American-Eurasian J. Agric. & Environ. Sci., 9 (5): 534-538, 2010
ISSN 1818-6769
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Oxidation of Toxic MTBE to Formaldehyde and TBA by *Pseudomonas* Grown on Naphthalene

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Abstract: With the current practice of amending gasoline with up to 15% by volume toxic MTBE, the contamination of groundwater and river by MTBE has become widespread since this compound are toxic and carcinogen and have been wide distributed in river water from car fuel. As a result, the biodegradation of MTBE-impacted aquifers has become an active area of research. Therefore, the aim of this work was to isolate and identify the effect of Naphthalene in oxidation of MTBE. Sea water was contaminated with Naphthalene (1000ppm) and after several subculture, three strains were isolated and identified as *Pseudomonas* by biochemical test. Naphthalene cell biomass was transferred to Teflon cape tube included 2 micro litres MTBE and incubated for 24 hr at different condition. The removals of MTBE have been assayed by GC. The results showed that strain SA86 remove 70% MTBE without any by-product but produced formaldehyde from TBA, however, Strain SA58 and SA90 produced alcohol and formaldehyde from MTBE respectively. The results emphasis that Naphthalene help for all 3 strains to reduced MTBE but heat shocked cells did not showed any MTBE oxidation.

Key words: Naphthalene · MTBE oxidation · co-metabolism · *Pseudomonas*

INTRODUCION

Among fuel oxygenates, MTBE is most commonly used because of its high octane level, low production cost, ease of blending with gasoline and ease of transfer and distribution [1, 2]. The addition of methyl tertiary butyl ether (MTBE) to gasoline began on a relatively small scale in the late 1970's 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 in part to its high aqueous solubility, low octanol water partition coefficient and chemical structure which is relatively resistant to microbial attack [3]. 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 (>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 and biodegradation. It can be treated biologically with special bacterial strains or natural isolates under aerobic condition. However, the bacteria grow slowly with low yields of biomass and are sometimes unstable. Some reports, showed mechanism for MTBE biodegradation [8]. According to them TBA and formaldehyde are the by-product of MTBE biodegradation. In this survey the ability of MTBE removal also formaldehyde and TBA production of three isolated strains were investigated.

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MATERIALS AND MEHODS

Bacterial cultures were grown in artificial sea water solution ONR contain3%NaCl [9] and BH (Bushnell–Haas, Brown and Braddock 1990) with naphthalene as sole source of carbon and energy.

Sampling and Strain Isolation: Contaminated sediments and sea water of Persian Gulf and Caspian Sea as well as activated sludge obtained and stored in sterile bottles at 4°C. 5ml of samples was added to artificial seawater (ONR) and shaken with rpm of 150 for 1 week at room temperature. After several subcultures, 100 µl from culture medium was spread on ONR plate solidified with 1.5% agar. 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 1 weeks. The resulting colonies were picked and inoculated into ONR containing naphthalene crystals with concentration of 1000 ppm. Purity was verified by restreaking colonies onto ONR agar naphthalene plates. In order to figure out whether or not growths of the bacteria are salt dependent, bacteria were inoculated on BH naphthalene solution medium. Bacteria grown in these media led to identification of salt tolerant bacteria.

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 O/F test. These were done according to standards for microbial identification written in Bergey's manual of systematic bacteriology. Carbohydrate acidification and fermentation test performed in PSS medium (peptone-succinate-salt, Hylemon 1973), acidification took up to 2 weeks to occur.

Growth of Bacteria in MTBE, TBA and Formaldehyde: Three ml of BH bacterial culture contains 1/5×107 per ml of medium of each bacteria, with 60µl of MTBE, TBA and Formaldehyde were added to different Teflon cap tubes.

Also in other test 20 micro liter of MTBE, TBA and formaldehyde as the only source of carbon and energy inject to different Teflon cap tube daily. After 24hr results were observed and recorded.

MTBE Removal Assay: MTBE removal assayed in cultures contain determined amount of MTBE and monitoring the disappearance of MTBE with Gas Chromatography (GC). 2ml of BH and ONR bacterial mass culture (OD:0.4) with 2 micro liter of MTBE added to each Teflon cape tube and were shaken in the darkness with 150 rpm, at room temperature for 24hr.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 GC (model 6890 USA) with HP-1 column. The initial oven temperature was 40°C and the oven temperature was increased at a rate of 15°c/5min until it reached 100°C. The Hiter and detector temperature were maintained at 100°C and 220°C Respectively. Carrier gas was helium [10].

