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Biodegradation of MTBE by Bacteria Isolated from oil Hydrocarbons-Contaminated Environments

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ABSTRACT: Methyl tertiary butyl ether (MTBE) belongs to the group of gasoline oxygenates and persistent environment contaminants, and shows potential for biodegradation in aerobic and anaerobic conditions, through application of pure microbial cultures. Presented research shows that indigenous bacterial isolates 6sy and 24p, selected from oil hydrocarbons-contaminated environments, were capable of utilizing MTBE as sole carbon and energy source. Based on 16S rDNA sequence analysis, bacterial isolates 6sy and 24p were identified as *Staphylococcus saprophyticus* subsp. *saprophyticus* and *Pseudomonas sp.*, respectively. The MTBE biodegradation rate was affected by longevity of incubation period and initial MTBE concentration. After 3 weeks of incubation at 25°C in a dark, the removal rates of initial 25 and 125 ppm MTBE concentrations by *Staphylococcus saprophyticus* 6sy were found to be 97, and 63%, respectively, while efficiency of *Pseudomonas sp.* in degradation of indicated concentrations was 96, and 40%, respectively. Both bacterial isolates were able to grow in MTBE-containing growth medium. Highest growth rate of bacterial isolates was observed at the end of incubation period. The presented results indicated the potential of these bacterial isolates in bioremediation of MTBE-contaminated environments.

Key words: Bioremediation, MTBE-degrading bacteria, *Staphylococcus*, *Pseudomonas*

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is the most commonly used oxygenate, added to gasoline to replace the tetraethyl lead and other toxic components in order to improve fuel combustion and reduce carbon monoxide and VOC emissions. Consequently, MTBE has become an important component of gasoline, with concentrations up to 15% (v/v) in USA (USEPA, 1994). Its production has risen in 1999 to 21 million tons, with 3.3 million tons in the EU countries (Krayer von Kraus and Harremoës, 2001). However, high water solubility, mobility, persistence and toxicity make it an important pollutant of surface soils, groundwaters and sediments. After a few years of intensive use, MTBE has become one of the most frequently detected soil and water contaminants in Europe (Klinger *et al.,* 2002) and USA (Baehr *et al.,* 1999). U.S. Geological Survey identified MTBE as the 2nd most common urban aquifers contaminant in USA, while USEPA classified it as a possible human carcinogen (Squillace *et al.,* 1996) and limited its concentration in drinking water below a limit of 20 to 40 ppb (USEPA, 1997). The consequence of widespread environment contamination with MTBE was development of remediation technologies (Hatzinger *et al.,* 2001), based on activity of living organisms, primarily microorganisms (Vidali, 2001).

Because of its chemical structure, MTBE is relatively recalcitrant. The most important characteristic of MTBE in biodegradation process is low biomass yield of microorganisms (Salanitro *et al.,* 1994), primarily because of ether bond and branched moiety, which makes the MTBE's aversion to enzymatic attack (Pirnik, 1977). Initial research indicated that microorganisms are not capable of

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MTBE degradation. Fujiwara et al. (1984) and Jensen and Arvin (1990) reported absence of aerobic MTBE degradation by activated sludge, and Suflita and Mormile (1993) concluded that MTBE degradation was not performed under methanogenic conditions.

A mixed microbial consortium BC-1 was first culture reported to degrade 120 μg of MTBE ml^{-1} as sole carbon and energy source in aerobic conditions (Salanitro *et al.,* 1994). This consortium metabolized MTBE to CO_2 (40%), with accumulation of *t*-butyl alcohol (TBA) as initial product of MTBE degradation (Smith *et al.,* 2003).

Until the present time, partial MTBE biodegradation has been reported in soils (Yeh and Novak, 1994), sediments (Bradley *et al.,* 2001) and biofilters (Fortin *et al.,* 1999) by a few pure cultures of bacteria (Mo *et al.,* 1997) and fungi (Magaña-Reyes *et al.,* 2005; Lalevic *et al.,* 2008). Several studies have reported the cometabolitic biodegradibility of MTBE (Steffan *et al.,* 1997; Garnier *et al.,* 1999), as well as potential for natural attenuation of MTBE in sediments (Bradley *et al.,* 1999).

