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EXPERIMENTAL ARTICLES

Analysis of the Anaerobic Microbial Community Capable of Degrading *p*-Toluene Sulfonate

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Abstract—Three strains of *Clostridium* sp., 14 (VKM B-2201), 42 (VKM B-2202), and 21 (VKM B-2279), two methanogens, *Methanobacterium formicicum* MH (VKM B-2198) and *Methanosarcina mazei* MM (VKM B-2199), and one sulfate-reducing bacterium, *Desulfovibrio* sp. SR1 (VKM B-2200), were isolated in pure cultures from an anaerobic microbial community capable of degrading *p*-toluene sulfonate. Strain 14 was able to degrade *p*-toluene sulfonate in the presence of yeast extract and bactotryptone and, like strain 42, to utilize *p*-toluene sulfonate as the sole sulfur source with the production of toluene. *p*-Toluene sulfonate stimulated the growth of *Ms. mazei* MM on acetate. The sulfate-reducing strain *Desulfovibrio* sp. SR1 utilized *p*-toluene sulfonate as an electron acceptor. The putative scheme of *p*-toluene sulfonate degradation by the anaerobic microbial community is discussed.

Key words: methanogenic microbial community, anaerobic biodegradation, *p*-toluene sulfonate, *Clostridium, Desulfovibrio, Methanosarcina, Methanobacterium.*

Hazardous aromatic compounds get into the environment in the form of diverse detergents with oil spills, sewage from petroleum refineries and chemical plants, and with municipal wastewaters. Aerobic processes in waste treatments plants may not completely remove aromatic compounds from wastewaters, turning researchers' interest to the study of the anaerobic metabolism of these compounds [1]. One of the most promising relevant anaerobic technologies employs the so-called UASB (upflow anaerobic sludge blanket) reactor with an ascending flow of liquid through a layer of anaerobic activated sludge in the form of granules produced by an active methanogenic microbial community adapted to particular pollutants [2]. The development and application of such technologies require a fundamental understanding of the composition of the methanogenic microbial community and the metabolism of the constituent microorganisms.

The aim of this work was to study the species composition of the anaerobic microbial community capable of degrading lower alkylbenzene sulfonates with the formation of methane.

MATERIALS AND METHODS

The anaerobic methanogenic microbial community capable of degrading lower alkylbenzene sulfonates was obtained from the UASB reactor granules incubated over an extended period of time in a medium with wastewaters from the Syktyvkar paper and pulp mill. The granules were disrupted by extruding the granule suspension through a syringe needle. The homogenate was inoculated into Hungate tubes or 125-ml flasks containing, respectively, 5 and 50 ml of PBBM medium of the following composition (g/l): K_2HPO_4, 0.29; KH_2PO_4, 0.29; NaCl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.2; NH_4Cl, 1.0; $CaCl_2$, 0.1; cysteine-HCl, 0.5; and yeast extract, 1.0. The medium was supplemented with 5 ml of the vitamin solution [3], 10 ml of the trace element solution [3], and 0.4 g/l of either benzene sulfonate or *p*-toluene sulfonate. The tubes and flasks were incubated anaerobically at 29 and 37°C. In some experiments, the medium was supplemented either with a mixture of the electron acceptors Na_2SO_4 and $NaNO_3$ in amounts of 2.0 g/l each or the amorphous oxide of Fe(III) in the same amount [4]. The microbial association was considered to be stable after eight passages into the fresh medium following the complete utilization of the aromatic sulfonates in the previous passage.

Microorganisms were isolated in pure cultures by the serial dilution method using the anaerobic Hungate technique [5].

Clostridia were isolated using PBBM medium with 2 g/l bactotryptone, 2 g/l yeast extract, and 0.2 to 0.4 g/l *p*-toluene sulfonate. Pure clostridial cultures were

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maintained in a medium containing (g/l) K_2HPO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 1.0; cysteine-HCl, 0.5; Na_2S, 0.5; yeast extract, 5.0; bactotryptone, 2.0; pesazurin, 0.002; *p*-toluene sulfonate, 0.2–0.4; and 10 ml/l of the trace element solution. This medium was also supplemented with either 5 g/l glucose (for strains 14 and 42) or 0.5 g/l toluene (for strain 21).

Sulfate-reducing bacteria were isolated using the Widdel and Pfennig medium [6] with lactate.

