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Biodegradation of Linear Alkylbenzenesulphonates (C_{11}LAS) by Mixed Methanotrophic/Heterotrophic and Mixed Heterotrophic Bacterial Cultures

Biološka razgradnja linearnih alkilbenzensulfonata (C_{11}LAS) pomoću mješovite metanotrofno-heterotrofne i mješovite heterotrofne kulture

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Summary

The objective of our work was to evaluate whether mixed methanotrophic/heterotrophic cultures are capable to degrade linear alkylbenzenesulphonates (LAS) major surfactants used in household detergents and widespread pollutants in waste water and natural waters. Mixed bacterial culture (MM1) containing a type II methanotroph and four heterotrophic strains, originating from an uncontaminated groundwater aquifer and a mixed bacterial culture consisting of five heterotrophic strains, isolated from the waste water of a detergent plant were used. Biodegradation experiments were conducted in shake flasks at 21 °C. Methane, carbon dioxide and oxygen concentrations were determined by headspace analysis on a Fisher-Hamilton gas partitioner. Concentrations of C_{11}LAS and their intermediates were measured by reversed-phase high-performance liquid chromatography (RP-HPLC). Comparison of the kinetic parameters shows that the mixed heterotrophic culture was more efficient in degradation of the alkyl chain than the mixed culture containing type II methanotroph. On the contrary, mixed culture MM1 was able to further the degradation of C_{11}LAS breakdown intermediates (sulphophenylalcanoic acids), while mixed culture with only heterotrophic species did not express this capability under the same experimental conditions. Occurrence of the same intermediates and only C-odd sulphophenylalcanoic acids suggested that the most probable mechanism for the degradation of the alkyl part of LAS molecule by both mixed cultures was ω- and β-oxidation. The rate studies for methane utilization demonstrate competitive inhibition of methane oxidation in the presence of C_{11}LAS.

Sažetak

Cilj našeg rada bio je istražiti ulogu metanotrofnih bakterija u razgradnji linearnih alkilbenzensulfonata (LAS), površinsko-aktivnih tvari koje se upotrebljavaju u proizvodnji detergenata, a koji su česti onečišćavači otpadnih i prirodnih voda. Upotrijebljene su dvije bakterijske kulture, i to: mješovita metanotrofno-heterotrofna kultura (MM1) porijeklom iz vodonosnog sloja podzemnih voda, koja sadržava metanotrof tipa II, te četiri soja heterotrofnih bakterija, i mješovita heterotrofna kultura (5 sojeva) porijeklom iz otpadne vode tvornice detergenata. Tijekom submerznog uzgoja kultura na tresilici pri temperaturi od 21 °C određivana je koncentracija metana, ugljik(IV)-oksida i kisika primjenom plinske kromatografije, te koncentracija LAS-a i nastalih međuproizvoda primjenom tekućinske kromatografije visoke djelotvornosti u sustavu s obratnom fazom (RP-HPLC). Analizom kinetičkih pokazatelja biološke razgradnje LAS-a utvrđeno je da je mješovita heterotrofna kultura djelotvornija u razgradnji alkilnog lanca od kulture MM1. Nasuprot tome, kultura MM1, koja sadržava metanotrof tipa II, nastavila je razgradnju nastalih međuproizvoda (kratkolančane sulfofenil-karboksilne kiseline), dok mješovita heterotrofna kultura nije pokazala to svojstvo tijekom pokusa. Pojava samo sulfofenil-karboksilnih kiselina s neparnim brojem C-atoma pokazuje da je najvjerojatniji mehanizam razgradnje C_{11}LAS-a primjenom obje kulture ω-, a zatim β-oksidacija. Analiza brzine potroška metana tijekom uzgoja kulture MM1 upućuje na kompetitivnu inhibiciju oksidacije metana u prisutnosti C_{11}LAS-a.

Introduction

The main objective of our work was to evaluate possible metabolic role of methanotrophs in the oxidation of linear alkylbenzenesulphonates (LAS), major anionic surfactants used in detergent industry. Methanotrophic bacteria constitute one of the major groups of free-living organisms, and their metabolic activities play an important role in the degradation of complex organic compounds, particularly hydrophobic organic compounds *(1)*. This ex-

traordinary and unexpected capability of methanotrophs is currently in the spotlight of research interest, implying that it is merely due to the lack of specificity of oxidative enzymes, whose primary function is to effect the oxidation of methane to carbon dioxide. Numerous investigations concerned with LAS biodegradability and environmental acceptability have been carried out and many papers published *(2-15)*. Despite these intensive studies and a general belief that we know more on LAS biodegradability than of any other synthetic chemical, this anionic surfactant is still a matter of environmental consideration and research interest. There are two main reasons for that: a) LAS is still most widely used synthetic anionic surfactant with the prospect of further increase in its use *(16)* and b) there is a high abundance of LAS in waste water treatment plants and the accumulation of LAS and/or their intermediates in anaerobically digested sewage sludges, river and sea sediments *(17,18)*.

It is well known and obvious from their chemical structure that LAS catabolism confronts microorganisms, generally heterotrophic bacteria, to convert different structures: namely the aliphatic chain with a non-uniform number of C atoms, and the aromatic ring substituted by sulphonate group distributed randomly over the alkyl chain. Therefore it is not surprising that mixed heterotrophic cultures are frequently more efficient in complete LAS mineralization than single cultures since they likely possess full enzymatic potential for conversion of all the structures mentioned. Furthermore, it has also been documented *(9,12,19)* that bacteria capable to degrade LAS, especially its aliphatic part including terminal or ω-oxidation followed by β-oxidation, are widespread in nature, while those capable to oxidize the aromatic part are not so common.

