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Technical Report

Bioaugmentation of anaerobic digesters treating lignocellulosic feedstock by enriched microbial consortia

Three different bioaugmentation cultures enriched from natural and engineered cellulolytic environments (cow and goat rumen, a biogas reactor digesting sorghum biomass) were compared for their enhancement potential on the anaerobic digestion of wheat straw. Methane yields were determined in batch tests using the Automatic Methane Potential Test System operated for 30 days under mesophilic conditions. All cultures had positive effects on substrate degradation, and higher methane yields were observed in the bioaugmented reactors compared to control reactors set up with standard inoculum. However, the level of enhancement differed according to the type of the enrichment culture. Methane yield in batch reactors augmented with 2% cow rumen derived enrichment culture was increased by only 6%. In contrast, reactors amended with 2% goat rumen derived enrichment culture or with the bioaugmentation culture obtained from the biogas reactor digesting sorghum biomass produced 27 and 20% more methane, respectively. The highest methane yield was recorded in reactors amended with 6% goat rumen derived enrichment culture, which represented an increase by 36%. The microbial communities were quite similar at the end of the batch tests independently of the bioaugmentation sources, indicating that the introduced microbial communities of the enrichment cultures did not dominate the reactors.

Keywords: Biogas / Biomethane potential / Cellulose degradation / Rumen microbiota / T-RFLP

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1 Introduction

Lignocellulose is the most abundant biomass on Earth and lignocellulosic feedstocks such as energy crops, agricultural waste and animal manure have a great potential for sustainable and economical production of energy carriers from renewable resources [1]. However, lignocellulose has a recalcitrant structure,

generally composed of lignin (5–25%), hemicelluloses (25–40%) and cellulose (35–45%) [2], and different groups of enzymes are required to degrade this robust compound [3].

Anaerobic digestion (AD) of organic matter is a complex microbial process that occurs in natural environments (e.g., anoxic sediments and gut systems) and is employed in engineered systems for the recycling of organic waste and the production of biogas as a renewable energy carrier. AD includes several interdependent steps such as hydrolysis, acidogenesis, acetogenesis and methanogenesis, in which various functional groups of microorganisms act synergistically and maintain process stability. The rate of biogas production depends on the balance of the individual steps and an inhibition or limitation on any step affects the overall process performance [4]. Hydrolysis is the rate-limiting step in AD of lignocellulose-rich feedstock due to its recalcitrant structure [5]. It has been estimated that around 50% of agricultural biomass cannot be converted in AD due to this limitation [6]. As AD plays an essential role in clean energy supply and waste management, studies on process optimization and

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Abbreviations: AD, anaerobic digestion; AMPTS, automatic methane potential test system; BMP, biomethane potential; CRE, cow rumen enrichment; GRE, goat rumen enrichment; NMDS, non-metric multidimensional scaling; SRE, enrichment culture from a biogas reactor digesting sorghum biomass; T-RFLP, terminal restriction fragment length polymorphism; T-RF, terminal restriction fragment; VS, volatile solids

Compiled in honour of the 80th birthday of Professor Wolfgang Babel.

innovative technologies to enhance methane production rate are of great importance [7]. Thus, various methods including physical pre-treatment (e.g., sonication, thermal treatment), chemical pre-treatment (alkali or acid addition), enzyme addition, ensiling, or bioaugmentation with pure or mixed cultures have been integrated in AD processes to enhance the breakdown of lignocellulosic biomass [8]. Since the range of lignocellolytic enzymes produced by a single strain is limited, mixed cultures have become more prominent due to several benefits such as containing synergistic microbiota, which produce enzyme mixtures for lignocellulose degradation and are more tolerant to stress conditions and environmental changes [4]. Although recent studies have recognized a positive effect of bioaugmentation with enriched cellulolytic consortia on AD processes [9–11], further investigations are required to find the most efficient cultures and the minimum effective concentration.

