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ORIGINAL PAPER

Anaerobic protozoa and their growth in biomethanation systems

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Abstract This study was to investigate growth of protozoa and its influence on biodegradation in anaerobic treatment systems. It was done by specifically controlling and monitoring growth of protozoa versus degradation in continuous stirred anaerobic reactors and batch anaerobic reactors. Occurrence of a diverse protozoa population such as the ciliates, *Prorodon, Vorticella, Cyclidium, Spathidium, Loxodes, Metopus* were observed in stable anaerobic systems and the flagellates, *Rhynchomonas, Naeglaria, Amoeboflagellates, Tetramitus, Trepomonas and Bodo* during increased VFA concentration and affected periods of biomethanation. The abundance of ciliates in the anaerobic system had significant correlation with the reduction of MLSS, increased rate of COD removal and higher methane production. The results of this study thus tend to relate increased anaerobic degradation with the abundance of protozoa, mainly ciliates, which indicate their possible involvement in the process. Present study also reveals that performance of anaerobic process can be assessed by monitoring the protozoa population in the system.

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Keywords Anaerobic ciliates · Anaerobic flagellates · Anaerobic protozoa · Biomethanation · Chemical oxygen demand · Mixed liquor suspended solids

Abbreviations

CSTAR	Continuous stirred tank anaerobic reactor
COD	Chemical oxygen demand
MLSS	Mixed liquor suspended solids
VFA	Volatile fatty acids

Introduction

Protozoa consortia are found in various anaerobic environments, including rumen ecosystem, marine and fresh water sediments, wet landfills and anaerobic sewage plants (Williams and Coleman 1991; Fenchel 1993; Fenchel et al. 1990; Finlay and Fenchel 1991; Fenchel et al. 1977). They are mainly considered as bacteriovorous and often represent main bacterial grazers in anoxic environments (Fenchel and Finlay 1991). The feeding rates of anaerobic ciliates on bacteria are comparable to those of aerobic ciliates (Massana and Pedros-alio 1994), but their net growth efficiency and gross growth efficiency are only about 20% and 25% respectively of the aerobic organisms (Fenchel and Finlay 1990).

Anaerobic protozoa lack mitochondria and instead possess redox organelles known as hydrogenosomes

(Finlay and Fenchel 1989), which are believed to be generating ATP and H_2 from the decarboxylation of pyruvate (Mueller 1988). The partial pressure of H_2 generated in the cells is managed by ecto and endosymbiotic bacteria mainly methanogens (Fenchel et al. 1977). It has been reported that anaerobic protozoa with endosymbiotic methanogens can contribute a substantial fraction of methane production (15–90%) in anaerobic marine sediments (Fenchel 1993) and 9–25% of methanogenesis in rumen fluid (Newbold et al. 1995).

The significance of protozoa in anaerobic environments has been less studied with respect to their role in biodegradation except in the rumen ecosystem where protozoa is said to enhance the degradation of organic material by direct uptake (Williams 1991; Santra and Karim 2002). The functional role of protozoa in biodegradation are of great interest as protozoan role is generally reported as antagonistic to bacterial growth from grazing activity and thereby unfavourable to bacterial growth and degradation (Kotta et al. 1999). A similar opinion prevails in the case of aerobic treatment, but positive impact of ciliates on the overall purification and performance of aerobic activated sludge has been shown, especially in the reduction of biological oxygen demand, concentration of suspended solids and control of viable bacteria in the treated effluent (Curds et al. 1968; Salvado et al. 1995; Madoni 1994). However, Finlay and Fenchel (1991) have reported that grazing and flocculation activity of anaerobic ciliates lead to an overall stimulation of anaerobic activity and to an increased turn over rate in wet anaerobic landfill sites. In order to understand the anaerobic degradation in detail studies are needed on the growth of anaerobic protozoa. The present study is on the growth of protozoa in biomethanation system related to their involvement in biodegradation.

