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Bioaugmentation with hydrolytic microbes to improve the anaerobic biodegradability of lignocellulosic agricultural residues

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HIGHLIGHTS

- Bioaugmentation with *Clostridium thermocellum* and *Melioribacter roseus* was examined.
- *C. thermocellum* enhanced the anaerobic biodegradability of wheat straw up to 34%.
- *M. roseus* had limited efficiency and boosted the methane yield by only 11%.
- *C. thermocellum* can be used to extract the residual methane of the reactor.
- Bioaugmentation did not alter markedly the indigenous microbial communities.

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ABSTRACT

Bioaugmentation with hydrolytic microbes was applied to improve the methane yield of bioreactors fed with agricultural wastes. The efficiency of *Clostridium thermocellum* and *Melioribacter roseus* to degrade lignocellulosic matter was evaluated in batch and continuously stirred tank reactors (CSTRs). Results from batch assays showed that *C. thermocellum* enhanced the methane yield by 34%. A similar increase was recorded in CSTR during the bioaugmentation period; however, at steady-state the effect was noticeably lower (7.5%). In contrast, the bioaugmentation with *M. roseus* did not promote markedly the anaerobic biodegradability, as the methane yield was increased up to 10% in batch and no effect was shown in CSTR. High-throughput 16S rRNA amplicon sequencing was used to assess the effect of bioaugmentation strategies on bacterial and archaeal populations. The microbial analysis revealed that both strains were not markedly resided into biogas microbiome. Additionally, the applied strategies did not alter significantly the microbial communities.

1. Introduction

Lignocellulosic substrates are considered as vital feedstock for economically feasible bioenergy production. These biomasses mainly consist of degradable polysaccharides (i.e. cellulose and hemicellulose) as well as the non-degradable fraction of lignin (Koch et al., 2010). Among the three major constituents, the most serious impediments for efficient biological deconstruction are derived from the nature of cellulose and lignin components.

Specifically, the monomers of cellulose (i.e. D-glucose) are linked with β-1,4-glycosidic bonds, forming a crystalline structure that is resistant to microbial attack (Jørgensen et al., 2007). The lignin polymer consists of aromatic building blocks forming covalent bonds with structural carbohydrates that act as the main barrier for anaerobic biodegradation (Ghaffar and Fan, 2013). On the contrary, the hemicellulose units can be hydrolysed rather easily due to their decreased molecular weight and their amorphous shape (Sawatdeenarunat et al., 2015). Thus, anaerobic digestion (AD) has limited efficiency if the lignin-carbohydrate complex is not disrupted properly and therefore pretreatment is a way for better utilization of lignocellulosic feedstock (Monlau et al., 2013; Tsapekos et al., 2015).

Apart from the well-established pretreatment technologies, methods applied directly to the digesters during the AD process are recognized as interesting solutions (Tsapekos et al., 2017b). On this topic, the enrichment of the indigenous microbiota with

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a culture of microorganisms able to efficiently execute the rate limiting step of hydrolysis under anaerobic conditions is explored. The bioaugmentation with hydrolytic microbes aims to bolster biomass depolymerisation, taking advantage of the abilities of the injected community to encode either specific cellulolytic enzymes (e.g. cellulase, β-glucosidase, xylanase) or multienzyme complexes (e.g. cellulosome) (Azman et al., 2015; Mason and Stuckey, 2016). The hydrolytic characteristics render mandatory the presence of the mentioned microbial group for the solubilization of lignocellulosic substrates. Previous metagenomic studies in the biogas genome demonstrated the existence of bacterial species, having in their genome the complete set of genes involved in degradation of complex polysaccharides (Campanaro et al., 2016). These species are common in biogas reactors, but probably their abundance and/or their activity should be improved in order to perform a prompt and efficient degradation of recalcitrant polysaccharides. Thus, the overall process is occasionally limited by the hydrolysis step, but the limitation may be alleviated through appropriate bioaugmentation (Čater et al., 2015).

Bioaugmentation is not a new approach and has been already applied in anaerobic digesters using a variety of cellulolytic microbes, achieving quite promising results on biomethanation (Zhang et al., 2015). For instance, the methane production of brewery spent grain was significantly increased (+17.8%) by introducing cultures of four different hydrolytic microbes in batch assays (Čater et al., 2015). Findings from this research indicated that both bacterial and archaeal populations were significantly affected through bioaugmentation. It was indeed shown that the metabolic products of injected strains (e.g. *Clostridium cellulovorans*) led to an increased diversity of the archaeal population. On the other hand, an unexpected result on the bacterial communities was observed. Specifically, the strains which are considered as the main determinants for the highest methane increment (i.e. *Pseudobutyrivibrio xylanivorans*) were not detected in increased relative abundance at the end of the experimental period. This result is particularly interesting, as it can be concluded that, during bioaugmentation, the tentative colonization of the bioreactor by newly introduced species is not always "successful and stable" but can be transient (Herrero and Stuckey, 2015; Tyagi et al., 2011). In order to overcome this problem, frequent pulses of the culture may favour and, ideally, reassure the cohabitation of the inoculated bacteria with the indigenous microbiota. Following this concept, recent findings indicated the need of applying routine pulses to the substrate in order to secure the benefits of bioaugmentation for enhanced methane yield (Martin-Ryals et al., 2015). Nevertheless, the authors did not examine the changes on the microbial diversity and dynamicity and thus, no in depth conclusion was drawn about the way that bioaugmentation affects the reactor performance.