Methanogenic bacteria were isolated using a medium containing (g/l): K_2HPO, 0.27; KH_2PO_4, 0.19; NaCl, 5.0; $MgCl_2 \cdot 6H_2O$, 0.5; NH_4Cl, 1.0; $CaCl_2$, 0.1; cysteine-HCl, 0.5; 5 ml/l of the vitamin solution [3]; and 10 ml/l of the trace element solution [3]. To isolate methanosarcinas, the medium was additionally supplemented with either 50 mM sodium acetate or 20 mM trimethylamine and either casamino acids or *p*-toluene sulfonate in amounts of 0.2 g/l. To isolate hydrogen-utilizing methanogens, the medium was supplemented with 0.2 g/l yeast extract and a gas mixture of H_2 and CO_2 in a proportion of 4 : 1.

Colonies were obtained on the media solidified with 20 g/l Difco agar (United States).

Cell morphology was studied using an Opton ICM 405 phase-contrast microscope (Germany) at a magnification of 100×3.2.

The physiological characteristics of isolates were studied by the conventional methods [7–10].

The isolation and analysis of DNA. DNA was isolated by the Marmur method [13]. The G+C content of DNA was determined from melting profiles recorded using a Pye-Unicam SP1800 spectrophotometer (United Kingdom). DNA hybridization values were derived from DNA renaturation rates [14].

Immunological studies were carried out by the method of indirect immunofluorescence using the bank of antisera to methanogenic bacteria described by Bezrukova *et al.* [15].

Analytical methods. Cytochromes in the sulfate-reducing strain SR1 were analyzed spectrophotometrically, by recording the redox difference absorption spectra and the CO-difference spectra of cells in a Shimadzu UV-160 double-beam spectrophotometer (Japan).

Desulfoviridin was determined by the Postgate method [11].

Hydrogen sulfide was analyzed by the spectrophotometric method of Cline [12].

Fatty acids and methane were analyzed using a Pye-Unicam series 304 gas chromatograph (United Kingdom) as described earlier [16].

Benzene sulfonate, *p*-toluene sulfonate, and toluene were analyzed by HPLC using a Laboratorni Pristroje high-pressure liquid chromatograph (Czech Republic) equipped with a 15-cm SGXC C18 (7 μm) column (Tessek, Czech Republic). The mobile phase was either 100 mM phosphate buffer (pH 6.7) with 15% methanol at a flow rate of 1.5 ml/min (for analysis of benzene sulfonate and *p*-toluene sulfonate) or an acetonitrile–*n*-butanol–water mixture (52 : 8 : 40) at a flow rate of 1 ml/min (for analysis of toluene). The substances eluted from the column were monitored at 254 nm. Before injection into the column, samples of the culture liquid were centrifuged at 8000 *g* for 5 min.

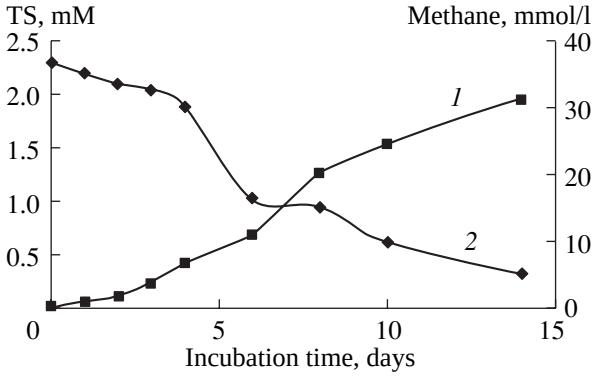

Fig. 1. The production of methane (*1*) from *p*-toluene sulfonate (*2*) by the anaerobic microbial association.

RESULTS AND DISCUSSION

The degradation of *p*-toluene sulfonate by the anaerobic consortium of microorganisms. Using different physicochemical conditions (E_h and pH) and electron acceptors, we succeeded in obtaining more than 30 anaerobic enrichment cultures capable of degrading benzene sulfonate (BS) and *p*-toluene sulfonate (TS) at different levels. The stable microbial association that completely degraded TS at 37°C with the formation of methane was chosen for further analysis and the isolation of constituent microbial species.

Figure 1 shows the dynamics of TS degradation and methane formation by the anaerobic microbial community incubated with TS for 14 days. As can be seen from this figure, in the course of the indicated period, the concentration of TS in the medium decreased from 2.3 to 0.4 mM, which was accompanied by the accumulation of 31.2 mM CH_4 in the gas phase. In the control medium containing no TS, the concentration of methane reached only 4.6 mM. In the second control medium, which contained TS but was autoclaved together with the microbial community, the concentration of TS decreased by no more than 4% (data not presented).

