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Isolation of acetic, propionic and butyric acid-forming bacteria from biogas plants

Katharina Gabriela Cibis*, Armin Gneipel, Helmut König

Institute of Microbiology and Wine Research (IMW), Johannes Gutenberg-Universität of Mainz, Johann-Joachim-Becherweg 15, 55128 Mainz, Germany

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ABSTRACT

In this study, acetic, propionic and butyric acid-forming bacteria were isolated from thermophilic and mesophilic biogas plants (BGP) located in Germany. The fermenters were fed with maize silage and cattle or swine manure. Furthermore, pressurized laboratory fermenters digesting maize silage were sampled. Enrichment cultures for the isolation of acid-forming bacteria were grown in minimal medium supplemented with one of the following carbon sources: Na^+-DL-lactate, succinate, ethanol, glycerol, glucose or a mixture of amino acids. These substrates could be converted by the isolates to acetic, propionic or butyric acid. In total, 49 isolates were obtained, which belonged to the phyla Firmicutes, Tenericutes or Thermotogae. According to 16S rRNA gene sequences, most isolates were related to *Clostridium sporosphaeroides*, *Defluviitoga tunisiensis* and *Dendrosporobacter quercicolus*. Acetic, propionic or butyric acid were produced in cultures of isolates affiliated to *Bacillus thermoamylovorans*, *Clostridium aminovalericum*, *Clostridium cochlearium*/*Clostridium tetani*, *C. sporosphaeroides*, *D. quercicolus*, *Proteiniborus ethanoligenes*, *Selenomonas bovis* and *Tepidanaerobacter* sp. Isolates related to *Thermoanaerobacterium thermosaccharolyticum* produced acetic, butyric and lactic acid, and isolates related to *D. tunisiensis* formed acetic acid. Specific primer sets targeting 16S rRNA gene sequences were designed and used for real-time quantitative PCR (qPCR). The isolates were physiologically characterized and their role in BGP discussed.
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1. Introduction

In biogas plants, a complex and dynamic microbial community produce biogas by anaerobic digestion of organic biomass. The process of anaerobic digestion is divided into four steps. In the first step (hydrolysis), organic polymers, such as polysaccharides, lipids and proteins, are converted into sugars, fatty acids, amino acids, alcohols, acids, other monomeric compounds, hydrogen and carbon dioxide. The hydrolysis products formed are degraded in the next step (acidogenesis) to volatile fatty acids (VFA), alcohols, carbon dioxide and hydrogen. The VFA are converted to acetic acid, carbon dioxide and hydrogen in the third step (acetogenesis). Methanogenic Archaea produce methane and carbon dioxide in the last step (methanogenesis) (Kaiser et al., 2008; Merlin Christy et al., 2014; Stantscheff et al., 2014).

Acetic, propionic and butyric acid are important VFA produced during anaerobic digestion. An accumulation of VFA often leads to a decrease of the pH value in biogas plants and causes process disturbances. To overcome this problem, stopping the supply of substrates into biogas plants can counteract the acidification, because microorganisms have time for degradation of excess acids. The upper value of acetic acid in agricultural biogas plants should be lower than 3000 mg l^{-1}, the value of propionic acid lower than 1000 mg l^{-1}, of *iso*-butyric acid below 500 mg l^{-1} and of VFA below 4000 mg l^{-1} (Kaiser et al., 2008). Furthermore, a process disturbance could be possible, if the ratio of propionic acid to acetic acid is higher than 1.4 (Hill et al., 1987) or higher than 1.0 (if the value of propionic acid is greater than 1000 mg l^{-1}; Weiland, 2010).