Despite these promising results, the majority of studies are focused on batch experiments and questions are still raised about the actual effect on continuous mode operations, as for example whether wash-out of the injected culture can occur due to competition with the original microbiome (Fotidis et al., 2014). The present study examined the effect of bioaugmentation with different strains of cellulolytic microbes in batch and continuous mode experiments and correlated it with the global community composition. Bioaugmentation with *Clostridium thermocellum*, typically found in lignocellulosic-based AD systems (Tsapekos et al., 2017a) and with the recently isolated *Melioribacter roseus*, never detected in AD systems (Azman et al., 2015), were performed in biogas reactors co-digesting cattle manure with wheat straw. Furthermore, characterization of bacterial and archaeal species was conducted using high throughput 16s rRNA amplicon sequencing prior to and after bioaugmentation in order to define the microbiological shifts.

2. Materials and methods

2.1. Inoculum

Thermophilic inoculum was obtained from Snertinge biogas plant, in which livestock manure and wastes from ethanol industry were co-digested. The inoculum was sieved to discard the remaining undigested materials and consequently placed in a thermophilic incubator for seven days to reduce the background biogas production before being used. The main physicochemical characteristics of the effluent after starvation period were: pH: 8.11, Total Solids (TS): 27.47 ± 0.24 g/L, Volatile Solids (VS): 17.11 ± 0.12 g/L, Total Kjeldahl Nitrogen (TKN): 3.63 ± 0.13 g-N/L, Ammonium Nitrogen: 3.51 ± 0.12 g NH_4^+ -N/L, and total Volatile Fatty Acids (TVFA): 0.90 ± 0.10 g/L.

2.2. Manure and wheat straw

Cattle manure was obtained from a livestock farm in Snertinge (Zealand, Denmark). Prior to usage, the manure was sieved in order to remove large lignocellulosic materials and then, was stored at −20 °C. The main physicochemical characteristics of the cattle manure were: pH: 7.69, TS: 31.75 ± 0.04 g/L, VS: 22.08 ± 0.03 g/L, TKN: 2.57 ± 0.10 g-N/L, Ammonium Nitrogen: 1.72 ± 0.05 g-NH_4^+-N/L and TVFA: 3.62 ± 0.12 g/L.

Wheat straw was provided from a farm in Zealand, Denmark. After arrival to the laboratory, the biomass was further processed with a cutting mill (SM 200, Retsch GmbH, Germany) until the size distribution was less than 0.5 cm. The TS and VS content were 928.43 ± 0.59 and 864.50 ± 0.39 g/kg, respectively. The cellulose, hemicellulose and Klason lignin content were 423.47 ± 5.13 g/kgTS, 309.43 ± 5.13 g/kgTS and 247.71 ± 3.55 g/kgTS, respectively. Also, the wheat straw contained 4.82 ± 0.08 g-N/kgTS and 0.763 ± 0.01 g- NH_4^+-N/kgTS, as TKN and ammonium nitrogen, respectively. The chemical oxygen demand (COD) of the lignocellulosic biomass was 1036.67 ± 14.70 gCOD/kgTS and the C:N ratio was 80.67 ± 1.14.

2.3. Hydrolytic cultures used for bioaugmentation

The hydrolytic bacteria were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The modified culture medium for the strain *C. thermocellum* (DSM 1237) contained per liter of Milli-Q water: 1.30 g of $(NH_4)_2SO_4$, 2.60 g of $MgCl_2{\cdot}6H_2O$, 1.43 g of KH_2PO_4, 5.50 g of K_2HPO_4, 0.13 g of $CaCl_2{\cdot}2H_2O$, 6.00 g of Na_2-β-glycerol phosphate$\cdot 4H_2O$, 1.10 mg of $FeSO_4{\cdot}7H_2O$, 0.25 g of L-Glutathione reduced, 4.50 g of yeast extract and 1 mg of resazurin. The pH was adjusted to 7.0–7.2 under gassing with N_2:CO_2 (80%:20%) and then, the medium was sterilized at 121 °C for 20 min. Cellobiose was always added to the autoclaved medium from an anoxic and sterilized 10% (w/v) solution directly before inoculation.

M. roseus (DSM 23840) was grown in a medium having the following composition per liter of Milli-Q water: 1.00 g of NaCl, 0.40 g of $MgCl_2{\cdot}6H_2O$, 0.50 g of KCl, 0.10 g of $CaCl_2{\cdot}2H_2O$, 0.25 g of NH_4Cl, 0.20 g of KH_2PO_4, 4.00 g of Na_2SO_4, 1.00 mL of trace element solution, 2.00 g of yeast extract and 0.10 g of $NaHCO_3$. The trace element solution composition per 990 mL of Milli-Q water was as follows: 784.0 mg of $FeSO_4(NH_4)_2{\cdot}6H_2O$, 143.6 mg of $ZnSO_4{\cdot}7H_2O$, 99.0 mg of $MnCl_2{\cdot}4H_2O$, 94.6 mg of Na_2SeO_4, 6.0 mg of H_3BO_3, 33.0 mg of $Na_2WO_4{\cdot}2H_2O$, 1.8 mg of $CuCl_2{\cdot}H_2O$, 238.0 mg of $CoCl_2{\cdot}6H_2O$, 395.0 mg of $NiSO_4(NH_4)2SO_4{\cdot}6H_2O$, 24.0 mg of $Na_2MoO_4{\cdot}2H_2O$ and 10.0 mL of concentrated HCl. The culture medium was autoclaved at 121 °C for 20 min and prior to