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Effect of different acetate:propionate ratios on the methanogenic community during thermophilic anaerobic digestion in batch experiments

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ABSTRACT

The present study investigated the metabolism of different acetate:propionate ratios (0.25, 0.33, 0.5, 1.0, 2.0, 3.0, 4.0) in equimolar carbon concentration during an anaerobic decomposition process under defined laboratory conditions and evaluated the engaged methanogenic community. Significant differences on a metabolic level (gas production, gas composition, volatile fatty acid (VFA) concentration) were observed between acetate:propionate ratios ≤1 and ≥2. Generally ratios ≥2 resulted in a faster methane production and VFA decomposition compared to ratios ≤1. Regarding the composition of the methanogenic community as well as the abundance of *Methanosarcinales* these differences were not reflected in an appropriate manner when DNA based methods (dHPLC and qPCR) were applied. However, by a culture based approach these differences could be documented showing a significant difference in the number of cultivable methanogens between initial acetate:propionate ratios ≤1 and ≥2.

1. Introduction

Biogas as a source of renewable energy has been paid increased attention during the last two decades. Therefore, the microbiology of anaerobic digestion is getting more and more into the spotlight, since anaerobic degradation processes are diverse, complex, depend on several trophic levels of bacteria and archaea [1] and still have some potential for further optimisation [2–4]. The anaerobic decomposition process itself can be divided into different phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis [5,6], during which various volatile fatty acids (VFAs) are known to play a central role. VFAs are intermediate products between complex substances and the end products of the microbial decomposition process H_2, CO_2, and CH_4. Especially acetate [8,9] but also propionate [7] are known as the most important intermediates in the anaerobic digestion process.

It is well known, that acetate, like other short chain fatty acids (VFAs), *per se* can serve as a toxic substance when prevalent in its undissociated form [10] and thermodynamic investigations could show that high acetate concentrations are hampering acetogenic bacteria as well as the propionate degradation during anaerobic degradation processes [11–15].

The last step in the anaerobic digestion cascade, when methane is formed either from acetate or CO_2/H_2, is often seen as the most important one [16]. For the latter reaction solely hydrogenotrophic methanogens are responsible whereas methane formation from acetate can be carried out via acetoclastic methanogenesis as well as syntrophic acetate oxidation (SAO). While SAO is a two-step reaction involving acetate oxidizing bacteria producing H_2/CO_2, which subsequently is utilized by a synthrophic, CO_2 reducing methanogenic partner-organism, acetate can be cleaved directly. In either pathway acetate is posing the key on the way to methane production.

Also propionate is an important intermediate during anaerobic digestion. When degraded into acetate and H_2/CO_2 and subsequently into methane it may account for 6% up to 35% of total methane in an anaerobic digestion process [17]. Under standard conditions propionate oxidation to H_2, formate and acetate is an endergonic reaction [18]. Therefore in anoxic environments propionate oxidation is only made possible by methanogens which are responsible for the maintenance of low concentrations in hydrogen, formate, and acetate [19]. The syntrophic degradation of fatty acids (acetate (SAO) and propionate (syntrophic propionate oxidation)) with its final result methane is believed to be a rate limiting step of methane production in the anaerobic degradation process [20,21].

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The aim of the present study was to investigate the effect of different Ac:Pr ratios on the overall biogas and methane production applying strictly defined conditions in a batch system. These data were connected to the abundance of methanogens as well as to the methanogenic community as derived from cultivation based and molecular biological investigations.

2. Materials and methods

2.1. Media

Serum flasks (with a total volume of 120 ml) were filled with 50 ml of minimal medium (pH 7.2) containing per liter: KH_2PO_4 0.50 g, $MgSO_4 \times 7\ H_2O$ 0.25 g, NaCl 0.40 g, NH_4Cl 0.500 g, $CaCl_2 \times 2\ H_2O$ 50 mg, $FeSO_4 \times 7\ H_2O$ 5 mg; additionally 1 ml of trace element-, vitamin-, Resazurine-, and Se-W solution and 2 ml of NaS solution [22] were added. The headspace was flushed with gas consisting of 70% N_2 and 30% CO_2. Flasks containing 50 ml of minimal medium were prepared in order to achieve different acetate: propionate (Ac:Pr) ratios. Ratios tested were (Ac:Pr): 0.25, 0.33, 0.50, 1.0, 2.0, 3.0, and 4.0. For this purpose 1.0 M aqueous stock solutions of sodium acetate and sodium propionate were prepared anaerobically and added to the flasks by a syringe. The carbon concentration for all samples was kept equimolar (45 mM carbon in the liquid phase in order to achieve a complete utilization of carbon within approximately 3 months based on previous experience).

2.2. Inoculum

As inoculum, digester sludge from the outlet sampling port of a 750,000-l anaerobic digestion plant in Roppen, Austria, running under thermophilic conditions (described by in [23]; running parameters can be found in [24]) was used. When samples for the inoculum were taken (09/2009) the acetate: propionate ratio within the reactor was 0.8 (±0.29) on average when taking a period of 3 months as a basis [25]. The preparation of the inoculum was carried out as follows: distilled water was autoclaved for 10 min and during the cooling down process the flask's headspace was flushed for 10 min with a gas mixture of 70% N_2 and 30% CO_2. Subsequently, approximately 100–150 g of sludge was added to obtain a dilution of 1:5, before the flask was flushed again and closed with a butyl rubber septum (Ochs, Germany). The flask containing the diluted fermenter sludge (DFS) was shaken at 200 rpm for 30 min at 52 °C.

