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Effects of carbon source on methanogenic activities and pathways incorporating metagenomic analysis of microbial community

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ABSTRACT

In this study, the effects of four types of organic compounds (tryptone, acetate/propionate, glucose and ethanol) on methanogenesis, electron transfer processes and microbial community structure were examined. When tryptone and acetate/propionate were used, the dominant methanogenic pathway was aceticlastic methanogenesis and *Methanosarcina* was the most abundant methanogen. When glucose or ethanol were provided as the external carbon source, the aceticlastic and hydrogenotrophic pathways were utilised simultaneously, and *Methanosarcina* and *Methanobacterium* were enriched. However, the reactor fed with glucose was prone to acidification because volatile fatty acids accumulated in the medium, which inhibited methane synthesis. *Geobacter* was dominant in the reactor fed with ethanol and 45% of genes encoding pili synthesis were attributable to *Geobacter*, indicating that direct interspecies electron transfer may be a possible mechanism during syntrophic methanogenesis.

1. Introduction

Anaerobic digestion is widely applied for removing organic compounds from various wastewaters because of its low cost, resilience to shock loading, and potential to produce methane (CH_4) and hydrogen (H_2) (Appels et al., 2008; Moraes et al., 2014; Xie et al., 2016). Anaerobic digestion occurs as a synergistic process between fermentative bacteria and methanogens. Here, direct and indirect interspecies electron transfer has been revealed as a key mechanism during organic matter degradation and methanogenesis (Malvankar & Lovley, 2014; Rotaru et al., 2014). Indirect electron transfer involves interspecies hydrogen transfer: IHT (Wolin, 1982), and interspecies formate transfer: IFT (Thiele and Zeikus, 1988). Direct interspecies electron transfer (DIET) is a syntrophic metabolism where electrons flow between cells without being shuttled via reduced compounds such as hydrogen or formate (Shrestha and Rotaru, 2014; Summers et al., 2010). Recent studies have shown that DIET can be predominant during syntrophic methanogenesis (Lovley, 2017; Zhao et al., 2016a; Zhao et al., 2017).

Interspecies electron transfer is affected by intermediate metabolites formed during the syntrophic metabolism of different organic carbon sources. Indirect electron transfer, IHT in particular, was the primary pathway of electron transfer when butyric acid was the carbon source, with *Methanosarcinacea* as the dominant methanogen (Li et al., 2015). Similarly, IHT was the primary electron transfer pathway during syntrophic propionic acid degradation, where a large proportion of the community was propionic acid oxidizing bacteria, and *Methanosaeta* was the dominant methanogen (Jing et al., 2017). In contrast, Morita et al. (2011) and Shrestha and Rotaru (2014) reported that *Geobacter* was enriched during anaerobic digestion of brewery wastes. They proposed that *Geobacter* participated in syntrophic methanogenesis via DIET.

Clearly, the type of organic compounds present affect the acclimated anaerobic microbial communities and their degradation pathways. Zhao et al. (2016b) observed that *Methanobacterium* and *Methanolinea* were the dominant methanogens when acclimatized to propionic acid, while *Methanolinea* and *Methanosaeta* were the dominant methanogens when using butyric acid. Antwi et al. (2017) observed aceticlastic methanogens, especially *Methanosaeta*, to be enriched when using starch as the substrate. *Methanothrix*, another aceticlastic methanogen, was abundant in the anaerobic community consuming cassava flour (Sun et al., 2012). Furthermore, Angelidaki and Batstone (2011) observed acidification when polysaccharides were used as the carbon source, and most methanogens were aceticlastic

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methanogens, with 60%–70% of CH_4 generated from the aceticlastic pathway. However, to date, few integrated studies have examined the effect of various carbon sources on methanogenesis, pathways, and community analyses using metagenomics and activity assays.

In this study, tryptone, acetate/propionate, glucose and ethanol, representing protein, volatile fatty acids (VFAs), carbohydrate and alcohol, respectively, were used as the carbon sources. Their effects on the microbial community structure and methanogenic activity were investigated. Metagenomic analyses were conducted to better understand the effect of the carbon source on prevalent methanogenic pathways within the enriched communities.

2. Materials and methods

2.1. Long term acclimation and operational conditions

Four 2-L anaerobic sequencing batch reactors (ASBRs) were fed tryptone (ASBR_Try), volatile fatty acids (ASBR_VFA), glucose (ASBR_Glu) or ethanol (ASBR_Eth). The mesophilic reactors (35 ± 1 °C) were operated at a hydraulic retention time of 48 h. The ASBR operation cycle consisted of 23 h of anaerobic mixing (including 5 min filling), 55 min for settling and 5 min for decanting (under anaerobic conditions). After each cycle, 1 L of water and 60 mL of excess sludge were discharged, with the sludge retention time of approximately 33 days. The total gas produced was measured using water displacement. The VFA concentrations, suspended solids (SS), volatile suspended solids (VSS) and gas production were periodically measured during the acclimation process.