Ruminants have a unique digestive system including a forestomach named rumen, which is considered one of the most effective environments for plant-biomass degradation [12]. Whereas ruminants cannot produce all the necessary enzymes to degrade plant fibers, the rumen microbiome largely contributes to the decomposition of plant polymers [13] and their fermentative conversion into short chain fatty acids, thus providing nutrients to the host animal. Consequently, rumen fluid was effectively used to inoculate anaerobic digesters in previous studies [14, 15]. However, it is not feasible and questionable from an ethical point of view to provide the necessary amount of rumen content to inoculate full-scale anaerobic digesters. Thus, the selective enrichment of lignocellulose-degrading consortia from high-performance cellulolytic systems such as rumen can be an alternative way for bioaugmentation applications.

This study aimed to determine the potential effects of various cellulolytic methanogenic consortia enriched from two ruminants (cow and goat) and from an engineered system (lab-scale biogas reactor adapted to fiber-rich plant biomass). Various ratios of autochthonous and the introduced microorganisms were investigated to address quantitative effects during bioaugmentation. Within this scope, batch tests were performed in triplicates using the Automatic Methane Potential Test System under mesophilic conditions. Molecular fingerprinting techniques were used to track the fate of the bioaugmented microorganisms.

2 Materials and methods

2.1 Enrichment cultures

Three different bioaugmentation cultures, which were enriched from cow rumen fluid (CRE culture), goat rumen fluid (GRE culture) and from a lab-scale continuous biogas reactor digesting sorghum biomass as mono-substrate (SRE culture) [16] were used as supplementary inocula in the bioaugmented batch experiments. In brief, the rumen fluid samples were collected from a Jersey cow (*Bos primigenius taurus*, 6 years old, 450 kg) via rumen fistula and from a domestic goat (*Capra aegagrus hircus*, 12 years old, 61 kg) via rumen cannula in accordance with the institutional animal care guidelines. Enrichment cultures were set up under anoxic mesophilic conditions (37°C) using modified DSMZ medium 1036 (pH 7.5 adjusted by flushing the

headspace with 75% N₂ and 25% CO₂) with wheat straw as a complex carbon source as previously described [9, 16]. To provide the necessary amount of inocula for the experiments, the enrichment cultures after the third transfer [16] were used to set up 450 mL batches with 4.5 g wheat straw.

2.2 Biomethane potential (BMP) tests

The bioaugmentation potential of the cellulolytic enrichment cultures was assessed using the Automatic Methane Potential Test System (AMPTS) II (Bioprocess Control, Sweden, Lund). BMP tests were set up as described in our previous study [9]. Briefly, the batch reactors were operated for 30 days at 37°C with wheat straw as a substrate and an inoculum/substrate ratio of 2 based on volatile solids (VS). Standard inoculum was obtained from a pilot-scale biogas plant digesting cow manure and maize silage under mesophilic conditions. The reactors were amended with the enrichment cultures corresponding to approximately 2, 4, 6 and 8% of the microbial cell number of the standard inoculum. Cell numbers were counted using a Thoma counting cell chamber (Laboroptik GmbH, Friedrichsdorf, Germany) and phase contrast microscopy (Axioplan-2-Imaging, Zeiss, Jena, Germany). Control reactors (non-bioaugmented; including only standard inoculum and wheat straw) were operated as well. In order to determine methane production from the standard inoculum itself, blank reactors (including only standard inoculum without substrate) were also set up and then the amounts of the methane produced were subtracted from the methane production obtained from the bioaugmented and control reactors. Each batch variant was run in triplicate. In total, three AMPTS runs were included in the study to cover all variants, each with independent control and blank reactors. Results of the BMP tests are reported based on the VS content of wheat straw.

Furthermore, to determine the effect of the bioaugmentation cultures in terms of additional biomass, triplicate reactors were operated with autoclaved SRE cultures corresponding to approximately 2% of the microbial cell number of the standard inoculum.

