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Hydrolysis of microalgal biomass using ruminal microorganisms as a pretreatment to increase methane recovery

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ABSTRACT

The use of ruminal fluid as a source of hydrolytic microorganisms for the pretreatment of a native consortium of microalgae (essentially *Senedesmus*) was investigated. The hydrolytic enzyme activity of the ruminal culture was first enriched in a bioreactor. Then, using the enriched culture, the effect of the microalgae to the ruminal fluid ratio (S/X) on the hydrolysis and subsequent production of methane was investigated. An S/X ratio of 0.5 showed the best hydrolysis efficiency (29%) reaching in a second stage process a methane yield of 193 mL CH_4 g COD^{-1}. The processing time (pretreatment plus methanization) was only 7 days. The predominant ruminal hydrolytic bacteria selected in the enrichment were principally *Clostridium*, *Proteocatella* and *Pseudomonas*.

1. Introduction

A lack of fossil fuels, climate change, and environmental degradation drives the search, development, and implementation of cleaner technologies for energy production. Biomass is a source of renewable energy that is widely used (Demirbas, 2009). In this sense, third generation biofuels obtained from microalgae have advantages, such as lower land use than second generation biofuels and the potential coupling to CO_2 mitigation and wastewater treatment. Microalgae are unicellular microorganisms that grow in aquatic environments and convert CO_2, water and sunlight into lipids, carbohydrates and proteins via photosynthesis, providing a greater production yield than terrestrial crops (Li et al., 2008). The definition of microalgae commonly includes all simple unicellular and multicellular photosynthetic microorganisms, both prokaryotic microalgae (cyanobacteria) and eukaryotic microalgae (green algae, red algae and diatoms) (Brennan and Owende, 2010).

Many of the technologies that employ microalgae as a substrate are focused on the production of biodiesel; however, the large-scale production of biodiesel is limited due to the high costs of downstream processing. Lipid extraction must be done from dry biomass, and this drying process consumes 50–80% of the energy required for the entire production process (González-Fernández et al., 2012a). Anaerobic digestion of microalgae is one of the most promising technologies for energy recovery from microalgal biomass, with the first study conducted in the 1950s (Golueke et al., 1957). In this study, a consortium of microalgae (*Chlorella* and *Scenedesmus*) were directly subjected to anaerobic digestion, reaching a yield of 0.17–0.32 L CH_4 g-volatile solids $(VS)^{-1}$. The low biodegradability of microalgae is due to the composition of their cell wall, which is rich in cellulose and hemicellulose (González-Fernández et al., 2012b). To increase the methane recovery from microalgae biomass, a pretreatment is usually carried out by chemical or enzymatic hydrolysis. The primary classes of enzymes used to hydrolyze microalgae biomass are glucanases, glycosidases, peptidases and lipases. These enzymes have been used both alone (Mahdy et al., 2016) and in enzymatic cocktails (Ciudad et al., 2014; Mahdy et al., 2016, 2014). Using the latter strategy, one of the best gaseous biofuel productions was achieved by Ciudad et al. (2014). In this study, the cell wall of the microalgae *Botryococcus braunii* was digested using an enzymatic extract (ligninolytic enzymes) obtained from *Anthracophyllum discolor*, a white-rot fungus, producing 521 mL of CH_4 g-VS^{-1}, representing a 90% of anaerobic biodegradability. Despite the clear advantage of enzymatic pretreatments to microalgae biomass, the use of pure enzymes is a limiting factor in the scaling-up of the process due to their high costs and lack of reusability (He et al., 2016; Muñoz et al., 2014).

To explore more cost-effective methods, the use of hydrolytic microbial cultures have been proposed for the pretreatment of microalgal biomass. Using this approach, Muñoz et al. (2014) reported the use of a bacterium with hydrolytic activity to perform the pretreatment of *Nannochloropsis gaditana* biomass, increasing methane production by up to 158%. He et al. (2016) pretreated *Chlorella* sp. biomass with the proteolytic bacterium *Bacillus licheniformis*, resulting in an increase in methane production of 22%, compared to raw biomass. Recently,

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Carrillo-Reyes et al. (2016) reviewed biological pretreatment methods for microalgal biomass and suggested that natural hydrolytic bacterial consortia can be used, such as those found in the digestive tracts of termites and ruminants.

