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Research paper

Effect of humic acids on the activity of pure and mixed methanogenic cultures

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ABSTRACT

The impact of humic acid (HA) on methanogenic activity was investigated. Methanogenic crushed granular sludge and pure cultures of mesophilic methanogens were incubated in batch cultures with HA. Initial methane production rates and substrate consumption rates were quantified. In the presence of 1 kg m^{-3} HA, the methane production rate of all hydrogenotrophic methanogens was inhibited by more than 75%, except *Methanospirillum hungatei* that was not inhibited up to 5 kg m^{-3} HA. The acetoclastic *Methanosarcina barkeri* was completely inhibited by HA ≥1 kg m^{-3}. However, *Methanosaeta concilii* was only slightly affected by HA up to 3 kg m^{-3}. When methanogenic granular sludge was incubated with HA, the specific methanogenic activity (SMA) tests showed less inhibition, when compared to the pure cultures of methanogens. The SMA test with H_2/CO_2, formate and acetate showed reduced initial methane production rate of 42%, 23% and 40%, respectively. Differences in HA susceptibility were explained by differences in cell wall structure.

1. Introduction

Methanogens are strictly anaerobic archaea that have diverse morphology and phylogeny. Their ecological niches are widely distributed. They can be found in aquatic sediments (marshes and swamps), stagnant soil (peat bogs and rice fields), marine geothermal vents, the digestive tract of animals (ruminants and termites) and in engineered anaerobic digesters [1]. Methanogens are sensitive to environmental factors. Wide range of organic compounds, such as long chain fatty acids, aromatic compounds, xenobiotics, and inorganic compounds such as ammonia and heavy metals have been described to affect the methanogenic activity [2].

Humic acids (HA) are charged polyelectrolyte complexes due to the presence of carboxylic, phenolic, ketonic, aromatic and aliphatic groups and interact with both living and non-living matter [3]. They can function as electron shuttles in anaerobic environments for fermentive, iron-reducing and sulphate-reducing bacteria, as well as for methanogenic archaea [4–8].

Although the role of HA in natural environments is known, their abundance, composition and effect in engineered systems (e.g. in anaerobic digesters) are not defined well in the literature. In an anaerobic digester environment, abundance and composition of HA mainly depend on the type of the feed [9]. HA concentrations can reach up to mass fraction of 1.5% of total solids in the treatment sludge and agricultural waste, such as manure and maize [9–11]. Abundance of HA in anaerobic digesters may negatively affect the overall conversion processes. Indeed, the negative effect of HA on hydrolysis step of anaerobic digestion was shown [9,12–14]. In addition, a decrease in methanogenic activity was observed in the presence of HA [12–15]. However, from these experiments it was not evident whether the methanogens were affected and if so, which physiological groups/phylotypes of methanogens were most vulnerable to HA inhibition. Thus, it is important to determine the physiological response of different methanogenic groups to get more information about the methane production in the anaerobic digesters, having higher HA concentrations.

In this study, important acetoclastic and hydrogenotrophic

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methanogenic groups, belonging to *Methanosaetaceae*, *Methanosarcinaceae*, *Methanospirillaceae*, and *Methanobacteriaceae*, were selected to test their methanogenic activity in the presence and absence of HA. These methanogenic groups were selected due to their high abundance in most of the anaerobic digesters [16]. The methanogenic activity of pure cultures was compared to anaerobic crushed methanogenic granular sludge from a full scale UASB (Upflow Anaerobic Sludge Blanket) reactor treating paper mill wastewater. In this scope, batch tests were set-up in identical conditions for both pure and mixed cultures. During the batch experiments, methanogenic activity of each experimental group was monitored with gas and organic acid measurements.

2. Materials and methods

2.1. Experimental set-up

The effect of humic acid (CAS Number 68131-04-4, Sigma-Aldrich, Zwijndrecht, The Netherlands) on mesophilic methanogens was investigated in batch tests. Crushed mesophilic anaerobic granular sludge and pure cultures of methanogens were tested. Batch incubations were performed in 120-cm^3 bottles with 50 cm^3 bicarbonate buffered mineral salts medium, supplemented with cysteine (0.96 kg m^{-3}), trace elements and a vitamin mixture. Additionally, 0.12 kg m^{-3} acetate was added to the hydrogenotrophic cultures (also when grown on formate) as additional carbon source [17,18]. The bottles were inoculated with a volume fraction of 10% of a culture pre-grown on the same substrate. Depending on the metabolic property of the strain, the growth substrates were H_2/CO_2 (a volume fraction of 80%/20%, respectively at 150 kPa), formate (final concentration: 1.68 kg m^{-3}) or acetate (final concentration: 1.2 kg m^{-3}), the latter two having a headspace of N_2/CO_2; a volume fraction of 80%/20%, respectively at150 kPa. In the assays 0, 1, 3 and 5 kg m^{-3} humic acid were tested, unless stated otherwise. The batch incubations were performed in duplicate and in the dark at 37 °C, pH 7. Methane (CH_4) production and hydrogen (H_2) consumption were monitored by gas chromatography. Liquid samples were collected to measure changes in acetate and formate concentrations.

