Pakistan Journal of Biological Sciences 8 (7): 974-977, 2005
ISSN 1028-8880

Isolation and Detection of MTBE Degrading Bacteria

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Abstract: This research was carried out to isolate and identify degrading microorganisms of MTBE in the soil samples taken from the adjacent ground of lead-free gasoline storage tanks as well as from drainage water of MTBE and gasoline storage tanks. Water and soil samples were prepared and microorganisms were inoculated and then harvested from mineral salt media. After several passages within 200 days, microorganisms capable of using MTBE as carbon and energy source were isolated. Isolated microorganisms inoculated in common culture media including R2A agar and BHI were identified as *Pseudomonas putida, Comamonas, Alcaligenes, Bacillus* and *Micrococcus* by specific kit of epi.

Key words: Microbial biodegradation, isolation, degradation microorganisms

INTRODUCTION

Methyl Tert-Butyl Ether (MTBE) use has been begun through the world since 1970. This compound is an octane enhancer agent as well as an oxygenate factor. Various researchers stated that using this compound is very beneficial in reducing air pollution. As with other petroleum products, transportation, storage and processing of MTBE may result in serious environmental pollution due to its molecular structure. This compound, contrasted with other petroleum products, can not be adsorbed by surface layer, thus it will move through deep soil and will lead to serious groundwater pollution. U.S. EPA has listed MTBE as possibly carcinogens, as a consequence strict maximum permissible concentration (5 μg L^{-1}) has been set for it[1].

Although various physical, chemical and biological methods exist for MTBE removal from water and soil, a cost-effective and suitable method is used microbial potential for MTBE elimination. By 1997, most researchers considered this compound as a refractory organic or in most cases as a non-degradable substance. Recently, many investigators have been focused on biological degradation of MTBE, resulting in considerable success in this regard[2]. Early investigations suggested that MTBE biodegradation was not considerable in aerobic conditions, but more recent studies indicate that biodegradation is occurring in aerobic condition in a slower rate than that of methanogenic, sulfate reducing, iron reducing and nitrate reducing conditions[3-7].

The results obtained from aerobic biodegradation of MTBE are of significant importance and it is believed that this process is based on two mechanisms. A group of microorganisms are capable of using MTBE carbon as energy source for growth. This group mostly has low growth rate in MTBE-bearing media and produce low biomass as well. The oxidation product of MTBE biodegradation is Tert Butyl Alcohol (TBA), which is not accumulative and readily undergoes degradation. As time proceeds, MTBE will act as a catalyst in the degradation process. Although monooxygenase has been reported as active enzyme, sufficient evidence does not exist. Enzymes can be early identification method for microbes, thus MTBE degradation is apparently known to be related to certain microbial species. Although all enzymes involved in MTBE degradation have not been known yet, perhaps due to the limited investigation on this subject, the results obtained from *Rubrivax* sp. (PM1) and *Hydrogenophage flava* ENV735 showed that MTBE and TBA degradation are taken place by this enzyme. This is in agreement with the results obtained from MTBE metabolizing organisms or *Mycobacetrium austroafricanum*[3,7-13].

Another group of aerobic bacteria are capable of cometaolizing MTBE in the presence of hydrocarbons. Most of hydrocarbons act as substrate in this mechanism

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and contribute to accelerate the MTBE metabolism. Most petroleum products such as branched, linear, aromatic and acyclic alkanes are included in the group of cometabolizer compounds. This idea seems to be very useful because in past it was thought that MTBE degradation individually is much simpler than its degradation together with gasoline, which is a solvent for many hydrocarbons. However, at present it is believed that MTBE carbon in gasoline for biological degradation is not much dissimilar with that in individual MTBE. As the majority of MTBE enters the environment as part of gasoline, this range of cosubstrates raises the possibility that organisms involved in gasoline biodegradation could contribute to MTBE degradation in gasoline-impacted environments.

The initial reactions in the pathway of cometabolic MTBE oxidation have been examined but incompletely recognized, in both alkane-utilizing fungi and propane oxidizing bacteria. Although both of these types of organisms generate TBA as an MTBE oxidation product, the fungal system also generates tert-butyl formate (TBF)[7-14].

MATERIALS AND METHODS

Sampling and sample preparation: Soil samples were taken from the adjacent ground of MTBE and gasoline-bearing MTBE storage tanks, during years of 2004-2005. The samples were taken from the depth of 50-70 cm of ground and kept and carried in screw-cap bottles to laboratory. Soil samples were passed though 2 mm sieve to prepare them for further microbiological examination.

Inoculation of microorganisms present in the drainage water of MTBE storage tanks: The prepared samples were passed through 0.45 mm membrane filters and then the filters were put in nutrient broth culture medium (100 mL) that was made and sterilized in advance. The culture media were kept at 30°C for 72 h in incubator. Then enriched culture media prepared for inoculation were poured into the MSM culture medium with MTBE.

Preparation of soil suspension for making microbial culture medium: Ten gram sieved soil samples were added to 100 mL distilled water and some drops of Tween 80 were poured into it. This solution was stirred for 15 min and then it was allowed to settle. This solution was used to count and determine microbial density of soil samples.

