3	41.85
		3×10^8	24.6	37.7	40	30.05
	150	1.5×10^8	5.3	142	11.9	132.15
		3×10^8	15.95	126	30.95	103.5
E. coli	50	3×10^8	24.3	12.5	33.5	99.5
		1.5×10^8	9.1	45.45	16.3	41.85
	150	3×10^8	24.6	37.7	40	30.05
		1.5×10^8	5.3	142	11.9	132.15

pure bacterial cultures and the growth rate of two bacterial species at different concentrations of MTBE included in soil samples are shown in Table 1.

Biodegradation of 50 ppm MTBE by *K. planticola* and *E. cloacae* with inoculum sizes of 3×10^8 CFU/mL, is displayed in Figure 1. As shown in Figure 1, *K. planticola* and *E. cloacae* respectively indicated 43% and 40% MTBE degradation, in 120 hours detention time. In addition, the bacteria showed 24.6% and 29% MTBE degradation after 48 hours.

Biodegradation of 150 ppm MTBE by *K. planticola* and *E. cloacae* with inoculum size of 3×10^8 CFU/mL, is displayed in Figure 2. As shown in Figure 2, *K. planticola* and *E. cloacae* respectively indicated 30.95% and 33.50% MTBE degradation, in 120 hours detention time. The bacteria also showed 19.95% and 24.30% MTBE degradation after 48 hours.

Biodegradation of 150 ppm MTBE by *E. coli* with inoculum size of 3×10^8 CFU/mL, is presented in Figure 3. As presented in Figure 3, E.coli with different inoculum sizes of 1.5×10^8 and 3×10^8 CFU/mL showed 13% and 19.8% MTBE degradation, respectively, in 120 hours detention time. The bacteria also showed 9% and 6.5% MTBE degradation after 48 hours.

Discussion

The bacteria were capable to grow in all samples while the growth rate of the bacteria and MTBE degradation rate were different. The results showed that both bacterial species, in the similar conditions, could use initial MTBE concentration of 50 ppm better than the concentration of 150 ppm. In a similar study by Okeke et al, concentration

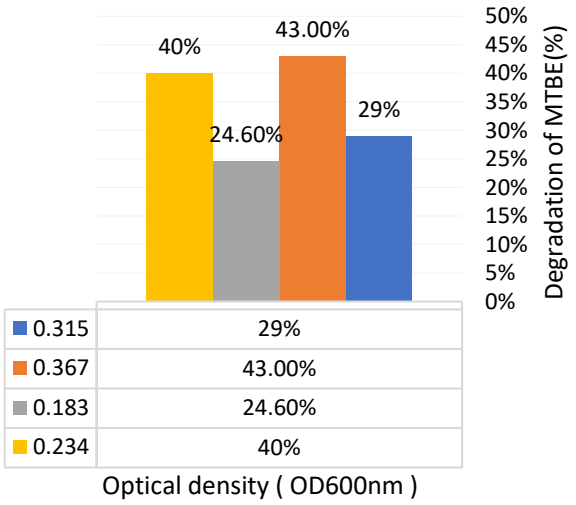

Figure 1. Biodegradation of 50 ppm MTBE by *Klebsiella planticola* and *Enterobacter cloacae* with inoculum size of 3×10^8 CFU/mL. White column, optical density of *K. planticola* after 48 and 120 hours and black column, optical density of *E. cloacae* after 48 and 120 hours.

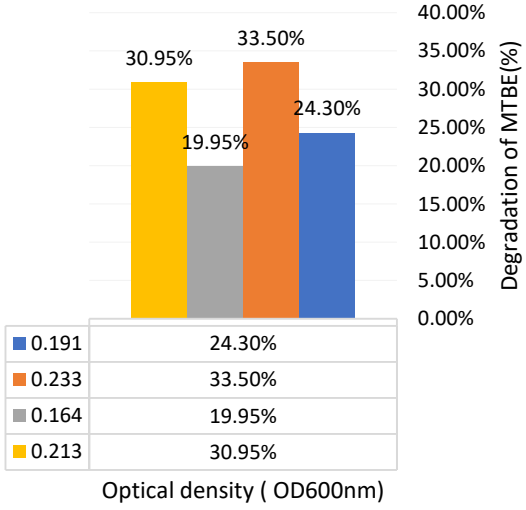

Figure 2. Biodegradation of 150 ppm MTBE by *Klebsiella planticola* and *Enterobacter cloacae* with inoculum size of 3×108 CFU/mL. White column, optical density of *K. planticola* after 48 and 120 hours and black column, optical density of *E. cloacae* after 48 and 120 hours.