Materials and methods

Continuous stirred tank anaerobic reactor

Two continuous stirred tank anaerobic reactors (CSTARs) with effective volume of 1 l each were fabricated using glass bottles and operated for this study. Initial seeding of the reactors was done with the anaerobic sludge collected from a dairy wastewater treatment plant. The CSTARs were fed continuously with carbon sources at a rate of 1 g COD l^{-1} d^{-1} and 2 g COD l^{-1} d^{-1}. One reactor was fed with sodium acetate (solution) and other with emulsified sodium oleate (particulate suspension) and studied the growth and population of anaerobic protozoa. Each of the carbon sources was mixed with the Vanderbilt media (Nuri et al. 2001) before feeding and was supplied to the reactors using a peristaltic pump (Autoclude VT, UK) at a rate to run experiments on the hydraulic retention time of 5 days. Alkalinity of the reactor liquid was adjusted by adding 1 N NaOH. Both reactors were operated at 30 ± 2°C. Cysteine HCl was added initially to remove residual oxygen in the reactors. Methane generated in the reactors was collected over 0.5 N NaOH and measured.

As a direct method for the contribution of protozoa to methanogenesis and COD removal, experimental set up was made with and without protozoa. Reactors without protozoa were obtained by adding cycloheximide (250 mg/l), which inhibits eukaryotic protein synthesis (Kotta et al. 1999). Control tests were conducted to study the effect of cycloheximide on anaerobic bacteria. Anaerobic bacteria were isolated from the same anaerobic reactor sludge. Isolates were incubated anaerobically in sodium acetate added mineral medium with and without cycloheximide in 50 ml amber Schott Duran bottles screw capped with silicon septa (Thomson Scientific, USA). Isolation and culturing of anaerobic bacteria was done as per Microbiological Aspects of Anaerobic Digestion, Laboratory Manual (1988). DAPI stained samples of culture were checked for the effect of cycloheximide on bacteria under epifluorescence microscope (Leica DM 2500). The results did not show any effect on bacterial number by dosing of cycloheximide and was same in cultures with and without protozoa.

Batch anaerobic reactors

Schot duran bottles with a capacity of 500 ml were used for batch reactor studies. The basal medium used was described by Harada et al. (1994). The initial COD concentration in the batch anaerobic reactor was set at 1 g g^{-1} VSS for sodium oleate suspension as substrate. Each bottle was capped with a rubber septum (Aldrich, USA) and the headspace was filled with nitrogen. Sample for analyses was

withdrawn with a sterile syringe needle inserted through the septum and the methane produced was measured by the displacement of 0.5 N NaOH solution in water.

Chemical analysis

Samples were drawn periodically from the reactors for chemical analyses. COD and MLSS were determined by following standard methods (APHA 1998). Total VFA and alkalinity concentrations were estimated titrimetrically (Anderson and Yang 1992). A pH meter attached with glass electrode was used for measuring pH of the samples (Systronic, India). Methane was determined in a gas chromatograph (FISIONS 8000, TCD, 2 mm-i.d. silica gel column, He 150 ml/min, oven 40°C, injector 110°C, and detector base 120°C).

Microscopic studies

The growth of protozoa was assessed daily with live samples. Observations were carried out using a compound microscope, Nikon—ALPH 4 YS2. Protozoan number was determined by direct counting on a haemocytometer. Fast moving protozoa were counted after fixing in Shaudinn's fixative and staining with Lugol's iodine of 1% (Patterson 1995). The protozoa were identified according to the schemes summarized by Patterson (1995) and Foissner and Berger (1996).

Results

Growth of anaerobic protozoa in CSTARs

The number and diversity of anaerobic protozoa were significantly varied with respect to feed composition and physicochemical conditions of laboratory scale CSTARs. During the startup of CSTARs the fluctuation of protozoa population was even more with abundance of flagellates and amoeboids.

