Methanogenic biodegradation of aromatic amines

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Abstract Batch methanogenic toxicity and biodegradability of 2-, 3- and 4-aminobenzoic acids (ABA) as well as 4- and 5-aminosalicylic acids (ASA) have been studied in the presence of two mesophilic (Shell and cattle) and one thermophilic sludges. The aminoaromatics tested practically did not inhibit methanogenesis up to concentrations of 3–7 g/l; moreover, some of them (2-ABA, 4-ABA and 5-ASA) even exert a stimulating effect on aceticlastic activity of the sludges. Concerning biodegradability, 5-ASA was completely mineralised by all the sludges tested; however, 4-ASA was not degraded at all by any of the sludges. Both mesophilic sludges were able to perform a complete mineralization of 2-ABA but this was not a case for the thermophilic sludge. 3-ABA was not biodegraded only in the presence of the Shell sludge. On the contrary, 4-ABA was quantitatively mineralised only by the Shell sludge. All the adapted sludges were able to mineralise the corresponding aminoaromatics in N-deprived media. Cross-acclimatisation trials showed that 2-ABA-, 5-ASA- and salicylic acid adapted sludges were unable to degrade any other aminoaromatics tested that manifest about a different nature of key bacteria responsible for primary decomposition of these substrates. The main possibility of continuous mineralization of 2-ABA as a unique source of carbon and nitrogen has been demonstrated using mesophilic UASB reactor inoculated by adapted sludge.
Keywords Aminobenzoic acids; aminosalicylic acids; biodegradability; toxicity; UASB reactor

Introduction

Nitro-, azo- and amino-substituted aromatics play an important role in the production of explosives, dyes, pesticides, polymers, pharmaceuticals etc. and, consequently, appear in wastes generated by the corresponding industries. They are very dangerous for the environment owing to their mutagenic and carcinogenic influence on all the living organisms and some of them (nitro- and azo-) are quite persistent to aerobic biodegradation. Recent research has highlighted the hitherto unsuspected ability of anaerobic bacteria to degrade various N-aromatics (Razo-Flores, 1997) but the internal mechanisms of the processes are poorly studied. Since the limiting step of anaerobic biomineralisation of any N-aromatic is usually degradation of aromatic amines, the objective of this work was to get more insight in both microbiological (the know) and technological (the how) aspects of methanogenic degradation of several widespread (both naturally occurring and xenobiotic) aromatic amines - aminosalicylic acids (ASA) and aminobenzoic acids (ABA).

Materials and methods

Aromatic substrates. The aminoaromatics tested included: 4-ASA, 5-ASA, 2-ABA, 3-ABA and 4-ABA. 5-ASA was purchased from Sigma-Aldrich (USA), the other amines as well as salicylic acid (SA) - from Reakhim (Russia). All aromatic compounds were of the highest purity available and were used without further purification.

Sludge. Three types of anaerobic methanogenic sludge were used:

(i) mesophilic granular sludge from a full-scale UASB reactor treating chemical industry wastewater of Shell Nederland Chemie at Moerdijk, The Netherlands (Shell sludge); VSS concentration – 25.5 g/l; specific aceticlastic activity – 0.3 g COD/l/g VSS (30ºC);

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(ii) mesophilic floccular sludge from a lab-scale UASB reactor treating cattle manure wastewater (Cattle sludge); VSS concentration – 20.5 g/l; specific aceticlastic activity – 0.05–0.06 g COD/l/g VSS (30ºC);
(iii) thermophilic sludge from a full-scale CSTR reactor digesting primary and secondary sludges at Kur'yanovskaya municipal aeration station, Moscow, Russia (Thermo sludge); VSS concentration – 20.5 g/l; specific aceticlastic activity – 0.1 g COD/l/g VSS (55ºC).

