Biofilm reactor technology for low temperature anaerobic waste treatment: microbiology and process characteristics

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Abstract The microbial composition, methanogenic activity and architecture of particulate and fixed biofilms within four anaerobic hybrid reactors, R1–R4, operating at psychrophilic temperatures were investigated. The reactors treated low-strength (1 g COD l^{-1}; R1) and high-strength (10 g COD l^{-1}; R2–R4) wastewaters from the food-processing sector (R1, R2 – whey; R3 – sucrose; R4 – volatile fatty acids). Successful start-up and long-term psychrophilic operation was observed for all four reactors, with COD removal efficiencies of 80–99% achieved at 12–20 °C at organic loading rates of 1.3–20 kg COD $m^{-3} d^{-1}$. The formation and maintenance of a well-settling granular sludge bed and an attached biofilm were shown to occur under psychrophilic conditions, an important consideration for the successful implementation of low temperature biofilm reactor technology. Culture-independent molecular techniques (terminal restriction fragment length polymorphism, clone library analysis and 16S rRNA gene sequencing) revealed that microbial population structure could be a key factor in reactor performance, with changes in the community structure of the three high-strength reactors preceding granular instability and a subsequent decline in COD removal efficiency. Biomonitoring of microbial population structure and dynamics within anaerobic reactors may, therefore, allow for the early recognition of potential operational problems.
Keywords 16S rRNA genes; biofilm reactors; granular sludge; psychrophilic wastewater treatment

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Introduction

Biofilm reactors are among the oldest and most efficient methods of wastewater treatment. High levels of active biomass retention within these systems, either by immobilisation onto inert carriers (anaerobic filter; Young, 1991) or by self-immobilisation of the microbes (upflow anaerobic sludge bed; Lettinga and Hulshoff Pol, 1991), allows the application of high organic loading rates at low hydraulic retention times. Recent advances in biofilm reactor design and operation have significantly increased the applicability of the technology and practical applications of biofilm-based systems for wastewater purification have become more common and varied (McHugh *et al.*, 2003a). However, due to the social and economic requirement for low-cost, low-technology treatment systems, there remains a constant state of, and need for, development and improvement, making this area one of the most active in environmental biotechnology (O'Flaherty and Lens, 2003).

Low temperature (psychrophilic) anaerobic digestion is one of the most promising future areas for the application of biofilm reactor technology, offering both technical and economical benefits, particularly for the treatment of the wide variety of industrial wastewaters discharged at low or ambient temperatures. Research to date in the area of psychrophilic waste treatment is, however, limited with almost all studies focusing on the more established areas of mesophilic (30–40 °C) and/or thermophilic (50–60 °C) treatment. In the present study, the feasibility of psychrophilic anaerobic digestion as a treatment option for the food-processing sector was investigated using four anaerobic biofilm reactors. The microbial community structure and dynamics of the four reactors were

monitored, over a long-term trial period, using molecular microbiological techniques, in order to ascertain basic information on the structure, function and biological properties of biofilms involved in psychrophilic anaerobic wastewater treatment. In addition, specific methanogenic activity (SMA) profiling, microscopy and physico-chemical (extracellular polysaccharide content) analysis of biofilm samples removed from all the reactors were carried out.

Methods

Reactor operation

Four 3.8 l anaerobic hybrid reactors (R1–R4) were used in this study, as described previously (McHugh *et al.*, 2004). The operating conditions and feeding regimes for the reactors are outlined in Table 1. R1 and R2 were fed a whey-based wastewater (R1 – 1 g COD l^{-1}; R2 – 10 g COD l^{-1}) and operated for 500 days. The initial organic loading rates (OLRs) applied to R1 and R2 were 0.5 kg COD $m^{-3} d^{-1}$ and 5 kg COD $m^{-3} d^{-1}$, respectively, and these were increased to 1 kg COD $m^{-3} d^{-1}$ and 1.3 kg COD $m^{-3} d^{-1}$ for R1 and to 10 kg COD $m^{-3} d^{-1}$ and 13.3 kg COD $m^{-3} d^{-1}$ for R2, on days 83 and 167 of the trial, respectively. This increase in OLR was achieved by a stepwise reduction of the hydraulic retention time (HRT) from 2 days to 1 day and, finally, to 18 h. The initial operating temperature of R1 and R2 was 20 °C and this was decreased during the trial to 18 °C, 16 °C, 14 °C and 12 °C on days 257, 340, 354 and 410, respectively. The initial upflow velocity applied to R1 and R2 was 5 m h^{-1} and this was increased, in a stepwise manner, to 7.5 m h^{-1}, 10 m h^{-1} and 12.5 m h^{-1} on days 4, 134 and 448 of the trial, respectively.

