The microbial diversity, methane production, operational routine of an anaerobic reactor treating maize processing wastewater

B. Ince, Z. Cetecioglu, S. Celikkol and O. Ince

Abstract

Industrial wastewaters have turned out to be a significant problem during the last decades. The best alternative for maize processing wastewaters due to high organic content and slow biodegradability characteristics is anaerobic treatment, which is successfully used in the treatment of medium-high strength industrial wastewaters. This study investigates a full-scale anaerobic expanded granular sludge bed reactor treating a maize industry wastewater in terms of reactor operation, methanogenic activity of reactor sludge, changes in composition and numbers of microbial populations during a 5-month period. Three samples were taken in 2-months intervals when the reactor was re-operated after a shut-down of 2 months. Combination of denaturing gradient gel electrophoresis (DGGE), quantitative real time polymerase chain reactions (Q-PCR) and specific methanogenic activity tests were used to investigate composition, diversity and quantity of microbial community with activity of acetoclastic methanogens. During monitoring period, COD removal efficiencies and organic loading rates varied in ranges of 79–95% and 0.65–3.85 kg COD/m³.d, respectively. Potential methane production rates of the reactor sludges decreased 27% with time that is from 394 to 287 mL CH₄/gVSS.d. Archaea, bacteria, and methanogens in the sludge samples were quantified by Q-PCR and the results showed that while amount of archaea was decreasing, quantity of methanogens increased during the monitoring time. The DGGE results of the anaerobic sludge revealed that the microbial diversity dramatically changed, particularly in the last sample. Among these, the differences in archaeal community were more remarkable. Although an average COD removal efficiency of 86%±8.2 which was quite a reasonably stable performance was maintained during the monitoring period, remarkable differences were observed on both acetoclastic methanogenic activity and the methanogenic community. The increase in the numbers of total methanogens despite the decrease of acetoclastic methanogenic activity might be an indicator of dominating hydrogenotrophic methanogenic pathway. Further, construction of clone library was necessary to identify the species and their changes within the all three samples.

Key words: anaerobic treatment, EGSB reactor, maize processing wastewater, molecular tools, microbial diversity, specific methanogenic activity

INTRODUCTION

Agricultural crop residues, which have high organic content and nutrients, are leading candidates of fermentation by-products and biogas production in terms of bioenergy recovery (Angenent et al. 2004). Biodegradability characteristics of wastes and wastewaters, which in turn lead to the decision of treatment method depend on not only raw material types but also production steps of agricultural crop processes. Due to high yield potential, maize is the most dominating energy crop for biogas production. In addition, maize-processing wastewater, which is rich in suspended solids and organic matter, is appropriate for anaerobic treatment (Amon et al. 2007). Because of these reasons, anaerobic
Biorreactors are commonly used in the treatment of maize process waste (water) with some unknown points such as effect of microbial community structure on pollutant removal yield and relation between sludge potential and treatment efficiency.

For decades, anaerobic treatment alternatives have been extensively used in the treatment of medium-high strength wastewaters generated from specific industrial processes and gained a considerable success in removal of organic compounds from wastewaters parallel to production of renewable energy. High organic loading rates (OLR) up to 25 kg COD/m³.day, less sludge production, lower energy requirements, biogas production as an alternative energy are considerable advantages of anaerobic systems in contrast to longer start up times (Chan et al. 2009). Nevertheless, anaerobic microbial community is less susceptible to operational system shut-downs compared to aerobic activated sludge systems.

Anaerobic biodegradation pathway is a synchronous chain of reactions conducted by a diverse community of archaea and bacteria. Maintenance of methanogenic population in an anaerobic reactor is of critical importance by means of treatment efficiency. For a stable reactor performance, monitoring of microbial population is essentially important along with operational parameters like sludge age, hydraulic retention time (HRT), OLR, F/M ratio, pH or temperature. Activity tests and molecular methods dependent on DNA and RNA such as cloning-sequencing, denaturing gradient gel electrophoresis (DGGE), quantitative real time polymerase chain reactions (Q-PCR) and fluorescent in situ hybridization (FISH) became more than an issue to monitor and better understand system stability.

The aim of this study is to investigate the microbial consortia related to the reactor performance after a 2-month shut-down of a full-scale mesophilic expanded granular sludge bed (EGSB) reactor treating maize-processing wastewaters. Combination of, DGGE, Q-PCR, methanogenic activity tests and also operational data were used to determine microbial population dynamics, quantity of microbial community on DNA basis and methane production, respectively.

