Methanosaeta fibers in anaerobic migrating blanket reactors

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Abstract An anaerobic migrating blanket reactor (AMBR) was seeded with flocculent biomass from a digester and fed a substrate consisting of volatile fatty acids and sucrose to study granulation. After three months of operation, a mature granular blanket developed in the reactor. Moreover, fibers of approximately 1 cm long had become prevalent in the AMBR. Scanning electron microscopy (SEM) and light microscopy revealed a very dense structure consisting of bundles of filaments resembling *Methanosaeta* cells. Further studies with fluorescence in-situ hybridization (FISH), showed that *Methanosaeta concilii* was the predominant microorganism in these fibers.

Keywords Anaerobic migrating blanket reactor; AMBR; fibers; oligonucleotide hybridization probes; ribosomal RNA; *Methanosaeta*

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Introduction

During the last 30 years, anaerobic systems that rely on the separation of solids retention time (SRT) from hydraulic retention time (HRT) have proven to be sustainable. Particularly, the upflow anaerobic sludge blanket (UASB) process, and its derivatives, have demonstrated excellent performance in numerous full-scale operations worldwide (Lettinga, 1995). These systems are dependent on the immobilization of biomass to retain slow growing microbial populations in the reactor. Immobilization of biomass without a carrier material was first observed in UASB reactors as the formation of dense, spherical biofilms, called granules. Granules consist of conglomerates of anaerobic microorganisms that are still visible as separate entities after settling (Dubourguier *et al.*, 1987). Despite the success of UASB reactors, the need for other systems that allow for biomass immobilization without carrier material has become obvious for a variety of reasons. For example, non-compartmentalized reactors, such as the UASB process, often lose biomass with the effluent due to excessive bed expansion or poor granulation (Guiot and van den Berg, 1985). Therefore, a new high-rate anaerobic compartmentalized system, called the anaerobic migrating blanket reactor (AMBR), was recently developed to address some of the concerns associated with UASB reactors (Angenent and Dague, 1996).

The AMBR is a flow-through reactor consisting of three to five compartments and is operated by reversing the flow periodically (Figure 1) (Angenent and Dague, 1996). No hydraulic upflow pattern is required for mixing and development of granules, which eliminates the gas-solids-separator and feed-distribution systems that are required for UASB reactors. Only intermittent, gentle mechanical mixing is required to accomplish sufficient contact between substrate and biomass. Non-acidified carbohydrate containing waste streams can be treated in the AMBR at much higher loading rates than in a UASB reactor. The higher shear of mechanical mixing compared to the shear generated by the upflow pattern in the UASB reactor sloughs off more acidogens that attach to the outside layers of granules. The abundance of acidogens attached to granules in UASB reactors can create bulking, which is defined here as low-buoyancy rising of the sludge blanket as clumps of

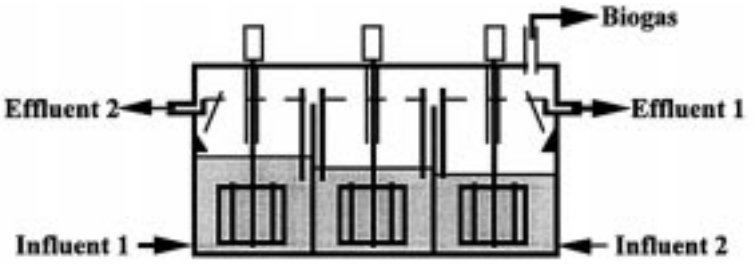

Figure 1 Schematic diagram of a three-compartment anaerobic migrating blanket reactor (AMBR)

granular biomass. Therefore, it is often necessary to pre-acidify carbohydrate wastewater before feeding to UASB reactors (Alphenaar, 1994). The flow-through design of the AMBR allows faster migration of flocculent biomass compared to granular biomass resulting in the wash out of less settleable, flocculent biomass (Angenent and Dague, 1996). To eliminate accumulation of biomass in the final compartment of the AMBR, the flow is reversed periodically. Reversing the flow is not only advantageous to accomplish migration of biomass, but also to maintain the volatile fatty acids concentrations relatively low and pH levels close to neutral in the initial compartments. Therefore, recycling of effluent is eliminated, which enhances plug-flow conditions in the reactor. Recent studies with a five-compartment AMBR, fed a carbohydrate-rich wastewater, resulted in very high removal efficiencies at a high volumetric loading rate of 45 g COD/litre/day (Angenent *et al.*, in preparation). To our knowledge, no other system that can treat a carbohydrate-rich, non-acidified waste stream at these loading rates has been described.