In our experiment two well defined mixed bacterial cultures were used, i.e., 1. the mixed methanotrophic/heterotrophic culture which was efficient in the oxidation of trichloroethylene *(20,21)* and halogenated aromatic hydrocarbons *(22)* and 2. the mixed heterotrophic culture which was previously used in the biodegradation of linear alkylbenzene sulphonate, showing much higher efficiency in the oxidation of alkyl chain than in the further oxidation formed ring-containing intermediates *(4)*.

Materials and Methods

Mixed bacterial cultures

Mixed methanotrophic/heterotrophic culture (MM1) was enriched from aquifer material collected from the Moffet Field, Naval Air Base, Mountain View, California, USA. This is a stable consortium consisting of one methanotroph and four heterotrophs. The methanotroph was a nonmotile irregularly-shaped coccobacillus that contained the internal membrane structure characteristic of type II methanotrophs, lipid inclusions and utilized serine pathway. Detailed description of the enrichment technique, culture conditions, morphological, physiological and other characteristics of this mixed culture have been described elsewhere *(20,21)*.

Mixed heterotrophic culture (MC) was isolated from the waste water of the detergent plant Saponia, Osijek, Croatia. It consisted of five strains belonging to the genus *Pseudomonas* and two strains each of the genera *Achromobacter* and *Acinetobacter*. Detailed characterization of this mixed culture during the enrichment experiments in continuous culture using a mineral medium with commercial linear alkylbenzene sulphonate (LAS) as the limiting carbon and energy source was described in previous reports *(4,23,24)*.

Media

Modified Whittenbury medium (mineral medium). A modification of Whittenbury medium (25) was made in our experiments by adding NH_4Cl (0.53 g/L) instead of KNO_3 (1 g/L) as nitrogen source. With the addition of the proper concentration of C_{11}LAS this mineral medium was used for biodegradation experiment carried out with both mixed cultures.

Enriched Whittenbury medium (enriched medium). This medium was prepared by adding 160 mg Bacto Peptone and 110 mg meat extract (both from Difco Laboratories, Detroit Mich.) in 1 L modified Whittenbury mineral medium and was used for the maintenance of the mixed heterotrophic culture.

Reagents

Undecylbenzenesulphonate (C_{11}LAS), pure homologue of chemical purity 64 %, determined by RP-HPLC method using 4-octylbenzene sulphonic acid, sodium salt (Aldrich Chemical Co., Inc., Deerfield, IL) as internal standard, was supplied by Unilever, Port Sunlight, UK. Structure of C_{11}LAS is presented in Fig. 1.

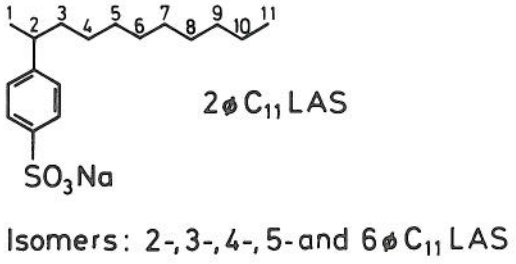

Fig. 1. Undecylbenzenesulphonate (C_{11}LAS) consisting of five phenyl positional isomers *(14)*
Slika 1. Undecilbenzensulfonat (C_{11}LAS) sadržava pet izomera *(14)*

4-Sulphophenylvaleric Acid Disodium salt (4-C_5SPC) and *3-Sulphophenylheptanoic acid disodium salt (3-C_7SPC)* were donated by Monsanto Co., St. Louis, MO and were used as standards for identification and quantitative determination of ring-containing C_{11}LAS intermediates.

Methane (99.3 % pure) and *Oxygen (Univ. Medical)* were purchased from Liquid Carbonic Specialty Gas Corp. Chicago, IL., USA.

All chemicals used for the growth media were of analytical reagent grade (Aldrich, Chemical Co., Milwaukee, WI, USA) except for the vitamins Sigma grade (Sigma Chemical Co., St. Louis, MO, USA). All solvents for HPLC analyses were of HPLC grade (Aldrich, Chemical Co., Milwaukee, WI, USA).