**MATERIALS AND METHODS**

**Description of the anaerobic reactor and seed sludge**

Wastewater treatment plant, located in Turkey, treats maize industry effluents consists of an anaerobic EGSB with a total reactor volume of 1,500 m³ and active volume of 1,250 m³. Total volatile solids (TVS) concentration in the anaerobic EGSB reactor was in a range of 27,000–29,000 mg/L.

The EGSB reactor was fed by wastewater with a COD in a range of 1,900–4,500 mg/L. OLR was maintained in a range of 0.65–3.85 kg COD/m³.d during the monitoring period, November 2008 to March 2009. The EGSB reactor was fed by the wastewater with TKN in the range of 11.5–67.6 mg/L, PO₄-P in the range of 2.5–24.1 mg/L during this period. Mesophilic temperature conditions were not successfully maintained in the EGSB reactor as larger than a few degrees celcius ups and downs were observed from the operational data.

**Analytical methods**

The full-scale EGSB reactor was monitored regularly in terms of temperature, pH, COD, BOD, biogas production and composition, total solids (TS) and TVS. Gas compositions for methanogenic activity tests were determined using a Hewlet Packard 6850 gas chromatograph (GC) with a thermal conductivity detector operated at 200 °C. The column used was a HP Plot Q 30 m × 530 μm. All analyses were carried out according to Standard Methods (APHA 2005).
Description of specific methanogenic test

The anaerobic EGSB reactor sludge samples for methanogenic activity tests were taken in November 2008, January 2009 and March 2009. These tests were performed using the pressure transducer technique (Colleran et al. 1992). The pressure increases in sealed vials fed with non-gaseous substrates as acetate were monitored. The hand-held pressure transducer (Lutron PM-9107, U.S.A.) was capable of measuring a pressure in a range of 5–7,000 mbar, corresponding to 0.01 mole biogas in 60 ml headspace. The liquid volume as well as the headspace volume was 60 ml; 2,000 mg/L TVS were added to the serum bottles. Acetate concentrations in a range of 1,000–5,000 mg/L were initially tested in order to reach maximum potential methane production (PMP) rate during the batch tests. Among those 2,000 mg/l acetate concentration was found to be optimum. The basal medium in the batch experiments was prepared based on OECD311 (2006) protocol under strict anaerobic conditions. During the 4-day test duration, the bottles were stored at 35±2 °C and shaken daily by hand. Head-space pressure was measured every day by hand-held pressure transducer.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification of 16S rRNA genes

Genomic DNA (GDNA) was extracted from 0.5 g sludge using the FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions. Amplification of 16S rDNA from the extracted DNA was performed with primers selective for the *Archaea* and *Bacteria*. The primers and their annealing temperatures are given in Table 1. A nested PCR approach was applied to enhance sensitivity and specificity in all cases for Archaeal amplifications. The first round of Archaeal 16S rDNA amplification employed primers Arch46f and Arch1017r. PCR amplification was done as described by Cetecioglu et al. (2009).

Denaturing gradient gel electrophoresis (DGGE)

Community profiles of *Archaea* and *Bacteria* within the sediments were obtained using DGGE analysis of PCR amplification products from primers Arch344f_GCb-Univ522r and Bact341f_GCb-Bact534r as described by Muyzer et al. (1993). DGGE analysis and statistical tools were applied as described by Cetecioglu et al. (2009).

Quantitative real time polymerase chain reaction (Q-PCR)

The procedure recommended by Roche was followed and a Light Cycler Master Kit (Roche, Applied Science, Switzerland) was used to set up the reaction (2.0 μl master mix, 1.6 μl MgCl2 1.0 μl Primer F and R, 13.4 μl H2O, 1 μl sample). Absolute quantification analysis of the GDNA was carried out with a LightCycler 480 Instrument (Roche Applied Science, Switzerland). Bac 519f- Bac 907r (Lane 1991),

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<th>Table 1</th>
<th>Primers used in PCR amplifications</th>
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<td><strong>Primer</strong></td>
<td><strong>Target</strong></td>
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<tr>
<td>Bact341f_GCb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Bacterial 16S rDNA</td>
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<tr>
<td>Bact534r</td>
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<tr>
<td>Arch46f</td>
<td>Archaeal 16S rDNA</td>
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<td>Arch1017r</td>
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<tr>
<td>Arch344f_GCb&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Univ522r</td>
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<sup>a</sup>Escherichia coli numbering.
<sup>b</sup>5'-GC clamp on Arch344f and Bact341f (GCCGCGCCGCGCCGCGCCGCGCCGCGGGCCGACGGGGGCGGGGG).