2.3 Microbial community analysis

The microbial community patterns of the reactor samples collected at the end of the AMPTS runs and the enrichment cultures used as bioaugmentation inocula were analyzed by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA genes or *mcrA* genes as described by [9, 17]. In brief, the restriction enzymes *RsaI* (New England Biolabs, Frankfurt/Main, Germany) with the standard MapMarker 1000 (Eurogentec) and *BstNI* (New England Biolabs, Frankfurt/Main, Germany) with the standard Red 500 DNA Size Standard (Nimagen, Nijmegen, The Netherlands) were used to analyze bacterial 16S rRNA gene amplicons and *mcrA* gene amplicons, respectively. T-RFLP peaks in the range of 50–1000 bp (16S rRNA gene amplicons) and 50–500 bp (*mcrA* gene amplicons) were kept in the further analysis. Taxonomic assignment of *mcrA* terminal restriction fragments (T-RF) was done using a T-RFLP database for methanogenic community members

Figure 1. Methane yields of BMP test cultures amended with (A) CRE culture, (B) GRE culture, (C) 2% SRE and (D) 4% SRE culture and control batches. The data are the average of triplicate reactors. Error bars indicate the standard deviation.

in AD [18]. The similarity of T-RFLP patterns of the samples was evaluated by non-metric multidimensional scaling (NMDS) applying the Bray–Curtis dissimilarity index, which considers presence/absence as well as relative abundance of terminal restriction fragments (T-RF).

3 Results and discussion

3.1 Bioaugmentation potential of different enrichment cultures in AD of wheat straw

Results of the controls with autoclaved cultures revealed that the biomass of the bioaugmentation cultures as the carbon source had a negligible influence on the methane yield and methane production was almost the same (Supporting Information Fig. 1).

Figure 1A shows the methane yield of the CRE culture amended batches at two different concentrations (containing 2 or 4% CRE culture) compared to the controls. The average methane yield was 173 mL_N CH₄ g_{VS}^{−1} in the control reactors (non-bioaugmented) at the end of the operation period. Bioaugmentation cultures had a positive effect on methane production as higher methane yields were observed in bioaugmented reactors. After 30 days of operation, 183 mL_N CH₄ g_{VS}^{−1} was generated in the 2% CRE culture amended reactors, representing an increase of only 6%. However, the reactors with 4% CRE culture represented an enhancement by 27% (219 mL_N CH₄ g_{VS}^{−1}). During the first three days of operation, there was only a minor difference between the methane production of bioaugmented

and control reactors. However, due to the faster kinetics of the bioaugmented reactors the difference in methane yield started to increase after day 3. Furthermore, doubling the concentration of the CRE culture resulted in a 4.5 times higher methane yield.

The methane yields of the reactors amended with GRE culture in three different concentrations in comparison to the controls are shown in Fig. 1B. While the amount of methane produced was similar in all reactors during the first four days, the difference between the reactors amended with 6% and the other reactors started to increase on day 5. At the end of the operation of 30 days, the average methane yield of the non-bioaugmented control reactors was 182 mL_N CH₄ g_{VS}^{−1}. Bioaugmentation with GRE culture resulted in an enhancement of methane production in general. However, the methane yield did not increase proportionally to the GRE culture concentration in the reactors. The lowest methane amount was produced in the 8% GRE culture amended reactors (224 mL_N CH₄ g_{VS}^{−1}; enhancement by 23%) and the highest methane yield was observed in the reactors amended with 6% GRE cultures, representing an increase by 36% (247 mL_N CH₄ g_{VS}^{−1}). In the reactors with 2% GRE culture, 233 mL_N CH₄ g_{VS}^{−1} was produced, which is an enhancement by 27% compared to the control.