Recent laboratory studies have indicated that MTBE release from environment is controlled by natural activity of microbial populations isolated from oil hydrocarbons-contaminated environments. According to increasing accidence of MTBE in the environments (Hansen *et al.,* 2002) and comparatively slow natural attenuation of at least 2 years (Johnson *et al.,* 2000), it is necessary to find microbial population capable of MTBE biodegradation. The aim of this research is isolation, identification and characterization of microorganisms naturaly capable of using MTBE as sole carbon and energy source.

MATERIALS & METHODS

Microorganisms used in this study were obtained from oil hydrocarbons-contaminated soil and wastewater samples collected in «Oil refinery Pancevo», Serbia. To obtain the enrichment cultures, a soil and wastewater samples were placed in flasks with 100 ml of liquid mineral medium (Salanitro *et al.,* 1994) with MTBE (Sigma-Aldrich, 99.9%) added as sole carbon and energy source. The flasks were incubated at 25 °C for 2 months in a dark.

The culturing of MTBE-degrading bacteria was performed in 500 ml flasks containing 100 ml of mineral medium (Salanitro *et al.,* 1994) with 25 or 125 μg MTBE/mL as sole carbon and energy source. All flasks were incubated on orbital shaker at 120 rpm at 25 °C in a dark. Flasks with the same medium containing killed cells, and kept under same conditions, were used as a control. All experiments were conducted in triplicate. The optical density of the liquid cultures was determined spectrophotometrically at 550 nm using a T70 UV/VIS spectrometer (PG Instruments) and 0.5-cm cuvettes.

The concentration of MTBE was measured by sampling a 200 μl of microcosm headspace (Agilent 7694E Head Sampler) and analyzed by GC system (Agilent Technologies 6890N Network) connected to a flame ionization detector (FID), with DB-624 (J&W Scientific) column (30 m x 0.53 mm ID). The temperature of injector was set at 170 °C. The column temperature was initially 50 °C for 2 minutes and was ramped to 100 °C at 8 °C/min. The detector temperature was set at 300 °C. Nitrogen was used as a carrier (4.5 ml min^{-1}) and a make up gas (25 ml min^{-1}). The retention time for MTBE was 3.31 min. The measurements of MTBE concentration, as well as optical density, were performed at the start of incubation period, and subsequently after 3, 6, 10, 16 and 21 days of incubation.

Isolated bacterial strains were analyzed in order to investigate their morphological properties by growing on agar medium and examined under a light microscope (Leica DMLS). Identification of pure cultures degrading MTBE was performed by sequence analyzes of 16S rDNA.

MTBE-degrading bacterial isolates were grown on 0,1x-TS agar plates. Total genomic DNA was extracted from pure culture according to previously described protocol (Hopwood *et al.,* 1985). Amplification of 16S rDNA was conducted with the universal bacterial primers 27F 5′-GAGAGTTTGATCCTGGCTCAG-3′ (Gürtler and Stanisich, 1996) and 1523R 5′-AGGAGGTGATCCAGCCG-3′ (Gonzales *et al.,* 1997) which are amplifying products of approximately 1500 bp in size. DNA amplification was performed in 20 μl total reaction volume. The reaction mixture contained as template 1 μl of the extracted DNA, 2 mM $MgCl_2$, 0.3 mM each dNTPs, 0.75 μM each primer, 0.75 U of Fermentas *Taq* polymerase (Lithuania) and the buffer supplied with the enzyme. After the initial denaturation of 5 min at 95°C, thirty-three amplification cycles in a thermal cycler (Eppendorf, Mastercycler ep gradient S, Hamburg, Germany) were performed according to the following thermal profile: 1 min at 95°C for denaturation step, 1 min at 50°C for annealing and 1 min at 72°C for primer extension. The final extension was carried out by a 7 min at 72°C. PCR products were separated on 1% agarose gel in TBE (Tris-Borate 90 mm, EDTA 1 mm) buffer, stained with ethidium bromide and visualized under a UV transilluminator.