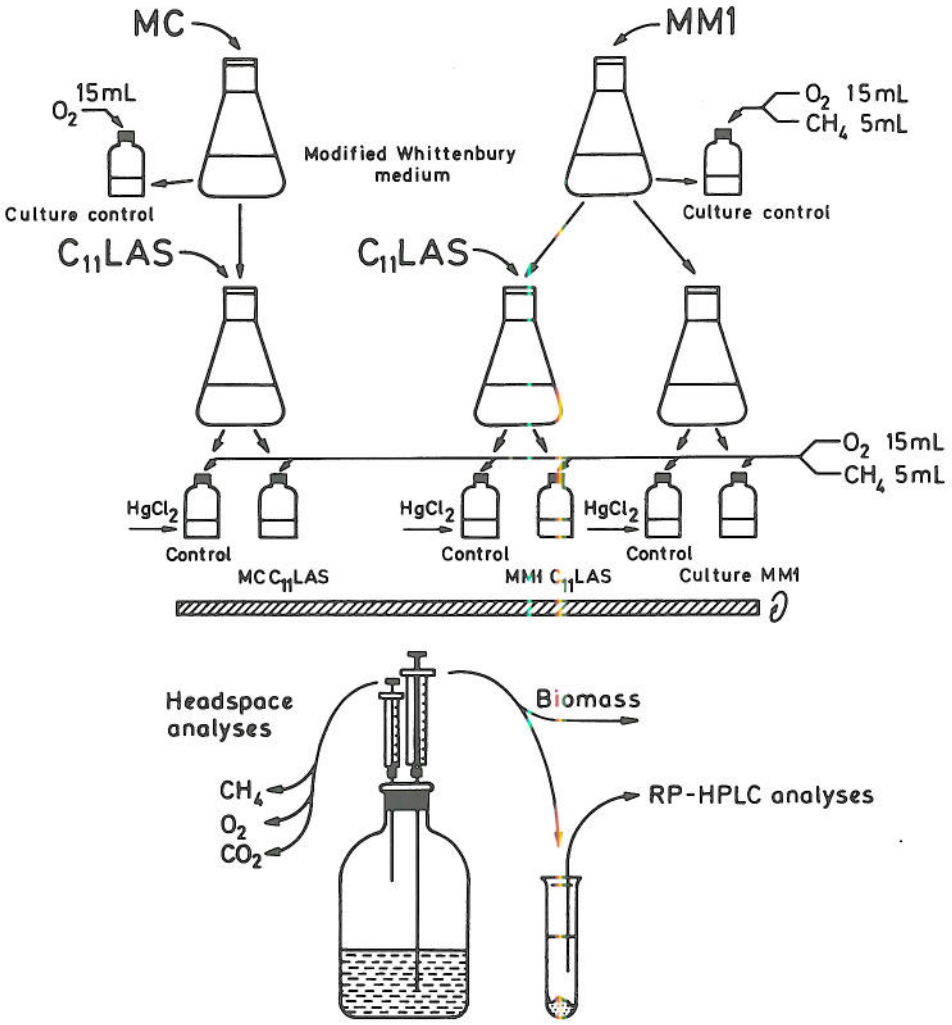

Fig. 2. Scheme of C_{11}LAS biodegradation assays
Slika 2. Shema pokusa odredivanja biološke razgradnje C_{11}LAS

C_{11}LAS transformation assays

Scheme of C_{11}LAS biodegradation experiments is presented in Fig. 2. The mixed cultures were prepared in the following way:

Original culture MM1 (grown in continuously-stirred reactor with magnetic stir bar agitation under the stream of 30-35 % methane in air and stored at 4 °C for 3 months without methane) was grown in Whittenbury medium to the mid-log phase of growth in shaken flasks (with addition of methane, 20 mL and oxygen, 20 mL). Then culture was transferred to a fresh medium with C_{11}LAS (5 mg/L) amended with 20 mL oxygen (approximately 30 % in headspace) and 10 mL methane (approximately 9 % in headspace) and incubated on rotary shaker (250 rpm). This was repeated three times over the period of 10-14 days. The culture grown to the mid-log phase was centrifuged for 5 min at 6000 g, resuspended in mineral medium to the turbidity of 0.08-0.09, and after C_{11}LAS addition (18 mg/L) thus prepared culture was used in biodegradation experiments.

MC culture, stored under glicerol (40 %) at –16 °C for a few years, was grown over a period of two months in shaken Erlenmeyer flasks (500 mL) in enriched Whittenbury medium with 10 mg/L of C_{11}LAS at 28 °C. Every two weeks 10 mL culture was transferred to the fresh medium (100 mL) and thus prepared one-week-old shaken culture (10 %) was used as inoculum.

Biodegradation experiments were performed in 100 mL serum bottles containing 40 mL cultures prepared under mentioned conditions. The bottles were sealed with butyl septa (Supelco, Inc., Bellefonte, PA) and amended with 15 mL oxygen (approximately 30 % oxygen in headspace). When mixed culture MM1 was used, 5 mL methane was also injected to achieve a starting aqueous concentration of approximately 1.4 mg/L (5.6 % methane in headspace). Methane injection (5 mL) was repeated twice (on the 7th and 12th day). The bottles were incubated on a rotary shaker (250 rpm) in a 21 °C environmental chamber.

All biodegradation experiments were performed in triplicate. Three types of controls were evaluated, i.e. cultures with addition of $HgCl_2$ (50 mg/L), sterile mineral medium blanks and culture control (without C_{11}LAS). The first two controls were used to evaluate the extent of sorption of C_{11}LAS to the glass and/or the cells. It was observed that less than 5 % C_{11}LAS was adsorbed to the cul-

ture and/or glass, suggesting that abiotic elimination of both compounds was negligible under the conditions of this study. The third control was conducted for the evaluation of the possible toxic effect of C_{11}LAS on the mixed culture MM1 and on the methane consumption.

Headspace analysis

Headspace samples (400 μL) were injected into a Fisher-Hamilton Gas partitioner model 25 V (Fisher Scientific Pittsburg PA) equipped with thermal conductivity detector, (helium as carrier gas) and methane, carbone dioxide, oxygen and nitrogen concentrations were determined. For the calibration certified gas standards (Liquid Carbonic Specialty Gas Corp., Chicago IL) were used. During C_{11}LAS transformation studies methane and oxygen uptake and carbon dioxide formation were expressed as a total amount of gases in the bottles (in mmol) and plotted against time (in days).

