Bioresource Technology 149 (2013) 318–326

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Biokinetic and molecular studies of methanogens in phased anaerobic digestion systems

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## HIGHLIGHTS

- Various phased anaerobic digesters were set up.
- Kinetics and composition of methanogens were investigated.
- 1st-stage and 2nd-stage digesters were kinetically different.
- 1st- and 2nd-stage digesters showed different archaeal community composition.
- Arrhenius-type equation was not applicable for all kinetic parameters.

## ARTICLE INFO

*Article history:*
Received 5 July 2013
Received in revised form 9 September 2013
Accepted 13 September 2013
Available online 21 September 2013

*Keywords:*
Anaerobic digestion
Acetoclastic methanogens
Kinetics
Modeling
DGGE

## ABSTRACT

The influence of differing operational conditions of two-stage digesters on biokinetic characteristics and communities of methanogenic archaea was evaluated. Operating temperature of each phase influenced the archaeal communities significantly. Also, a strong correlation was observed between community composition and temperature and pH. The maximum specific substrate utilization rates ($k_{max}$) of acetoclastic methanogens in the mesophilic and thermophilic 1st phases were 11.4 and 22.0 mgCOD mgCOD$^{-1}$ d$^{-1}$, respectively, whereas significantly lower $k_{max}$ values were estimated for the mesophilic and thermophilic 2nd-phase digesters which were 7.6 and 16.6 mgCOD mgCOD$^{-1}$ d$^{-1}$, respectively. It appeared that the biokinetic characteristics of the acetoclastic methanogen communities were reliant on digester loading rates. Also, higher temperature dependency coefficients ($\theta$) were observed for the long retention time digesters when compared to the values computed for the 1st-phase digesters. Accordingly, the implementation of two sets of biokinetic parameters for acetoclastic methanogen will improve modeling of phased anaerobic digesters.



## 1. Introduction

Anaerobic digestion technology exhibits several advantages such as low sludge production, high organic loading, low energy consumption and a promising renewable energy source. Anaerobic digestion is a process of sequential metabolic reactions including hydrolysis, acidogenesis, acetogenesis and methanogenesis. These reactions are performed by complex microbial communities, resulting in the eventual production of methane and carbon dioxide. Recently, phased anaerobic digestion systems have gained attention as a sustainable technology for sludge digestion and methane production (De Vrieze et al., 2012). Operation of anaerobic digesters in series with either identical or different temperatures has several advantages compared to conventional single-stage digestion processes (Han and Dague, 1997). In phased digestion systems, solubilization and acid production processes are completely or partially separated from acetogenesis and methanogenesis processes, such that the former reactions become dominant in the first phase and the latter in the second phase. Process partitioning into differing stages results in increased conversion of organics to biogas when compared to single stage digesters operated at the same hydraulic retention time. The underlying causes of increased performance are not well understood because of the complex nature of anaerobic systems.

Modeling is a valuable tool for better understanding microbial reactions occurring within an anaerobic digester. Most anaerobic models describe the anaerobic digestion process in a continuous-flow stirred tank reactor in which biological degradation reactions are described by microorganisms with characterized metabolic functions (Batstone et al., 2002). In this regard, modeling of phased anaerobic digestion systems has typically been based on the same modeling concepts as those employed for single-phase digesters, where a single set of kinetic parameters are employed and they

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http://dx.doi.org/10.1016/j.biortech.2013.09.058

are adjusted for temperature by an Arrhenius-like relationship if the stages are operated at different temperatures (Parker, 2005).

Methane production, as the final anaerobic process step, is carried out by methanogenic archaea and may be the rate-limiting reaction in digestion systems. Acetoclastic methanogenesis typically accounts for 65% of digester methane production and is typically mediated by two methanogenic genera (*Methanosarcina* and *Methanosaeta*), with relative abundances depending on digester acetate concentration (Zinder et al., 1984). Hence, it appears that kinetic parameters for acetoclastic methanogens will differ between the first and second stages of phased digesters due to differences in organic loading and acetate availability. Accurate prediction of the growth-kinetics of methanogens is therefore important, particularly for modeling the first stage of staged anaerobic digestion processes where kinetic limitations might influence performance.

Even though several studies have investigated archaeal ecology in anaerobic sludge digesters, limited research has focused on phased anaerobic digestion systems (Raskin et al., 1995; Shin et al., 2010). These studies have reported the dominance of kinetically distinct acetoclastic methanogens in separate stages of phased digestion systems. *Methanosarcinia* spp. have high maximum growth rates and are more resistant to stressors such as low pH, elevated volatile fatty acid (VFAs) and $NH_3$ concentration. In contrast, *Methanosaeta* spp. have low maximum growth rates and are more sensitive to pH change and $NH_3$ concentration (Conklin, 2004). These distinct characteristics are reflected in growth kinetic ($\mu_{max}$ and $K_s$) and morphological differences between these two genera (De Vrieze et al., 2012). Consequently, interspecies competition due to the differing operating conditions in each stage of phased digestion systems may lead to the proliferation of kinetically different species in separate phases.