Figure 1C and 1D show the methane yields of reactors amended with the SRE culture and the controls obtained in two independent AMPTS runs. The difference in the methane production between the control reactors and the reactors amended with 2% SRE culture started to increase at the beginning of the operation period. The methane production was almost completed within two weeks. Whereas the average methane yield was 153 mL_N CH₄ g_{VS}^{−1} in the non-bioaugmented control

Figure 2. NMDS plot of T-RFLP profiles of bacterial 16S rRNA gene amplicons showing the similarity of the bacterial communities based on the Bray-Curtis dissimilarity index.

reactors, $185 \text{ mL}_N \text{ CH}_4 \text{ g}_{\text{VS}}^{-1}$ was produced in the reactors amended with 2% SRE culture, representing an enhancement by 20%. The methane production rates of the control reactors and the reactors amended with 4% SRE cultures were quite similar in the first two weeks of the operation period. Over 30 days, $182 \text{ mL}_N \text{ CH}_4 \text{ g}_{\text{VS}}^{-1}$ was produced in the control reactors, while the average methane yield was $202 \text{ mL}_N \text{ CH}_4 \text{ g}_{\text{VS}}^{-1}$ in the reactors amended with 4% SRE culture, representing an increase by 11%.

Earlier studies have demonstrated a strong association between the concentration of the bioaugmentation culture and the level of enhancement. While the study carried out by Sträuber and colleagues [17] showed that addition of 1% of an alkali-tolerant cellulolytic enrichment culture as a co-inoculum in the reactors treating pre-treated wheat straw did not influence the absolute methane yield, our previous study [9] indicated that increasing the concentration of the bioaugmentation culture to 2 or 4% (sheep rumen-derived enrichment culture) led to a considerable increase of methane yields by 7.8 and 27%, respectively. Besides the concentration effect, the efficiency of bioaugmen-

tation with cellulolytic cultures depends on the type of the bioaugmentation culture. Čater and colleagues [19] examined the bioaugmentation effects of different pure cultures of hydrolytic bacteria in AD of brewery spent grains, which is a lignocellulose-rich substrate as well. The authors found that *Pseudobutyrvibrio xylanivorans* Mz5^T was the most efficient strain and a 5% (v/v) addition of this strain revealed an 18% increase in methane production. In another study, a pure culture of *Melioribacter roseus* had a limited enhancing effect on methane yields of anaerobic reactors treating wheat straw under thermophilic conditions ($53 \pm 1^\circ\text{C}$) representing an increase by 11%, whereas a pure culture of *Clostridium thermocellum* increased the methane yield by 34% [1]. Different from these pure culture studies, Poszytek and co-workers obtained hydrolytic enrichment cultures from different sources to enhance methane production in AD of maize silage [11]. In accordance with our results, they showed that the level of enhancement depended on the original inocula and that a 10% (v/v) addition of bioaugmentation cultures increased the biogas production by 10–29%.

Figure 3. Methanogenic community composition of all samples. (A) T-RFLP profiles of *mcrA* gene amplicons, (B) NMDS plot of T-RFLP profiles of *mcrA* gene amplicons showing the similarity of the methanogenic communities based on the Bray-Curtis dissimilarity index.

3.2 Microbial community profiles

Bacterial and methanogenic community compositions in the reactor samples were profiled by T-RFLP fingerprinting. The bacterial communities were quite similar at the end of the BMP tests independent of the concentration and type of bioaugmentation cultures added (Supporting Information Fig. 2). The NMDS plot of the bacterial community profiles shows that data points for the different bioaugmented reactors clustered together with the data points for the corresponding control reactors (Fig. 2). This result indicates that the bacterial communities of the enrichment cultures added could not dominate the reactors. Instead, the bacterial communities in all reactors set up with the same standard inoculum underwent a similar succession during the BMP test and their composition was first and foremost determined by the standard inoculum, whereas the bioaugmentation cultures added had only minor effects on the community assembly. This result is in agreement with our previous study [9] in which the community patterns of the reactors bioaugmented with sheep rumen derived enrichment cultures and controls showed higher similarity at the end of the BMP tests compared to the initial communities.

