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Synergistic degradation of acetamide by methanogens and an aerobic Gram-positive rod

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Abstract Methanogenesis from acetamide occurs through a two-step reaction in methanogenic sludges. First, acetamide is transformed to acetate and ammonia, then, acetate is converted to methane. There is evidence that the first step is performed by an aerobic sporulating rod that co-exists in the same sludge with methanogens. This finding provides new perspectives for further technological developments in the field of waste-water treatment, on the basis of the establishment of controlled relationships between aerobes and strict anaerobes.

Introduction

Acetamide is a widely used chemical in the lacquer, cosmetics, explosives, textile and pharmaceutical industries. Miscellaneous applications include its use as a stabilizer in peroxides and as a raw material in organic synthesis (Moretti 1978). It is a product of the bacterial degradation of nitrile compounds such as acetonitrile (DiGeronimo and Antoine 1976).

In anaerobic environments no information is available on the biodegradation of acetamide, particularly in anaerobic digestors. In this study, the biodegradation of acetamide by methanogenic sludges is investigated. Evidence is provided to show the necessary co-existence of aerobic and anaerobic metabolisms in the same closed environment in order to degrade acetamide.

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Materials and methods

Sources of inocula

Two types of sludges were used throughout this study. The first sludge (NT sludge) was from the neutralization tank of a sugar cane factory in the state of Michoacan (Mexico). This sludge was described previously by Guyot et al. (1993) and presents good anaerobic characteristics in terms of microbial composition. This sludge was maintained during 2 years, in batch conditions without continuous mixing except when necessary to dissolve the substrate added as a powder. Weekly, acetamide was added and one-third of the basal liquid medium (salts and vitamins, see below) was renewed. The second sludge (UASB sludge) was taken from a lab-scale upflow anaerobic sludge blanket (UASB) reactor previously fed continuously during a 2·5 month period with acetamide as the only substrate at a loading rate of 1.13 kg chemical O_2 demand (COD)/m^3 per day (Torres et al. 1992). The reactor sludge came from a 40-m^3 pilot UASB reactor treating waste-waters from a baker's yeast factory.

The enrichment procedure is important for the discussion of the results. The medium used [medium no. 1 of Balch et al. (1979) without yeast extract and biotrypcase] was boiled under an N_2 atmosphere in order to exclude dissolved O_2 (without addition of reducing agents). The medium was poured onto the sludges under an N_2 atmosphere. Gas exchange (gas products leaving and air entering) was provided by means of a needle inserted through the stopper. Therefore, while no strictly anaerobic conditions were maintained, the preliminary anaerobic precautions ensured a significant methanogenic population in the sludges, since methanogenic bacteria (fluorescent pseudosarcina and rods) were maintained during the time of the enrichment, as demonstrated by frequent direct epifluorescence microscopic observations at 420 nm. During the enrichment, an active gas production, containing methane and CO_2, was observed.

Methanosarcina barkeri 227 (OCM 35) and MS (OCM 38) and *M. mazei* S-6 (OCM 26) were obtained from the Oregon Collection of Methanogens (OCM). They were cultivated several times with acetate, before acetamide biodegradation was tried with them.

Biodegradation tests

Sixteen millilitres of Balch medium no. 1 (Balch et al. 1979), acetamide as substrate from an anaerobic stock solution, and 4 ml sludge as inoculum were anaerobically introduced into 60-ml serum bottles. The initial acetamide concentration was greater than 10 mM; the exact initial concentrations were determined by gas chromatography

and are reported in each figure. The medium was prepared anaerobically (Hungate 1969; Balch et al. 1979) and dispensed inside an anaerobic chamber (MacCoy) into the serum bottles (16 ml/bottle). The serum bottles were then closed with butyl rubber stoppers and secured with aluminium caps. The serum-bottle gas phase was a mixture of N_2/CO_2 (80:20) at 1 atm. The initial pH was between 6.8 and 7.2.

Controls with sterilized sludges as inocula were performed in order to check for possible abiotic degradation of acetamide. Under these conditions, no disappearance of acetamide and no methane production were observed during the period of the experiments. Controls without substrate but with active inocula were also undertaken. All experiments were performed in triplicate.

Analytical methods

A 0.5-ml aliquot of the contents of each serum bottle was transferred into 1.5-ml eppendorf centrifugation tubes. To this, 30 µl concentrated formic acid was added. The mixture was centrifuged at 13,000 rmp for 15 min and the supernatant liquid was frozen until the moment of the analysis. Acetamide and acetate were simultaneously determined by gas liquid chromatography using flame ionisation detection and a non-packed capillary (wide bore) column (0.54 mm, 10 m) (AT-1000, Alltech). The carrier gas was N_2 at a flow rate of 2 ml/min. The oven temperature was programmed from 120 to 170°C at a rate of 10°C/min. Integration and calibration of the peaks were carried out with a Shimadzu Chromatopac C-R3A.