C_{11}LAS analysis

Culture samples (3 mL) were centrifuged for 5 min. at 6000 g to remove the cells and supernatants were submitted to the direct analysis of C_{11}LAS concentrations and evaluation of their ring-containing intermediates formed by subsequent shortening of alkyl chain. Two intermediates, 4-C_5SPC and 3-C_7SPC, were identified and their further transformation evaluated. The former analyses were performed by RP-HPLC method.

The HPLC system consisted of a Perkin Elmer Series 3B liquid chromatograph (Norwalk, Connecticut, USA) equipped with Rheodyne injector (Model 7152) and SP 4000 Chromatography data system (Spectra-Physics, Santa Clara, CA). Detection was performed by the spectrophotometric detector (Perkin Elmer LC-75) at 223 nm.

Initial and residual concentrations of C_{11}LAS were determined at isocratic conditions using a column 250 x 2.1 mm ID packed with octylsilica particles of 3 μm (Supelcosil LC-18-DB, Supelco, Inc., Bellefonte, PA) and fitted with a 20 mm long precolumn of the same packing material. The mobile phase (0.2 mL/min) consisted of a mixture of acetonitrile/water of volume ratio 51/49 containing 10 g/L sodium perchlorate.

The separation of ring-containing intermediates formed during C_{11}LAS degradation was performed on a column 250 x 4.6 mm ID packed with octylsilica particles of 5 μm (Supercosil LC-18) and equipped with a 20 mm long precolumn of the same packing material. For these analyses ion suppression chromatography and a gradient mode using the eluents A (mixture of water/acetonitrile of volume ratio 85/15 containing trichloroacetic acid, 0.125 mM) and B (water containing trichloroacetic acid 0.125 mM) were used. During the gradient elution the composition of the mobile phase was changed linearly from 10 to 15 % A in 5 min, then to 77.2 % A in further 10 min, this final composition being kept for another 7 min. The HPLC was operated at ambient temperature at a flow rate of 1.0 mL/min.

Biomass concentration

Cell biomass was determined on the optical density basis by measuring the absorbance at 600 nm (A_{600}) on UV-spectrophotometer. Data were converted to a biomass dry weight (mg/L) using calibration curves generated on a dry weight of the cultures of known optical density. To ensure as accurate a measure of biomass dry weight as possible, specially prepared 0.2 μm Supor filters (Gelman Sciences Inc., Ann Arbor, MI) were used as described previously *(7)*.

Methane oxidation rate and growth rate of culture MM1

Specific methane oxidation rates (k) were determined for a particular cultivation period ($t_{n-1} - t_n$) by using the equation:

$$k = -\frac{\Delta M}{x \cdot V_l} \cdot \frac{1}{t_{n-1} - t_n} \qquad (1/\text{day})$$

where: ΔM = mass of CH_4 consumed (mg), x = average biomass concentration (mg/L), and V_l =volume of liquid (L).

Specific growth rates of culture MM1 (μ), mean values, for a particular cultivation period ($t_{n-1} - t_n$) were calculated by using the equation:

$$\mu = \frac{1}{t_{n-1} - t_n} \ln \frac{x_{n-1}}{x} \qquad (1/\text{day})$$

where: x_n and x_{n-1} are biomass (dry weight in mg/L) at the time t_n and t_{n-1}, respectively.

Results and Discussion

Comparison of C_{11}LAS transformation by mixed MM1 and MC cultures

In order to gain more insight into the mechanism of C_{11}LAS transformation by mixed culture MM1, a comparative study with well defined mixed heterotrophic culture (MC) previously used in the biodegradation experiment of LAS *(4,23)* was performed. To ensure good comparison of C_{11}LAS transformation, the culture MC was grown under the same conditions as the culture MM1 except for the addition of CH_4. Results on primary degradation of individual 2-phenyl isomer and a mixture of other C_{11}LAS containing isomers, presented in Fig. 3. confirmed our previous observation of preferential transformation of 2--phenyl positional isomer by the culture MC *(4)*, and suggested that culture MM1 exhibited the same capability. This clearly indicated that both cultures transformed external isomers faster than the isomers with a sulphophenyl group situated closer to the middle of the alkyl chain. It is also evident that under the conditions of this study the culture MC degraded C_{11}LAS significantly faster than the culture MM1 (all isomers were transformed into transient intermediates after 30 and 96 h, respectively). RP--HPLC analyses of culture samples during C_{11}LAS degradation (some characteristic chromatograms presented in Fig. 4) showed that four major peaks of intermediates which did not degrade as fast as they were formed, appeared in chromatograms of both cultures. Identification of the peaks belonging to 4-C_5SPC and 3-C_7SPC, developed from 2- and 5-phenyl isomers, and appearance of two other peaks with similar elution time (probably developed from 3- and 4-phenyl isomers) suggested that the terminal oxidation, followed by β-oxidation, might be the most probable mechanism for the transformation of alkyl part