2.3. Experimental setup and procedure

The flasks prepared as in the way described in 2.1 were inoculated with 5% of diluted sludge (refer to Section 2.2) and incubated at 52 °C for 75 days. As a control, a sample without any additional carbon source was treated analogously in order to quantify the endogenic activity (methane production) from the inoculum only. All variants were prepared in triplicate.

Samples for downstream analysis were taken at day 0, 4, 11, 18, 30, 42, 56, and 75. Samples for VFA analysis were taken separately (refer to Section 2.4). An aliquot sample for molecular biological investigations was deep frozen, whereas analysis of cultivable methanogens was done immediately at the sampling date. Pressure and headspace gas analysis was performed at day 0, 4, 11, 18, 30, 42, 56, and 75 prior to sampling as described in Section 2.4.

2.4. Gas, pH and VFA analysis

Liquid samples for organic acid analysis were taken by removing 0.5 ml of the liquid with a syringe after intense shaking. Before further treatment one drop of the liquid was used for pH measurement using pH-strips (pH 5–10; Merck, Germany). Subsequently, the samples were centrifuged at 20,000 × *g* for 15 min, the supernatant filtered through a 0.2 μm filter (RC, regenerated cellulose), and injected (20 μl) directly onto the HPLC system. Samples for organic acid determination were used immediately after drawing them or deep frozen until analysis day. The concentrations of VFAs in the liquid phase (formic-, acetic-, propionic-, and butyric-acid) were measured via HPLC analysis on a BioRad AMINEX HPX-87H column as described before [26]. Pressure in the headspace was measured with a pressure sensor (Greisinger, Germany). The measurement of the pressure allowed the calculation of the total volume of the produced gases. To obtain correct values overpressure resulting from the increase in temperature at the beginning of the incubation and after each gas measurement was drained with a cannula after the liquid had reached the incubation temperature of 52 °C. Analysis of methane, hydrogen and carbon dioxide in the headspace was carried out using a Shimadzu GC2010 with a Wide Bore Injector (WBI) equipped with a Restek ShinCarbon 100/120 packed column and TCD (Thermal Conductivity Detector) as described before [22].

2.5. Cultivable methanogens

The most probable number of cultivable methanogens was evaluated using method and equipment as described in [27] with the following modifications: as a dilution solution a modified medium was used containing 0.5 g KH_2PO_4, 0.4 g $MgSO_4$, 0.4 g NaCl, 0.4 g NH_4Cl, 0.05 g $CaCl_2 \times 2H_2O$, 2 mg $FeSO_4 \times 7H_2O$, 3.0 g yeast extract, 2.0 g Na-acetate, 4.0 g Na-formate, 2.0 $NaHCO_3$, 0.5 g cysteine–HCl, trace element-, Se-W-, and resazurine solution as described above (Section 2.2). A fatty acid mixture (20 ml per liter medium) was added after autoclaving containing 0.5 g valeric acid, 0.5 g isovaleric acid, 0.5 g 2-methylbutyric acid, 0.5 g iso-butyric acid, 20 ml distilled water. For further reduction an anaerobic, 1% (v/v) of a 0.5% $TiCl_3$ solution was used under anoxic conditions. 96-well plates were filled with 180 μl of medium, 20 μl of sample was added, diluted 10-fold up to 10^{-11}, and sealed with adhesive aluminum-foil. An anaerobic incubation of 14 days followed and the plates were analyzed towards methane production via GC measurement as described in [27]. The last row of the plates was used to determine the CH_4 concentration in the surrounding atmosphere (control). A well was considered positive when the methane concentration 3-fold exceeded the control (on the basis of area counts).

2.6. DNA-extraction, PCR, and PCR conditions

An aliquot of 500 μl liquid from each replicate was taken out of the flasks by a syringe, pooled and centrifuged at 20,000 × *g*. Supernatant of 250 μl liquid was discarded, the pellet re-suspended in the remaining rest of the liquid and transferred into DNA extraction matrix tubes using the NucleoSpinSoil DNA extraction Kit (Machery & Nagel). All extraction steps were carried out according to manufacturer's protocols including enhanced lysis buffer. DNA was eluted in 50 μl of elution buffer, reserve-aliquots stored at −20 °C, working stocks at 4 °C. DNA concentrations were measured spectrophotometrically using NanoDrop 2000 (Thermo Fisher Scientific).

PCR with universal primers targeting the 16s rRNA gene (109f/1492r) [28,29] and subsequently with methanogen specific primers (nested PCR) (O357f/O691r) [30] was carried out according to standard protocols. The reaction mixture contained 25 μl MyTaq™ 2 × Mix PCR mixture (VWR, Germany), primers in a final concentration of 0.5 μM, 50 μg bovine serum albumin (aqueous solution, filter sterilized), PCR grade water to achieve a final volume of 50, and 2 μl of template DNA. Following PCR-programs were used for amplification of DNA: or primer 109f/1492r 10 min of initial denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 52 °C and 45 s at 72 °C and a final elongation at 72 °C (10 min);