The rumen is the first chamber of the alimentary canal of ruminants and is colonized by a complex population of anaerobic microbes, including bacteria, protozoa, fungi and archaea. Members of the genera *Fibrobacter* and *Ruminococcus* are the most abundant cellulolytic bacteria in the rumen and are additionally able to degrade xylan. Other ruminal bacteria have amylolytic activity (*Ruminobacter* species and *Succinomonas amylolytica*), and species of the *Lachnospira* genus are able to digest pectin. Ruminal microorganisms can degrade lignocellulosic material generating short chain fatty acids and biogas (22–29% methane) as the main products of metabolism (Van Soest, 1994). In this sense, very recent studies report the use of ruminal microorganisms to produce methane from microalgae biomass in one step processes. Giménez et al. (2017) used the ruminal fluid as inoculum for the semi-continuous methane production from *Scenedesmus* biomass, achieving a methane yield of 214 mL CH_4 g-chemical oxygen demand $(COD)^{-1}$ with a hydraulic and solid retention times of 31 and 100 days, respectively. In addition, Aydin et al. (2017) used an isolated hydrolytic fungus from the ruminal fluid for the bioaugmentation of a granular anaerobic sludge. The latter work increased 41% the biogas production compared to the anaerobic sludge without fungus, using *Haematococcus pluviali* as substrate. These previous studies demonstrate the potential of ruminal microorganisms to increase the methane recovery from microalgae biomass. However, there are no reports that indicate the use of ruminal fluid for a pretreatment step which can improve the cell wall disruption rate before the methanogenic step. In this regard, it is necessary to evaluate the operational parameters such as the substrate to inoculum ratio and the understanding of the ruminal community enrichment.

Therefore, the present work aimed to study the use of ruminal fluid as a source of hydrolytic microorganisms for the pretreatment of a native consortium of microalgae (essentially *Senedesmus*). In this sense, the hydrolytic enzyme activity of the ruminal culture was first enriched in a bioreactor. Then, using the enriched culture, the effect of the microalgae to the ruminal fluid ratio (S/X) on the hydrolysis and subsequent production of methane was investigated. In addition, the selected ruminal bacteria and the endogenous bacteria from the microalgae culture was characterized by 16S rDNA using next generation sequencing techniques.

2. Materials and methods

2.1. Microalgal biomass and ruminal fluid

2.1.1. Native microalgae consortium

The microalgae biomass used as substrate was a native consortium from a lake located in Queretaro, Mexico (20° 42′ 07.0″ N, 100° 27′ 36.7″ W and 1900 m above sea level). The microalgae culture was enriched in Bold medium using plastic tubular bags (8 L) as reactors. Once the desired microalgae density was reached (0.74, absorbance at 685 nm; or 0.58 g VS L^{-1}), it was concentrated by centrifugation (4500 rpm, 10 min). The reactors were aerated with a constant flow of 1 L min^{-1} (0.035% CO_2) using stone diffusers and maintaining a light–dark period of 12 × 12 h. The light was provided with 54 W neon lamps (LT 300 Extech Instruments, USA), with a light intensity of 100 μmol m^{-2} s^{-1} (Cea-Barcia et al., 2014). The culture composition was determined by optical microscopy (Leica DM500, Japan) and direct counting in a 0.1 mm Neubauer chamber according the method described by Wehr and Sheath (2003). Considering the number of cells per milliliter, the primary genera identified were *Scenedesmus* (98%), *Keratococcus* (1%).

