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Isolation of lactic acid-forming bacteria from biogas plants

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ABSTRACT

Direct molecular approaches provide hints that lactic acid bacteria play an important role in the degradation process of organic material to methanogenetic substrates in biogas plants. However, their diversity in biogas fermenter samples has not been analyzed in detail yet. For that reason, five different biogas fermenters, which were fed mainly with maize silage and manure from cattle or pigs, were examined for the occurrence of lactic acid-forming bacteria. A total of 197 lactic acid-forming bacterial strains were isolated, which we assigned to 21 species, belonging to the genera *Bacillus*, *Clostridium*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Pseudoramibacter*-related. A qualitative multiplex system and a real-time quantitative PCR could be developed for most isolates, realized by the selection of specific primers. Their role in biogas plants was discussed on the basis of the quantitative results and on physiological data of the isolates.

1. Introduction

The anaerobic conversion of polymeric organic biomass to methane and carbon dioxide in biogas plants (BGPs) is performed by a complex bacterial and archaeal community. The anaerobic degradation of biomass, consisting mainly of plant material and manure, is divided into four steps. Firstly, the polymeric materials are hydrolyzed into mono- and oligosaccharides, amino acids and long chain fatty acids (hydrolytic step). In the second step (acidogenesis), these compounds are converted into volatile fatty acids (VFA), alcohols and other products, such as lactic acid. These, in turn, are further degraded in the third step (acetogenesis), to the methanogenic substrates acetic acid, carbon dioxide and hydrogen. In the last step (methanogenesis), methane and carbon dioxide are produced by methanogenic archaea.

Under certain conditions, lactic acid is an important precursor for VFA, such as propionic acid or acetic acid. If the concentrations of these VFA exceed certain limits, propionic acid 900 mg l^{-1} and acetic acid 3000 mg l^{-1}, a disturbance of the microbial activity and the methane production is documented (Kaiser et al., 2008; Wang et al., 2009). Several isolates from BGPs have already been obtained which form propionic acid and acetic acid from lactic acid (Cibis et al., 2016). In higher concentrations, propionic acid especially leads to a disruption of the whole biogas process and it is, therefore, suitable as an indicator of fermenter failure (Nielsen et al., 2007).

The occurrence of some lactic acid bacteria in biogas plants has already been proven by 454 pyrosequencing or SOLiD technology (Eikmeyer et al., 2013a; Li et al., 2013; Wirth et al., 2012). Lactic acid bacteria can be divided into three groups regarding their fermentation type: obligately homofermentative, facultatively heterofermentative and obligately heterofermentative species (König and Fröhlich, 2009). Obligately homofermentatives use the Embden-Meyerhof-Parnas pathway to transform glucose to pyruvate and then reduced the latter to lactic acid with the enzyme lactate dehydrogenase. More than 85% of the glucose is transferred into lactic acid. Furthermore, these species are not able to ferment any pentoses due to the lack of phosphoketolase. Obligately heterofermentative species use the phosphoketolase pathway to degrade hexoses and pentoses. Facultatively heterofermentatives degrade hexoses by the Embden-Meyerhof-Parnas pathway and pentoses by the phosphoketolase pathway, on the basis of possessing both aldose and phosphoketolase. Thereby, in addition to lactic acid, acetic acid or ethanol and carbon dioxide are produced (Gottschalk, 1979). Lactic acid bacteria get into the maize silage with plant material and develop extensively by fermenting the water-soluble carbohydrates to organic acids, mainly lactic acid under anaerobic

Abbreviations: BGP, biogas plant; *B.*, *Bacillus*; *C.*, *Clostridium*; HPLC, high performance liquid chromatography; *Lb.*, *Lactobacillus*; MPN, most probable number; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; *P.*, *Pediococcus*; *Ps.*, *Pseudoramibacter*; *S.*, *Streptococcus*; SAPD-PCR, specifically amplified polymorphic DNA-PCR; TJM, tomato juice medium; VFA, volatile fatty acids.

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conditions. Consequently, the pH of the silage decreases and the growth of spoilage microorganisms are prevented (Weinberg and Muck, 1996).

Apart from the lactic acid bacteria, some other species are able to form lactic acid in BGPs. Bacillales and Clostridiales particularly occur frequently in biogas fermenter samples (Hanreich et al., 2013; Krause et al., 2008). There are numerous reports on lactic acid production by some *Bacillus* (*B.*) species, including *B. subtilis* (Gao et al., 2012; Ramos et al., 2000), *B. coagulans* (Michelson et al., 2006; Payot et al., 1999) and *B. licheniformis* (Sakai and Yamanami, 2006). Lactic acid-forming is also known for *Clostridium* (*C.*) species, for example, *C. cellulolyticum* (Petitdemange et al., 1984) and *C. thermocellum* (Levin et al., 2006). The latter two have been identified in biogas-producing microbial communities by Schlüter et al. (2008). Furthermore, Wirth et al. (2012) identified Bacteroidia species in higher quantities from biogas fermenter samples. *Bacteroides fragilis*, for example, is able to form lactic acid, acetic acid and propionic acid (Macy et al., 1975; Mayhew et al., 1975). Other genera also include lactic acid-forming species, such as *Staphylococcus*, *Pseudomonas*, *Enterobacter* or *Escherichia* (Smith et al., 1986). The community of lactic acid bacteria has been characterized in samples of grass silaging (Eikmeyer et al., 2013b), but up to now, it has not been systematically investigated in the context of biogas production plants. Therefore, in this study, lactic acid-forming bacteria were isolated, physiologically characterized and a species-specific system for their detection in BGPs was developed.