R3 was fed a sucrose-based wastewater (10 g COD l^{-1}) and R4 was fed a volatile fatty acid (VFA)-based wastewater, composed of acetate, ethanol, propionate and butyrate (1:1:1:1; 10 g COD l^{-1}), over a 300 day trial period. Both reactors were operated at a HRT of 12 h for the duration of the trial, corresponding to an OLR of 20 kg COD $m^{-3} d^{-1}$. The initial temperature of both reactors was 37 °C and this was decreased to 30 °C, 25 °C, 20 °C, 18 °C and 16 °C on days 45, 98, 167, 184 and 198 of the study, respectively. The liquid upflow velocity applied to R3 and R4 was increased from 3.5 m h^{-1} to 5 m h^{-1} to 7.5 m h^{-1} on days 118 and 236 of the study, respectively (McHugh *et al.*, 2004).

The lower section of each reactor contained a granular sludge bed, which was expanded using an effluent recirculation facility. A fixed-film section, comprised of randomly packed polyethylene rings, was added to R1 and R2 on day 238 of the study and to R3 and R4 on day 1 of the study. Biogas and effluent samples were routinely removed from the reactors and methane, volatile fatty acid and ethanol content were analysed by gas chromatography (McHugh *et al.*, 2004). Volatile suspended solids (VSS), pH and COD were determined according to *Standard Methods* (APHA, 1992).

Molecular analyses of sludge biomass

Biomass samples were routinely removed from the upper (fixed biofilm) and lower (granular sludge bed) chambers of the reactors and DNA was extracted using the MoBio Soil

Table 1 Operational and performance characteristics of R1, R2, R3 and R4

	Temp (°C)	Feed	OLR (kg COD $m^{-3} d^{-1}$)	HRT (h)	Upflow velocity (m h^{-1})	COD removal (%)	Biogas CH_4 (%)
R1	12–20	Whey (1 g COD l^{-1})	0.5–1.3	18–48	5–12.5	60–90	60–80
R2	12–20	Whey (10 g COD l^{-1})	5–13.3	18–48	5–12.5	40–98	40–60
R3	16–37	Sucrose (10 g COD l^{-1})	20	12	3.5–7.5	40–90	25–60
R4	16–37	VFA (10 g COD l^{-1})	20	12	3.5–7.5	40–99	40–90

DNA extraction kit (Cambio), according to the manufacturer's instructions. Terminal restriction fragment length polymorphism (TRFLP) analysis was carried out on all samples, as described previously (Collins *et al.*, 2003; McHugh *et al.*, 2004). Archaeal and bacterial clone libraries were generated on the seed sludge and on the final biomass samples removed from R1–R4 (McHugh *et al.*, 2004) and 16S rRNA gene sequencing of the clones obtained and phylogenetic analysis of the retrieved sequences were carried out, as described previously (McHugh *et al.*, 2003b, 2004).

Aggregate and fixed biofilm characteristics

Specific methanogenic activity (SMA) was determined for aggregate and fixed biofilm biomass, as described by Collins *et al.* (2003). Tests were carried out, in triplicate, at 37 °C, 22 °C, 15 °C and 12 °C. The EPS content of the sludge biomass samples was quantified by chemical analysis of the uronic acid content (De Beer *et al.*, 1996). Flotability and settling velocity tests, size distribution and scanning electron microscopy were also performed (O'Flaherty, 1997).