2.1.2. Ruminal fluid

For the source of the hydrolytic bacteria inoculum, 3 L of ruminal content was taken from a fistulated adult caw (National Institute of Forestry and Agriculture Research, Queretaro, Mexico). The ruminal fluid was collected in the morning before the cattle were fed and was preserved at 40 °C until its use. Prior to being used, the ruminal fluid was liquefied to ensure homogenization and was bubbled with N_2 to maintain the reducing medium. The typical ratio of VS/total solids (ST) was 0.73, with an average content of 0.05 g TS (mL ruminal fluid)$^{-1}$.

2.1.3. Evaluation of the hydrolytic activity of ruminal microorganisms

To initially evaluate the hydrolytic capability of the ruminal fluid using microalgae as the substrate, a batch test was performed for 7 days. A 4-L bioreactor (Applikon Bioreactor Systems, Netherlands) was used with constant stirring of 100 rpm, at 40 °C, and a maintained pH of 7, under anaerobic conditions. The reactor was inoculated with 20% (v/v) ruminal fluid and 3.5*g* VS L^{-1} of microalgae. A sample was taken every 24 h to evaluate the extracellular enzymatic activity of carboxymethylcellulase (CMCase), xylanase and amylase. In this, and subsequent experiments the medium reported by McDougall (1948), which is similar in composition to the saliva of ruminants, and contained (mg L^{-1}) $(NH_4)_2SO_4$, 1300; K_2HPO_4, 2040; $NaHCO_3$, 400; NaCl, 80; $MgSO_4{\cdot}7H_2O$, 19.2; $FeSO_4{\cdot}7H_2O$, 1.1; $CaCl_2$, 8; KH_2PO_4, 40.

2.1.4. Anaerobic sludge

In the methane production experiments, a mesophilic anaerobic sludge that was obtained from a brewing industry reactor was used as an inoculum. The contents of TS and VS of the inoculum were 28 g TS L^{-1} and 19 g VS L^{-1}, respectively.

2.2. Design of experiments

2.2.1. Enrichment of hydrolytic bacteria in the rumen

The enrichment of ruminal fluid was performed in 120-mL glass serum bottles with an 80-mL of working volume; the headspace was purged with N_2 for 15 s, and then bottles were incubated at 40 °C with shaking at 100 rpm for 7 days. Two microalgae concentrations (3.5 and 7 g TS L^{-1}), and two carbon sources (carboxymethylcellulose, CMC, and xylan, both at 1 g L^{-1}) as positive controls were tested. As a negative control, microalgae (3.5 and 7 g TS L^{-1}) without ruminal fluid, and a sample containing only ruminal fluid (0.5 g TS L^{-1}) were evaluated. Three inoculations were carried out. For the first inoculation, 0.5 g TS L^{-1} of ruminal fluid was added to the bottles. The second and the third inoculations were conducted by transferring 8 mL of the content from the first bottles to the next ones, with the volume adjusted to 80 mL (0.05 g TS L^{-1}). For the second and third tests, the microalgal concentration and mineral medium were the same as in the first experiment. At the end of the tests, biomass samples were taken and stored at −20 °C for the subsequent characterization of the bacterial communities. All conditions evaluated were performed in triplicate.

2.2.2. Effect of the initial substrate to inoculum ratio (S/X) on methane production

2.2.2.1. Pretreatment of microalgal biomass. Different initial S/X ratios (0.3, 0.5, 1 and 2) were evaluated by varying the initial microalgae TS concentration and keeping the ruminal fluid constant at 5 g TS L^{-1}. In addition, controls containing only ruminal fluid or microalgae were used (both at 5 g TS L^{-1}). The tests were carried out in batch cultures in glass serum bottles, using the same volume, temperature and mixing as described in the enrichment experiment (Section 2.2.1). The gas production was quantified daily by liquid displacement using an inverted test tube containing an acidic solution (pH < 2). At the end of the tests, the soluble fraction was recovered by centrifugation at 3500 rpm for 10 min and was characterized. Biomass samples were stored at −20 °C for further characterization of bacterial communities. All the tests were performed in triplicate.