Population dynamics of rumen microbes using modern techniques in rumen enhanced solid incubation

N. Raizada, V. Sonakya, R. Dalhoff, M. Hausner and P.A. Wilderer

Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, D-85748 Garching, Germany

Abstract The microbial ecology of the rumen is very complex. Different species of bacteria, protozoa, and fungi are involved in digestion of plant material in ruminants. In spite of complicated interrelationships among the various groups of microorganisms in the rumen ecosystem, *Bacteria* and *Archaea* are believed to play a major role because of their numerical predominance and metabolic diversity. In this work we are presenting the results for microbial population dynamics of rumen microbes during two-stage anaerobic digestion of grass. The reactors were inoculated with fresh rumen content. Fluorescent in situ hybridization, confocal laser scanning microscopy and epifluorescence microscopy were employed for microbial investigation. It was observed that *Bacteria* dominated in the hydrolytic reactor (1st stage) whereas *Archaea* were predominant in the methanogenic reactor (2nd stage). The stability of the methanogenic reactor was result of the dominance of *Methanosaeta* species (mainly the filamentous type).

Keywords Anaerobic digestion; CLSM; FISH; methanogens; rumen microorganisms

Water Science and Technology **Vol 48 No 4 pp 113–119** © IWA Publishing 2003

Introduction

Anaerobic bioreactors are used to treat various organic wastes, to convert them into methane and solid materials of agricultural value (McCarty, 1982). The most important merits of anaerobic treatment are the capacity to degrade efficiently high COD concentrations, low energy demand, low operating cost, 10–20 times less sludge production as compared to aerobic methods, production of biogas containing methane and the applicability of the solids effluent as fertilizer. In anaerobic digestion of particulate substances hydrolysis is considered to be the rate limiting step. Thus it is essential to understand the microbiology of the hydrolytic step to achieve higher efficiency and stability of the reactor.

Anaerobic digestion involves interaction between four major metabolic groups of microorganisms that are generally assumed to be present in anaerobic digesters (Zehnder, 1978). These groups consist of hydrolytic-fermentative bacteria, proton reducing acetogenic bacteria, aceticlastic methanogens, and hydrogenotrophic methanogens. At higher substrate loadings, such as during start-up and periods of overload, more reduced metabolites (e.g. propionate, butyrate, lactate, ethanol) may accumulate because hydrogenotrophs fail to consume all of the hydrogen produced during fermentation and acetogenesis. The presence of lipids in some feedstocks (e.g. food waste) may result in the production of excess amounts of fatty acids through hydrolysis of triglycerides causing inhibitory effects. Volatile fatty acid accumulation can lead to a drop in pH and inhibit methanogenesis, causing an even greater imbalance. Methanogens, sulfate reducing bacteria (SRB) and acetogens are believed to be responsible for the removal of hydrogen in most anaerobic systems (Zehnder and Stumm, 1988). The presence of methanogens is required in order to generate methane, which can be used to produce energy. It is commonly accepted that during methanogenesis 70–75% methane is produced via the acetate route from complex substrates in biogas reactors (Boone, 1982). Consequently, it is important to develop processes which allow complete conversion of acetate into methane. No members of the domain *Archaea* other than methanogens are likely to exist in anaerobic digesters (Sørensen *et al*., 1997).

The advent of the fluorescent in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes in combination with confocal laser-scanning microscopy (CLSM) allows direct analysis of the microbial community structure of such systems and information to be obtained on the localisation of specific microbial communities (Amann, 1995). CLSM is a powerful and advanced tool in microbiological studies, since microorganisms may be examined in their natural morphological state. Viability studies can help to assess changes in microbial populations during fermentation.

The aims of the present study were: (i) to investigate microbial populations in a two-stage mesophilic reactor; (ii) to observe shifts in the microbial population; and (iii) to correlate these results with reactor parameters (e.g. biogas, pH, COD, fatty acids) for a better understanding and control of the performance of a two-stage reactor system consisting of a hydrolysis and methanogenesis reactor.

Methods

Feed

Fresh grass was used as the only feed source. It was collected from the open landscape and cut into small pieces (1–2 cm in length) with a grass cutter.

Reactor set up

The reactors were constructed from glass (6 L volume) with a lid having four openings which were fitted with plastic pipes (Figure 1).