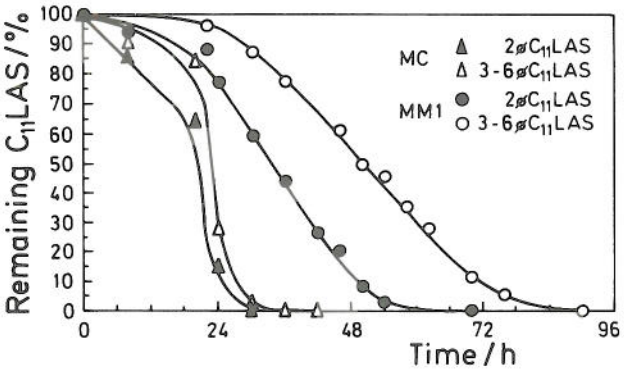

Fig. 3. Comparison of C_{11}LAS primary degradation by mixed methanotrophic/heterotrophic culture, MM1 (circles) and mixed heterotrophic culture, MC (triangles) determined by RP-HPLC method. Degradation of individual 2-phenyl isomer is shown as full symbols and of a mixture of 3- to 6-phenyl isomers as open symbols.

Slika 3. Usporedba početne razgradnje C_{11}LAS pomoću mješovite metanotrofno-heterotrofne kulture, MM1 (kružići) i mješovite heterotrofne kulture, MC (trokuti) određene RP-HPLC metodom. Razgradnja 2-fenil izomera prikazana je praznim simbolima, a razgradnja smjese 3- do 6-fenil izomera punim simbolima.

of C_{11}LAS molecule by both cultures. It is well documented and there is a general belief that this is the most probable and most common mechanism for the initiation and partial transformation of LAS molecules by heterotrophic bacteria *(2,5,9,14,27)*, although some other methabolic routes are also possible *(12,19)*.

Gradual decrease of characteristic peaks observed after extended incubation (7 days) with the culture MM1 and their complete disappearance on the 20th day (Fig. 4) indicated that the culture MM1 continued to transform ring-containing intermediates. On the contrary, persistence of all the peaks developed at 44 h until the end of experiment (20 days) with the culture MC, showed that under the conditions of this study mixed culture containing only heterotrophic species did not exhibit the capability to continue the transformation of formed key intermediates, i.e. short chain sulphophenylcarboxylic acids.

Methane consumption and growth kinetics of mixed culture

RP-HPLC measurements of culture samples and headspace analyses of methane consumption during the growth of mixed culture MM1 under the conditions of this study, i.e modified Whittenbury medium with the addition of C_{11}LAS, 18 mg/L, initial biomass dry weight 65

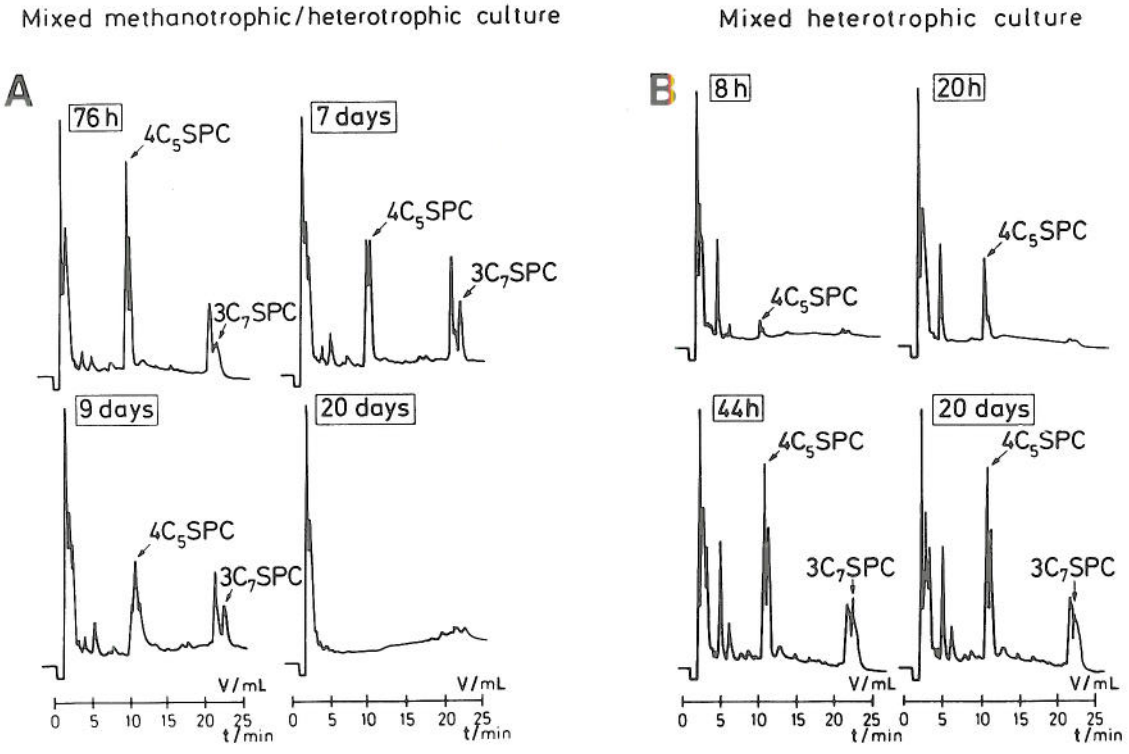

Fig. 4. RP-HPLC chromatograms of C_{11}LAS intermediates during biodegradation experiment with mixed methanotrophic/heterotrophic culture and mixed heterotrophic culture. Initial C_{11}LAS concentration was 18 mg/L, and remaining C_{11}LAS as follows: MM1 – 1.0 mg/L at 76 h; MC – 16.3 mg/L at 8 h, and 15.2 mg/L at 20 h. Peaks of the two identified intermediates (4-C_5SPC and 3-C_7SPC) are indicated on the chromatograms.