Figure 3A provides an overview of the methanogenic community composition of the enrichment cultures and the reactor samples collected at the end of the operation period. The communities in all samples were dominated by hydrogenotrophic methanogens. The enrichment cultures revealed some differences according to their origin. The cow rumen derived CRE culture was dominated by *Methanomicrobiales* (T-RF 95 and T-RF 340), while *Methanobacteriales* (T-RF 464) and *Methanobacteriales/Methanomassiliicoccales* (T-RF 470) were the dominant methanogens in the goat rumen derived GRE culture. The SRE culture was dominated by *Methanomicrobiales* (T-RF 95) and *Methanobacteriales* (T-RF 467). The methanogenic communities of the bioaugmented reactors and the corresponding controls were quite similar to each other, which is also illustrated in the NMDS plot of the *mcrA* profiles (Fig. 3B). In contrast to the enrichment cultures, the reactor samples contained also acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*), which originated from the standard inoculum. *Methanosarcina* (T-RF 56/57), *Methanomicrobiales* (T-RF 94/95) and *Methanobacteriaceae* (T-RF 123) dominated the methanogenic communities in the bioaugmented reactors and their controls.

Furthermore, *Methanosaeta* (T-RF 128) was detected in most of the bioaugmented reactors and their controls. In general, the community development of methanogens in the BMP test cultures followed a similar trend as observed for the bacterial communities (Fig. 2), meaning that the methanogens contained in the bioaugmentation cultures did not establish in the reactors but methanogenic community assembly was mainly determined by the development of the standard inoculum.

In agreement with our results, Tsapekos and co-workers showed in a previous study [1] that bioaugmentation cultures did not significantly affect the microbial community composition of anaerobic digesters and the abundance of used strains did not increase after bioaugmentation. These results match those observed in an earlier study carried out by Martin-Ryals and colleagues, in which bioaugmentation cultures could not be sustained in semi-continuous reactors [5]. This effect might be

due to competition with the seed microbiota and/or the lack of the ability to adapt to the given environment [20]. In contrast, Yang and colleagues observed a significant shift in bacterial community compositions between bioaugmented and non-bioaugmented reactors operated in batch and semi-continuous mode [21].

4 Concluding remarks

Bioaugmentation with cellulolytic enrichment cultures from rumen and a biogas reactor adapted to fiber-rich feedstock improved the degradation of wheat straw and increased the methane yields from this lignocellulosic substrate by up to 36%, which is clearly a substantial progress compared to previous bioaugmentation studies with cellulolytic consortia from other sources. Although the effect of bioaugmentation was remarkable, the mechanisms behind this enhanced process performance are still unclear. We can exclude that it was due to the added microbial biomass, which is eventually converted to methane. The enhancement might be due to the hydrolytic enzymes delivered by the bioaugmentation cultures. It is probably a transient effect of the increased overall cellulolytic activity in the bioaugmented reactors, which is not followed by effective and competitive proliferation and did therefore not affect the community structure at the end of the BMP tests. The long-term effects of bioaugmentation in continuous reactors and the implications for practical applications in AD technology are subject of further investigations.

Practical application

Microbial consortia enriched from natural and engineered high performance cellulose-degrading systems (cow or goat rumen, anaerobic digesters adapted to lignocellulosic feedstock) can be applied for inoculation or bioaugmentation of biogas reactors digesting plant biomass with high fiber content. Due to the increased hydrolytic activity, substrate conversion rate and methane yield of such augmented reactors are enhanced. However, the level of enhancement depends on the concentration and type of the enrichment culture added. In biomethane potential tests with wheat straw as substrate, reactors amended with 6% goat rumen derived enrichment culture produced 36% more methane compared to control reactors. The usage of enrichment cultures for inoculation and/or bioaugmentation does not require ethical approval and it is more feasible to access these cultures in sufficient amounts compared to the raw rumen fluid. Thus, commercial large-scale production of such bioaugmentation cultures has the potential to increase the productivity of agricultural biogas plants.

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