2. Methods

2.1. Sampling

Samples from five mesophilic (40 °C) BGPs were examined for the analysis of the lactic acid-forming bacteria: BGP Friedrich (Hochdorf-Assenheim, Germany), BGP Gebel (Oberthal, Germany), BGP Glahn (Zweibrücken, Germany), BGP Lieser (Arenrath, Germany) and BGP Neumann (Herschberg, Germany). In some cases, the investigations included hydrolysis-fermenters, and primary and secondary fermenters, depending on the construction of the BGPs. The hydrolysis-fermenter is a preliminary fermenter type which supports the degradation of polymeric biomass. The substrates for biogas production are fed into the hydrolysis-fermenter or, if it is not available, into the primary fermenter, whereas a part of the fermenter content are transferred, after a certain time, to the secondary or post-fermenter. Substrates for biogas production were mainly maize and grass silage, liquid manure from cattle or pigs, whole crop silage, solid dung or chicken dung. Further information about the BGPs is given in Table S1.

2.2. Isolation of lactic acid-forming bacteria

The following aerobic media were used for the isolation of lactic acid-forming bacteria from the samples from BGPs: tomato juice medium (TJM) (Petri et al., 2013), medium 1, medium 11 and medium 381 (DSMZ, 2016). The anaerobic media 58, 104, 143 (DSMZ, 2016) and medium MD (K_2HPO4 0.05%; KH_2PO_4 0.05%; $NaHCO_3$ 0.2%; NaCl 0.05%; $MgSO_4$ 0.05%; NH_4Cl 0.1%; yeast extract 0.02%; starch 0.2%;filtrated biomass sludge from BGP Neumann 2.5%; resazurin strain solution (0.1% w/v) 0.1%; trace element solution 318 (DSMZ, 2016) 0.5%; vitamin solution 141 (DSMZ, 2016) 0.5%; cysteine x HCL 0.05%; Na_2S x $9H_2O$ 0.01%; pH 7.3) were prepared and used for the isolation in an anaerobic chamber (Coy Laboratory Products, Michigan, USA). Different isolation techniques were used to receive pure cultures. The streak plate technique was mainly applied. In addition, plating with a sterilized spreader rod and dilution series or the deep agar shake method were applied. The latter was previously described by Cibis et al. (2016). Anaerobic culture vessels were pressurized to 50 kPa with N_2 or a mixture of N_2/CO_2 (80:20). Special stainless steel cylinders were used for the incubation of anaerobic agar plates. The cultures were incubated at 40 ± 1 °C.

2.3. Detection of substrates and fermentation products in bacterial cultures

A total of thirteen substrates and fermentation products, such as sugars, organic acids, lactic acid and alcohols were determined by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan). The separation of the compounds were carried out with the column HPX-87H (300 × 7.8 mm; BioRad, Munich, Germany) and their identification was ascertained with a refractive detector 156 (Beckman, Krefeld, Germany). The mobile phase was sulfuric acid (0.013 M), the injection volume was 5 μl, the flow rate was 0.6 ml min^{-1} and the column oven was constantly heated at 65 °C.

2.4. Titer determination of lactic acid-forming bacteria

The concentration of viable cells (titer) of lactic acid-forming bacteria in samples from BGPs and in maize silage samples was determined by the most probable number (MPN) method (McCrady, 1915). Therefore, TJM was modified to contain 0.67 g l^{-1} potassium sorbate at pH 5.0. Firstly, 15 g silage sample was added to 50 ml 0.9% (w/v) NaCl solution and shaken for 30 min. After that, 1 ml fermenter or liquefied silage sample was added to 9 ml of TJM. The samples were diluted in a decadic serial dilution (10^{-1}–10^{-10}) for further analysis. Three dilution series were carried out for each sample. The incubation of the test tubes occurred at 40 ± 1 °C for 14 days. After incubation, the test tubes were examined for bacterial growth. The MPN was calculated according to McCrady (1918). The highest dilution steps, which showed bacterial growth, were investigated to ensure that the microorganisms grown were able to form lactic acid.

2.5. DNA extraction

Cells from bacterial cultures were harvested by centrifugation at 5000 × g for 10 min and washed twice in 0.9% (w/v) NaCl solution. The DNA was extracted via a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the protocol for Gram-positive bacteria. The DNA from fermenter samples from BGPs was isolated using a GeneMATRIX Stool DNA Purification Kit Eur_x (Roboklon, Berlin, Germany).

2.6. Identification of lactic acid-forming bacteria

The 16S rRNA genes of the isolated lactic acid-forming bacteria were amplified by a polymerase chain reaction (PCR) for the purposes of identification. A 50 μl 16S rDNA PCR reaction contained solutions of 1 μl PurEubak5 (5′-GAGTTTGATCMTGGCT-3′; 10 μM), 1 μl primer PurEubak3 (5′- GAAAGGAGGTGATCC-3′; 10 μM), 1 μl dNTPs (10 mM), 2 μl $MgCl_2$ (25 mM), 5 μl reaction buffer Y (10x), 5 μl enhancer solution (5x), 31 μl sterile PCR water (Roth, St. Leon, Germany), 2 μl Taq-DNA-Polymerase (1 U μl^{-1}) and 2 μl template DNA. Primers were from Sigma–Aldrich (Steinheim, Germany). All other PCR components were purchased from peqlab (Erlangen, Germany). The following PCR program was used: initial DNA denaturation for 5 min at 95 °C, followed by a cycle of 35 repetitions consisting of 94 °C for 1 min, then at 56 °C for 1 min, and finally, at 72 °C for 1.5 min. The PCR program was finished with an extension step at 72 °C for 10 min. The PCR products were checked by agarose gel electrophoresis on 1.5% (w/v) gels at 120 mV for 45 min. The