Protozoa of different genera were observed in CSTARs during continuous operation. The commonly observed ciliates in the CSTARs fed with acetate and oleate were *Prorodon, Cyclidium, Metopus, Spathidium, Loxodes and Vorticella*, with the maximum occurrence of *Prorodon, Cyclidium, Metopus* and *Spathidum*. In addition, the oleate fed reactor also had *Loxophyllum* and *Brachonella* and *Discomorphella* occasionally. The flagellates present throughout in the reactors were *Rhynchomonas, Naeglaria, Amoeboflagellates, Tetramitus, Trepomonas* and *Bodo*, and *Menoidium* occasionally. *Peranema* was also found rarely in the oleate fed reactor. Number of protozoa was relatively higher in suspended feed (oleate) compared to solution (acetate) throughout the period of operation. As shown in Fig. 1 the maximum number of protozoa were indexed in the oleate fed reactor ($2.3 \times 10^6 \pm 0.55$ ml^{-1}) compared to the acetate fed one ($1.95 \times 10^6 \pm 0.52$ ml^{-1}) at early period of reactor operation.

Neutral pH was the essential condition for maximum growth of ciliates in both the CSTARs fed with acetate and oleate. A decline of ciliate count from 4.5×10^4 ml^{-1} – 2.5×10^3 ml^{-1} was observed in the CSTARs with the decrease in pH from 7.0 to 6.5 (Fig. 2). Compared to ciliates, flagellates were less affected in the anaerobic reactors by the acidic condition particularly during the early period of operations where VFA concentration was higher (>8 meq l^{-1}) and the pH was below 7.0. Flagellates reached a maximum number of $1.5 \times 10^6 \pm 0.56$ ml^{-1} in the acetate fed anaerobic reactor and $1.3 \times 10^6 \pm 0.50$ ml^{-1} in oleate fed at early days of reactor operation (Fig. 3). On reaching the anaerobic process stable the density of flagellates declined to the range of 2.0×10^4 – $6.5 \times 10^4 \pm 0.4$ ml^{-1} and maintained a steady state thereafter in both CSTARs. The growth of ciliates was predominant in the reactors from steady state and the total ciliate count

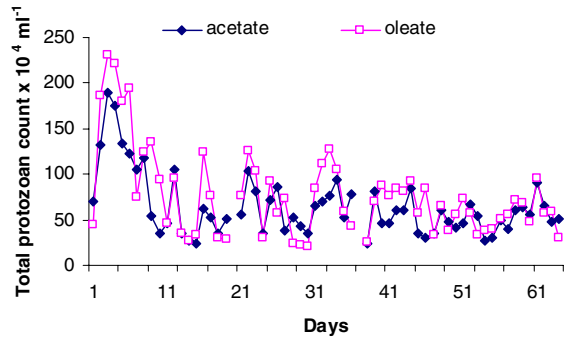

Fig. 1 Total protozoan count in acetate fed and oleate fed CSTARs operated with loading rate of 1 g COD l^{-1} d^{-1} at 5 days HRT

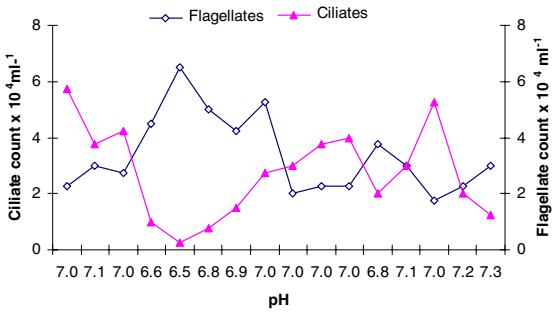

Fig. 2 Effect of pH on protozoa in CSTARs supplemented with sodium oleate feed

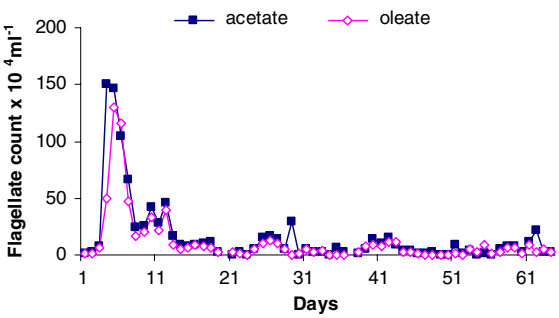

Fig. 3 Total flagellate count in acetate fed and oleate fed CSTARs operated with loading rate of 1 g COD l^{-1} d^{-1} at 5 days HRT

in the CSTARs were in the range of 2.5×10^3–$9.25 \times 10^4 \pm 0.3$ ml^{-1} (Fig. 4).