The first two openings of the methanogenic reactor for inlet and outlet of feed were connected with a two-way peristaltic pump. The next two openings were used for pH adjustment and for gas outlet. In the first reactor (first stage) the chopped grass and during the start-up phase of the experiment fresh rumen content were mixed and incubated at 37°C for hydrolysis. The gas counter was attached to the methane reactor (second stage) which continuously monitored the biogas production during methanogenesis.

Reactor operation

The two-stage anaerobic digestion process was performed at mesophilic conditions (37°C), at an overall hydraulic retention time (HRT) of 4 days. Grass hydrolysis was carried out for 2 days. The second-stage reactor (HRT of 2 days) was fed in a semi-continuous manner using hydrolysed grass (effluent from the first stage) as organic substrate. Reactor performance was evaluated on the basis of effluent COD and volatile fatty acids, methane production and pH measurement.

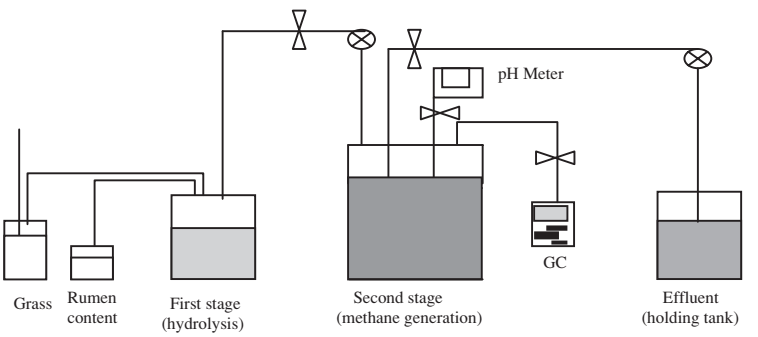

Figure 1 Schematic representation of the experimental unit

Analyses

Organic acids were analysed by gas chromatography (Carlo Erba, HRGC 5300 Mega series) using a DB-FFAP column (30 m in length) and helium as a carrier gas. Total solids (TS) were analysed by the standard procedures given in the Handbook of German procedures for water, wastewater and sludge analyses (Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, Band VII, (DIN 38414), 50. Lieferung 2001). Gases were analysed by an infra-red gas analyser (Geotech, UK). Chemical oxygen demand (COD) was estimated according to the Dr. Lange cuvett test.

Inoculum

During the initial period of time, fresh rumen content, obtained from the rumen of fistulated cow from a slaughter house, was filtered through two-layered cheese cloth and used as a source of inoculum for the first-stage reactor.

The enrichment of methanogens in reactors

The reactor was initially filled with 4 L of active sludge from an already running anaerobic reactor. In addition, 1 L feed (hydrolysed grass) was also added to provide minerals and nutrients to microorganisms. Nitrogen gas was passed for 10 min at a flow rate of 0.1 l/min to provide an oxygen-free atmosphere for the microorganisms in the second-stage reactor. The reactors were then kept at 37°C for 15 to 30 days for acclimatisation and enrichment of methanogens in the anaerobic reactor. The pH, availability of minerals and nutrients and other parameters were monitored continuously.

Fixation protocols

The PFA fixation and ethanol fixation (EtOH) protocols as developed by Amann *et al.* (1990) were used. Fixed samples were applied directly to the wells on gelatin-coated slides (Raskin *et al.*, 1994b) or diluted prior to their application on the slides. The dilution was obtained by adding 10 µl of sample to 490 µl of a 0.1% solution of sodium pyrophosphate (Zarda *et al.*, 1997). Samples were immobilized on glass slides by air-drying followed by ethanol dehydration in serial immersion 50, 80 and 100% and allowed to air dry (Raskin *et al.*,1994a). These slides were directly used for hybridization.

In situ hybridization

In situ hybridization was performed as described by Raskin *et al.* (1994b) with some modifications in terms of temperature, incubation time and the concentration of formamide in the hybridization buffer (Fall *et al.*, in preparation). Samples from all the stages were hybridized at 46°C for 2 h. The formamide concentration in the hybridization buffer used was 25% (Zarda *et al.*, 1997). Five fluorescently labelled oligonucleotide probes were used: ARC915 (Domain *Archaea*; Stahl and Amann, 1991), ARC344 (Domain *Archaea*; Raskin *et al.*, 1994a), EUB338 (Domain *Bacteria*; Amann *et al.*, 1990), MSMX860 (Family *Methanosarcinaceae*; Raskin *et al.*, 1994a). These probes were labelled with fluorescent cyanine dyes CY3 or CY5. All probes were purchased from MWG Biotech (Ebersberg, Germany).