Mineral media. The basal N-supplemented medium contained (mg/l): NH_4Cl – 280, $CaCl_2 \cdot 2H_2O$ – 10, K_2HPO_4 – 250, $MgSO_4 \cdot 7H_2O$ – 100, EDTA – 1, resazurin – 0.2, $NaHCO_3$ – 5000, H_3BO_3 – 0.05, $FeCl_3 \cdot 4H_2O$ – 2, $ZnCl_2$ – 0.05, $MnCl_2 \cdot 4H_2O$ – 0.05, $CuCl_2 \cdot 2H_2O$ – 0.03, $AlCl_3 \cdot 6H_2O$ – 2, $NiCl_2 \cdot 6H_2O$ – 0.05, $Na_2SeO_3 \cdot 5H_20$ – 0.1; yeast extract – 100; pH 7.2. The N-deprived medium contained the same components, except ammonium chloride. The solutions of microelements and vitamins prepared as described by Razo-Flores (1997) were added to the basal medium.

Batch toxicity and biodegradability experiments. The batch assays were carried out in serum bottles (0.12 l) in mesophilic (30ºC) and thermophilic (55ºC) conditions as described elsewhere (Sklyar *et al*., 1999). During the toxicity assays, acetate (2.1–2.3 g COD/l) was used as a methanogenic substrate in the mineral medium, to where the tested aromatic amines were also added in different concentrations. During the biodegradability assays, only the amine was the source of carbon.

Continuous experiments with 2-ABA. Investigations were carried out in a laboratory UASB reactor (diameter – 6.8 cm, height – 85 cm, total working volume – 2.6 l) made from transparent plastic and equipped with 6 sampling ports along the reactor height. Operating temperature of 30±1ºC was maintained by placing the reactor into thermostat "TS-80" (Russia). The mesophilic granular sludge from laboratory UASB reactor treating winery wastewater up-graded by addition (5% by volume) of 2-ABA adapted sludge was used as a seed sludge. The feed consisted of N-deprived medium and 2-ABA (2 g/l) as a unique source of carbon and nitrogen. Feeding of the reactor as well as recycling of effluent (1:1) was provided by peristaltic pump "P-3" (Pharmacia, Sweden).

Analyses. Gas composition, ethanol and volatile fatty acids (VFA) were analysed by gas chromatography (Kalyuzhnyi *et al.*, 1996). Aromatic compounds were identified and quantified by their UV-absorption at 275 (4-ABA), 295 (4-ASA), 300 (3-ABA and SA), 310 (2-ABA) and 330 (5-ASA) nm using a Shimadzu UV-1202 spectrophotometer (Japan). Ammonia concentration was also monitored spectrophotometrically with Nessler reagent at 425 nm (Lurie, 1984). Biogas production from UASB reactor was recorded by a wet gas meter "GSB-400" (Gaspribor, USSR). All gas measurements are expressed at 0ºC and standard pressure (760 mm Hg). Feed input in UASB reactor was monitored by measuring the accumulated outflow on a daily basis. Determination of specific sludge activities of the reactor sludge was performed in batch tests as described previously (Kalyuzhnyi *et al.*, 1996). All other analyses were performed using *Standard Methods* (APHA, 1985).

Results and discussion

Toxicity

According to Razo-Flores (1997), toxicity of aromatic amines toward aceticlastic methanogens is moderate - at least it was on average less than one five-hundredth than the corresponding nitroaromatics. However, our investigation showed that all the

Table 1 Relative aceticlastic activity (%) of the various sludges in the presence of aminoaromatics (means of triplicates/duplicate ± standard deviation)*