Results and discussion

Reactor performance

A summary of the performance characteristics of R1–R4 is shown in Table 1. The feasibility of psychrophilic anaerobic digestion for the treatment of low strength (1 g COD l^{-1}) wastewaters was demonstrated by R1, with COD removal efficiencies of 80% and biogas methane values of 70% obtained at 12 °C ((OLR): 0.5–1.3 kg COD $m^{-3} d^{-1}$; Figure 1a). The successful anaerobic treatment of high strength (10 g COD l^{-1}) wastewaters was also demonstrated, with COD removal efficiencies of 95% achieved for the treatment of volatile fatty acid (VFA)-based wastewaters (OLR: 20 kg COD $m^{-3} d^{-1}$) at 18 °C, 80% achieved for the treatment of sucrose-based wastewaters (OLR: 20 kg COD $m^{-3} d^{-1}$) at 18 °C and 98% achieved for the treatment of whey-based wastewaters (OLR: 5–13.3 kg COD $m^{-3} d^{-1}$; Figure 1b) at 14 °C. Rapid start-up and stable long-term reactor performance was demonstrated in all four reactors, following start-up at both low temperatures (20 °C – R1, R2) and at mesophilic temperatures (37 °C – R3, R4), followed by a stepwise reduction in operating temperature. Towards the end of the trial, however, following a shift in the microbial population structure of the biofilms within the systems, a decline in reactor performance was observed in the three reactors treating the high-strength wastewaters (Figure 1b; McHugh *et al.*, 2004).

Reactor design and, in particular, effective biomass retention appeared to be an important factor in the successful implementation of psychrophilic anaerobic digestion. Elevated liquid upflow velocities visibly enhanced mixing within the systems, increasing substrate–biomass contact, and resulting, generally, in improved reactor performance. Addition of the fixed-film section to the upper chambers of the reactors served to stabilise and increase COD removal efficiency, primarily by decreasing effluent propionate levels. This was illustrated, for example, by a decrease in the propionate concentration in R2 effluent from 500–1,500 mg l^{-1}, prior to addition of the fixed-film section, to <200 mg l^{-1} propionate, subsequent to addition of the fixed film section (data not shown). The bulk of influent mineralisation, however, occurred within the granular sludge bed in the bottom chamber of the reactors, as determined by COD measurements of effluent discharged from both the middle and upper sections of R1–R4. The fixed-film section allowed for increased biomass retention within the system, as indicated by lower suspended solids concentrations in reactor effluents (total COD values; data not shown), an important factor in anaerobic wastewater treatment, due to the slow growth rates of acetogens and methanogens, particularly at psychrophilic temperatures.

Microbial composition of sludge biomass

High levels of *Methanosaeta* sp. were detected in all four reactors at the beginning of the trial (Figures 1–3). The predominance of methanogens closely related to *Methanosaeta concilii* in anaerobic sludges is widely reported and these filamentous organisms are regarded as being important for the formation and maintenance of granular sludge (McHugh *et al.*, 2003b). High levels of *Methanosaeta* sp. persisted in R1, the low-strength reactor, throughout the trial (Figures 1a, 2a), corresponding to the granular nature of R1 biomass and, also, to the low acetate levels prevailing within the reactor throughout the trial (data not shown).

In the three high-strength reactors, R2–R4, a distinct shift in archaeal population structure was noted, with a decrease in the relative abundance of *Methanosaeta* sp. and a proliferation of the hydrogenotrophic methanogen, *Methanomicrobiales* sp. (*Methanocorpusculum parvum*-like sp.; Figures 1b, 2b, 3). This shift was associated with stressed conditions within the reactors (McHugh *et al.*, 2004). The reduction in the relative abundance of acetoclastic methanogens present in R2–R4, as illustrated by TRFLP (Figure 2b), clone library analysis (Figure 3) and specific methanogenic activity tests (Table 2), appeared to confer an increased susceptibility to process disturbance on the system, with a decline in reactor efficiency observed subsequent to the population change (Figure 1b; McHugh *et al.*, 2004). Balanced and successful anaerobic treatment appears to require the presence of both methanogenic groups, particularly during perturbations to the system.

As the shift in population structure occurred prior to the decline in reactor performance (Figure 1b), it would also appear that, to some extent, the biomonitoring of microbial populations within reactor sludge may allow for the early recognition of potential granulation or operational problems. In R2, the high-strength whey reactor, a re-emergence of *Methanosaeta* sp. was observed towards the end of the trial (Figures 1b, 2b), associated with the formation of granules from the disintegrated sludge within the reactor, and a decrease in effluent acetate concentrations over the final days of the study (data not shown).