Microscopy and image processing

Single optical sections or image z-series were digitised in selected optical planes, either directly or by using the average filtration, with a CLSM 410 confocal laser scanning microscope coupled to an Axiovert 135M inverse microscope (both instruments from C. Zeiss, Jena, Germany) and operated with a Carl Zeiss LSM software package (version 3.95).

Results and discussion

Reactor performance

Experiments were conducted for 14 weeks. During the initial period the VFA production was low but increased steadily. The hydrolysed feed (effluent of the first-stage reactor) was filtered through a sieve of 0.4 mm pore size. The filtered liquid was used as feed of the second-stage reactor. The methane reactor (second stage) was fed on daily basis and 2 days HRT was maintained. During initial period (startup time) the biogas production was low (less than 40 L of biogas) with 60–65% CH_4 and 20–25% CO_2 (Figure 2). After 6–7 weeks the methane production increased as a result of the enhanced VFA production in the hydrolytic reactor (Figure 3). The methane content also increased up to 75% and CO_2 decreased to 15%.

Microbial analysis

In this study the change in microbial population in the two stages of the reactor system was investigated in correlation with reactor performance.

In the fresh rumen content we observed more members of *Bacteria* than *Archaea* (Figure 4a,b). Protozoa were also observed, but their abundance was rather small. In the hydrolytic reactor effluent members of *Bacteria* and some methanogens (Figure 4c,d) were observed but no protozoa were detected. The presence of methanogens in the hydrolytic reactor effluent could be explained by the fact that few methanogens from the rumen content adapted themselves to the environment in the hydrolytic reactor although no biogas production was observed during this stage. The drop of pH could explain that no protozoa were detected in the hydrolytic reactor effluent (pH 4–4.5) compared to fresh rumen

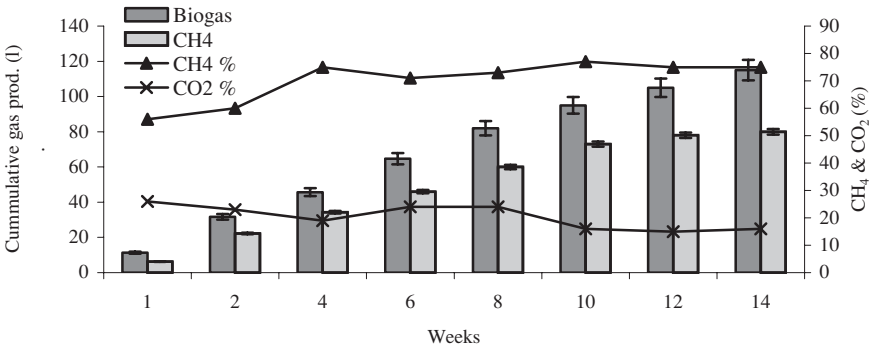

Figure 2 Cumulative biogas production during the experiment

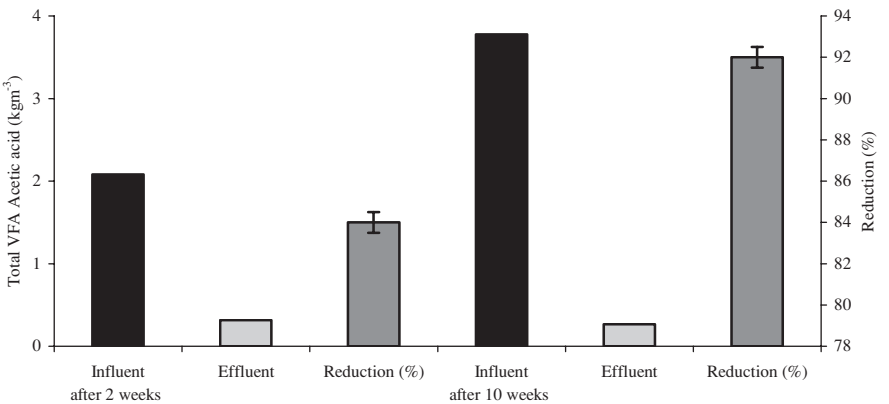

Figure 3 Total VFA removal during methanogenesis at steady-state condition

content (pH 5.5–6.0). In the methanogenic reactor the population of methanogens increased and drastic reduction of members of *Bacteria* was observed. This methanogenic population was dominated by *Methanosaeta*-resembling cells which were in the filamen-