The potential for development of distinct methanogenic communities and consequently differing growth characteristics for separate phases has been addressed in modeling phased anaerobic digesters. Our objective was to assess the biokinetics of acetoclastic methanogens in various temperature phased anaerobic digesters. Importantly, archaeal community patterns were also investigated to confirm distinct methanogen populations associated with particular reactor conditions used in phased anaerobic digesters. The composition of archaeal communities was monitored using denaturing gradient gel electrophoresis. Batch experiments were used to estimate the biokinetic parameters of acetoclastic methanogens in the digesters. The interpretation of the biokinetic results was complemented by using the archaeal fingerprints. Practically, the biokinetic parameters estimated in this study can be used in process modeling and optimization of phased anaerobic digesters.

## 2. Methods

### 2.1. Operation of source digesters

The lab-scale digesters employed in this research were previously described in detail (Zamanzadeh et al., 2013). Briefly, six digesters were operated in series as phased digestion systems and two additional digesters were used as single-phase digesters (Fig. 1). Various temperature combinations were used for the phased digesters, including mesophilic–mesophilic (M1–M2), mesophilic–thermophilic (M1–T3), thermophilic–mesophilic (T1–M3) and thermophilic–thermophilic (T1–T2) temperatures to determine the effect of temperature on biokinetic characteristics and archaeal community composition. Two single-stage digesters were operated at mesophilic (C1) and thermophilic (C2) temperatures as a reference. The mesophilic digesters were set at 35 °C and the thermophilic ones at 55 °C. The seed inoculum was taken from a mesophilic anaerobic digester at the Waterloo wastewater treatment plant (WWTP; Ontario, Canada). Initially, the temperature was set at 35 °C for all the digesters. However, temperature of the thermophilic digesters was immediately raised to 55 °C according to Han and Dague (1997). A mixed of primary and secondary sludge as feed were taken from the Waterloo WWTP. All digesters were fed three times daily, with an overall organic loading rate of 1.7 kgVS $m^{-3}$ $d^{-1}$. The digesters were operated for an extended time of 300 day.

### 2.2. DNA extraction and PCR

Samples from the influent and effluent of the digesters collected and stored at −80 °C until molecular analysis. An aliquot of 0.5-mL was used for DNA extraction using a FastDNA Spin kit (MP Biomedicals, OH, USA) and a FastPrep-24 Instrument (MP Biomedicals) for lysis. 100 μL of DES (DNase/Pyrogen-Free Water) was added to the purified DNA for elution and then quantified on a 1% agarose gel and stored at −20 °C prior to the polymerase chain reaction (PCR).

Archaeal 16S rRNA sequences were amplified by nested PCR. Initially, a primer set consisting of 109f as forward and 958r as reverse primers were employed for a first amplification (Grobkopf et al., 1998). The PCR mixture used for this first amplification contained 1.5 μL of bovine serum albumin (BSA; 10 μg/mL), 2.5 μL Thermopol buffer (10×), 0.2 μL deoxyribonucleotide triphosphate (dNTPs; 100 μM), 0.125 μL forward primer 109f (100 μM), 0.125 reverse primer 958r (100 μM) and 0.125 μL *Taq* DNA polymerase. Sludge sample DNA (1 μL; 1–10 ng) was added and the final PCR mixture volume was adjusted to 25 μL with sterile water. Along with DNA of the sludge samples, the DNA of *Methanosarcina barkeri* was used as a positive control for PCR amplification. The PCR program for the first amplification was as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min. The final extension was conducted at 72 °C for 7 min.

For the second amplification, the PCR mixture contained 1.5 μL bovine serum albumin (BSA; 10 μg/mL), 2.5 μL Thermopol buffer (10×), 0.2 μL deoxyribonucleotide triphosphate (dNTPs; 100 μM), 0.125 μL (SA1f-GC + SA2f-GC) as forward primer (100 μM), 0.125 PARCH 519r as reverse primer (100 μM) and 0.125 μL *Taq* DNA polymerase (Nicol et al., 2003). A volume of 1 μL of the PCR product obtained in the first round was used as a template and added to the PCR mixture. The final volume of the mixture was brought to 25 μL by adding 19.5 μL of sterile water. The PCR program for the second round was as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 53.5 °C for 1 min, and extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The presence of PCR products for each round was assessed by running product reaction aliquots on a 1% agarose gel.

### 2.3. Denaturing gradient gel electrophoresis (DGGE)

A detailed description of DGGE procedure and conditions was explained previously in Zamanzadeh et al. (2013). A DGGEK-2001-110 system (C.B.S. Scientific Inc., California, USA) was used to carry out DGGE analysis following Green et al. (2010). An aliquot of 5 μL PCR product was added to 10% denaturing polyacrylamide gels (gradient of 30–70%, where 100% denaturant is 7 M urea and 40% formamide). Following electrophoresis at 85 V and 60 °C for 14 h was followed by staining gels for 45 min in SYBR Green I nucleic acid gel stain (Invitrogen, Ontario, Canada). Gels were scanned using a Typhoon 9400 Variable Mode Imager system (GE Healthcare Life Sciences, Quebec, Canada).