Slika 4. RP-HPLC kromatogrami međuproizvoda nastalih razgradnjom C_{11}LAS-a primjenom metanotrofno-heterotrofne kulture i mješovite heterotrofne kulture. Početna koncentracija C_{11}LAS-a iznosila je 18 mg/L, a zaostala kako slijedi: MM1 – 1.0 mg/L pri 76 h; MC – 16.3 mg/L pri 8 h i 15.2 mg/L pri 20 h. Pikovi identificiranih međuproizvoda (4-C_5SPC i 3-C_7SPC) naznačeni su na kromatogramima.

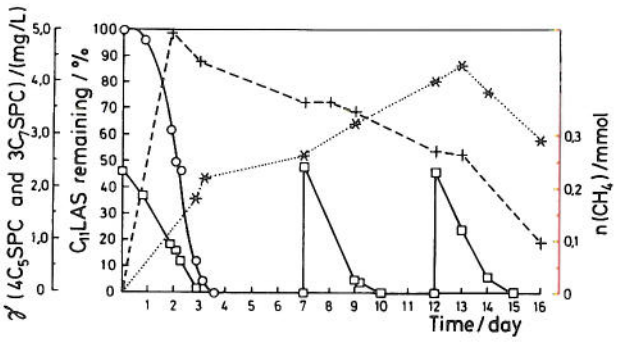

Fig. 5. Degradation of C_{11}LAS by mixed culture MM1 expressed as degradation of alkyl chain (o-o) and as degradation of benzenering containing intermediates (4-C_5SPC, +-+, and 3-C_7SPC, *-*). Methane consumption (□-□) during biodegradation experiments is also shown.

Slika 5. Razgradnja C_{11}LAS-a primjenom mješovite metanotrofno-heterotrofne bakterijske kulture, izražena kao razgradnja alkilnog lanca (o-o) i kao razgradnja nastalih međuproizvoda (4-C_5SPC, +-+, i 3- C_7SPC, *-*). Također je prikazan potrošak metana (□-□) tijekom pokusa.

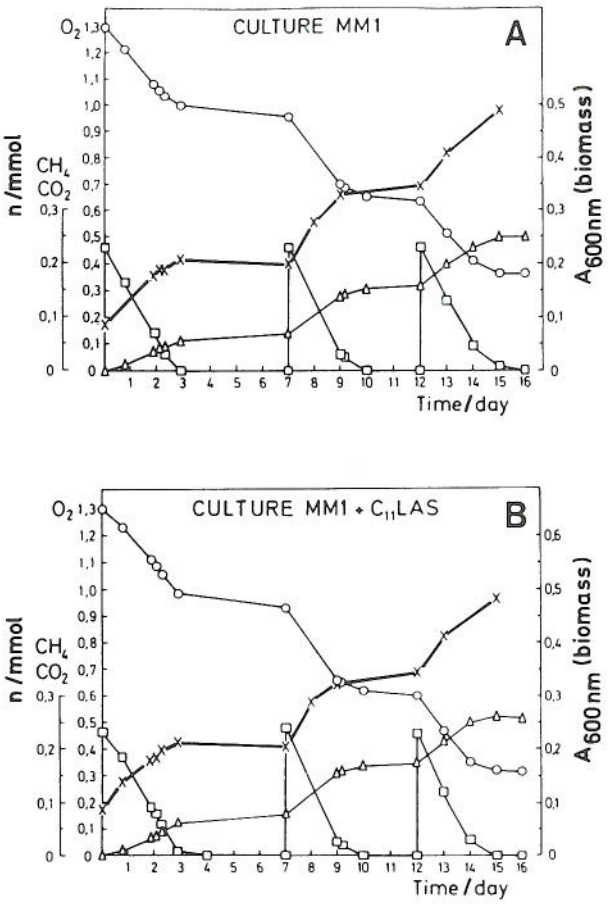

Fig. 6. Methane (□-□) and oxygen (o-o) consumption and carbon dioxide formation (△-△) during growth of mixed culture MM1 on methane only (A), or during C_{11}LAS degradation (B). Growth curves of mixed culture MM1 (x-x) are also shown.

Slika 6. Potrošak metana (□-□) i kisika (o-o) te nastanak ugljik(IV)-oksida (△-△) tijekom rasta mješovite kulture MM1 samo u prisutnosti metana (A) i uz dodatak C_{11}LAS (B). Također su prikazane krivulje rasta mješovite kulture MM1 (x-x).

mg/L and with methane amendment (5.6 % in headspace), are presented in Fig. 5. It is evident that mixed culture containing type II methanotroph revealed the ability of simultaneous methane oxidation and C_{11}LAS degradation. The shape of the curves suggested that the culture MM1 possesses the capability of immediate methane oxidation, while C_{11}LAS degradation started after a few hours delay. Similar stagnation in degradation was observed for transient intermediate 4-C_5SPC after extended incubation (16 days) at two repeated methane amendments (the 7th and 12th day). This indicated that the competition for the same enzyme methane monooxygenase (MMO), which could be responsible for the oxidation of terminal C-atom in the alkyl chain or hydroxylation of transient intermediates, could be one of possible reasons for this observation. Results presented in Fig. 5 also show that despite the 4 and 2 days starvation (without methane) between the second and the third CH_4 amendment, the culture MM1 retained the ability of methane oxidation.