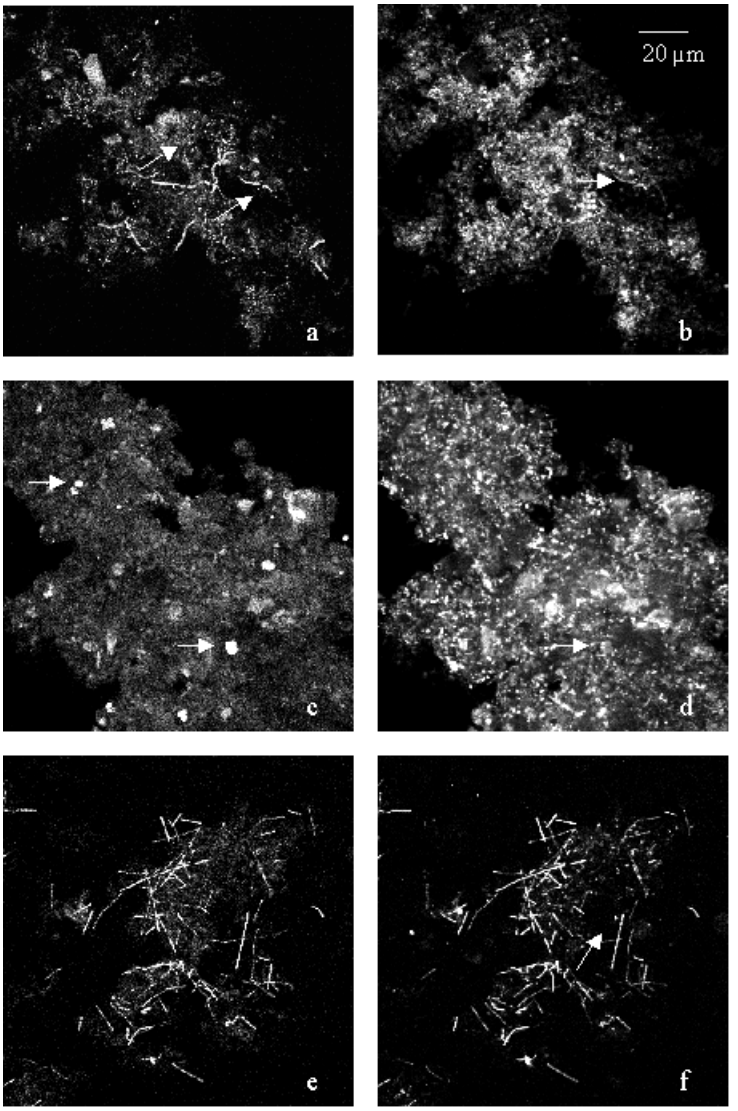

Figure 4 Confocal laser scanning microscopic images (single x–y sections) of PFA-fixed samples. Figures (a), (c) and (e) show members of *Archaea* (bright signals) hybridized with probe ARC915-CY3. Due to hybridization with an additional *Archaea* specific probe (ARC344-CY5), Figures (b), (d) and (f) depict archaeal cells corresponding to those shown in Figure (a), (c) and (e). In addition, Figures (b), (d) and (f) show members of *Bacteria* hybridized with EUB338-CY5. Probe positive microorganisms gave strong hybridization signals whereas autofluorescence resulted in weak signals
(a, b) Rumen: (a) Members of *Archaea* (rods, cocci) (↑); (b) Members of *Archaea* corresponding to those shown in Figure (a) (↑). In addition, this figure illustrates the abundancy of *Bacteria* (bright signals, mostly cocci)
(c, d) Hydrolytic reactor: (c) Single cells and clusters (↑) of *Archaea*; (d) as (b) above, *Archaea* shown in Figure (c) (↑)) and *Bacteria*
(e, f) Methanogenic reactor: (e) Filamentous *Archaea*; (f) Filamentous *Archaea* shown in Figure (e). Note the drastic reduction of *Bacteria*. Only a few weakly fluorescing cells were detected (↑)

tous form (Figure 4e,f). It was also observed that the acetic acid production was increased in the hydrolytic reactor, which resulted in a higher substrate loading and subsequently the dominance of methanogens in the methanogenic reactor.

