Biodegradation of methyl-*tert*-butyl ether by *Kocuria* sp.

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Abstract
Methyl-*tert*-butyl ether (MTBE) has been used to replace the toxic compounds from gasoline and to reduce emission of air pollutants. Due to its intensive use, MTBE has become one of the most important environment pollutants. The aim of this study was the isolation and identification of the bacteria from wastewater sample of "HIP Petrohemija", Pančevo (Serbia), capable of MTBE biodegradation. The results of the investigation showed that only the bacterial isolate 27/1 was capable of growth on MTBE. The result of sequence analyzes of 16S rDNA showed that this bacterial isolate belongs to the *Kocuria sp*. After the incubation period of 86 days, the degradation rates of initial MTBE concentration of 25 and 125 μg/ml were 55 and 36%, respectively. These results indicated that bacteria *Kocuria* sp. is successfully adapted on MTBE and can be potentially used in bioremediation of soils and waters contaminated with MTBE.

Keywords: Biodegradation; bioremediation; MTBE; *Kocuria*.

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In recent years, oil has been used as a primary energy source. Its value and consumption of its final products have caused a significant increase of crude oil production [1], especially after development of internal combustion engines [2]. However, production, refinement, transport and storage lead to release of crude oil and its products into the ecosystem, causing problems for the environment [3]. Petroleum and its derivates are one of most important final products of the oil industry, and their presence in large concentrations in the environment is toxic to living organisms [4].

Fuel oxygenates have been used as a gasoline additive in order to increase the octane number and combustion efficiency [5]. One of the most commonly used fuel oxygenates is methyl-*tert*-butyl ether (MTBE). Because of its useful properties, MTBE was one of the organic chemicals with the highest world production [6]. Nevertheless, its widespread use has led to discharge into environment [7], *i.e.*, to soil and groundwater pollution. To date, many techniques are being developed for removal of organic pollutants from environment. The biological methods are more efficient and cheaper compared with physical or chemical treatments [8]. The principal biological agents in degradation of organic pollutants are microorganisms [9], especially microbial isolates exposed to hydrocarbon contamination [10].

Several studies have addressed the potential of microorganisms to use MTBE as a sole carbon and energy source. MTBE biodegradation has been reported under oxic and anoxic conditions [6], in river sediments [11], by pure bacterial cultures [7,12,13] or microbial consortium [14]. However, according to the increasing of MTBE worldwide accidence [15], comparatively low rate of its natural attenuation [16] and numeorus microorganisms capable of MTBE biodegradation, it is necessairy to find other microorganisms with similar capability and potential efficiency in bioremediaton of MTBE-contaminated environments. The objective of this research was to isolate, identifiy and characterize a bacterial pure culture capable of utilizing MTBE as the sole carbon and energy source.

MATERIAL AND METHODS

Hydrocarbon-contaminated wastewater collected from "HIP Petrohemija", Pančevo district (Serbia), was used as the sample for this study. Enrichment cultures were obtained after placing the wastewater sample in flasks with liquid mineral medium [17] with MTBE (Sigma-Aldrich, 99.9%) for 2 months at 25 °C. The culturing of MTBE-degrading bacteria was conducted in flasks with same mineral liquid medium supplemented with 25 and 125 μg MTBE/ml. Incubation was performed at 25 °C in the dark on an orbital shaker (120 rpm). Flasks with same mineral medium containing killed cells were used as the control variant. All experiments were performed in triplicate.

The optical density (OD_{550}) was measured using a T70 UV/Vis spectrometer (PG instruments). The num-

ber of bacteria during incubation was determined on 0.1×TSA and expressed as colony forming units (CFU/ml).

The concentration of MTBE (retention time was 3.31 min) in all experiments was measured by sampling of 0.2 mL of headspace (Agilent 7694E Head Sampler) and analyzed by a GC system (Agilent Technologies 6890N Network) connected to a flame ionization detector with DB-624 column (JandW Scientific, 30 m× ×0.53 mm ID). During the experiment, MTBE concentration was measured in gas phase above the suspension and compared with initial MTBE concentration. The injector temperature was 170 °C, while the column temperature was initially 50 °C for 2 min and was then ramped to 100 °C at 8 °C/min. The temperature of detector was set at 300 °C. Nitrogen was used as the carrier (4.5 ml/min) and make-up gas (25 ml/min).

