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Process performance and comparative metagenomic analysis during co-digestion of manure and lignocellulosic biomass for biogas production

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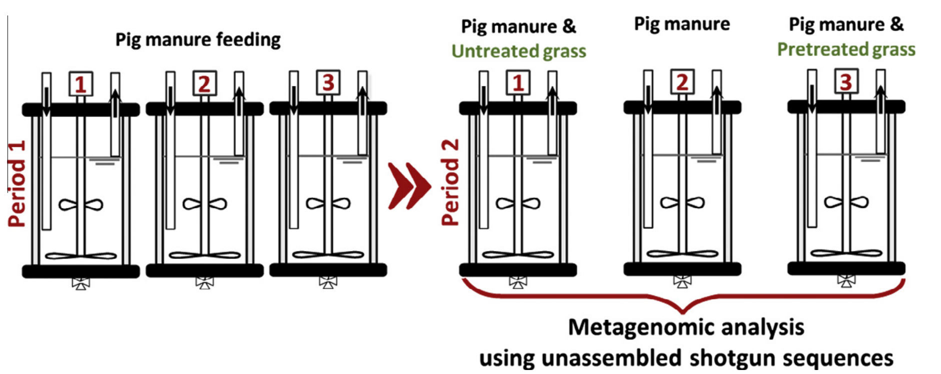
HIGHLIGHTS

- Pig manure and ensiled meadow grass were examined in co-digestion process.
- Mechanical pretreatment increased the methane yield by 6.4%.
- *Coprothermobacter proteolyticus* was firmly bounded to the digested grass.
- *Clostridium thermocellum* was enriched in the firmly attached grass samples.
- The abundance of methanogens was higher in the liquid fraction of digestate.

GRAPHICAL ABSTRACT



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ABSTRACT

Mechanical pretreatment is considered to be a fast and easily applicable method to prepare the biomass for anaerobic digestion. In the present study, the effect of mechanical pretreatment on lignocellulosic silages biodegradability was elucidated in batch reactors. Moreover, co-digestion of the silages with pig manure in continuously fed biogas reactors was examined. Metagenomic analysis for determining the microbial communities in the pig manure digestion system was performed by analysing unassembled shotgun genomic sequences. A comparative analysis allowed to identify the microbial species firmly attached to the digested grass particles and to distinguish them from the planktonic microbes floating in the liquid medium. It was shown that the methane yield of ensiled grass was significantly increased by 12.3% due to mechanical pretreatment in batch experiments. Similarly, the increment of the methane yield in the co-digestion system reached 6.4%. Regarding the metagenomic study, species similar to *Coprothermobacter proteolyticus* and to *Clostridium thermocellum*, known for high proteolytic and cellulolytic activity respectively, were found firmly attached to the solid fraction of digested feedstock. Results from liquid samples revealed clear differences in microbial community composition, mainly dominated by *Proteobacteria*. The archaeal community was found in higher relative abundance in the liquid fraction of co-digestion experiment compared to the solid fraction. Finally, an unclassified *Alkaliphilus* sp. was found in high relative abundance in all samples.

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1. Introduction

The rapid growth of the human population, during the previous century, resulted in the increased demand for food supply. Consequently, the sector of industrialized livestock production was hugely expanded in order to accomplish the needs of humanity for adequate nutrition. However, the animal breeding sector is known to provoke serious environmental problems due to the huge production of waste amounts. For example, in Europe, the population of pigs is approximately 161 millions and hence, >295 millions tonnes of pig manure are produced annually [1], amount that can result in significant adverse impacts if not treated properly.

Traditionally manures are applied untreated on agricultural soils as fertilisers but when treated, anaerobic digestion (AD) is the most common treatment method, which results in energy generation in the form of heat or power as an additional benefit; apart from the main target of sufficient organic matter removal and subsequently, environmental pollution control [2]. However, the efficiency of AD process utilizing pig manure as sole substrate is limited mainly for two reasons [3]. Firstly, this biomass has low organic matter content, typically less than 5% [4], and thus low biogas production can be achieved from such diluted samples [5]. Regarding the second reason, pig manure is associated with high concentrations of ammonia, ionized (NH_4^+) and free ammonia (NH_3) [6,7], which is potentially accumulated during the process and thus, lead to process failure. Specifically, concentrations higher than 4.0 g NH_4^+-N/L [8] and 1.10 g NH_3-N/L [9] can lead to inhibition or toxicity problems. Though the course of ammonia rich feedstock is well known, Danish full-scale biogas plants are in many cases operating without alleviating this problem. In such cases, stable methane productivity is observed under increased ammonia levels (i.e. the so-called "inhibited steady-state" conditions), but typically the overall profitability is up to 30% decreased due to the suboptimal operational conditions [10]. Therefore, a lot of research is focused on avoiding or dealing with the ammonia toxicity and various techniques are available to counteract this problem [11].