Methane was determined as previously described (Guyot et al. 1990).

Ammonia was quantified colorimetrically by the Nessler method as described by Rodier (1978).

Volatile suspended solids (VSS) were analysed according to APHA (1985).

Isolation methods

The anaerobic roll-tube technique (Hungate 1969) was used to isolate the methanogens present in the enrichments. Antibiotics (penicillin, 0.5 g/l, and cycloserine, 0.1 g/l) were added to the medium for the isolation of acetoclastic methanogens. For the aerobic acetamide-degrading bacteria the roll-tube method was also used, but the medium was prepared aerobically and the bacteria isolated in the presence of air. Isolations were performed with a sludge that had been transferred twice under strictly anaerobic conditions. Microscopic observations were done with an epifluorescence Olympus BH2-RFCA microscope. Methanogens were observed at 420 nm.

Results

First transfer of the enrichments under strictly anaerobic cultivation conditions

With the sludges from the enrichments, the kinetics of the acetamide degradation and product formation were determined under strictly anaerobic conditions. For both sludges, methane production from acetamide is characterized by ammonia accumulation and a transient production of acetate (Fig. 1a and b). According to the kinetics profiles, methane is not directly coupled to acetamide removal. Therefore, a two-step conversion reaction may be postulated. First, the transformation of acetamide to ammonia and acetate, and, second, the methanogenesis from acetate. The UASB sludge presented a higher specific activity for acetamide degradation and methanogenesis than the NT sludge (Table 1).

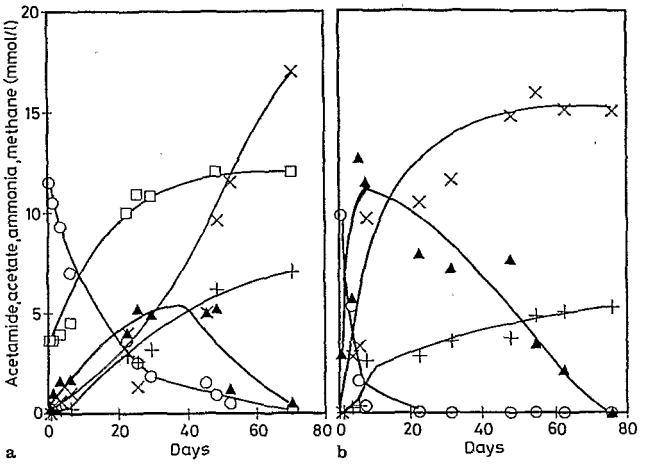

Fig. 1a, b Kinetics of methanogenesis from acetamide by enriched sludges after a first transfer under anaerobic conditions: o acetamide, ▲ acetate, □ ammonia, × methane, + methane control. **a** Neutralization tank (NT) sludge. **b** Upflow araerboic sludge blanket (UASB) sludge

With NT sludge, an additional experiment was performed using the same conditions, except that the medium was not supplemented with ammonia. Similar specific activities of acetamide degradation and methane production were obtained with and without ammonia (Table 1). The apparent substrate saturation constant (K_s) for acetamide degradation by the UASB sludge was 115.5 mM (Fig. 2).

Successive transfers under strictly anaerobic cultivation conditions of the sludges from the first experiments

Sludges from the previous kinetics experiments were successively transferred to anaerobic media in serum bottles. For each transfer, the time-course degradation of acetamide was followed. Surprisingly, as shown in Fig. 3a and b, a progressive decrease in acetamide degradation was observed. At the fourth kinetics experiment with both sludges, acetamide was not degraded.

Table 1 Specific activities (*Asp*) of acetamide degradation and methane production by the sludges at the first transfer in anaerobiosis (*VSS* volatile suspended solids, *NT* neutralization tank, *UASB* upflow araerobic sludge blanket)

	Asp (mmol/h/g VSS)	
Sludges	Acetamide	Methane
NT sludge		
With ammonia	0.117	0.087
Without ammonia	0.110	0.083
UASB sludge	0.514	0.140

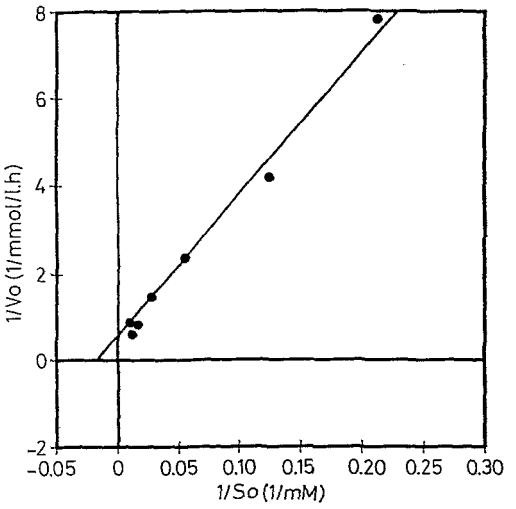

Fig. 2 Lineweaver–Burk plot of the kinetics of acetamide degradation by UASB sludge after a first transfer under anaerobic conditions (V_0, initial reaction rate per unit volume, S_0, initial substrate concentration)