In order to gain more insight into the mechanisms of C_{11}LAS degradation, a detailed analysis of growth kinetics of the culture MM1 was performed together with the kinetics of methane and oxygen uptake, and carbone dioxide formation. Data on headspace analyses presented in Fig. 6. showed that almost similar trends of methane and oxygen consumption and carbone dioxide formation were observed when culture MM1 was growing in mineral medium with or without addition of C_{11}LAS. The same could be said for the growth curves, which showed similar pattern in control experiment (with methane only) and in the presence of both methane and C_{11}LAS. However, a detailed analysis of specific rate of methane oxidation and growth kinetics of the culture MM1 (Tab. 1) showed slightly impaired methane oxidation rate under the first 48 h of experiment (1st CH_4 amendment, when C_{11}LAS and methane were simultaneously degraded) compared with the control experiment. This suggested that the competition for the same enzyme (MMO) could be a possible explanation for this observation. Other possibilities, such as toxic effect of C_{11}LAS on the methanotroph in culture MM1 and the changes in the composition of mixed culture, may also account. Decreased specific methane oxidation rate, specific growth rate and growth yield at the 2nd and especially the 3rd methane addition in the control experiment, which were also observed although pronounced in the presence of C_{11}LAS, indicated that depletion of reductant and of some nutrients might have occurred during extended incubation of the culture MM1 under the conditions of transformation experiment.

Mechanisms of C_{11}LAS degradation by mixed culture MM1

Comparative study of C_{11}LAS transformation by two well defined and characterized mixed cultures showed that despite faster primary C_{11}LAS degradation obtained by mixed heterotrophic culture (MC), mixed methanotrophic/heterotrophic culture (MM1) revealed the ability of a more complete oxidation of these molecules. At first sight, it could be surprising that the culture MM1, characterized as very efficient in the transformation of trichloroethylene

Table 1. Specific rate of methane consumption (k), specific growth rate (μ) and growth yield (y) of culture MM1 during C_{11}LAS degradation experiment

Tablica 1. Specifična brzina potrošnje metana (k), specifična brzina rasta (μ) i prirast biomase (y) kulture MM1 tijekom pokusa razgradnje C_{11}LAS-a

Medium Podloga	CH_4 addition CH_4 dodatak	k^a /(1/day) k^a /(1/dan)	μ^b /(1/day) μ^b /(1/dan)	y^c /mg dry wt/mg CH_4) y^c/(mg suhe tvari/mg CH_4)
Control Kontrola	1st prvi	0.34±0.033	0.38±0.026	0.66±0.045
	2nd drugi	0.24±0.022	0.25±0.017	0.68±0.038
	3rd treći	0.17±0.013	0.13±0.009	0.36±0.021
C_{11}LAS amendment C_{11}LAS dodatak	1st prvi	0.27±0.015	0.38±0.031	0.78±0.052
	2nd drugi	0.25±0.012	0.23±0.014	0.57±0.042
	3rd treći	0.23±0.014	0.14±0.007	0.35±0.025

[a] Average specific methane oxidation rate of three replicates determined at the following cultivation intervals: 0-48 h (1st CH_4 addition), 5-7 days (2nd CH_4 addition) and 12-14 days (3rd CH_4 addition).
Prosječna brzina oksidacije metana odredjena u 3 uzorka u slijedećem razdobljima: 0-48 h (prvi dodatak CH_4), 5-7 dan (drugi dodatak CH_4) i 12-14 dan (treći dodatak CH_4).

[b] Average specific growth rates of MM1 culture (three replicates) at same cultivation intervals as in [a].
Prosječna specifična brzina rasta kulture MM1 (3 uzorka) odredjena u istim vremenskim razdobljima kao u [a].

[c] Average growth yield expressed as mg biomass dry weight/mg CH_4 normalized to C. For the evaluation of carbon in biomass, typical empirical formulation for bacterial cells $C_5H_7O_2N$ was used.
Prosječni prirast biomase, izražen kao ugljik (C). Za izračunavanje udjela C u biomasi primijenjena je empirijska formula za bakterije $C_5H_7O_2N$.

and chloroform *(20,21,26)*, exhibited this ability, while the culture MC, adapted to LAS and characterized as efficient in degradation of commercial mixture and pure linear alkylbenzene sulphonate *(3,4,23)*, did not. The fact that the same and only C-odd short-chain sulphophenylalcanoic acids, resulted from C_{11}LAS primary degradation, suggested that mixed culture MM1, containing type II methanotroph, degraded alkyl chain by the same mechanisms, i.e. oxidation of terminal methyl group followed by β-oxidation, as mixed culture containing only heterotrophs. Although we are fully aware that the activity of other members (heterothrophs) of mixed culture MM1 should not be overlooked, owing to the analysis of methane consumption, and to the growth kinetics of mixed culture MM1 obtained in this study, and based on the available literature *(1,11,20,28,29)* it seems likely to propose that type II methanotroph of mixed culture possesses the ability to initiate C_{11}LAS transformation. The possibility of this methanotroph to continue the shortening of the alkyl chain by β-oxidation still remains uncertain, making a valuable area for further research. An equally interesting field of investigation is a possible role of type II methanotroph in transformation of short-chain sulphophenylalcanoic acids that can be expected based on more complete oxidation of C_{11}LAS molecules by mixed culture MM1 than by mixed culture containing only heterotrophic bacteria. In this case simple co-metabolic reaction of hydroxylation, analogous to the oxidation of methane to methanol and/or desulphonation of the aromatic ring, might be essential for further degradation of key intermediates by heterotrophic species in mixed culture.