The reactors were fed with synthetic wastewater containing the respective organic compound (i.e. tryptone, acetate/propionate, glucose, or ethanol), and nutrient elements at a chemical oxygen demand (COD): nitrogen: phosphorus ratio of 200:5:1. Influent COD concentration was about 2000 mg/L, which consisted of either 1880 mg/L tryptone, 1280 mg/L sodium acetate and 860 mg/L sodium propionate, 1870 mg/L glucose, or 1.22 mL/L ethanol. In this study, a synthetic wastewater was used to better understand the underlying phenomena and minimize confounding results. It should be noted that results in real wastewaters may differ, due to heterogeneity in nutrients, carbon availability and pH. The synthetic wastewater also contained 192 mg/L NH_4Cl, 48 mg/L Na_2HPO_4, 100 mg/L $CaCl_2$, 200 mg/L $MgCl_2$, 1000 mg/L $KHCO_3$ and 1 mL/L trace elements. The trace elements were prepared according to Yin et al. (2017).

At steady-state, COD removal and methanogenic activity from a typical ASBR reaction cycle were determined. A 125-mL mixed liquor sample taken from the reactor was mixed with 125-mL of synthetic wastewater, and placed in a 310 mL serum bottle. Prior to the experiment, the headspace was flushed with nitrogen gas for 5 min to remove residual oxygen. The bottles were sealed with rubber stoppers and placed in an air bath shaker at 170 rpm at 35 °C. Liquid and gas samples were collected at 2-h intervals and analyzed for COD, VFAs and CH_4. Gas production was measured using a differential pressure meter. Gas samples were stored in evacuated vials prior to analysis by gas chromatography.

2.2. Methanogenic activity experiments

The mechanism of methane production was assessed using acetate or H_2/CO_2 as substrates. A flask with an effective volume of 310 mL was used as a reactor. A 125 mL mixed liquor from the parent reactor was mixed with 125 mL of synthetic wastewater containing acetate. The protocol was the same used for methanogenic activity during the ASBR cycle experiment.

When H_2/CO_2 were used as electron donors, a flask with an effective volume of 310 mL was also used as the reactor, and 100 mL mixed liquor from the parent reactor was mixed with 100 mL of synthetic wastewater without any carbon source (the low volume was adopted to enhance the substrate loading in the gas phase). The headspace was sparged with a mixture of H_2 and CO_2 at a ratio of 4:1 (v:v) at ambient pressure. The protocol was also the same as the methanogenic activity during the ASBR cycle experiment.

2.3. Analytical methods

COD, SS and VSS were measured according to standard methods (APHA, 1995). CH_4 was determined by gas chromatography (GC2014, Shimadzu, Japan) equipped with a thermal conductivity detector (Yin et al., 2017). The modified Gompertz equation was applied to quantitatively analyze the production of CH_4 (Zwietering et al., 1990):

$$P = P_{max} exp\left\{-exp\left[\frac{R_{max} e}{P_{max}}(\lambda - t) + 1\right]\right\}$$

where P is cumulative CH_4 production (mL); P_{max} is maximum cumulative CH_4 (mL) potential by the end of incubation; R_{max} is the maximum CH_4 production rate (mL/h); λ is the lag time (h); e is the natural base (2.71828).

Microbial DNA was extracted from the sludge using a PowerSoil DNA Kit (Laboratories Inc., CA, USA). The extracted DNA was amplified by PCR in the V4 region of the target fragment 16S RNA gene, and analyzed by high-throughput sequencing using the Illumina Miseq platform. After screening, species classification of the representative spliced sequences for each OTU was performed using Qiime software. Results were classified to the phylum, class, order, family and genus levels (Navarro et al., 2015).

The protocol for metagenomic sequencing was as follows. The DNA samples were extracted using TruSeq DNA Sample Prep Kit (Illumina Inc, USA) and analyzed using the Illumina Miseq platform. The sequences assembled by the SOAPdenovo program were then predicted by the MetaGene program (Noguchi et al., 2006). To further estimate the function and metabolic pathway of genes, the Evolutionary Genealogy of Genes_Non-supervised Orthologous Groups (EggNOG) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were used via the BLASTP program with an E-value cutoff of 10^{-5}. The relative abundance of a gene was estimated by comparing the number of high-quality reads assigned to a specific gene in the gene set (95% identity) versus the number of total high-quality reads obtained for that sample using the SOAPaligner program (Li et al., 2008).

3. Results and discussion

3.1. Long-term system performance

In this study, COD removal, VFA fluctuation and biogas production represent the key system performance indicators. The four reactors were operated for more than 80 days. Over an acclimation period of 30 days, ASBR_Try, ASBR_VFA and ASBR_Eth reached steady state, while the steady state for ASBR_Glu was achieved after 60 days. Under steady state conditions, the COD removal in ASBR_Try, ASBR_VFA and ASBR_Eth exceeded 90%. The lowest COD removal in ASBR_Glu was 72%.

The COD removal in a typical ASBR cycle are presented in Fig. 1a. The highest COD removal rate was obtained in ASBR_Glu (323 mg/L·h) during the initial stage, indicating the ease of glucose assimilation compared to other carbon sources. However, the COD removal rate declined after 2 h, and the lowest overall COD removal was obtained in ASBR_Glu (71.93%), probably due to the accumulation of the VFAs. The COD removal for reactors fed with other carbon sources were all greater than 90%. The maximum COD removal rates for ASBR_Try, ASBR_VFA and ASBR_Eth were 195, 102 and 104 mg/L·h, respectively.

Dynamics of the VFA fluctuations are shown in Fig. 1b. Acetic and propionic acids were the dominant VFAs in ASBR_Try and ASBR_Glu, with acetic acid accumulation higher than that of propionic acid in