Concentration, g/l	2-ABA	3-ABA	4-ABA	4-ASA	5-ASA
Mesophilic sludge (cattle sludge)					
0	100±1	100±4	100±1	100±1	100±1
0.5	301±3	61±4	271±2	93±3	229±3
1	313±2	70±4	292±4	94±2	261±2
2	325±3	127±6	345±3	106±2	169±2
4	325±2	95±5	276±2	102±3	175±3
5	ND	ND	ND	ND	251±3
Mesophilic sludge (Shell sludge)					
0	ND	ND	ND	ND	100±2
0.1	ND	ND	ND	ND	120±2
0.5	ND	ND	ND	ND	121±2
1	ND	ND	ND	ND	137±2
3	ND	ND	ND	ND	96±4
Thermophilic sludge (thermo sludge)					
0	100±4/100±2#	100±4/100±2#	100±6/100±2#	100±4/100±2#	100±4/100±3#
0.5	ND/84±2#	ND/78±2#	49±5/138±2#	ND/108±3#	ND/74±1.3#
1	129±3/132±5#	51±3/79±2#	90±6/98±3#	43±4/88±2#	ND/58±4#
2	ND	ND/90±2#	ND	ND	ND/60±2#
3	ND	ND/120±4#	60±4/120±3#	ND	ND
3.5	ND/125±3#	ND	ND	/71±2#	ND/65±2#
4	ND	ND/94±1#	ND	ND	ND
5	88±3/179±4#	105±3/ND	173±5/132±3#	0±0/56±3#	71±1/64±3#
7	ND	ND	ND	ND	ND/111±8#

*Calculated from the slope of the steepest linear segment of cumulative methane concentration curve (initial acetate concentration – 2.14 g COD/l; the activity of the sludge not treated with a toxicant was taken as 100%);
ND – not determined;
#sludge pre-grown with acetate

aminoaromatics tested practically did not inhibit aceticlastic methanogenesis up to their concentrations of 3–7 g/l (Table 1). Moreover, some of them (2-ABA, 4-ABA and 5-ASA) even exert a stimulating effect for the Cattle and Thermo sludges. Figure 1 represents a typical example of such behaviour. It is seen that an addition of 5-ASA in the concentrations of 0.1–1 g/l led to a substantial enhancement of methane production rate (Figure 1a, points). Further increase of 5-ASA concentration also resulted in more steeper methane production curves in comparison with the control; however, it is accompanied by increase of lag-period, especially for 5-ASA concentration of 5 g/l (Figure 1b, points).

For better visualisation of the tendencies above mentioned, the experimental data of Figure 1 were treated by fitting to the integral Monod equation using nonlinear least-squares analysis (Varfolomeyev and Kalyuzhnyi, 1990). It is seen that the theoretical curves (Figure 1, lines) agree quite satisfactorily with the experimentally observed methane production curves (Figure 1, points) and the estimated values of maximum specific aceticlastic activities of the sludge and lag-periods do increase with an increase of 5-ASA concentrations (Table 2). However, it should be noted that the stimulating effect of 5-ASA and other aminoaromatics tested had a pronounced effect mainly on the cattle sludge having relatively low initial aceticlastic activity (Table 1, cattle sludge). The Shell sludge exerting moderate intial aceticlastic activity was only slightly stimulated by the presence of 5-ASA (Table 1). Analogously, the stimulating effect of aminoaromatics was significantly less pronounced for the Thermo sludge. The enhancement of methane

Table 2 Kinetic parameters estimated from the experimental data of Figure 1 by fitting to the integral Monod equation

Parameter	5-ASA concentration, g/l					
	0	0.1	0.5	1.5	3	5
*A_{max}, mg COD/g VSS/day	54.2±2.4	70.3±4.8	65.7±3.6	101.8±2.1	87.3±3.9	114.2±13.5
Lag-period, days	0.7±0.1	0.7±0.1	0.2±0.1	2.0±0.1	3.7±0.2	17.7±1.8

*A_{max} – maximum specific aceticlastic activity

Table 3 Anaerobic biodegradability of aminoaromatic acids by various anaerobic sludges

Compound	Structure	Sludge type	Biodegradability, %	Duration of incubation, days	Lag period, days
		cattle sludge	100	90	72
2-ABA		Shell sludge	100	90	60
		thermo sludge	0	120	
		cattle sludge	100	300	200
3-ABA		Shell sludge	0	120	
		thermo sludge	100	70	48
		cattle sludge	0	300	
4-ABA		Shell sludge	100	90	60
		thermo sludge	0	120	
		cattle sludge	0	180	
4-ASA		Shell sludge	0	300	
		thermo sludge	0	95	
		cattle sludge	100	60	28
5-ASA		Shell sludge	100	30	20
		thermo sludge	100	48	34