Obtained amplimers of the expected size were purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Both

strands were sequenced on automated equipment (BMR Service, Padova, Italy), and sequence deposited in NCBI GenBank database under accession numbers GU370938 and GU370939. Sequence identity was determined by comparison with bacterial sequences from GenBank using BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS & DISCUSSION

The environment is usually contaminated by different gasoline compounds simultaneously. Because the MTBE is one of frequently present pollutants in soils and groundwaters, it is necessary to stimulate the autochthonous microflora to degrade this contaminant. The role of microbial population in degradation of organic compounds is well known (Bagherzadeh-Namazi *et al.,* 2008; Nasrollahzadeh *et al.,* 2007; Talaie *et al.,* 2010). Several investigations have addressed the biotransformation of MTBE by few pure bacterial cultures, e.g. *Methylobacterium, Rhodococcus*, *Arthrobacter* (Mo *et al.,* 1997), *Mycobacterium* (François *et al.,* 2002; Smith *et al.,* 2003) etc. Many of these pure cultures are belonging to *Pseudomonas* genus (Morales *et al.,* 2009; Steffan *et al.,* 1997). However, the role of *Staphylococcus saprophyticus* in biodegradation processes has not been well studied so far, except for the biodegradation of MTBE added as an additional carbon and energy source (Lalevic *et al.,* 2007), some polycyclic aromatic hydrocarbons (Bidaud and Tran-Minh, 1998), and plasticizers (Ogawa *et al.,* 2009). On the other hand, some species of genus *Staphylococcus* are found to be capable of utilizing dibenzofuran and fluorene (Monna *et al.,* 1993), BTX compounds (Machnicka and Suschka, 2001) and drilling fluid base oil in biodegradation process (Nweke and Okpokwasili, 2003). Out of thirty bacterial strains, isolated from enrichment cultures obtained from oil hydrocarbons-contaminated soil and wastewater samples, only two were capable to utilize MTBE as a sole carbon and energy source. One of these two strains, named 6sy, formed a small yellow to yellowish colonies on TS agar. In young as well as old cultures, cells were spheral, single or in pairs. This strain was gram-positive and non-spore forming. Second strain 24p formed small yellow slimy colonies on TS agar, was gram-negative, having short-rod shape and did not produce spore. The obtained sequences of 16S rDNA of both strains were analyzed using BLAST. Sequence of isolate 6sy consisted of 1326 nucleotides (nt), from 61 to 1386 nucleotide position of 16S rDNA gene. Comparison with sequences available in data library showed the maximal similarity (100%) with 16S rDNA gene sequence of the species *Staphylococcus saprophyticus* subsp. *saprophyticus* (GenBank Acc. No. AP008934). Obtained sequence of bacterial isolate 24p consisted of 1291 nt, from 76 to 1366 base pair (bp) position of 16S rDNA gene sequence of *Pseudomonas* sp. (FJ821774) with whom it shares 100% identity. A comparison of this sequence with sequences from data library showed the 99% identity with 16S rDNA sequence of the *Saltmarsh* clone strain LCP-79 (AF286035), isolated from sediments contaminated with PCB and mercury. The differences between sequences of isolate 24p and *Saltmarsh* clone LCP-79 are presented with only two mutations. Also, the sequence of isolate 24p showed 99% identity with 16S rDNA sequence of *Pseudomonas pseudoalcaligenes* strain KS-1 (EU815635) and *Pseudomonas mendocina* species (DQ178221) with difference in 5 nt. Other recently published studies have also confirmed presence of some *Staphylococcus* (Nweke and Okpokwasili, 2003) and *Pseudomonas* species (Garnier *et al.,* 1999) in similar environments, which were found to be able to utilize organic pollutants.

Strains *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p were capable of utilization of MTBE added as the sole source of carbon and energy. During the incubation period of three weeks, decreasing of initial MTBE concentrations was recorded. The degradation rate of MTBE was affected by initial MTBE concentrations, bacterial strains and incubation time.

Table 1. MTBE biodegradation by pure cultures of *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p

Microorganisms	MTBE initial concentration (µg/ml)	MTBE remaining (%)				
		After 3d	After 6d	After 10d	After 16d	After 21d
Staphylococcus saprophyticus 6sy	25	93.8	85.7	77.1	47.6	2.7
	125	97.4	92.2	81.9	63.8	36.7
Pseudomonas sp. 24p	25	83.3	55.0	4.7	4.4	4.2
	125	98.5	94.9	87.6	75.9	59.9
control	25	96.2	92.9	87.3	83.2	76.8
	125	99.1	98.3	97.4	96.6	96.6