2.2. Composition and the source of the humic acid

Humic acid (CAS Number 68131-04-4, Sigma-Aldrich, Zwijndrecht, The Netherlands) was used in the experiments. Only one batch of the humic acid was used throughout the experiments to avoid the composition changes during the production phase of the product. According to the manufacturer, the product may be produced from dead plants and brown coal. Alkaline extraction methods are applied to recover the humic acid. Molecular weight of the product is in the range of 2000–500000. The composition of the product includes polysaccharides, proteins, simple phenols, and chelated metal ions. Washing steps with deionized water are applied to remove the excess amount of the organic contaminants. Addition of HA to the experiments introduces a maximum amount of sodium (approximately 0.3 kg m^{-3}) and small amounts of calcium and iron in the anaerobic media [16]. Although an excess of sodium could potentially inhibit anaerobic digestion, sodium in HA, was still 10 folds lower than inhibitory sodium concentrations that were previously reported [19–21]. Moreover, the presence of the other cations such as potassium, magnesium and calcium, are likely to show antagonistic effects to sodium inhibition [19].

2.3. Growth conditions of methanogenic cultures and anaerobic sludge

In this study, *Methanosaeta concilii* (DSM 2139), *Methanosarcina barkeri* (DSM 800), *Methanobacterium formicicum* (DSM 1535), *Methanospirillum hungatei* (DSM 864) and *Methanobrevibacter arboriphilicus* (DSM 744) were used as pure cultures. All cultures were routinely grown at 37 °C in an anaerobic bicarbonate buffered medium [17,18]. Three subsequent transfers of each strain were made to ensure optimum growth conditions in the defined medium. After successful transfers, the microorganisms were used in the batch activity tests.

Granular methanogenic sludge was obtained from a UASB reactor treating pulp and paper industry effluents (Industriewater Eerbeek, The Netherlands). Sludge samples were collected on 10th of April 2014. Immediately after collection, granules were crushed under nitrogen gas flow in a 500-cm^3 serum bottle that contained 250 cm^3 phosphate saline buffer solution (0.1 kg m^{-3}, pH 7). The slurry obtained was transferred to a 500-cm^3 serum bottle and flushed with nitrogen gas. About 5 cm^3 of the prepared slurry (1 kg m^{-3} volatile solids) was used for the batch activity tests.

2.4. Analytical methods

2.4.1. Gas measurements

CH_4 and H_2 content of the gas phase was analysed with a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 2 m long, 3 mm internal diameter and 60–80 mesh packed column (Molsieve 13X) (Varian, Middelburg, The Netherlands). The column had a thermal conductivity detector that was operated at 70 mA, 150 °C. Argon was the carrier gas at a flow rate of 30 cm^3 min^{-1}. Gas samples (0.2 cm^3) were taken by syringe and the gas content was expanded to 1 cm^3 while the needles were in the rubber stopper, and injected to the column. All measurements were performed in duplicate and data was analysed using ChromQuest software (Thermo Scientific, Waltham, MA).

2.4.2. Organic acid measurements

Liquid samples were collected to determine acetate and formate concentrations. Liquid samples were centrifuged (11200 RCF, room temperature, 10 min) and filtered through a polypropylene filter (0.45 μm). The obtained supernatants were analysed by Thermo Scientific Spectrasystem HPLC system, equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 45 °C, running with 0.5 kg m^{-3} sulphuric acid as eluent. The eluent had a flow rate of 0.8 cm^3 min^{-1}. The detector was a refractive index detector. Data was analysed using ChromQuest (Thermo Scientific, Waltham, MA).

3. Results and discussion

3.1. Effect of humic acid on methanogenic cultures

For all methanogenic pure cultures used in this study, the recovery of reducing equivalents in the form of CH_4, produced from H_2/CO_2, acetate and formate, was always higher than 85%.

3.1.1. Hydrogenotrophic methanogenesis

When *Methanobacterium formicicum* was grown on formate in the absence of HA, the maximum total amount of methane (0.2 mmol) was produced within one day (Fig. 2a). In the presence of HA, methane was also produced, but after a long lag phase of 20 days (Fig. 2b). The duration of the lag phase was similar for the cultures grown with different HA concentrations. During the lag phase, accumulation of trace amounts of H_2 was observed (to 0.027–0.035 mmol, Table 1). After day 20, the trace amounts of H_2