The optical density and concentration of MTBE were measured at time zero, and subsequently after 15, 30, 50 and 86 days of incubation.

Morphological properties of isolated bacteria, after growth on agar medium, were analyzed under a light microscope (Leica, DMLS).

Identification of MTBE-degrading bacteria was conducted by API and APIWEB system (bioMerieux, Inc., France) and by sequence analyzes of 16S rDNA. After growth on 0.1×TSA, extraction of total genomic DNA was performed [18]. For amplification of 16S rDNA, universal bacterial primers 27F 5′-GAGAGTTTGATCCT-GGCTCAG-3′ [19] and 1523R 5′-AGGAGGTGATCCA-GCCG-3′ [20] were used, with the size of amplifying products about 1500 bp. The reaction mixture (20 μl) contained as template 1 μl of extracted DNA, 2 mM MgCl$_2$, 0.3 mM of each dNTPs, 0.75 μM of each primer, 0.75 U of Fermentas *Taq* polymerase (Lithuania) and the buffer supplied with enzyme. The initial denaturation was performed in a thermal cycler (Eppendorf, Mastercycler ep gradient S, Hamburg, Germany) at 95 °C for 5 min, and 33 amplification cycles according to the following thermal profile: denaturation step (at 95 °C for 1 min.), annealing (50 °C for 1 min), primer extension (at 72 °C for 1 min), and final extension (at 72 °C for 7 min). The product was separated on 1% agarose gel in TBE (Tris-Borate 90 mm, EDTA 1 mm) buffer, stained with ethidium bromide and visualized under a UV transiluminator. Purification was performed using a QIAquick PCR Purification Kit (QIAGEN) and sequencing on automated equipment (BMR service, Padova, Italy). The sequence was deposited in the NCBI GenBank database under accession number JQ219670. The sequence identity was compared with sequences from GenBank using BLAST analyses (http:// //www.blast.ncbi.nlm.nih.gov/ Blast.cgi).

RESULTS AND DISCUSSION

Eleven bacterial strains classified in three colony morphologies were observed from enrichment cultures. Of these eleven isolates, three forming yellow pinpoint colonies, seven forming white large colonies and one, which was the only isolate with capability of utilizing MTBE as a sole source of carbon and energy, was marked as 27/1. Its morphological properties are shown in Table 1.

Table 1. Morphological properties of Kocuria sp. 27/1

Morphological property	Result
Characteristics of colonies on 0.1×TSA	Small, red to reddish
Shape of cells	Cocci, single or in pairs
Size of cells, μm	0.64–0.96
Spore forming	–
Gram staining	+
Presence of capsule	–

After incubation on API STAPH and using by APIWEB technique, the results showed maximal similarity with *Kocuria rosea* (Table 2).

Table 2. Results of API STAPH test for bacterial isolate 27/1.

Test	Substrate	Isolate 27/1
0	–	–
GLU	D-Glucose	+
FRU	D-Fructose	+
MNE	D-Mannose	–
MAL	D-Maltose	–
LAC	D-Lactose	–
TRE	D-Trehalose	–
MAN	D-Mannitol	–
XLT	Xylitol	–
MEL	D-Melibiose	–
NIT	KNO$_3$	+
PAL	β-Naphthyl phosphate	–
VP	Na pyruvate	–
RAF	D-Raffinose	–
XYL	D-Xylose	–
SAC	D-Saccharose	–
MDG	Methyl-α-D-glucopyranoside	–
NAG	*N*-Acetyl glucosamine	–
ADH	L-Arginine	–
URE	Urea	–