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Arc 349f- Arc 806r primers (Takai & Horikoshi 2000) and Met 348f- Met 786r (Sawayama et al. 2006) were used for quantification of Bacteria, Archaea and methanogens, respectively.

RESULTS AND DISCUSSION

Performance of the full-scale anaerobic EGSB reactor

Changes in COD removal efficiency and OLR of the anaerobic EGSB reactor between November 2008 and March 2009, duration of 135 days, are shown in Figure 1. The anaerobic reactor was out of operation from September 2008 to November 2008 because of technical problems. First sample was collected just before the re-start of the anaerobic reactor. The other two samples were collected 2 months apart. While COD removal efficiencies varied from 79 to 95%, OLR and influent COD concentration changed 0.65–3.85 kg COD/m³.d and 1,900–4,500 mg/L, respectively. Fang et al. (2011) showed that these kinds of wastewaters were sufficiently treated in lab-scale EGSB reactor operated at considerably low OLRs such as 2.5–4.2 kg COD/m³.d. During the monitoring period, the anaerobic EGSB reactor was operated at temperature as 32 ± 4 °C which is near to lower limit of the mesophilic conditions. Another operational parameter, pH of 7.0 ± 0.1 was maintained in the reactor. EGSB reactors are able to treat low-strength wastewaters, particularly at low to mid temperatures (Lettinga et al. 1997). However, such temperature oscillations at the full-scale EGSB reactor might have caused instabilities in treatment performance. Considering oscillation in wastewater characteristics and operational parameters, COD removal efficiency of the EGSB reactor was comparatively successful.

Specific methanogenic activity test

Methanogenic activity tests were carried out to evaluate the PMP capacity of reactor sludge and to determine possible effects of the short-term shut-down on the reactor sludge. According to the activity test results, which are shown in Figure 2, a decrease of 27% in average PMP capacity of the sludge was observed between November 2008 and March 2009 sludge samples. However, the average actual methane production (AMP) rates obtained from the full-scale EGSB reactor were 165 ± 35 mL CH₄/gTVS.d. The AMP/PMP ratios were 0.41, 0.42 and 0.56, of November 2008, January 2009 and March 2009, respectively. The reason of this increase in the ratio was the loss of potential methanogenic activity (PMP) of the sludge. Based on the decrease in the PMP rate to 287 mL CH₄/gVSS.d, we considered that the potential methanogenic activity of the anaerobic sludge reached lower limits for good quality sludges reported in literature (>500 mL CH₄/gTVS.d) (Rintala et al. 1999; Ince et al. 2007). Ince et al. (1995) advised that optimum AMP/PMP ratios should vary 0.6–0.7 to achieve high COD removal efficiency and maintain high methanogenic activity. Accordingly, the OLR maintained
at the EGSB reactor can slightly be increased to maintain AMP/PMP ratio of 0.6–0.7. It can be said that, the short term shut-down of the EGSB reactor did not cause a major drawback on methanogenic activity but there is a considerable risk for maintenance of unsafe operational parameters. If these unfavorable operational conditions continue the reactor, sludge can lose its activity.

DGGE and cluster analysis of prokaryotic community composition

Results of DGGE analysis suggested that bacterial diversity was higher than archaeal diversity. It was found that the profile of bacterial community was very similar in all three sampling times whereas the archaeal community showed more different profile for each sample. A total of 30 different bacterial species and 15 different archaeal species were detected in the sludge samples. The most diverse sample was observed in March 2009 in which 25 different bacterial and 13 different archaeal bands were determined. However, the least diverse sludge was in January 2009, sample with 16 bacterial and 6 archaeal bands. As given in Table 2, both bacterial and archaeal diversity as measured by the Shannon–Weaver diversity index and species richness as measured by Margalef’s species richness measure, vary between 1.75–3.12 and 1.09–5.21, respectively. The lowest bacterial and archaeal diversity were in January 2009 sample in taxa results. The species evenness indices of bacteria and archaea varied between 0.83 and 0.98. This variation demonstrated that the relative abundances of the detected species were equally distributed within the DGGE profiles. High microbial diversity in the complex ecosystems provides more stability and various biochemical processes (Fernandez et al. 2000; Hunter-Cevera et al. 2005).