Stimulation of anaerobic degradation by protozoan activity in CSTARs

The presence of protozoan community had significant influence on removal of COD. The removal of COD was higher in both acetate and oleate fed CSTARs possessing abundant ciliates (Fig. 4) in studied loading rates and retention time. The maximum COD removal of more than 72% (± 2) was observed in the reactors having the highest number of ciliates (in the range of $2.5 – 9.25 \times 10^4 \pm 0.3$ ml^{-1}). While in unsteady states, during reactor start up and change in loading rates the number of ciliates was less than 2.5×10^3 ml^{-1} and obtained less COD removal. In experiments with control, enhanced COD removal was observed in CSTARs with protozoa (more than 75%) compared to the protozoa controlled CSTARs at the same loading rate and retention time (Fig. 5). The decreased COD reduction could not have resulted from any adverse effect of cycloheximide on anaerobic bacteria, the selective inhibitor used in this study for protozoa did not exhibit any deleterious effect on bacteria as the bacterial number was the same in the presence and absence of cycloheximide.

Along with stimulated COD removal, substantial reduction of MLSS was observed with abundance of ciliates in the both acetate and oleate fed anaerobic CSTARs. A negative correlation was obtained in correlation analysis between MLSS concentration and ciliate density and a reduction of more than 25% (±5) MLSS obtained with 5×10^4 ml^{-1}ciliates (Fig. 6).

Growth of protozoa and stimulation of anaerobic degradation in anaerobic batch system

The presence of protozoan community also had significant influence on biodegradation in anaerobic batch systems. The similar trend was observed in batch experiments with development of organisms and enhanced biodegradation. Flagellates reached to a maximum of $12.5 \times 10^4 \pm 0.5$ ml^{-1} at initial stage and declined to less than $3 \times 10^4 \pm 0.3$ ml^{-1} with the development ciliates. Ciliates number reached to a

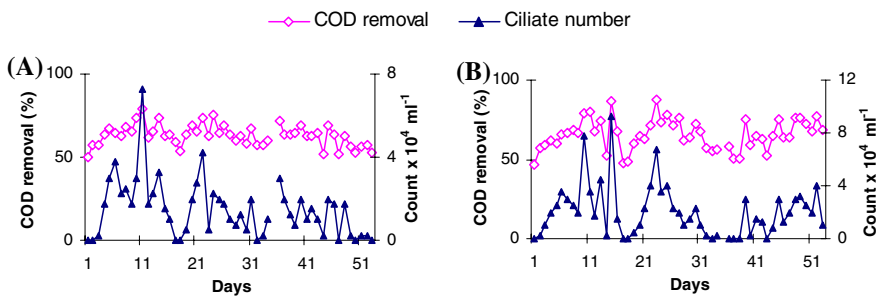

Fig. 4 COD removal and ciliate density in CSTARs with loading rate of 1 g COD l^{-1} d^{-1} at 5 days HRT (**A**) acetate fed reactor and (**B**) oleate fed reactor

maximum of $8.75 \times 10^4 \pm 0.34$ ml^{-1} (Fig. 7). Like in the CSTARs the enhanced COD removal and methane production in batch anaerobic study also had positive correlation with the abundance of ciliates. The maximum rate of methane production was obtained in the range of 28–42 ml d^{-1} in the anaerobic batch system when the ciliates count was noted high, in the range of 6.25×10^4–$8.75 \times 10^4 \pm 0.34$ ml^{-1} (Fig. 8).