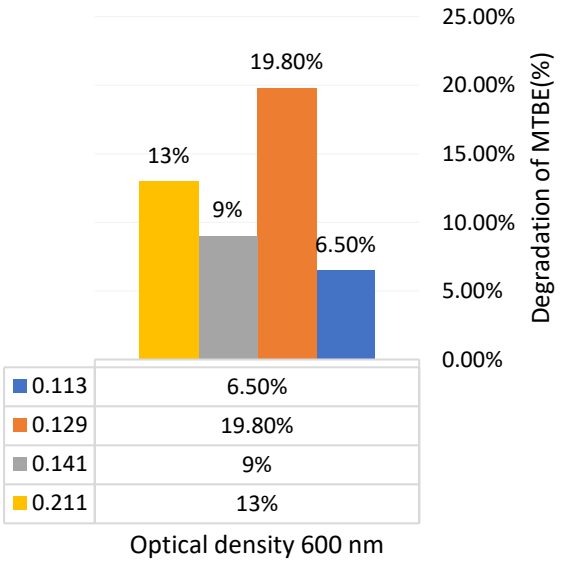

Figure 3. Biodegradation of 150 ppm MTBE by *E. coli* with different inoculum sizes of 1.5×108 and 3×108 CFU/mL. White column, optical density of *E. coli* after 48 hours. .

of 50 ppm has been reported as the best concentration of MTBE. It seems that toxicity increases at higher concentrations (18). Also, in the study by Zhang et al, on the bioremediation of MTBE-contaminated soil, the results showed that MTBE degradation rate increased from 50 to below 100 ppm (12). Another study also showed that *Staphylococcus saprophyticus* 6sy and *Pseudomonas* sp. 24p were capable of degrading 25 µg/mL MTBE better than 125 µg/mL in 21 days. Other researchers insisted on the role of different concentrations of MTBE on bioremediation of MTBE (37). Abbaspour et al showed that *Bacillus cereus* strain RJ1 can survive at different concentrations of MTBE while the initial concentration of 200 ppm is rapidly degraded (38). In this study, the highest rate of MTBE degradation was recorded after 5 days while there were no significant MTBE disappearances in the blank samples. It was also revealed that pure culture of *K. planticola* is more capable of degrading MTBE in the initial concentration of 50 ppm comparing with *E. cloacae*, especially after the end of the period. *K. planticola* showed a significant difference (student's *t* test) in different concentrations ($P=0.005$) compared to *E. cloacae* ($P=0.0117$). The initial absorbance at 600 nm was 0.1 and the inoculum size was 3×10^8 CFU/mL. According to Figure 1, MTBE degradation rate by both strains increased with increasing the microbial density. The results are consistent with the results of the study by Zhang et al (12). They reported that there is a direct relationship between the removal of MTBE and time. According to Figures 1 to 3, all bacterial species had the potential for degradation of 50 ppm and 150 ppm MTBE. Biodegradation rate of MTBE was 29% and 43% while the absorbance at a wavelength of 600 nm was 0.315 and 0.367, respectively (Figure 1). *E. cloacae* is also a good candidate. Jose Barbera et al confirmed that *E. cloacae* species play an important role in the MTBE biodegradation. They also showed that *E. cloacae* MCM2/1 has a high potential for utilizing MTBE (39). In the first hours, degradation rate by *K. planticola* was higher than other strains, and it seems that *E. coli* needs more time for degrading MTBE (Figure 3), it can be due to the complex molecular structure of MTBE, while the cultures containing yeast extract, this compound makes a good condition for better growth of the strains (19). Generally, there was no difference in the growth rate of both strains (*K. planticola* and *E. cloacae*) at different concentrations of MTBE. Besides, degradation rate was lower when the inoculum size was 1.5×10^8 CFU/mL (Table 1). The results of this study show that there is a direct relation between inoculum size and MTBE degradation. It should be noted that when the inoculum size increases, it uses more oxygen (12), which is consistent with the study by Rui-Ling et al. They reported that when cell density was 2×10^8 CFU/mL, the species had better performance than when it was 4.5×10^8 CFU/mL.

Conclusion

Klebsiella planticola, E. cloacae and *E. coli* could degrade MTBE at different concentrations and different cell densities in different times. Therefore, these strains are good candidates for removing MTBE from the environment. However, further studies using optimized media with other compounds for MTBE removal by these strains are suggested.

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Ethical issues

It is confirmed that this manuscript is the original work of the authors. The authors certify that all data collected during the study are presented in this manuscript, and no data from the study has been or will be published separately.

Competing interests

The authors declare that they have no conflicts of interests.

Authors' contribution

All authors contributed in data collection, analysis, and interpretation. All authors reviewed, refined, and approved the manuscript.

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