The growth of the microbial community on TS was accompanied by the production of fatty acids, including acetic, propionic, butyric, isobutyric acids and small amounts of valeric and hexanoic acids. The growth of the microbial community on BS was accompanied by the production of only acetic, propionic, and butyric acids. In both cases, acetic acid was produced in twofold greater amounts than any of the other fatty acids. The concentration of acetic acid in the medium grew

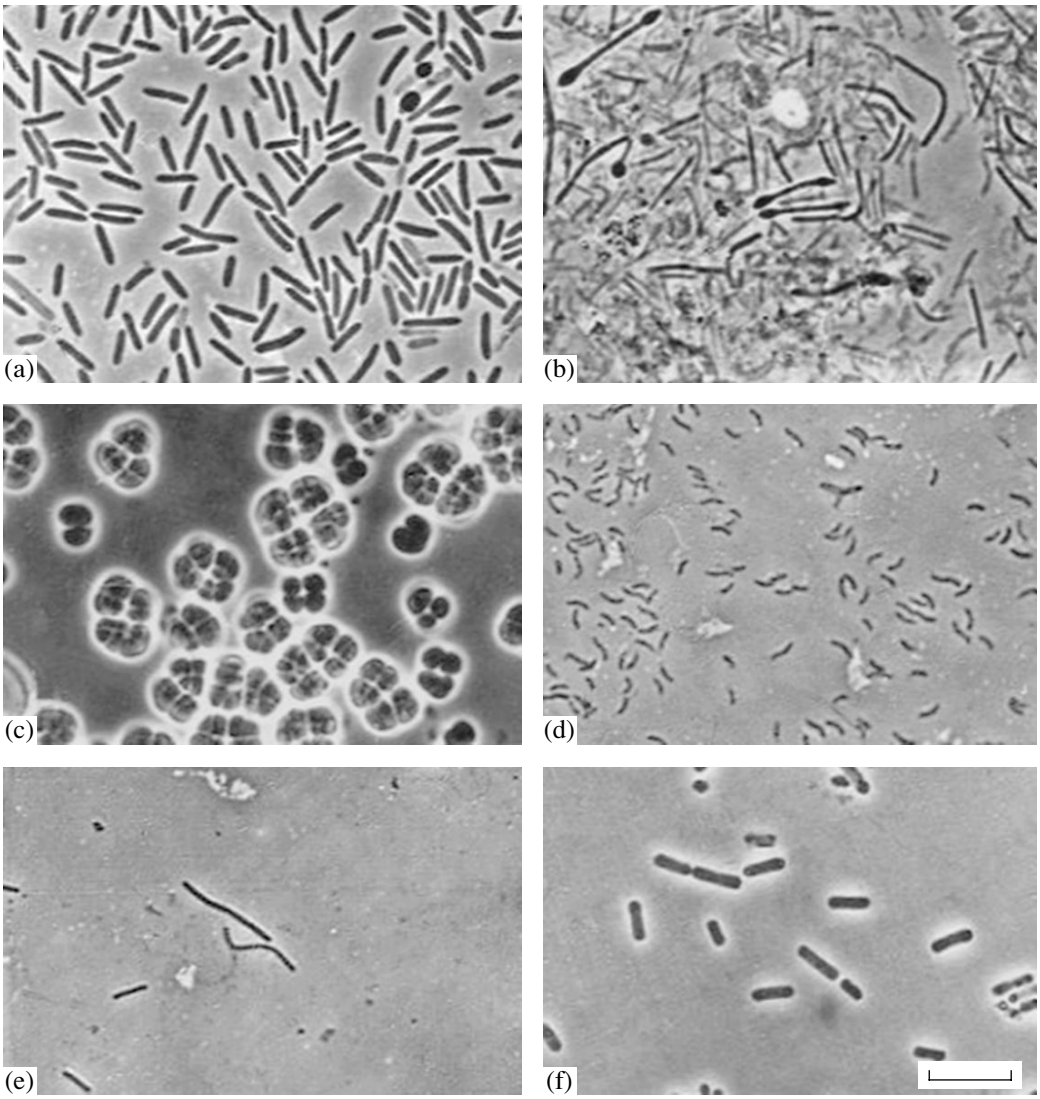

Fig. 2. The micrographs of cells of (a) strain 14 (VKM B-2201), (b) strain 42 (VKM B-2202), (c) strain MM (VKM B-2199), (d) strain SR1 (VKM B-2200), (e) strain MH (VKM B-2198), and (f) strain 21 (VKM B-2279) isolated from the anaerobic microbial community capable of degrading *p*-toluene sulfonate.

during the first 3–4 days of incubation to reach 0.53–0.73 g/l. In the course of further incubation, the concentration of this acid gradually decreased, likely because of the development of acetate-consuming microflora.