Effect of Cell Mass on Formaldehyde Production: For production of formaldehyde, the studied bacteria were grown on Naphthalene broth (1000ppm) incubated at 28°C with 150 rpm shaking (Shaking Incubator, n-Biotek) for 24 hr. Cells were harvested by centrifugation at 3000 rpm (microcentrifuge, Sigma-110) for 10 min, washed twice with phosphate buffer saline (PBS contained (per liter), 45 g NaCl, 15.2 g Na2HPO4, 3.93 g NaH2PO4 and pH=7.4) and resuspended in the same buffer to adjust the cell mass to an O.D. 600 nm of 30. This cell suspension was inoculated to BH containing 4000 ppm of MTBE without another carbon source and incubated at 28°C in shaker. At 24 hr intervals formaldehyde assay was performed.

Determination of Formaldehyde Production: The concentration of produced formaldehyde by studied bacteria were measured by Hantzsch method [11]. Equal volumes of Hantzsch reagent (2M ammonium acetate, 50 mM acetic acid, 20 mM acetyle aceton) were added to 2ml of centrifuge cell biomass (CFU=10^7) grown on two condition (naphthalene broth and nutrient broth) for 24 hr and induced with MTBE (4000 ppm) for 24hr, this mixed incubated at 60°C for 10 minutes. The obtained yellow color of 3,5 diacetyl 1.4 dihydrolotidin which produced from reaction between formaldehye and pentane 2,4 dion (acetyle aceton) in the present of ammonium acetate was centrifuged and measured at 412 nm against blank. Also different concentration of formaldehyde used for calibration line drawing.

RESULS AND DISCUSSION

Three Gram negative strains isolated in Naphthalene rich media from see water. This isolated are salt tolerant and could grow in BH medium. All growth of these isolates was studied on Naphthalene, MTBE, TBA and formaldehyde. They have good growth only on Naphthalene as only source of carbon and energy.

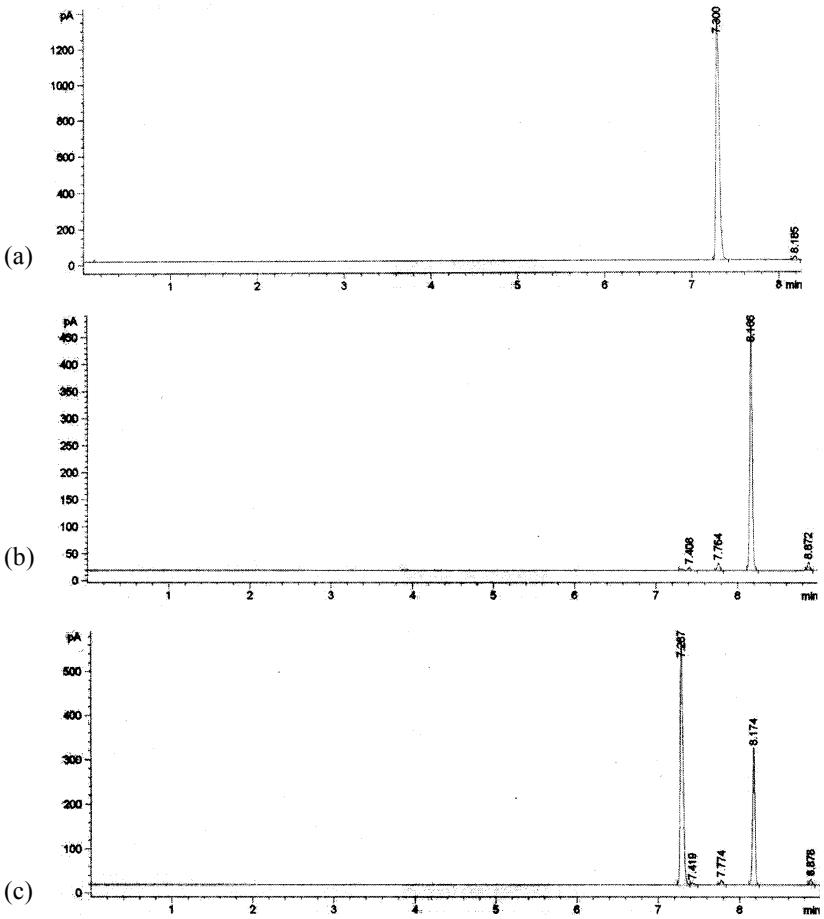

Fig. 1: GC analysis for production of formaldehyde by strain 90. A= MTBE blank, B= formaldehyde blank,C= Oxidation of MTBE to formaldehyde by strain 90