Efficient granulation is critical for the performance of the AMBR and is the result of microbial and hydraulic selection processes. The substrate types, substrate concentrations, and other environmental factors in each compartment determine which microorganisms will be present (microbial selection processes). As discussed above, selection of granules is accomplished through separation of flocculent biomass from better settling biomass due to settleability differences and shear generated by mixing (hydraulic selection processes). In a previous study, granules were observed as early as two months after startup in an AMBR seeded with flocculent anaerobic digester sludge and fed a carbohydrate-rich, non-acidified wastewater (Angenent *et al.*, 1997). This study demonstrated that fast granulation was possible with an AMBR. The treatment of this waste stream with a UASB reactor would likely have resulted in bulking problems.

Microscopic evaluation of samples taken from an AMBR fed a mixture of sucrose, acetate, propionate, and butyrate indicated that very dense and long fibers consisting of *Methanosaeta*-like cells were very prevalent in the biomass of this reactor. Since the conditions in the AMBR were not consistent with conditions believed to provide a competitive advantage for *Methanosaeta* species, we used a phylogenetic approach, including small-subunit (SSU) ribosomal RNA (rRNA) targeted oligonucleotide probe hybridizations, to confirm the presence of these organisms and to determine their importance in the AMBR process.

Materials and methods

A three-compartment, 54 litre AMBR was inoculated with flocculent anaerobic digester sludge (primary digester sludge, City of Ames wastewater treatment plant, Ames, IA, USA). The AMBR was operated at 35ºC and was fed a synthetic wastewater consisting of sucrose, acetate, propionate, and butyrate in a 1:1:1:1 ratio based on mass of COD

(Angenent *et al.*, 1997). A minimum of three compartments was required for the AMBR to feed the middle compartment for a certain amount of time before the flow was reversed. In this way, a breakthrough of substrate was prevented. Therefore, the middle compartment was fed for two hours between reversing the flow. The flow was reversed three times per day. Two automatic ball valves, with an internal diameter of 2.54 cm, were used to open and close effluent ports (True blue electric actuator model EBV-6, Plast-o-matic valves Inc., Cedar Groove, NJ, USA). Sufficient contact between biomass and substrate was maintained using intermittent mixing. Mixers (Model 5vb, EMI Inc., Clinton, CT, USA) were able to start and operate at a slow speed (30 rotations per minute; rpm) and the use of paddles further enhanced gentle mixing. All pumps used were Masterflex pumps of Cole Parmer Instrument Co., Chicago, IL, USA. Timers (ChronTrol Corporation, San Diego, CA, USA) regulated the operation. The specific methanogenic activity of fibers taken from the AMBR was assessed with the "headspace method" according to tests described by Rinzema *et al.* (1988).

For fluorescence *in situ* hybridizations (FISH), samples with high levels of fibers consisting of Methanosaeta-like cells were fixed in 4% paraformaldehyde (de los Reyes *et al.*, 1997). A tetra-methyl-rhodamine-isothiocynate (TRITC) labeled domain-specific probe for Archaea and a fluorescein isothiocyanate (FITC) labeled species-specific probe for *Methanosaeta concilii* were used for FISH and the fluorescent signal was analyzed with an epifluorescence microscope (Axioskop, Carl Zeiss, Germany) and a charge coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ, USA) (de los Reyes *et al.*, 1997).

Sample preparation for scanning electron microscopy (SEM) involved fixation over night at 4ºC by placing the fibers and granules in 2% paraformaldehyde, 2% glutaraldehyde, and anaerobic 0.05 M cacodylate buffer. The fixed samples were then washed with the same buffer three times and again fixed with 1% osmium tetroxide for one hour. Next, the samples were dehydrated with a graded series of ethanol in distilled water from 50 to 100% (v/v). Then the specimens were placed in 100% ethanol and critical point dried in CO_2. The prepared specimens were mounted on aluminium stubs and were sputter-coated in a Polaron E5100 (VG Microtech, UK), with platinum/palladium target (60:40). A Jeol JSM-5800LV scanning electron microscope (Japan) was used for the analysis.