According to the literature, certain bacteria can convert sugars, acids, alcohols or amino acids to propionic or butyric acid under anaerobic conditions. Glucose can be oxidized to propionic acid by *Propionibacterium* sp. or *Arachnia propionica* (Allen and Linehan, 1977; Pine and George, 1969). Glucose is converted to butyric acid, carbon dioxide and hydrogen during butyric fermentation. This reaction is performed, for example, by *Clostridium butyricum*, *Clostridium pasteurianum* or *Butyrivibrio fibrisolvens* (Gottschalk, 1979). Furthermore, microorganisms can metabolize various alcohols to propionic acid: *Propionibacterium acidipropionici* (glycerol

Abbreviations: BGP, biogas plant; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; SAPD-PCR, specifically amplified polymorphic DNA-PCR; VFA, volatile fatty acids.

* Corresponding author.
E-mail address: cibisk@uni-mainz.de (K.G. Cibis).

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to propionic acid; Barbirato et al., 1997), *Pelobacter propionicus* (ethanol and carbon dioxide to propionic and acetic acid; Schink et al., 1987). *Clostridium kluyveri* is able to form butyrate and caproate from ethanol and acetic acid, respectively (Seedorf et al., 2008). Propionic and butyric acid can also be produced by the conversion of acids, which can be intermediates or end-products of the previous steps of anaerobic digestion. *Propionigenium modestum* and *Selenomonas acidaminovorans* are examples of bacteria, which can decarboxylate succinate to propionic acid (Guangsheng et al., 1992; Schink and Pfennig, 1982). Gottschalk (1979) and Seeliger et al. (2002) reported on the conversion of lactic acid to propionic acid, acetic acid and carbon dioxide by *Propionibacterium* sp., *Veillonella parvula*, *Clostridium propionicum*, *Megasphaera elsdenii* and *Clostridium homopropionicum*. Microorganisms can also use some amino acids for the formation of the three acids mentioned. Glutamate, for example, is converted to butyric and acetic acid by *Clostridium tetanomorphum*, *Clostridium tetani*, *Clostridium cochlearium* and *Peptococcus aerogenes* (Buckel and Barker, 1974). Furthermore, butyric and acetic acid are formed with the substrate lysine by *Clostridium sticklandii* (Fonknechten et al., 2010). *C. propionicum*, *C. pasteurianum* and *P. aerogenes* are capable of the formation of propionic acid, carbon dioxide and hydrogen by the degradation of threonine (Gottschalk, 1979).

Up to now, the majority of microorganisms in biogas plants have been unexplored. In the past, molecular biological methods have been applied to get an insight into the diversity of microbiota. Metagenome analyses have revealed that many microorganisms have not yet been classified (Kröber et al., 2009). Because of the small number of isolates from biogas plants, there is a lack of microbial reference data about the physiology of these bacteria involved in the conversion of biomass to methane. Recently, a few isolates were obtained from biogas plants or laboratory biogas reactors: Koeck et al. (2014) identified isolated strains of *Clostridium thermocellum*, Hahnke et al. (2014), the new species *Clostridium bornimense*, and Stantscheff et al. (2014) methanogenic Archaea.

Since bacteria, especially those which form acetic, propionic and butyric acid in biogas plants, have been weakly characterized in detail so far, we focused on the investigation of acid-forming bacteria in the present paper. Therefore, bacteria were isolated from four biogas plants and two pressurized laboratory biogas fermenters and were characterized physiologically.

2. Methods

2.1. Microorganisms

The bacterial reference strains *C. kluyveri* (DSM-555), *Clostridium sporosphaeroides* (DSM-1294), *Defluviitoga tunisiensis* (DSM-23805), *Dendrosporobacter quercicolus* (DSM-1736), *Proteiniborus ethanoligenes* (DSM-21650), *Selenomonas bovis* (DSM-23594) and *Tepidanaerobacter acetatoxydans* (DSM-21804) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Selected acid-forming isolates from biogas plants used for physiological characterization were deposited in the culture collection of the Institute of Microbiology and Wine Research (IMW, Mainz, Germany). Additionally, isolate *D. tunisiensis* L3 was deposited with the DSMZ (DSM-29926).