Dynamics of MTBE degradation by pure cultures of bacteria is shown in Table 1. Initial concentration of 25 µg MTBE/mL was rapidly degraded by selected bacteria. The presented data show that this concentration of MTBE was almost completely consumed by *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p during the three weeks period of incubation. Initial slow degradation was followed by a fast transformation rate by *Staphylococcus saprophyticus* 6sy. Also, in the same conditions, rapid decreasing of indicated concentration was noticed for *Pseudomonas sp*. 24p as well. Similar dynamics of MTBE degradation by *Pseudomonas sp*. 24p has been reported previously (Garnier *et al.*, 1999) in initial phases of cometabolic MTBE degradation by *Pseudomonas aeruginosa*, as well as in complete mineralization of MTBE by *Pseudomonads* consortium (Morales *et al.*, 2009). MTBE biodegradation capability was also noticed for *Pseudomonas mendocina* KR-1 isolated from gasoline-contaminated soils (Hyman *et al.*, 2000). Selected bacteria were able to partially and slowly degrade the initial concentration of 125 µg MTBE ml^{-1}. Similar data for degradation rate were obtained with both bacterial isolates with small differences in degradation efficiencies during the first 10 days of incubation period. The highest efficiency of MTBE biodegradation was registered at the end of the incubation period (after 16 and 21 days of incubation). Significant differences of degradation rate by selected bacterial pure cultures were observed. According to our results pure culture of *Staphylococcus saprophyticus* is more efficient in degradation of 125 µg MTBE/mL comparing with pure culture of *Pseudomonas sp*., especially at the end of incubation period. Thus, the experiments showed that *Staphylococcus saprophyticus* isolate 6sy and *Pseudomonas sp*. isolate 24p were capable of MTBE biodegradation, but the degradation rate was low. Presented results suggest that MTBE may be potential inhibitor of microbial metabolism, a fact which is in accordance with other reports (Salanitro *et al.*, 1994; Mo *et al.*, 1997). They suggested that MTBE is possible electron-transport inhibitor and/or uncoupler of ATP synthesis. Also, the inhibition of microbial activity can be caused by intermediate(s) during MTBE mineralization (Liu *et al.*, 2001).

In abiotic controls (25 and 125 µg MTBE ml^{-1}, respectively), the MTBE depletion from the headspace was insignificant, compared with inoculated treatments.

As can bee seen from presented data (Table 2), both bacterial isolates were able to grow in MTBE-containing growth medium, but growth of selected isolates was slow. The initial concentration of MTBE in the medium was shown to have an effect on the growth of selected bacterial isolates *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p. Increasing in the MTBE initial concentration resulted in decreasing of optical density. The results of investigation also showed the similar trends in growth curve characteristics: rapid increase of growth rate in the beginning of incubation period, while in the second part of growth curve the values of optical density did not exhibit significant changes. However, at the end of incubation period, highest growth rate of bacterial isolates *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p were observed.

During the incubation period, relatively slow cell growth on different initial MTBE concentrations was

Table 2 .The effect of incubation time and initial MTBE concentrations on growth characteristics of *Staphylococcus saprophyticus* 6sy and *Pseudomonas sp*. 24p

Bacterial isolate	Incubation time (h)	OD_{550}	
		Initial MTBE concentration (µg/ml)	
		25	125
Staphylococcus saprophyticus 6sy	0	0.010	0.010
	3	0.059	0.034
	6	0.054	0.036
	10	0.061	0.036
	16	0.061	0.045
	21	0.065	0.046
Pseudomonas sp. 24p	0	0.040	0.040
	3	0.251	0.202
	6	0.354	0.255
	10	0.343	0.253
	16	0.372	0.271
	21	0.382	0.275

recorded. Slow microbial growth was also noticed in previous investigations (Hatzinger *et al.,* 2001; Steffan *et al.,* 1997) and is probably result of slow initial MTBE oxidation (Hatzinger *et al.,* 2001), utilizing only the methoxy carbon (Eweis *et al.,* 1998), recalcitrance of tertiary/quaternary C-atom (Suflita and Mormile, 1993) or a combination of these facts. Some previous studies similarly indicated that low bacterial growth is result of metabolite(s) occurrence during degradation (Hatzinger *et al.,* 2001).

CONCLUSION

The results of our research showed the potential of pure bacterial cultures of *Staphylococcus saprophyticus* and *Pseudomonas sp*. for biodegradation of the MTBE-contaminated sites and confirmed the significant ecological role of bacteria in petroleum-polluted environments. In addition, this is a first report concerning species *Staphylococcus saprophyticus* ability to utilize MTBE as a sole carbon and energy source and its role in natural biodegradation process of MTBE. These results should be useful for solving practical problems in degradation of recalcitrant contaminants such as oxygenates.

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