Microscopic analysis showed that the community consisted of 9–10 microorganisms of different morphology, six of which were isolated in pure cultures as described in the *Materials and Methods* section. Based on the analysis of the micrographs of these isolates (Fig. 2), they were presumptively referred to the genera *Clostridium* (Figs. 2a, 2b, 2f), *Methanosarcina* (Fig. 2c), *Desulfovibrio* (Fig. 2d), and *Methanobacterium* (Fig. 2e).

The characterization of strains isolated from the microbial community degrading *p*-toluene sulfonate. The basic characteristics of bacterial isolates belonging to different morphological and trophic groups are presented in Table 1.

Spore-forming microorganisms were represented by at least four species, three of which (strains 14, 42, and 21) were isolated in pure cultures. Strains 14 and 42 were isolated from the enrichment culture incubated in the medium with TS, whereas strain 21 was isolated from the enrichment culture incubated in the medium with toluene. Cells of all three strains were spore-forming rods differing in size and the characteristics of spore formation (Figs. 2a, 2b, 2f). The strains were obligate anaerobes, did not reduce sulfate and nitrate, and did not require NaCl for growth. These properties allowed strains 14, 42, and 21 to be referred to the genus *Clostridium.*

Table 1. The physiological and morphological properties of the isolates

Characteristics	Isolates					
	14 VKM B-2201	42 VKM B-2202	21 VKM B-2279	MM VKM B-2199	MH VKM B-2198	SR1 VKM B-2200
Cell morphology	Endospore-producing rods	Endospore-producing rods	Endospore-producing rods	Small aggregates of spheric cells	Rods forming filaments	Vibrios
Gram staining	+	+	+	+	–	–
Motility	+	+	+	–	–	+
Optimum pH for growth	7.8–8.0	7.8–9.0	7.8–8.6	6.3–6.9	7.0–7.2	7.0–7.2
pH range for growth	5.0–10.0	5.0–10.0	5.0–9.0	5.5–7.7	6.0–9.5	5.5–8.5
Optimum growth temperature	37	37	37	37	35–40	37–40
Temperature range for growth	13–40	13–45	ND	15–50	12–55	20–50
Substrates utilized	Histidine, glutamine, tyrosine	Glucose	Glucose, sucrose	Methanol, acetate, methylamines	$CO_2 + H_2$, formate	Lactate, propionate, formate, $CO_2 + H_2$
Products*	Acetate, isobutyrate, propionate, CO_2, H_2	Acetate, isobutyrate, CO_2, H_2	Acetate, butyrate, isobutyrate, propionate, valerate	Methane	Methane	Acetate, CO_2
Autotrophy	–	–	–	–	+	+
Growth factors	Yeast extract	Yeast extract	Yeast extract, peptone	Casamino acids, TS	Yeast extract	–
G+C content, mol %	30.3	27.9	36.1	40.3	38.4	66.5
Diagnosis	*Clostridium* sp.	*Clostridium* sp.	*Clostridium* sp.	*Methanosarcina* sp.	*Methanobacterium* sp.	*Desulfovibrio* sp.

* The metabolic products of strains 14, 42, and 21 were determined by growing them in the PYG medium.

Strains 14, 42, and 21 were able to grow at temperatures between 13 and 45°C and pH between 5.0 and 10.0 (Table 1). Strain 14 could grow on glutamic acid, histidine, and tyrosine. Strain 21 was able to hydrolyze gelatin. All three strains required yeast extract for growth and differed in the range and amount of fatty acids produced in the PYG medium (Table 1). All this made it possible to identify strain 14 as a glutamate-fermenting species of the genus *Clostridium.* This strain has specific physiological and biochemical properties and may represent a new species of this genus. Strains 42 and 21 may represent, respectively, saccharolytic and proteolytic species of the genus *Clostridium.*

The degradation of *p*-toluene sulfonate by the clostridial isolates. Experiments showed that strains 14 and 42 were fairly tolerant to high concentrations of TS in the medium: the 50% inhibition of growth of these strains was observed, respectively, at 30 and 20 g/l TS in the medium.