A common approach to avoid the ammonia toxicity and simultaneously increase the biogas production is to provide the digester with an improved nutrient balance and an increased carbon-to-nitrogen (C:N) ratio. To achieve this predetermined target, the co-digestion of ammonia-rich with carbon-rich feedstocks is an interesting option. In the past, lignocellulosic materials (e.g. maize, straw) were efficiently used as supplemental carbon sources in order to be co-digested with ammonia-rich substrates, so as to reduce the danger of ammonia inhibition [12,13]. In this concept, meadow grass, directly harvested from non-cultivated lands and thus, composed of a huge variety of different species, can be considered as a potential co-substrate, as it poses the desired characteristics; it has considerable higher carbon content and theoretically higher biogas potential than livestock manure [14]. Meanwhile, similarly to pig manure, the usage of grass as substrate for AD is more preferable to be performed in a mixed feedstock instead of being the sole substrate, in order to avoid operational problems. It was proven that feeding with ensiled grass for a long digestion period as an individual substrate was not efficient due to absence of essential trace elements for the microbial community [15]. Livestock manure could efficiently provide the digester with the limited chemical elements to overcome this obstacle.

At the same time, the utilization of meadow grass in full-scale is advantageous from numerous points of view. Notably, it can be implemented as additional feedstock in full-scale applications, as the grasslands are one of the most dominant single land use in EU and huge areas are available to provide organic feedstock for industrial processes [16]. Hence, it is a cheap source of biomass as no cultivation cost is needed compared to the alternative cost-demanding energy crops. In addition, meadow grass can be easily ensiled and stored in order to be preserved and subsequently, be supplied constantly all year long to the biogas plants [17].

Despite the aforementioned advantages, the utilization of such lignocellulosic substrates for AD is ambiguous. Primarily, the dilemma lies in the presence of lignin, which protects the plant from the microbial attack leading to limited biodegradation. However, previous study showed that the biodegradation of meadow grass can be improved by mechanical pretreatment using shearing forces in order to extensively damage the recalcitrant lignocellulosic structure [18]. Mechanically pretreated ensiled meadow grass was also examined in co-digestion with ammonia rich livestock manure (i.e. mink and chicken manure) and it was concluded that it can efficiently boost the methane production in batch assays [17]. The effect of mechanical pretreatment on meadow grass biodegradability has been studied mainly in batch assays, but these experiments are not suitable to predict real practice AD performance. In contrary, limited information is available about the impact of mechanical pretreatment methods in continuous mode operations. Recently, continuous trials of grass based-AD showed that the particle's size reduction is pivotal to avoid substantial mechanical problems, such as accumulation of grass substrate inside the reactor and wrapping of longer grass particles around the stirring device causing excess strain on the motor, which in turn leads to electrical shutdowns [19]. Nevertheless, by reducing the particle's size only, the surface damage is not markedly induced and consequently, the access to the degradable organic matter is not improved significantly. Thus, mechanical methods that provide more efficient disruption of the plant structure are required, taking into consideration their bioenergy output. Moreover, a more comprehensive study targeting on their impact under continuous reactor operation would be beneficial, in order to evaluate the process performance and decipher the real effect of these methods.

Because of the aforementioned benefits, the co-digestion of livestock manure with lignocellulosic substrates is a widely established practice. It is well known that the lignocellulosic substrates are rather recalcitrant to AD due to their complexity and especially, hydrolysis is considered as the rate limiting step before succeeding an efficient AD process [20,21]. Hydrolysis is mediated by hydrolytic bacteria which transform the complex organic substrates into monomers by the production of extracellular enzymes and it can be hypothesized that in efficiently operating reactors, which digest lignocellulosic biomass, the hydrolytic bacteria are present in abundance so as to hydrolyse the large molecules. Of course, apart from the hydrolytic bacteria, in a typical anaerobic reactor a huge variety of specialized microorganisms is co-existing. As a continuation of the hydrolysis step, the hydrolysed small molecules will be further utilized by fermentative bacteria to produce volatile fatty acids (VFAs) and alcohols. Subsequently, the acetogenic bacteria will transform the intermediate molecules into acetate, carbon dioxide and hydrogen in order to be used by methanogenic archaea to produce methane. However, that overall description presents the process stages quite simplified and does not take into account the relationship between the different microorganisms. Conversely, the interactions among the microbial consortium are extremely complex and not known yet in depth. So, in order to result in high rate of methane production, the knowledge of microorganisms' interactions is of central importance. Moreover, it is generally accepted that the microbial composition depends significantly on feedstock composition. For instance, during the AD of ammonium rich substrates, the hydrogenotrophic methanogenesis can be the main metabolic pathway due to the increased sensitivity of acetoclastic methanogens to inhibitors [22]. How-

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