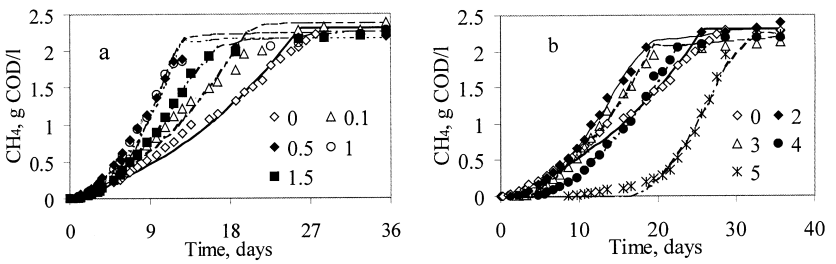

Figure 1 Influence of various concentrations of 5-ASA (g/l, figures on the graphs) on methane production from acetate (cattle sludge, 30°C). Points – experimental data corrected for blank (means of triplicates), lines – fitted curves obtained by using an integral Monod equation

production under the presence of tested aminoaromatics may be attributed to the fact that they represent the important bacterial vitamins (e.g., 2-ABA is vitamin L_1; 4-ABA is vitamin H^1 or B_x) or resemble their structures (The Merck Index, 1989). Probably other mechanisms (e.g., redox mediating capacity) play a role as well. Thus, the previously suspected toxicity of aromatic amines is not relevant for aceticlastic methanogenic bacteria.

Effect of sludge source on biodegradability

The results on anaerobic biodegradability of tested aminoaromatic (both naturally occurring (2- and 4-ABA) and xenobiotic (3-ABA, 4- and 5-ASA)) are generalised in Table 3. It

is seen that 5-ASA was completely mineralised by all the sludges used with lag periods lasting from 20 to 34 days. This relative susceptibility of 5-ASA to anaerobic mineralization by unadapted sludges was a little surprising taking into account the xenobiotic origin of this compound. On the other hand, 4-ASA was not degraded at all by any of the sludges used during 95–300 days. This finding emphasises an extreme importance of substituent position in xenobiotic compounds to be biodegraded. The different behaviour was observed for aminobenzoates. Both mesophilic sludges were able to perform 100% mineralization of 2-ABA (lag periods of 60–72 days) but this was not the case with the thermophilic sludge under duration of incubation as long as 120 days. 3-ABA was not biodegraded only in the presence of the Shell sludge, though the duration of incubation might be not long enough for the sludge adaptation. On the contrary, 4-ABA was quantitatively decomposed into methane, carbon dioxide and ammonia only by the Shell sludge.

In general, our results with the mesophilic sludges coincide with available published data. Usually 2-ABA is easily degraded under methanogenic conditions by unadapted sludge (Balba and Evans, 1980; Schelton and Tiedje, 1984). 4-ABA is partly degradable and the adaptation can be achieved (Razo-Flores, 1997). Several researches have shown that 3-ABA can be mineralised under methanogenic conditions (Kuhn and Suflita, 1989; Schnell and Schink, 1992; Razo-Flores, 1997), but the other data are conflicting (Battersby and Wilson, 1989; Horowitz *et al.*, 1981). With regard to thermophilic conditions, it should be noted that this study constitutes the first report of complete methanogenic mineralization of 5-ASA and 3-ABA.

Sludge adaptation and bioaugmentation of unadapted sludge

The duration of lag period for biodegradation of 5-ASA by unadapted Shell sludge was found to be independent of the concentrations of 5-ASA and seed sludge, addition of vitamins and microelements, co-substrates (VFA, glucose, and benzoic acid). However, once the sludge became adapted, it demonstrated relatively high rates of decomposition of target compounds (Figure 2a). For the cattle sludge (Tables 3–4), the lag phase before the start of mineralization of 5-ASA (concentration of 400 mg COD/l) was 28 days. Initial rate of methane production was 10 mg COD/l/day, the rate of 5-ASA degradation was also 10 mg COD/l/day. After 85% conversion, 5-ASA was added in a triple concentration (1,180 mg COD/l). The methane production rate increased to 22 mg COD/l/day, whereas 5-ASA degradation rate increased to 25 mg COD/l/day. When 85% of this portion was