The obtained sequences of 16S rDNA of isolate 27/1 were analyzed by BLAST. The sequence of this strain consisted of 1407 nucleotides (nt), from 54 to 1460 nucleotide position of 16S rDNA gene. Comparison with sequences in database library showed the maximal si-

milarity (100%) and sequence query coverage (100%) with 16S rDNA sequences of the *Kocuria* sp. strains 29Y1zhy (GenBank Acc. No. AM418390), ZS2-6 (FJ889675) and E7 (EU372971), while 100% identity with 97–99% coverage was obtained for additional four 16S rDNA sequences of the *Kocuria* sp. strains SeLB8 (HM352419), bk_17 (HQ538676), MC10-F2 (HM196769) and II_Gauze_W_10_8 (FJ267552). BLAST analyses also showed maximum sequence homology and query coverage with sequences of two *Kocuria* strains identified to the species level: *Kocuria rosea* strain P27-24 (DQ060382) and BCT-6 (DQ015980), and *Kocuria erythromyxa* strain ATCC 187T (Y11330). An additional three 16 rDNA sequences of *Kocuria rosea* strains T1-8 (JF798383), CE7 (JN084134) and Y16 (JN084149) confirmed 100% identity with sequences of our isolate 27/1, although with sequence coverage of 97-98%. Based on results of 16S rDNA sequencing, the presence of *Kocuria* species in oil hydrocarbon-contaminated environments has been also previously reported [21].

Utilizing of microorganisms as biodegradation agents is continually increasing, because of their biodiversity and enormous catabolitic potential [9]. The role of microorganisms in biodegradation of organic pollutants is well described [22–24]. To date, MTBE biodegradation by different bacterial pure cultures, e.g. *Mycobacterium* [25], *Pseudomonas* [13,26], *Hydrogenophaga* [7], *Aquincola* [27] etc., was observed. However, biodegradation potential of *Kocuria* sp. has not been well studied, except for the degradation of some polycyclic aromates [28], chlorophenolic compounds [29], lindane [30] or some BTEX compounds [31].

The experiments showed that *Kocuria sp*. 27/1 was capable of utilization of MTBE as sole carbon and energy source. MTBE concentration above the suspension in gas phase during incubation was controlled by its initial concentration and incubation time (Table 3).

Dynamics of MTBE biodegradation are shown in Table 1. The initial concentration of 25 µg MTBE/ml was partially degraded by *Kocuria* sp. 27/1. The low degradation rate of this initial MTBE concentration in gas phase was followed by rapid transformation at the end of incubation period. At the same initial concentration, slow abiotic loss of MTBE during incubation was noticed. Similar dynamics of MTBE biodegradation has been observed previously [32] during cometabolic degradation by *Pseudomonas aeruginosa*.

Kocuria sp. 27/1 was also capable of partially degradation of initial 125 µg MTBE/ml concentration in gas phase. The highest efficiency of MTBE degradation was observed at the end of the incubation period. However, the degradation rate was low, which may be coupled with slow initial oxidation of MTBE [7] and/or recalcitrance of tertiary/quaternary C-atom [33]. In abiotic control (125 µg MTBE/ml) MTBE depletion from headspace was insignificant compared with biotic treatment.

As can be seen from Tables 4 and 5, *Kocuria sp*. 27/1 was able to grow in liquid media containing 25 and 125 µg MTBE/ml.

Table 4. Optical density (OD$_{550}$) of Kocuria sp. 27/1 in MTBE-containing liquid medium during incubation

Bacterium	Incubation time, days	Initial MTBE concentration, µg/ ml	
		25	125
Kocuria sp. 27/1	0	0.212	0.216
	15	0.134	0.095
	30	0.142	0.077
	50	0.155	0.088
	86	0.178	0.105

Table 5. Number of Kocuria sp. 27/1 during incubation ($\times 10^6$ CFU/ml)

Bacterium	Incubation time, days	Initial MTBE concentration, µg/ml	
		25	125
Kocuria sp. 27/1	0	38.0	42.5
	15	15.5	11.0
	30	15.7	9.5
	50	16.0	10.1
	86	21.0	13.3

The growth of *Kocuria* sp. 27/1 was controlled by initial concentrations of MTBE in the liquid media. Increasing of MTBE initial concentration was shown to have an effect on decreasing of optical density, as well as number of bacteria. These results also showed the similar dynamics of *Kocuria sp*. 27/1 growth: rapid decrease of growth rate until the 30th day of incubation was noticed, which was followed by the increase of optical density and bacterial number until the end of the incubation period. Indepentently from initial MTBE concentrations, growth of Kocuria sp. 27/1 was slow.