Although there is controversy about the importance of methanotrophs in the transformation of multi-carbon compounds due to the lack of specificity of oxidative enzymes *(11)*, it is well authenticated that these bacteria are able to introduce oxygen into many compounds including long-chain alkanes, alkenes, aromatic and alicyclic hydrocarbons *(1,22,28,29)*. Furthermore, based on transformation experiment with 1-(^{14}C)-n-hexadecane, Higgins et al. *(1)* reported that *Methylosinus trichosporium* can slowly degrade long-chain alkanes to CO_2, suggesting that the degradation process probably involves ω- and β-oxidation. It has also been generally accepted *(1,11,29)* that acetate once formed can serve as a carbon source for methanotrophs and that in the case of type II species, which possess an intact tricarboxylic acid cycle, acetate can be oxidized to CO_2, yielding reducing equivalent and ATP.

More essential work should be done to test the abovementioned hypotheses about substantial role of methanotrophs in the oxidation of complex LAS molecules, including, study of competitive inhibition, transformation study of pure LAS isomers and sulphophenylalcanoic acids by pure methanotrophic culture and also growth kinetics study of pure culture of methanotrophs during LAS transformation.

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References

1. I. J. Higgins, D. J. Best, R. C. Hammond, *Nature, 286* (1980) 561.
2. J. J. Heyman, A. H. Molof, *J. Water Pollut. Control Fed. 39* (1967) 50.
3. D. Hršak, M. Bošnjak, V. Johanides, *Tenside Deterg. 18*(3) (1981) 137.
4. D. Hršak, M. Bošnjak, V. Johanides, *J. Appl. Bacteriol. 53* (1982) 413.
5. R. L. Huddleston, R. C. Allred, *Develop. Ind. Microbiol. 4* (1963) 24.
6. R. L. Huddleston, A. M. Nielsen, *Soap, Cosmetics, Chemical Specialities*, March (1979) 34.
7. R. J. Larson, A. G. Payne, *Appl. Environ. Microbiol. 41* (1981) 621.
8. R. J. Larson, *Environ. Sci. Technol. 24* (1990) 1241.
9. P. Schöberl, *Tenside Deterg. 26* (1989) 86.
10. J. C. Sigoillot, M. H. Nguyen, *Appl. Environ. Microbiol. 58* (4) (1992) 1308.
11. D. I. Stirling, H. Dalton, *Nature, 291* (1981) 169.
12. R.D. Swisher: »Surfactant Biodegradation«, Marcel Dekker Inc., New York (1970).
13. R. D. Swisher, *Yukagaku, 21* (1972) 130.
14. R. D. Swisher, W. E. Gledhill, R. A. Kimerle, T. A. Taulli, in Proceedings of the 7^{th} *International Congress on Surface Active Substances* 4. Moscow: Vneshtorgizdat. Izd. No 700 M. (1978) pp. 218-230.
15. S. Terzić, D. Hršak, M. Ahel, *Water Res. 26*(5) (1992) 585.
16. P. Berth, P. Jeschke, *Tenside Deterg. 26* (1989) 75.
17. W. Giger, A. C. Alder, P. H. Brunner, A. Marcomini, M. Siegrist, *Tenside Deterg. 26* (1989) 95.
18. A. Marcomini, W. Giger, *Tenside Deterg. 25* (1988) 226.
19. R. S. Horvath, B. W. Koft, *Appl. Microbiol.* Feb. 1972 (1972) 407.
20. S. M. Henry, D. Grbić-Galić, *Microbiol Ecol. 20* (1990) 151.
21. S. M. Henry, D. Grbić-Galić, *Appl. Environ. Microbiol. 57*(1) (1991) 236.
22. P. Adriaens, Stanford University, California, personal communication (1991).
23. D. Hršak, V. Johanides, M. Starčević, in Proceedings of 7^{th} *International Congress on Surface Active Substances* 4, Moscow: Vneshtorgizdat. Izd. No. 7090 M. (1978) pp. 149-159.
24. V. Johanides, D. Hršak, in 5^{th} *International Symposium on Fermentation*, Dellweg, H. (Ed), Berlin: Westkreuz (1976) pp. 426.
25. R. Whittenbury, K. C. Phillips, J. F. Wilkinson, *J. Gen. Microbiol. 24* (1970) 225.
26. L. Alvarez-Cohen, Cometabolic biotransformation of trichloroethylene and chloroform by methanotrops. *Dissertation*, Stanford University, California, USA (1991).
27. E. C. Donaldson, G. V. Chilingarian, Teafuyen (Ed): »*Microbial Enhanced Oil Recovery*«, Elsevier, Amsterdam-Oxford-New York-Tokyo (1989) pp. 194-207.
28. S. C. Baker, D. P. Kelly, J. C. Murrell, *Nature, 350* (1991) 627.
29. A. M. Wadzinski, D. W. Ribbons, *J. Bacteriol. 123*(1) (1975) 380.