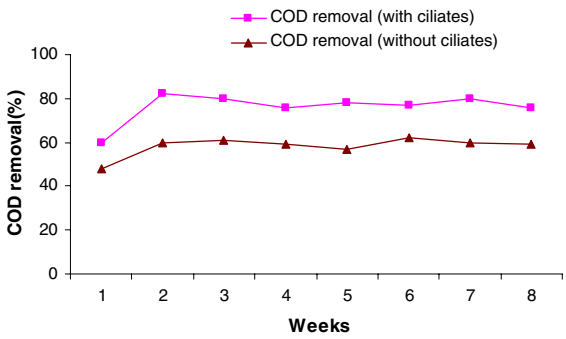

Fig. 5 Comparison of COD removal in CSTARs with and without protozoa with loading rate of 1 g COD l^{-1} d^{-1} at 5 days HRT

Discussion

In recent years, considerable attention has been paid towards the development of reactors for anaerobic treatment of wastewater leading to the conversion of organic materials in to biogas. All modern high rate biomethanation processes are based on the concept of retaining high viable biomass by bacterial sludge immobilization (Hulshoff and Lettinga 1986) and works were mainly focused on bacteria in anaerobic treatment process. But the role of protozoa in anaerobic digestion process is hardly explained. Gijzen et al. (1987a, b) have developed a two-phase anaerobic system based on processes and microorganisms including ciliates from the ruminant (Rumen Derived Anaerobic Digestion—RUDAD), however, the specific role of ciliates has not been investigated. This study is aimed at protozoa mediated biodegradation in anaerobic reactor and the results indicate that protozoa play important roles in anaerobic degradation.

The composition and size of protozoa population is considerably influenced by the nature of feed and conditions of system operation. Flagellates and amoeboids were flourished at early stages (Fig. 3)

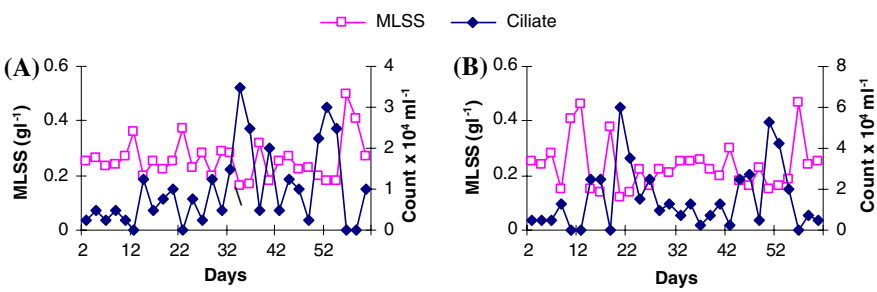

Fig. 6 Correlation between MLSS and abundance of ciliates in CSTARs operated with loading rate of 1 g COD l^{-1} d^{-1} at 5 days HRT (**A**) acetate fed reactor and (**B**) oleate fed reactor

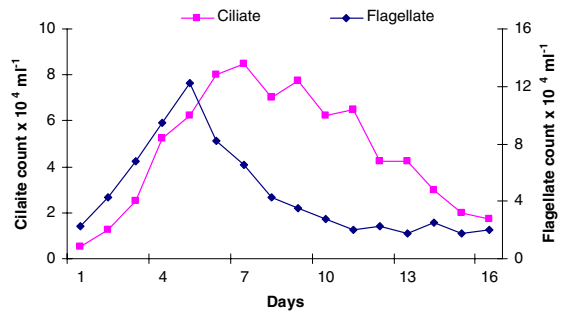

Fig. 7 Growth of flagellates and ciliates in anaerobic batch reactor fed with oleate feed