Mineral salt medium: The mineral salt medium contained the following salts (g L^{-1}): $MgSO_4.7H_2O$ (0.25), KNO_3 (0.5), $CaCl_2.2H_2O$ (0.009), KH_2PO_4 (0.5), K_2HPO_4 (0.5), NaCl (1.0) as well as trace elements solution $FeCl_2.4H_2O$ (1.5), $CuCl_2.2H_2O$ (0.015), $NiCl_2.6H_2O$ (0.025), $MnCl_2.4H_2O$ (0.1), $CoCl_2.6H_2O$ (0.12), $ZnCl_2$ (0.07), $NaMoO_4.2H_2O$ (0.025), H_3BO_3 (0.06) and $EDTA.4H_2O$ (5.2). The final pH was 4.2.

Analytical methods: One of the essential indicators showing biodegradation of MTBE by aerobic microorganisms is to measure MTBE concentration in the various time intervals and compare them with primary concentration. The analysis was carried out according to the modified method of ChrompPack 1313.

MTBE was quantified by gas chromatography with a Varian 3800 Gas Chromatography fitted by a Flame Ionization Detector (FID) and equipped with a capillary column (0.25 mm by 30 m, ID coating cp-select 624 CB, DF=1.4 μm). Headspace sampler was used (Model ComBIpal). The vials were incubated at 60°C with shaking (500 rpm) for 5 min. The injection temperature was 140°C at a flow rate of 250 μL^{-1} and a spill ratio of 60. The pressure of the carrier gas was 10 psi for 20 min. The column was maintained at 40°C and then increased to 180°C with 20°C/min. The capillary column temperature was kept 1 min at 180°C. The detector temperature was 250°C and Helium was used as the carrier gas at a flow rate of 30 mL min^{-1} and the folw rate of air was 300 mL min^{-1}. The minimum limit of this method was 5 μg L^{-1} and can be used for Headspace and Direct Injection methods. Star 6 software of Varian was used to process the collected data.

RESULTS AND DISCUSSION

After 90 days, MTBE degrading strains were isolated from MTBE-contaminated soil taken from MTBE and lead-free gasoline storage tanks as well as from drainage water of the storage tanks and transferred into MTBE-enriched media as substrate source in laboratory conditions. The maximum amount of MTBE in which microbial consortium was isolated was 3000 mg L^{-1}; however, earlier studies showed that 8000 mg L^{-1} of MTBE was inhibitor for microbial growth. In the second stage of this study, isolated microbial consortia were added to MSM culture media with 50, 200 and 3000 mg L^{-1} MTBE as carbon source. Controls contained 1 g of sodium azide per liter as growth inhibitor. Samples were kept in laboratory conditions and were incubated with shaking (150 rpm) at 25°C for about 150 days[15-24].

MTBE degradation was measured at the beginning and various time intervals (Table 1). Samples were kept in screw-cap bottles with Teflon-lined septa to prevent

Table 1: Variation in MTBE concentration during investigation in different samples

Run No.	1	2	3	4	5	6	7	8	9
	Detention time								
Samples	21	35	49	64	77	106	116	143	152
Residual MTBE in control	209.1301	208.1200	207.9113	208.1449	209.1019	209.1041	209.0010	208.8910	209.0141
Residual MTBE in inoculated samples during study	4.0627	3.7819	3.5337	3.1016	3.0592	3.0490	2.8682	2.5958	2.4930
Variation in MTBE concentration after inoculation of passaged and acclimatized microbial samples (Conc. of MTBE 3000 mg L^{-1})	2778.2612	2066.1150	1399.7345	526.1829	490.6351	367.3823	365.1814	374.0454	374.0110
Variation in MTBE concentration after inoculation of passaged and acclimatized microbial samples (Conc. of MTBE 200 mg L^{-1})	195.9043	194.0698	183.1389	161.2361	154.2019	153.2252	153.0095	151.5753	150.4231

MTBE loss and the sole of carbon source for existing microorganisms was MTBE. Although oxidation products were not determined in this study, comparison of GC analysis revealed that the early peaks of MTBE examination were considerably different with that of day 120. Because MTBE was used as the sole carbon source in samples and control, this shows MTBE degradation by microbial consortia of the samples. It should be noted that biomass was increased during MTBE degradation. Although due to high cell density, microbial counting was not preformed. Five genus including *Pseudomonas putida, Comamonas, Alcaligenes, Bacillus* and *Micrococcus* were isolated in this study that confirms the biodegradation of MTBE. The five isolates were identified by using epi microbial kit. Earlier studies reported *Pseudomonas putida, Comamonas* and *Alcaligenes* as MTBE degrading bacteria, relating them with enzymatic activities such as monooxygenates. In addition, a genus was identified to be effective in MTBE degradation in this study that earlier studies have not reported this genus. Further study should be carried out regarding this genus.

ACKNOWLEDGMENTS

The authors wish to thank the Dr. Ramin Nabizadhe and personnel of Environmental Health Engineering Department Laboratories, Tehran University of Medical Sciences and Center for Environmental Research for providing facilities. We acknowledge the assistance and support of Iranian Petrochemistry Company, National Iranian Oil Refining and Distribution Company, especially Mr. Amirsardari, Research Institute of Petroleum Industry, especially Mr. Ahmadi during this research.

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