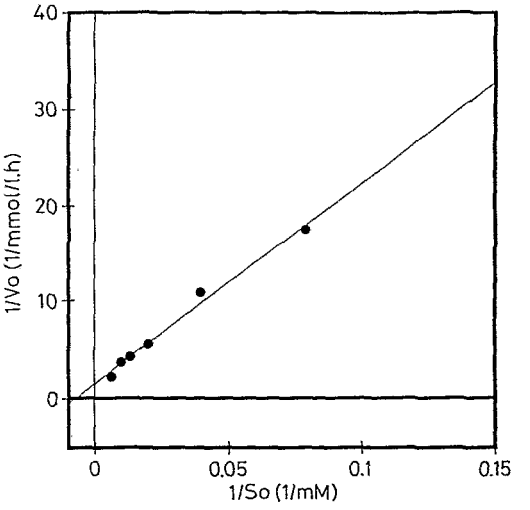

Fig. 4 Lineweaver–Burk plot of the kinetics of acetamide degradation by the strictly aerobic sporulating rod isolated from UASB sludge

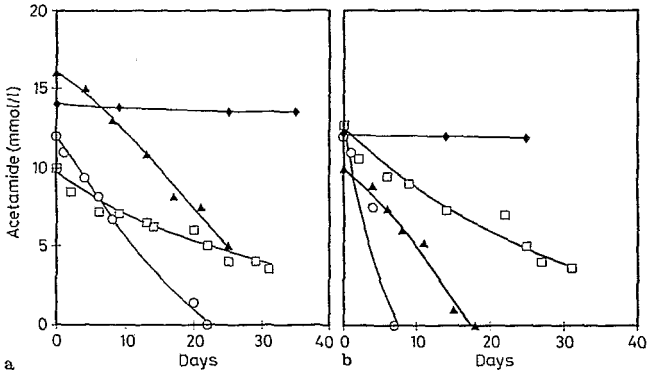

Fig. 3a, b Effect of successive anaerobic transfers from enrichments on acetamide degradation o 1st, ▲ 2nd, □ 3rd, ◆ 4th transfers. a NT sludge. b UASB sludge

The introduction of air at this moment did not restore the acetamide degradation. Similar experiments made with different yeast extract and biotrypcase concentrations, different reducing conditions (with and without cysteine), and with the addition of sulphate, gave exactly the same results: the acetamide degradation always stopped after several transfers.

Isolation of the acetamide-degrading bacteria

Both sludges contained methanogens (rods and pseudosarcina) as observed by epifluorescence microscopy. Using visible light microscopy, a sporulated rod was also observed. *Methanosarcina* and *Methanobacterium* species were isolated from both sludges with acetate and H_2/CO_2 as substrates, respectively. Both were unable to use acetamide. Furthermore, because of the ability of *Methanosarcina* species to use acetate and methylamines, an effort was made to cultivate pure cultures of *M. mazei* S-6 and *M. barkeri* 227 and MS with acetamide as the substrate. No growth or methane production was observed.

The isolation of anaerobic bacteria growing on acetamide was unsuccessful. The isolation of acetamide-degrading bacteria under aerobic conditions was attempted with sludges previously transferred twice under anaerobic conditions. An aerobic Gram-positive sporulating rod, which was unable to grow anaerobically, was isolated from each sludge. Both isolates were very similar. The detailed characterization of the rod from the UASB sludge is presented elsewhere.This bacterium had a K_s for acetamide of 100 mM (Fig. 4).

Discussion

At first, it might appear that the mechanism of acetamide degradation was entirely anaerobic, since methane was evolved during the experiments. The specific activities of acetamide degradation and methane production were low. In the case of the NT sludge, the absence of ammonia did not affect the kinetics. The fact that the UASB sludge presented higher specific activities than the NT sludge might be explained by their origin, since UASB reactors are known to promote a very dense bacterial population (Lettinga et al. 1980). This is supported by the observation that the acetamide-fed bench-scale UASB reactor had a high COD removal efficiency of 96% (Torres et al. 1992).

The presence of hydrogenophilic methanogens in the sludges cannot be explained on the basis of the two-step reaction described here. Interspecies hydrogen transfer from acetamide is not expected because of the

kinetics characteristics. Zinder (1990) described a thermophilic methanogenic syntrophic association that degrades acetate by interspecies hydrogen transfer. The first member of this co-culture was a rod that transformed acetate to H_2CO_2, the second member was a hydrogenophilic methanogen. It would be probable that such a reaction occurs in mesophilic anaerobic environments, but our isolations did not focus on this hypothesis. Furthermore, according to the results, consumption of acetamide by *Methanosarcina* species must be discarded.