Only one of the three clostridial isolates, strain 14, was able to partially degrade TS in the PBBM medium with yeast extract (Fig. 3a). The addition of 5 g/l glutamate led to the complete degradation of TS (Fig. 3b). It should be noted that the ranges and the amounts of the fatty acids produced by strain 14 in these two cases were different.

In the absence of other sulfur sources, strains 14 and 42 could utilize TS as the sulfur source, producing, respectively, 0.3 and 0.34 mmol toluene per mmol TS consumed. In the control experiments (incubation with killed cells or with cysteine and sulfate as sulfur sources) toluene was not detected in the medium.

Sulfate-reducing strain SR1. Strain SR1 represented small motile single vibrios (Fig. 2d), which did not produce spores and were gram-negative. Strain SR1 was strictly anaerobic and tolerant to NaCl at concentrations up to 30 g/l. The optimal values of pH and temperature for growth were 7.0–7.2 and 37–40°C, respectively. The strain could grow on lactate, pyruvate, $H_2 + CO_2$, and formate with doubling times of 6.3, 6.8, 7.2, and 10.3 h, respectively. In the absence of electron acceptors, strain SR1 fermented pyruvate, producing acetate and a small amount of valerate.

The strain could utilize *p*-toluene sulfonate (as well as sulfate, sulfite, S^0, but not benzene sulfonate) as the terminal electron acceptor. The replacement of 2 mM

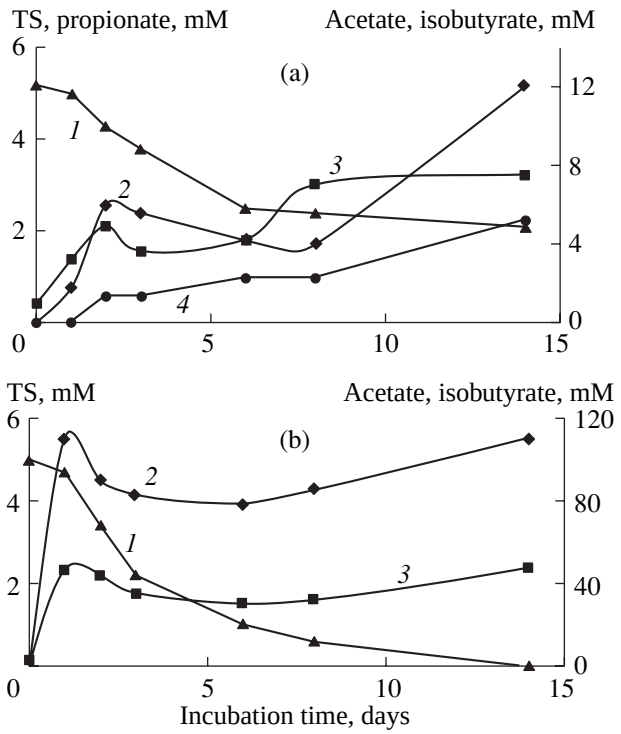

Fig. 3. The degradation of (*1*) *p*-toluene sulfonate and the production of (*2*) acetate; (*3*) isobutyrate; and (*4*) propionate by the *Clostridium* sp. strain 14 incubated in PBBM medium (a) in the absence and (b) presence of glutamate.

sulfate in the medium by 2 mM TS did not affect the growth of strain SR1. With either of the sulfur sources (sulfate and TS), the strain produced 1.6 mM sulfide. This indicated that strain SR1 is a sulfate-reducing bacterium with a unique ability to utilize the sulfur-containing aromatic compound TS as an electron acceptor.

The cell homogenate of strain SR1 was found to contain cytochrome *c* with the α, β, and γ absorption peaks at 552, 522.5 and 419 nm, respectively. Cytochrome *c* is typical of many sulfate-reducing bacteria. The strain also contained desulfoviridin, the enzyme that is typical of the genus *Desulfovibrio* [12].

All these properties (the dissimilatory reduction of sulfate, absence of spores, specific shape of cells, tolerance to NaCl, and presence of cytochrome *c* and desulfoviridin) allowed the strain SR1 to be assigned to the genus *Desulfovibrio.* In some properties, however, strain SR1 differs from the known species of *Desulfovibrio.* Among these are the production of valerate (along with acetate) during the growth of SR1 in the absence of sulfate and the ability of this strain to grow on $H_2 + CO_2$ mixtures in the absence of acetate or yeast extract in the medium.