Table 3. Degradation of MTBE (MTBE remaining±SD, %) by pure culture of Kocuria sp. *27/1*

Bacterium	MTBE initial concentration, µg/ml	Time, days			
		15	30	50	86
Kocuria sp. 27/1	25	90.9±6.4	98.3±5.3	98.8±5.7	45.2±5.5
	125	83.1±7.5	85.8±6.5	82.3±6.8	64.1±7.5
Control	25	94.0±4.8	97.7±4.2	96.8±5.2	81.9±4.9
	125	96.6±6.6	96.5±4.3	92.3±5.5	88.3±5.8

A similar conclusion was also reached in past research studies [7,34], which suggested that MTBE is a potential inhibitor of microbial metabolism [35] and/or uncoupler of ATP synthesis. Also, the low growth rate of *Kocuria* sp. 27/1 can be caused by occurrence of metabolite(s) during MTBE biodegradation [7], which inhibited the microbial activity [36].

CONCLUSION

The results of this research study showed the efficiency of pure bacterial culture of *Kocuria sp*. in biodegradation of the MTBE in laboratory conditions and its potential use for bioremediation of MTBE-contaminated sites. Also, this is the first report of capability of *Kocuria* sp. to utilize MTBE as a unique carbon and energy source. These results should be useful for practical application in oxygenates-polluted environments.

Acknowledgements

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IZVOD

BIODEGRADACIJA METIL TERCIJARNOG BUTIL ETRA POMOĆU *Kocuria* sp.

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(Naučni rad)

Metil tercijarni butil etar (MTBE) uveden je u proizvodnju s ciljem zamene toksičnih komponenti iz benzina, povećanja oktanskog broja goriva i redukcije aerozagađenja. Usled intenzivne primene, perzistentnosti i mobilnosti, MTBE je posle svega nekoliko godina postao značajan polutant u životnoj sredini. Cilj ovog rada je izolacija i identifikacija bakterija iz uzorka otpadne vode poreklom iz „HIP Petrohemije", Pančevo, sposobnih da vrše degradaciju MTBE-a. Od 11 bakterijskih izolata, svrstanih u tri grupe po morfološkim osobinama, samo je jedan izolat, označen kao 27/1, pokazao sposobnost rasta na MTBE-u kao jedinstvenom izvoru ugljenika i energije i odabran je za dalja istraživanja. Ovaj izolat ima sitne crvenkaste kolonije na podlozi 0,1×TSA. Ćelije su okruglastog oblika, ne stvaraju spore a po Gramu se boje pozitivno. Primenom API i APIWEB sistema, ovaj izolat pokazuje najveći stepen sličnosti sa bakterijskom vrstom *Kocuria rosea*. Sekvenca izolata 27/1 dobijena sekvencionom 16S rDNA analizom se sastoji od 1407 nukleotida, na osnovu čega je ovaj izolat identifikovan kao *Kocuria sp*. Nakon inkubacije od 86 dana, stepen degradacije početnih koncentracija MTBE-a od 25 i 125 µg/ml iznosio je 55, odnosno 36%. Pri početnoj koncentraciji od 25 µg/ml optička gustina i brojnost bakterija se smanjuju u prvih 15 dana inkubacije, dok je pri početnoj koncentraciji od 125 µg/ml zabeleženo smanjenje optičke gustine i broja bakterija u prvih 30 dana. Nakon ovog perioda stepen bakterijskog rasta se povećava. U početnim fazama inkubacije stepen degradacije MTBE-a nije visok, dok je krajem inkubacionog perioda konstatovan najveći stepen degradacije. Ovi rezultati ukazuju na uspešnu adaptaciju bakterije *Kocuria sp*. na prisustvo MTBE-a, što opravdava njenu primenu u bioremedijaciji zemljišta i voda kontaminiranih MTBE-om.