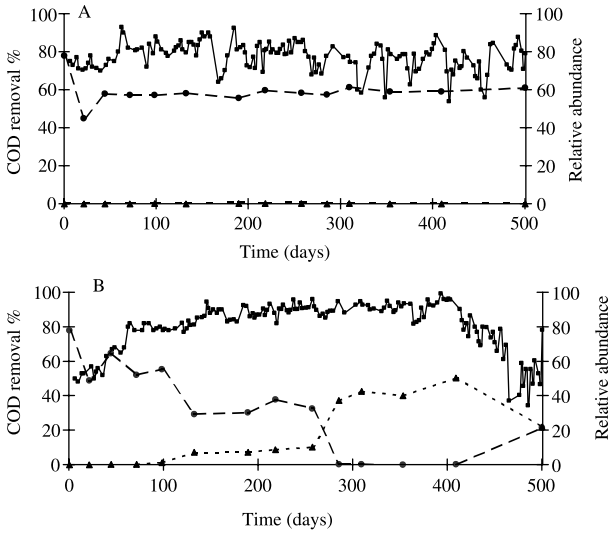

Figure 1 COD removal efficiencies achieved (–■–), relative abundance of *Methanosaeta* sp. within the archaeal community (–•–) and relative abundance of *Methanomicrobiales* sp. within the archaeal community (…▲…) of (A) R1 and (B) R2 during the trial

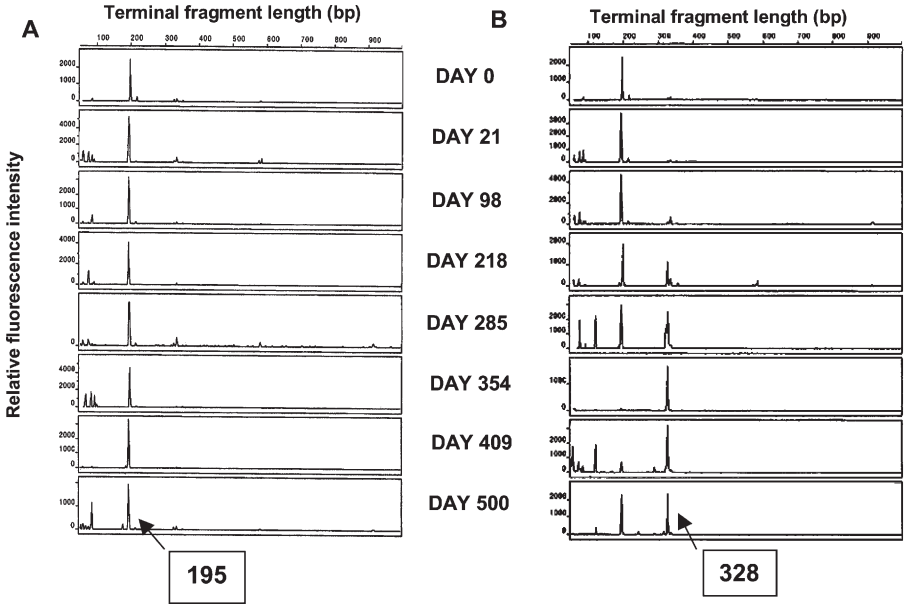

Figure 2 Archaeal TRFLP profiles generated from the restriction of the forward primer generated PCR products with *Hha* for (A) R1 biomass and (B) R2 biomass removed from the base of the reactors. (195 – *Methanosaeta* sp.; 328 – *Methanomicrobiales* sp.)