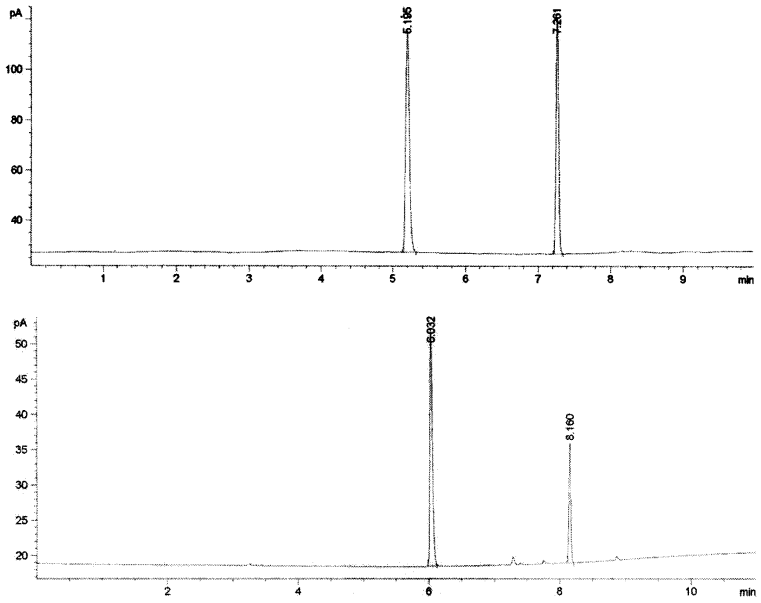

Fig. 2: GC analyses for production of TBA by strain 58. A= Oxidation of MTBE to TBA by strain 58, B= TBA blank.

Table 1: Oxidation of MTBE to formaldehyde and TBA by isolated *Pseudomonas*

Strains	%MTBE removal	Temperature °C	Formaldehyde Production FromTBA	Formaldehyde Production FromMTBE	TBA Production
Pseudomonas SA90	75	25	-	+	-
	10	100			
	43	25(ONR)			
Pseudomonas SA58	53	25	-	-	+
	0	100			
	49	25(ONR)			
Pseudomonas SA86	70	25	+	_	_
	0	100			

However they did not have any growth on MTBE, TBA or formaldehyde as the only source of carbon and energy (even with addition of 20 micro liter substrate by injection into Teflon cap tube daily). The cell biomass of all isolates could oxidize MTBE to formaldehyde by strain SA90 and TBA by strain SA58 (Figure 1 and 2).This reaction stopped by heating. Oxidation of MTBE to formaldehyde is shown in Table 1.

As it has been shown Strain SA90 was removed maximum 75% of MTBE.

Although they have been isolated from see water but addition of salts in ONR media decreased the oxidation of MTBE significantly. Production of formaldehyde only has been seen in Strain 90 by GC analysis and calorimeter reaction. The Naphthalene is important to reduced MTBE and those that have been grown on another substrate like nutrient broth could not change MTBE to formaldehyde.

The biodegradation of MTBE can either be enhanced or repressed by the presence of other organic contaminants in groundwater plumes. It has been reported that several propane-grown pure cultures were able to co metabolically biodegrade MTBE under aerobic conditions [12]. A pure culture (*Pseudomonas aeruginosa*) capable of co metabolically degrading MTBE in the presence of pentane is reported too [13]. On the other hand, MTBE biodegradation may be inhibited in the presence of more easily biodegradable compounds [14]. This inhibition can occur when MTBE degrading cultures preferentially utilize easily degradable hydrocarbons instead of MTBE. MTBE degradation rates decreased significantly in the presence of other carbon sources including TBA, TBF, iso-propanol, acetone and pyruvate [14]. It has been shown that the microbial community changed when toluene or benzene with MTBE is added to water [15]. In our research we showed that all 3 naphthalene utilizing bacteria oxidized MTBE to TBA or formaldehyde. So Naphthalene can induced MTBE oxidation enzyme in all 3 isolates.

ACKNOWLEDGMENTS

The financial support from the oil and gas company in Iran made this research possible.

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