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Effect of bioaugmentation by cellulolytic bacteria enriched from sheep rumen on methane production from wheat straw

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ABSTRACT

The aim of this study was to determine the potential of bioaugmentation with cellulolytic rumen microbiota to enhance the anaerobic digestion of lignocellulosic feedstock. An anaerobic cellulolytic culture was enriched from sheep rumen fluid using wheat straw as substrate under mesophilic conditions. To investigate the effects of bioaugmentation on methane production from straw, the enrichment culture was added to batch reactors in proportions of 2% (Set-1) and 4% (Set-2) of the microbial cell number of the standard inoculum slurry. The methane production in the bioaugmented reactors was higher than in the control reactors. After 30 days of batch incubation, the average methane yield was 154 $mL_N\ CH_4\ g\overline{vs}^1$ in the control reactors. Addition of 2% enrichment culture did not enhance methane production, whereas in Set-2 the methane yield was increased by 27%. The bacterial communities were examined by 454 amplicon sequencing of 16S rRNA genes, while terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of *mcrA* genes was applied to analyze the methanogenic communities. The results highlighted that relative abundances of *Ruminococcaceae* and *Lachnospiraceae* increased during the enrichment. However, *Cloacamonaceae*, which were abundant in the standard inoculum, dominated the bacterial communities of all batch reactors. T-RFLP profiles revealed that *Methanobacteriales* were predominant in the rumen fluid, whereas the enrichment culture was dominated by *Methanosarcinales*. In the batch rectors, the most abundant methanogens were affiliated to *Methanobacteriales* and *Methanomicrobiales*. Our results suggest that bioaugmentation with sheep rumen enrichment cultures can enhance the performance of digesters treating lignocellulosic feedstock.

1. Introduction

Anaerobic digestion (AD) is a proven sustainable technology to recover energy from many biodegradable wastes such as livestock manure, agricultural residues, food and brewery wastes, sewage sludge, industrial and municipal wastewaters, wood industry residues etc. [1,2]. Among these waste streams, agricultural residues belong to the most attractive substrates for AD due to the high abundance, high energy content and low costs [3]. As payback periods are major issues for biogas production plants, efficient methane production is of great concern [2]. Besides the many advantages of AD, the main challenge of this process is the hydrolysis of lignocellulose-rich feedstock, which is generally considered as the rate-limiting step. Thus, pretreatment technologies and/or other enhancement strategies are frequently integrated with the AD process.

The mutualistic interactions between different functional groups including hydrolytic, acidogenic and acetogenic bacteria and methanogenic archaea are crucial for efficient digestion [4]. Bioaugmentation is a promising option to introduce specific microorganisms into the autochthonous digester community to improve certain stages of the AD process. Recently, researchers have shown an increased interest in the bioaugmentation strategy to enhance the hydrolysis rate and consequently methane production from many plant residues, such as wheat straw [5,6], sweet corn processing waste [7], corn straw [3] and cattail [8].

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Lignocellulosic compounds are degraded in the gut system of animals by specific microorganisms excreting hydrolytic enzymes such as cellulases, hemicellulases and lignin-degrading enzymes [9]. Ruminants such as cattle, goat and sheep have the ability to feed on plant fibers with the help of their intestinal symbionts [10]. The unique rumen microbiota can easily adapt to a different diet and gain energy to sustain necessary metabolic functions [11]. Previous studies suggested that microorganisms in rumen fluid are more efficient in lignocellulosic feedstock degradation, thanks to their higher cellulolytic activities, than inocula from anaerobic digesters [12,13]. As the production of volatile fatty acids (VFA) is a key parameter to determine the efficiency of hydrolysis, reactors inoculated with rumen fluid can reach higher VFA concentrations during digestion [2]. Despite the fact that commercial enzyme addition has a positive effect on the hydrolysis rate, using lignocellulose degrading microorganisms is a sustainable and more efficient strategy with regard to their ability to produce the necessary enzymes depending on the given feedstock [14]. However, what is not yet clear in the literature is the fate of the rumen bacteria in anaerobic digesters and the mechanisms how they contribute to more efficient hydrolysis.

In the present study, we aimed to enrich cellulolytic microorganisms from sheep rumen fluid and to determine their bioaugmentation potential in biogas production from wheat straw. The fate of the rumen microbiota and their potential role in the bioaugmented digesters were investigated by microbial community analysis.

2. Material and methods

2.1. Sampling

Rumen fluid was taken from a healthy non-medicated East-Friesian milk sheep (10 years old, 85 kg). The sheep was cared for and handled in a barn of the Veterinary Faculty of Leipzig University, Germany. Its diet consisted of basically pelleted grass, root chips and hay. The rumen sample was collected via a rumen fistula in accordance with the institutional animal care guidelines, and the sample was immediately transferred to the laboratory for the enrichment procedure. Aliquots of the sample were stored at −20 °C for DNA extraction.