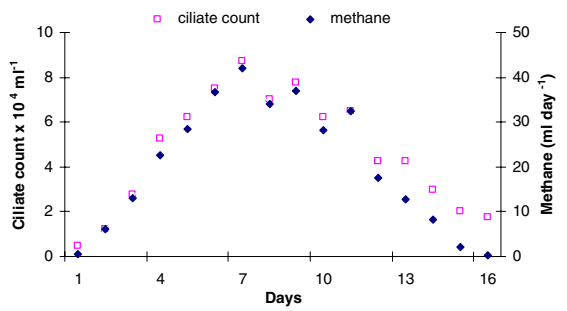

Fig. 8 Correlation between ciliate density and methane production in anaerobic batch reactor

and then ciliates, after reaching steady state in VFA generation and utilization by biomethanation (Fig. 4). The abundance of flagellates at initial stage could be due to its shorter generation time compared to ciliates and the energy advantage from the direct utilization of dissolved substrate like VFA as noticed higher at initial stage (>8 meq l^{-1}). Direct uptake of organic matter through osmotrophic nutrition has previously been reported in some of the soil flagellates (Ekelund and Ronn 1994).

Protozoa number and species composition showed notable difference with respect to feed composition. Relatively high density and diversity of protozoa obtained in oleate fed CSTARs supports their adaptation on particulate feeding along with bacterial grazing (Fig. 1). Again, oleate being complex substrate compared to acetate, larger diversity of bacterial population needed to degrade oleate particles, so can expect larger diversity of flagellates and ciliates. Protozoa are mainly considered as main bacterial consumers in anaerobic environments (Fenchel and Finlay 1991).

During the course of treatment protozoa changed its population along with changing physical conditions. Anaerobic protozoa are highly dependant on physical conditions of reactor and the most affected group was ciliates. A sudden change in population was obtained with ciliates. Flagellates and amoeboids were dominated at unsteady conditions such as change in pH (Fig. 2) and VFA concentration and during the change in loading rate. The results indicate that protozoa can be considered as good indicators of anaerobic system performance. However, changes in the community structure of protozoa have been reported in the aerobic treatment systems by the changes in pH and temperature (Fried et al. 2000).

Anaerobic CSTARs and batch system showed an obvious relation between abundance of ciliates and enhanced biodegradation. The correlation coefficients between COD removal and abundance of ciliates were significantly high ($R^2 > 0.95$) in the studied conditions of hydraulic retention time and feed loading (Fig. 4). Stimulation of terminal activity by the presence of protozoa has been reported in natural *aerobic* systems (Fenchel and Harrison 1976) and in *aerobic* treatment processes (Curds et al. 1968), but it is unknown earlier in anaerobic systems. Importantly, higher growth of ciliates in the anaerobic reactors had increased COD removal and at the same time reduction of MLSS was also found significantly higher (Fig. 6). This is a contradiction to the general view on the growth of protozoa, the grazers of bacteria, which are considered responsible for biodegradation. It means protozoa may be playing some role in the anaerobic degradation through consumption of biomass for the generation of methane. Protozoa grazing have previously been reported to reduce bacterial biomass to 12–43% in *aerobic* chemostat cultures (Ratsak et al. 1994) and control abundance of bacteria in fresh water and marine environments (Sherr and Sherr 1987).

The stimulation of methane production with higher counts of ciliates in anaerobic systems (Fig. 8) also indicating the possible direct involvement of protozoa in anaerobic degradation. The production of methane through endosymbiotic methanogens has been reported in anaerobic ciliates (Fenchel and Finlay 1992). Further more, symbiotic relation between protozoa and bacteria enhancing the anaerobic degradation and observed high rate methane production in the presence of ciliate *Metopus palaeformis* (Biagini et al. 1998). The present study thus confirms enhanced anaerobic degradation and methane production by the presence of protozoa, especially ciliates in anaerobic systems.

Conclusions

The information on eukaryotes in anaerobic degradation is scanty to describe their role in such systems. The present study on the growth of protozoa in anaerobic system tries to explain their presence and significance beyond bacterial grazing. The populations of protozoa vary considerably with the changes in the anaerobic reactors, which can be considered as an indicator of system performance in anaerobic process. The abundance of ciliates stimulates the rate of COD removal and methane production. Absence of protozoa affects the COD removal in the anaerobic system substantially.

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