Table 4 Degradation rates of various aminoaromatics in the presence of adapted sludges

Substrate	Sludge type	Substrate concentration, mg COD/l	Rate of substrate consumption, mg COD/l/day	Rate of methane production, mg COD/l/day
		400	10.1±0.9	10.1±0.2
	cattle sludge	1,180	25.4±0.4	22.1±0.3
5-ASA		2,256	18.4±0.5	15.4±0.2
		490	15.6±1.3	17.9±2.3
	thermo sludge	490	11.9±1.0	13.0±0.7
		490	13.0±0.7	4.6±0.3
		630	27.6±5.2	28.1±2.3
2-ABA	cattle sludge	630	60.1±4.3	ND
		630	77.5±5.8	ND
		900	101.6±13.4	94.3±2.8
3-ABA	thermo sludge	680	11.4±1.6	8.1±0.3
		680	29.0±5.3	2.5±0.3

ND – not determined

Table 5 Comparison of average times for 5-ASA depletion with adapted (A), unadapted (UA) and bioaugmented (UA+A) Shell sludges (different percentages of adapted sludge were added to the unadapted one)

	Average times for 5-ASA depletion/storage unfed, days									
Sludge	**A**	**UA**	**UA+ 0.01%A**	**UA+ 0.05%A**	**UA+ 0.1%A**	**UA+ 0.2%A**	**UA+ 0.5%A**	**UA+ 1%A**	**UA+ 2%A**	**UA+ 5% A**
1st feeding	10	30	27	26	23	23	21	21	20	15
Storage unfed	20	0	0	1	4	4	6	6	7	12
2nd feeding	16	5	4	6	6	8	9	11	16	21

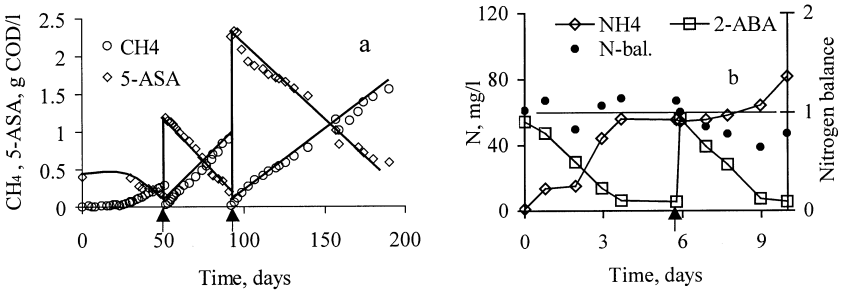

Figure 2 Anaerobic biodegradation of 5-ASA (a) and 2-ABA (b) by the cattle sludge. Arrows indicate an addition of new portions of substrate

biodegraded, a third portion of 5-ASA was added (final concentration of 2,256 mg COD/l), however, the rates of methane production and 5-ASA degradation did not increase further and were 15 and 18 mg COD/l/day, respectively (Table 4).

The enhancement of degradation of 5-ASA using a bioaugmentation of unadapted sludge scheme was studied with the Shell sludge. It is seen (Table 5, row "1st feeding") that an addition of even a small percentage of adapted sludge to the unadapted one decreased the average time of substrate depletion. However, 5-ASA adapted culture was found to be sensitive to the storage at 30ºC without supplying the additional portions of substrate (Table 5, row "2nd feeding". The delay in 5-ASA degradation after the unfed storage of adapted sludge might be attributed to the lysis of bacteria responsible for initial stage of decomposition of the target compound.

The other trends were observed for mesophilic 2-ABA adapted sludge. First, the rates of 2-ABA degradation and methane production increased from passage to passage as well as with increase of initial substrate concentration (Table 4). The other principal difference between mesophilic 5-ASA- and 2-ABA-adapted sludges was that the former quickly lost its ability to degrade 5-ASA after short (~1 day) period of absence of target compound in cultivation medium. The re-activation of such sludge required lag periods comparable to those for unadapted sludges (Table 5). On the contrary, the 2-ABA-adapted sludge can be kept unfed during several weeks without noticeable decrease of target activity.