The G+C content of the DNA of strain SR1 was 66.5 mol %, which is close to the G+C content of *Desulfovibrio vulgaris* (64.0 mol %). It should, however, be noted that strain SR1 showed only 10% DNA homology with the type strain VKM B-1760 of *Ds. vulgaris.*

Methanogenic bacteria. The enrichment cultures incubated in the presence of acetate, methanol, or $H_2 + CO_2$ were found to contain viable cells which, when observed under a luminescence microscope, had green fluorescence typical of methanogenic bacteria. Two methanogenic strains, MH and MM, were isolated in pure cultures.

The physiological and biochemical properties of the methanogenic strain MM are shown in Table 1. Strain MM represented gram-positive spheric cells aggregated in small packets (Fig. 2c). Based on its physiological, biochemical, immunological, and genotypic properties, strain MM was identified as the species *Methanosarcina mazei* [17]. *p*-Toluene sulfonate stimulated the methanogenesis of strain MM grown on acetate. In this case, the carbon atom of methane was shown to be derived from the *p*-toluene sulfonate molecule [17].

The hydrogen-consuming methanogenic strain MH represented gram-negative nonmotile rod-shaped cells (Fig. 2e). The addition of yeast extract to the medium prevented cell lysis and stimulated the growth of this strain with a doubling time of 5.9 h. The optimal concentration of sulfide for the growth and methanogenesis of strain MH was 0.4 mM. NaCl at concentrations higher than 0.15 mM suppressed the growth of this strain.

The G+C content of the DNA of strain MH comprised 38.4 mol %, being close to that of the species *Methanobacterium formicicum* (41–42 mol %). The DNA homology level between strain MH and *Mb. formicicum* 48 (VKM B-1632) was 61%. According to the data of indirect immunofluorescence analysis, strain MH was serologically similar to *Mb. formicicum* 48. At the same time, it was dissimilar to the other known rod-shaped methanogens whose immune antisera are available in the Laboratory of the Anaerobic Metabolism of Microorganisms [15]. All this made it possible to identify strain MH as belonging to the species *Mb. formicicum.*

The role of particular isolates in the anaerobic degradation of *p*-toluene sulfonate to methane. The initial anaerobic methanogenic association incubated in the medium without TS degraded the organic compounds of yeast extract with the formation of 4.6 mmol/l methane. The addition of TS to this medium augmented the production of methane to 46.7 mmol/l (in this case, the amount of TS consumed was 2.6 mmol/l). A mixed culture composed of the six strains isolated from the initial microbial association was also able to degrade TS with the production of methane, although the amounts of TS consumed (0.89 mmol/l) and methane produced (11.3 mmol/l) were less than in the case of the initial association (Table 2). It should be noted that the mixed culture produced more C_3–C_4 fatty acids than did the initial association (Table 2). This finding can be explained by the absence of microorganisms that are able to utilize these

Table 2. The production of fatty acids and methane associated with the degradation of *p*-toluene sulfonate

	TS consumed, mmol/l	Acetate, mmol/l	Propionate, mmol/l	Butyrate, mmol/l	Isobutyrate, mmol/l	Methane, mmol/l
Control (without TS)	0	ND	ND	ND	ND	4.6
Initial microbial association	2.6	5.93	1.09	0.69	0.69	46.7
Mixed culture composed of strains 14, 42, 21, SR1, MH, and MM	0.89	2.88	1.64	2.68	4.83	11.3

Note: All incubations were performed at 37°C in the PBBM medium with 1 g/l yeast extract for 14 days. ND stands for "not determined".

fatty acids in the mixed culture. The absence of such microorganisms may also be responsible for the lower efficiency of TS transformation by the mixed culture.

The transformation of *p*-toluene sulfonate by the studied methanogenic microbial community can be described as follows: *Clostridium* sp. strains 14 and 42 degrade *p*-toluene sulfonate to toluene or to more simple products. Toluene can be used by *Clostridium* sp. strain 21 as a carbon source. Alternatively, *p*-toluene sulfonate is used by the sulfate-reducing bacterium *Desulfovibrio* sp. SR1 as an electron acceptor or can be utilized by the methanogen *Ms. mazei* MM as the source of carbon and energy [17]. To gain deeper insight into the metabolism of *p*-toluene sulfonate in the anaerobic methanogenic community, further studies of the physiological and biochemical properties of the isolates and the comprehensive analysis of the *p*-toluene sulfonate intermediates are necessary.

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