2.2. Sampling

In this study, three mesophilic (40 °C) biogas plants were sampled: BGP Glahn (Zweibrücken, Germany), BGP Gebel (Oberthal, Germany) and BGP Wagner (Steinweiler, Germany). Furthermore, one thermophilic (54 °C) biogas plant (BGP Butschen; Viersen, Germany) and two mesophilic (39 °C) laboratory biogas fermenters (University of Hohenheim, Germany) were sampled. The mesophilic biogas plants were fed with the renewable resources maize silage and grass silage, and were partly supplemented with swine or cattle manure. The substrates in the thermophilic biogas plant were maize silage, barley, solid cattle manure and liquid swine manure. Further information about the biogas plants sampled are given in Table S1. In addition, two laboratory biogas fermenters pressurized to 5000 or 10,000 kPa were investigated. These fermenters were designed for investigating of the direct supply of purified biogas into the natural gas grid. They were fed with maize silage.

2.3. Isolation of acid-forming bacteria

In order to isolate acid-forming bacteria, a 500 mg sludge sample of the corresponding biogas plants was added to 9 ml minimal medium (modified DSMZ medium 287; Stantscheff et al., 2014), supplemented with one carbon source (Na^+-DL-lactate, glucose, glycerol, succinate, ethanol or a mixture of the six amino acids: L-alanine, L-serine, L-threonine, L-cysteine, L-glutamic acid and L-methionine). The DSMZ medium 287 mentioned was modified to contain 5 ml trace element solution, 5 ml vitamin solution (DSMZ 141; DSMZ, 2012a), 6 g l^{-1} of one of the carbon sources mentioned and 1.5% (w/v) agar (if required). Sodium acetate were not added. Cultures vessels were pressurized to 50–100 kPa with a gas mixture (80:20; N_2/CO_2). Preparation of nutrition media and isolation procedures were performed in an anaerobic chamber (Coy Laboratory Products, Michigan, USA). The mesophilic and thermophilic enrichment cultures were incubated at 39 °C or 54 °C, respectively. Different methods were used to achieve pure cultures of acid-forming bacteria. At first, the enrichment cultures were diluted in a decadical serial dilution (10^{-1}–10^{-6}). These bacterial cultures were plated onto Petri dishes, which were incubated in a special anaerobic stainless steel cylinder. For the deep agar shake method, 5 ml minimal medium with one carbon source and 1.5% agar were put into short culture tubes, which were covered with caps and placed into a gas-tight bottle of 1 l volume. This gas-tight bottle served as an anaerobic vessel and was pressurized to 50 kPa with N_2. After autoclaving for 20 min at 121 °C, the media were cooled down to 50 °C and inoculated and mixed with 0.1 ml enrichment culture or a bacterial culture with the aid of a sterile syringe. Then the agar shake was put into an ice-water bath for fast cooling. The cultures were incubated upside down in the gas-tight bottle at 39 °C or 54 °C. Colonies grown in the agar shake were withdrawn with a sterile needle and transferred into liquid media. This procedure was repeated at least three times for the preparation of pure cultures. Finally, the cultures were microscopically controlled. Further cultivations of the isolates obtained were performed in DSMZ medium 104b (DSMZ, 2014), DSMZ medium 1328 (DSMZ, 2012b) or modified DSMZ medium 287 with the respective carbon source.

2.4. Detection of acids in bacterial cultures

Acids in enrichment and pure cultures were detected by high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan). Samples were separated with the column Hpx87 (size: 300 × 7.8 mm, 9 μm particle size; BioRad, Munich, Germany) and analysed with the RI-Detector RID-10A (Shimadzu, Duisburg, Germany). Sulphuric acid (0.005 M) was used as a mobile phase. The injection volume was 10 μl, the flow rate was 0.6 ml min^{-1} and the temperature of the column oven amounted to 65 °C. The measurement samples were previously centrifuged at 14,300 × *g* for 2 min and then filtered (filter 0.2 μm pore size; Macherey-Nagel, Düren, Germany).

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