DGGE analysis of 16S rRNA genes were performed to compare the microbial composition of the three sludge samples and DGGE banding patterns were used to construct the dendograms as seen from Figure 3. The distribution and compositional changes of the bacteria and archaea reflect the

| Table 2 | Shannon–Weaver diversity indices, Margalef’s species richness measure, and evenness measure estimates for both bacterial and archaeal DGGE profile |
|---------|------------------|-----------------|-----------------|
|         | November 2008    | January 2009    | March 2009      |
| Bacteria| Taxa             |                 |                 |
|         | 18               | 16              | 25              |
|         | Shannon–Weaver diversity | 2.87          | 2.64            | 3.12            |
|         | Margalef’s richness | 3.69           | 3.25            | 5.21            |
|         | Evenness         | 0.98            | 0.88            | 0.90            |
| Archaea | Taxa             | 8               | 6               | 13              |
|         | Shannon–Weaver diversity | 2.01          | 1.75            | 2.37            |
|         | Margalef’s richness | 1.52           | 1.09            | 2.61            |
|         | Evenness         | 0.94            | 0.96            | 0.83            |
clusters obtained from the DGGE band patterns, which were divided into two clusters based on their sampling time. While the bacterial clusters were defined by more than 90% pattern similarity, the archaeal one were defined by less than 80%. Specific primers targeting bacterial and archaeal diversities yielded two main clusters, one of them including November 2008 and January 2009, and the second cluster for only March 2009. The most similar bacterial and archaeal DGGE band patterns, which had 93 and 95% similarities; respectively, belong to November 2008 and January 2009. March 2009 had different patterns. Also, AMP/PMP ratio of the March 2009 sample was more different than others. These clusters appeared to be very stable and almost showed no changes within the triplicate samples (data not shown). These results indicated that there is no significant shift in the microbial community pattern of November 2008 and January 2009. However, the microbial community was more diverse in March 2009.

Quantitative polymerase chain reaction (Q-PCR)

Quantitative changes in the 16S rRNA gene concentrations were also determined by real-time PCR (Figure 4). The concentration profile of bacterial community firstly decreased from $2.54 \times 10^{10}$ to
1.95×10^10 (approx. 33%) between November 2008 and January 2009, however the bacterial population increased around 56% after January 2009. While methanogenic community profile showed the same profile in the population, archaeal population had a decrease during the time. These results can indicate that a part of methanogenic population increased in the archaeal population from 0.01 to 0.6% during the monitoring period.

While SMA test results during the operating time showed that there was a change in the acetoclastic methanogenic activity of the anaerobic sludge, DGGE and Q-PCR results showed that there is an increase in the archaeal OTUs and methanogenic population portion in the archaeal community, respectively. It can be speculated that hydrogenotrophic methanogenic population might have become dominant in the archaeal population and hydrogenotrophic methanogenesis turned out to be primary pathway in this reactor during the monitoring time. Munk et al. (2010) showed that Methanobacteriales, Methanospirillum hungatei and Methanoculleus spp. were the dominant hydrogenotrophic methanogens in anaerobic digester where the maize waste was digested. In another study, the authors implied that hydrogenotrophic methanogenesis is only active metabolic pathway in herbal waste anaerobic digestor (Nettmann et al. 2008). Furthermore, low temperature operation (18 °C) of EGSB reactor cause to shift to hydrogenotrophic methanogenic population (Connaughton et al. 2006). Also the oscillation in the operational parameters such as temperature, pH firstly affects acetoclastic methanogens and give an advantage to hydrogenotrophic methanogens (McHugh et al. 2004).

**CONCLUSION**

After 2-month shut-down period, the COD removal efficiency of the anaerobic EGSB reactor can be considered as desirable while the acetoclastic methanogenic activity of the reactor sludge can be considered low-mid quality compared to literature. Microbiological studies showed that a change in methanogenic community composition in terms of quantity and diversity started after 4 months of re-start of the anaerobic reactor. We concluded that hyrogenotrophic methanogens might have become dominant because of the unstable operation conditions and 2-month shut-down. For further information, clone library should be constructed to determine the changes on species level. Also, the reactor was operated at a considerably low OLR, and temperature for anaerobic EGSB reactors. For long-term improvement on methanogenic activity and microbial characteristics/consortia of the sludge can be achievable at a higher F/M ratio, optimum mesophilic conditions and more consistant wastewater compositions.

**REFERENCES**


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