2.2. Enrichment culture

The enrichment and cultivation procedure was performed according to the protocol described by Porsch et al. [15] with some modifications. Briefly, we used the modified DSMZ medium 1036 containing 0.5 g NH_4Cl, 0.2 g KH_2PO_4, 0.1 g $MgCl_2 \times 6H_2O$, 0.2 g KCl, 2.0 g NaCl, 0.2 g yeast extract, 1.0 mL trace element solution SL10 (DSMZ medium 320) and 0.5 mg resazurin dissolved in 850 mL high-purity water. For making the medium anoxic, it was stirred for 30 min in an anaerobic chamber (98% N_2, 2% H_2) and then autoclaved (20 min, 121 °C). Wheat straw was used as complex carbon and energy source. For 50 mL cultures, 0.5 g straw and 1 mL high-purity water were added to 100 mL serum bottles, then autoclaved (30 min, 121 °C) and stored overnight in an anaerobic chamber for changing the bottle's conditions to anoxic. In the anaerobic chamber, high-purity water was added once again, and the bottles were closed with butyl rubber stoppers and autoclaved for a second time (30 min, 121 °C). Then, sterile anoxic medium was filled into the culture bottles. All bottles were flushed with a mixture of 75% N_2 and 25% CO_2 to adjust the pH to 7.5.

Before the first inoculation, pre-cultures were prepared from freshly sampled rumen fluid under anaerobic conditions. For the pre-cultures, rumen fluid was diluted 1:4 with water and 3 mL of the diluted sample was added to bottles and incubated at 37 °C. Two days later, fresh medium bottles were inoculated with 1 mL of the pre-culture and incubated at 37 °C. Sterile controls were prepared in the same way as the bottles for the cultures except that they were not inoculated. Biogas production, gas composition, VFA concentration and pH were monitored as described by Porsch et al. (2015). After the third transfer, cultures were up-scaled in 450 mL batches with 4.5 g wheat straw to get enough inoculum for biomethane potential (BMP) analysis.

2.3. BMP assay

The potential effects of bioaugmentation with cellulolytic mixed cultures to enhance AD of wheat straw was assessed in batch tests at 37 °C using the Automatic Methane Potential Test System (AMPTS) II (Bioprocess Control). The amount of substrate and inoculum applied in the experiment was calculated according to the manufacturer's instructions and the inoculum/substrate ratio was 2 on the basis of volatile solids (VS).

After the third transfer, the enrichment culture was scaled up and used as a supplementary inoculum (bioaugmentation) in the BMP experiment. The amount of enrichment culture added to the reactors corresponded to approximately 2% and 4% of the microbial cell number of the standard inoculum, which was taken from a pilot-scale biogas plant treating cow manure and maize silage under mesophilic conditions. The cell numbers were determined according to Sträuber et al. [6]. Non-bioaugmented controls (straw inoculated with standard inoculum) and blank reactors including only standard inoculum without straw were also set up. Each set-up tested in AMPTS II was run in triplicates, thus the experiment comprised 12 batch reactors.

In the anaerobic chamber, 50 mL of anoxic sterile growth medium was added to the controls and 50 mL of one of the concentrated microbial cultures to the experimental set-ups. The bottles with wheat straw were closed and each reactor was connected with the CO_2 trap and counting units of the AMPTS. The headspace of each reactor and its peripheral installation were flushed with 300 mL N_2. The produced methane volume was measured online and automatically corrected for the N_2 present in the system and normalized to standard conditions ($p = 101.325$ kPa, $T = 273.15$ K, no humidity). The reactors were operated with enrichment cultures and wheat straw for 1 day, and then standard inoculum was added to the reactors and further operated for 29 days.

2.4. Microbial community analysis

Samples from sheep rumen fluid, the enrichment culture, the standard inoculum and samples taken at the end of the BMP assays were analyzed for their microbial community composition. DNA was extracted from frozen cell pellets using the NucleoSpin® Soil Kit (Macherey-Nagel) applying buffer SL1 and enhancer solution SX according to the manufacturer's instructions. After checking the quality by agarose electrophoresis and photometric quantification, DNA samples were stored at −20 °C until further analysis.

2.5. 454 pyrosequencing

Bacterial community composition was determined by 454 pyrosequencing of 16S rRNA amplicons as described by Ziganshin et al. [16]. Polymerase chain reaction (PCR) amplification with 25 cycles was performed using the Phire Hot Start II DNA Polymerase (Thermo Scientific) with the primers Bac27F (5′-GAG TTT GAT CMT GGC TCA G-3′) and Bac519R (5′-GWA TTA CCG CGG CKG CTG-3′) specific for bacterial 16S rRNA gene fragments. A nested PCR with 10 cycles was performed using 454 fusion primers tagged with