It has been reported in earlier studies that two acetate-utilizing methanogenic genera, *Methanosarcina* and *Methanosaeta* are present in anaerobic reactors (Schmidt and Ahring, 1999). *Methanosaeta* spp. are filamentous organisms which are known to grow only on acetate while *Methanosarcina* spp. besides acetate, are also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide (Griffin *et al*., 1997; Schmidt and Ahring, 1999). *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than do *Methanosarcina* spp., but their affinity for acetate is 5 to 10 times higher. It is generally assumed that *Methanosaeta* spp. result in more stable reactor performance; consequently, *Methanosaeta* spp. should have been favored over *Methanosarcina* spp.

This, in turn, improved reactor stability and resulted in the higher removal of COD, TS and VFA such as acetate as well as in higher biogas production during the steady-state condition.

Conclusions

1. The FISH technique allowed us to characterize the microbial population in the rumen content, hydrolytic reactor and methanogenic reactor.
2. The hydrolytic reactor, dominated by members of *Bacteria*, allowed high VFA production, particularly of acetic acid.
3. Subsequently, the size of the Archaeal population increased in the methanogenic reactor where *Methanosaeta* spp. were observed to be predominant. Thus, long-term stability of the second-stage reactor was achieved. This resulted in a high removal of VFA and high biogas production.
4. Use of microscopic and molecular biological techniques (FISH) combined with conventional process monitoring were helpful in better understanding the micro-ecology of anaerobic reactors at the genus or species level.

Acknowledgements

The authors gratefully acknowledge BayFORREST (Bayerischer Forschungsverbund für Abfallforschung und Reststoffverwertung), for funding the project (No. F-213). We are also grateful to DAAD for providing financial support for V. Sonakya and N. Raizada.

References

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990). Combination of 16 S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. *Appl. Environ. Microbiol.*, **5**, 1919–1925.

Amann, R.I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA–targeted nucleic acid probes. In: *Molecular Microbial Ecology Manual*, A.D.L. Akkerman, J.D. van Elsas, and F.J. de Bruijn (eds), Kluwer Academic Publishers, Dordrect, The Netherlands, pp. 1–15.

Boone, D.R. (1982). Terminal reactions in the anaerobic digestion of animal waste. *Appl. Environ. Microbiol.*, **43**, 57–64.

Fall, P.A., Wuertz, S., Letsiou, I., Pfleiderer, P. and Hausner, M. (in preparation). Comparative study of fixation, pretreatment and hybridization procedures for the detection of microbial populations in anaerobic reactors.

Griffin, M.E., McMahon, K.D., Mackie, R.I. and Raskin, L. (1998). Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. *Biotech. Bioeng.*, **57**(3), 342–355.

McCarty, P.L. (1982). One-hundred years of anaerobic treatment. In: *Anaerobic digestion*, D.E. Hughes (ed.), Elsevier, Amsterdam, pp. 3–22.

Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A. (1994a). Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.*, **60**, 1232–1240.

Raskin, L., Poulsen, L.K., Nouera, D.R., Rittmann, B.E. and Stahl, D.A. (1994b). Quantification of methanogenic groups in anaerobical biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.*, **60**, 1241–1248.

Schmidt, J.E. and Ahring, B.K. (1999). Immobilization patterns and dynamics of acetate-utilizing methanogens immobilized in sterile granular sludge in upflow anaerobic sludge blanket reactors. *Appl. Environ. Microbiol.*, **65**(3), 1050–1054.

Sørensen, A.H., Torsvik, V.L., Torsvik, T., Poulsen, L.K. and Ahring, B.K. (1997). Whole-cell hybridization of *Methanosarcina* cells with two oligonucleotide probes. *Appl. Environ. Microbiol.*, **63**(8), 3043–3050.

Stahl, D.A. and Amann, R.I. (1991). Development and application of nucleic acid probes. In : *Nucleic Acid Techniques in Bacterial Systematics*, E. Stackebrandt and M. Goodfellow (eds.), v.8. Wiley, Chichester, England, pp. 207–248.

Zarda, B., Hahn, D., Chatzinotas, A., Schönhuber, W., Neef, A., Amann, R.I. and Zeyer, J. (1997). Analysis of bacterial community structure in bulk soil by in situ hybridization. *Arch. Microbiol.*, **168**, 185–192.

Zehnder, A.J.B. (1978). Ecology of methane formation. In : *Water pollution microbiology*, R. Mitchell (ed.), v.2. John Wiley & Sons Ltd., Chichester, England, pp. 349–376.

Zehnder, A.J.B. and Stumm, W. (1988). Geochemistry and biogeochemistry of anaerobic habitats. In: *Biology of Anaerobic Microorganisms*, A.J.B. Zehnder (ed.), Wiley, New York, pp. 1–38.