Concerning the thermophilic sludges degrading 2-ABA and 5-ASA, the rates of substrate degradation were more-or-less constant from passage to passage, whereas the rates of methane production decreased (Table 4). No acetate or other detectable (by analytical equipment used) intermediates were found to be accumulated. More powerful analytical technique is needed to elucidate this observation.

N-requirements

All adapted mesophilic sludges were able to grow in N-free media satisfying their nitrogen needs by deamination of the corresponding aminoaromatics. An almost quantitative correlation between the disappearance of substrate and the release of ammonia has been detected. Figure 2b represents an example of nitrogen transformations when 2-ABA was used as unique source of carbon and nitrogen in the medium. The rates of substrate degradation and methane accumulation were the same as in the basal (with ammonia) medium. In the literature, there are only the data on nitro- and aminophenols degradation in the N-deprived media under methanogenic conditions (O'Connor and Young, 1993). It was shown that the initial rates of biodegradation were four times lower and the methane production was by 25% lower in the N-deprived medium in comparison with the control medium.

Cross-acclimatisation

Mesophilic 2-ABA-, 5-ASA- and SA-adapted sludges did not show an ability to degrade any other aminoaromatics tested. This fact may manifest about the different nature of key bacteria responsible for primary decomposition of various aminoaromatics. The mechanisms of biodegradation of aminoaromatic compounds under anaerobic conditions are not well studied yet and the results are contradictory. For sulphate reducing and denitrifying bacteria, the biodegradation of 2-ABA, SA and phenol was shown to have a mechanism differing from that for 4-ABA and aniline (Tschech and Fuchs, 1987; Schnell and Schink, 1991). The enriched methanogenic culture degrading 2-ABA was also able to degrade 4-ABA to a small extent, but not SA and 3-ABA (Tschech and Schink, 1988). The initial step in 2-, 3-, and 4-ABA degradation under different anaerobic conditions was found to involve the activation to the corresponding aminobenzoil-CoA, but the further metabolism is still unknown (Ziegler *et al.*, 1989; Schnell and Schink, 1991). In methanogenic cultures inhibited with bromoethanesulfonate, 2-ABA was demonstrated to be first deaminated to benzoate before ring cleavage (Tschech and Schink, 1988). In our experiments, we have not observed the accumulation of any aromatic intermediates of the biodegradation. On the contrary, the release of methane and ammonia in mesophilic conditions almost quantitatively corresponded and coincided with the disappearance of initial substrates.

Continuous mineralization of 2-ABA

The principal possibility of continuous mineralization of 2-ABA as a unique source of carbon and nitrogen has been demonstrated using a mesophilic UASB reactor inoculated with the 2-ABA-adapted sludge. 90% removal of 2-ABA (2 g/l) has been achieved under hydraulic retention time of 2 days.

Conclusions

The following conclusions can be drawn based on this study.

- All the aminoaromatics tested practically did not have any toxic effect on methanogenesis up to their concentrations of 3–7 g/l; moreover, some of them even exert a stimulating effect on aceticlastic activity, especially when not very active sludges are used.
- 5-ASA is completely mineralised independently on the sludge used though the sludge adaptation is required. On the contrary, 4-ASA was not degraded at all by any of the sludges applied. All three aminobenzoates are principally biodegradable; however, sludge source and adaptation are essential. Concerning thermophilic conditions, this study constitutes the first report of methanogenic mineralization of 5-ASA and 3-ABA.
- All the adapted sludges were able to mineralise the corresponding aminoaromatics in N-deprived media.

- Cross-acclimatisation trials show that 2-ABA-, 5-ASA- and SA- adapted sludges are unable to degrade any other tested aminoaromatics that manifest about the different nature of key bacteria responsible for primary decomposition of 2-ABA, 5-ASA, and SA.
- The principal possibility of continuous mineralization of 2-ABA as a unique source of carbon and nitrogen has been demonstrated using mesophilic UASB reactor.

Acknowledgements

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