The strictly aerobic Gram-positive spore-forming rod that degrades acetamide, apparently belongs to the genus *Bacillus*. This bacterium presented a similar K_s to that found for the UASB sludge. Mass-transfer resistance can account for the difference. With acetate, similar results were found: disrupted granular sludges had a higher affinity for acetate than raw granular sludges (Dolfing 1985). Nawaz et al. (1989) isolated a different type of acetamide degrading bacterium from soil, *Pseudomonas putida*, which also consumed acetonitrile with a K_s value of 40.61 mM, but no data were presented in relation to acetamide.

Considering the bacteria observed in the sludge, the results of the kinetic experiments and the K_s values, it may be assumed that the isolated strictly aerobic rod was responsible for the acetamide degradation in both sludges. This would explain the progressive decrease in acetamide degradation activity after several successive transfers under strictly anaerobic conditions. At the first anaerobic transfer of the enrichments, enough O_2 was present in the sludges. At the next transfers, the small amount of O_2 was progressively depleted, but sufficient deamidase activity might still have been present in the inocula to allow the degradation of acetamide at decreasing rates up to the fourth anaerobic sludge transfer.

The fact that a strict aerobe survived for several months in a methanogenic sludge may be explained by the enrichment conditions. For the NT sludge, no strictly anaerobic conditions were established. For the UASB sludge, the reactor was continuously fed an oxygenated medium. Furthermore, preliminary results indicate that the aerobic rod is able to grow with acetate (final optical density of growth experiments: with acetate, 0.24; control without acetate, 0.03). Other carbon sources might have been provided by hydrolysed dead bacteria. It is also worthwhile mentioning that this rod grows very well with yeast extract and biotrypcase.

Previous works corroborated the co-existence of both aerobic and strictly anaerobic bacteria in the same environment. The presence of different kinds of strict anaerobes in aerobic activated sludges (Guyot et al. 1993) and their ability to grow in oxygenated liquid medium, protected by the structure of activated sludge, have been demonstrated (Guyot and Fajardo 1993). This was explained by the existence of anaerobic micro-niches in aerobic sludges, due to the production of exopolymeric substances, and the adjustment of the O_2 consumption rates to the O_2 diffusion rates into the liquid phase (Guyot and Fajardo 1993). Methanogens within a defined immobilized consortium of bacteria produced methane when cultivated in an aerobically prepared medium, in which *Escherichia coli* was pregrown in order to remove the dissolved O_2 (Jones et al. 1984). Gerritse et al. (1990) were able to co-cultivate in a chemostat aerobic and anaerobic (methanogenic) bacteria.

The presence of aerobes in anaerobic sludge is well known (Toerien 1967; Garcia et al. 1982). Toerien (1967) isolated several aerobic *Bacillus* and *Pseudomonas* species from anaerobic digestors fed with oxygenated waste-water. It has been recently shown that sulphate-reducing bacteria, usually known as strict anaerobes, were able to use O_2 as an electron acceptor (Dilling and Cypionka 1990), which may open new perspectives about their ecological significance in digestors. However, the different possible roles of aerobic and microaerophilic reactions in anaerobic digestors have not been emphasized. In many cases it is obvious that their action is limited to being O_2 scavengers, but the present study demonstrates that they may have other physiological roles.

The consequences of this work, in the field of anaerobic digestion, is important because it is the first time that in a similar ecosystem (UASB reactor or NT sludge), evidence is presented to show that methanogenesis can only proceed when it is necessarily coupled with an aerobic reaction as a first step. Since most of the waste-waters feeding anaerobic digestors are not previously deoxygenated, some hydrolytic reaction may be performed or initiated in the presence of dissolved O_2. These reactions will be more significant in the field of anaerobic treatment of xenobiotic and recalcitrant compounds. In view of these results, it is not surprising that a bacterium of the genus *Pseudomonas* that degrades terephthalic acid (PTA) was isolated from an UASB digestor treating PTA-manufacturing-plant waste-waters in China (Ton and Bai 1990).

From the microbiological evidence, it may be assumed that further developments of the available technologies for treating waste-water could emerge, which will closely associate aerobic and methanogenic reactions within the same reactor, by means of controlled aeration of waste-waters before feeding the digestor. Furthermore, adding exogenic aerobes with peculiar hydrolytic properties (e.g. *Pseudomonas*) to anaerobic sludges might provide a way of improving digestor efficiencies. Notwithstanding, a comparative screening of the kinetics of aerobic and anaerobic degradation reactions of pollutants would be needed in order to evaluate the range of applications of this concept.

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