Results and discussions

After three months of operating a lab-scale AMBR with a synthetic wastewater consisting of sucrose, acetate, propionate, and butyrate, fibers of approximately 1 cm long had become prevalent SEM and light microscopy revealed a very dense structure consisting of bundles of filaments resembling *Methanosaeta* cells (Figure 2b). The specific methanogenic activity (using acetate as the substrate) of a sample consisting of these fibers was found to be 1.3 g COD/g VSS/day. Bundles of *Methanosaeta* have previously been observed in chemostats fed only acetic acid (e.g., Huser *et al.*, 1982). However, the AMBR was fed a mixture of several substrates, suggesting that other trophic groups also should have been abundant. Furthermore, acetate concentrations were generally above the level believed to provide a competitive advantage for *Methanosaeta* (over *Methanosarcina*). Therefore, we were surprised to find a high prevalence of fibers apparently consisting of only *Methanosaeta*-like cells.

Two oligonucleotide probes, one for Archaea and one for the species *M. concilii*, were combined for FISH analysis of a fiber sample. This experiment confirmed that the fibers consisted of *M. concilii* (Figure 2f). We previously determined that granules from a five-compartment AMBR fed non-acidified sucrose contained up to 45% and about 15–20% methanogen rRNA in the middle and outside compartments, respectively (Angenent *et al.*, in preparation). None of the compartments contained *Methanosarcina* species, despite relatively high levels of acetate in all compartments (the AMBR was operated at minimum

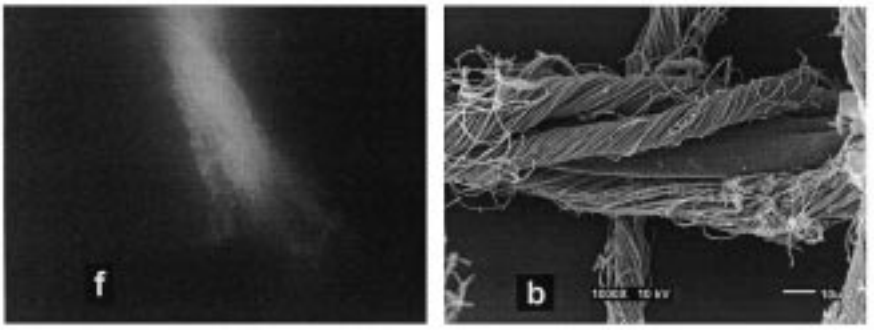

Figure 2 Epifluorescence and SEM images of microbial fiber samples from an AMBR. (f) epifluorescence micrograph of a FISH experiment with a TRITC (red) labeled probe for Archaea (S-D-Arch-0915-a-A-20) and a FITC (green) labeled probe for *M. concilii* (S-S-M.con-0381-a-A-22); superimposition of image fields obtained with the TRITC and FITC filter sets results in a predominantly yellow fiber, indicating that most cells comprising the fiber belong to the species *M. concilii*; (b) SEM micrograph, showing the compact structure of the fibers, which were twisted around a non-microbial object

acetate concentrations of 600 mg/litre for a period of three months). *M. concilii* was the most prevalent methanogen in this AMBR and its relative rRNA abundance was equal to the relative rRNA abundance of the methanogens belonging to the order Methanosarcinales, indicating that all aceticlastic methanogens were of the genus *Methanosaeta*.

The AMBR is a high-rate, continuously-fed anaerobic reactor in which granules are formed without a hydraulic upflow pattern. This finding is important because it was previously believed that a hydraulic upflow pattern was necessary to select for a granular blanket. The results obtained so far suggest that the unique reactor configuration and operational conditions of the AMBR select for the formation of granules and biofilms with non-conventional characteristics. For example, the finding that *Methanosarcina* species were absent and *Methanosaeta* species were very abundant, while acetate concentrations were relatively high in some compartments was unanticipated. This is significant from an operational standpoint, because *Methanosaeta* cells exhibit better settleability than *Methanosarcina* cells. Furthermore, the growth of Methanosaeta cells as long fibers was unexpected. The intermittent, mechanical mixing and altering of high and low acetate concentrations (feast and famine conditions) may have induced the formation and stability of the fibers, but further work is needed to elucidate the ecological strategies responsible for this phenomenon. Molecular tools will continue to be used to determine how abundant *Methanosaeta* spp. are relative to other microbial populations, and what the role of the fibers are in the process of granulation and reactor operation.

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