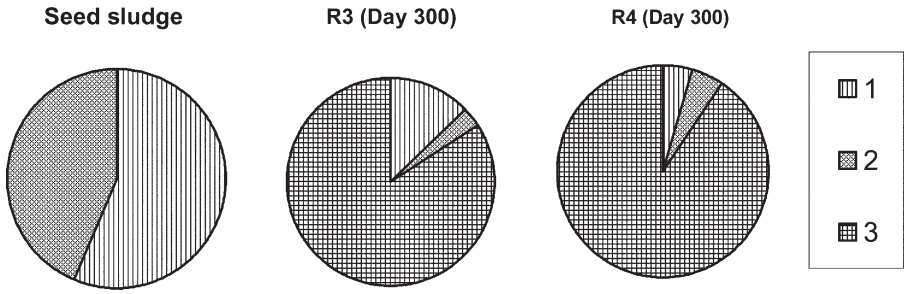

Figure 3 Percentage abundance of different archaeal species present within the seed sludge used to inoculate R3 and R4 and R3 and R4 biomass at conclusion of the trial, as determined by clone library analysis. 1 – *Methanosaeta* sp.; 2 – other archaeal species; 3 – *Methanomicrobiales* sp.

Significant levels of *Crenarchaeota*-like sequences, affiliated with the thermophilic *Crenarchaeota*, were detected in the majority of sludge samples, as were clones which showed strong similarity to the uncultured archaeons, 39-2 and ASDS 14. *Methanomicrobiales* sp. was the predominant methanogen detected in biomass samples removed from the fixed-film sections of all four reactors, perhaps due to the high levels of hydrogen prevailing in this section as a result of the degradation of substrate, and concomitant generation of hydrogen, in the first chamber. The bacterial populations of all four reactors were shown to be considerably more dynamic and diverse than the archaeal populations, with the main species detected belonging to genera classically identified within anaerobic digesters, such as *Bacteroides*, *Clostridium* and *Proteobacteria* or difficult-to-cultivate groups detected through the use of molecular techniques, such as *Spirochetes* and WCHD1-31.

Table 2 Average specific methanogenic activity values for sludge biomass against acetate at 37 °C (ml CH_4 g VSS^{-1} d^{-1} (STP); $n = 3$)

	Day 165	Day 188	Final sludge bed sample	Final fixed biofilm sample
R1	ND	133.6	199.7	12.9
R2	ND	264.7	60.2	18.3
R3	104.3	ND	32.2	16.8
R4	255.6	ND	151.7	15.5

ND; not determined

Aggregate and metabolic characteristics of particulate and fixed biofilm samples

The fixed-film biomass samples displayed a much lower specific methanogenic activity (SMA) than the granular biomass removed from R1–R4 (Table 2). The development of true psychrophilic microbial populations was not observed during the trials, with the SMA profiles for all sludge samples removed from the reactors on completion of the study displaying mesophilic (37 °C) temperature optima against all substrates tested (data not shown). The development of psychrotolerant biomass was, however, observed. The granular seed sludge used to inoculate the four reactors displayed high sludge settleability, even at elevated upflow velocities. In R1, the granular nature of the sludge bed remained intact throughout the trial, with a size distribution similar to that of the seed sludge observed at the conclusion of the trial. In the three high-strength reactors, a loss of granular sludge bed integrity occurred during the trial, following a decline in reactor performance (data not shown). The low levels of the filamentous *Methanosaeta* sp. within the sludge biomass may have promoted granular disintegration within the reactors. Granule regeneration was, however, noted in R2 towards the end of the trial, indicating that anaerobic sludge granulation is possible under psychrophilic conditions. The EPS content of granular sludge biomass was lower than that of both the disintegrated sludge and the fixed-film biomass, with the sucrose-fed biomass containing the highest EPS content (data not shown). A thick biofilm developed on the polyethylene rings in the fixed-film section of the reactors during the trial.

Conclusions

The feasibility of psychrophilic anaerobic digestion for the treatment of high- and low-strength food processing wastewaters was demonstrated in this study, with high COD removal efficiencies and stable reactor performance achieved at 12–25 °C. Due to current economic, social and environmental necessity, the use of this biofilm-based wastewater treatment technology should undoubtedly increase in the future. However, the system should not be operated merely as a 'black box', as microbial composition is a key factor in the successful implementation of this technology. It is apparent that a greater insight into the structure, function and biological properties of the microbial populations involved in psychrophilic anaerobic digestion is required in order to fully develop and improve the technology, both by providing a greater understanding of the degradation process and by potentially predicting reactor disturbance and failure.

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