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BIOMETHANE POTENTIAL FROM SUGARCANE STRAW IN VERACRUZ, MEXICO: COMBINED LIQUID HOT WATER PRETREATMENT AND ENZYMATIC OR BIOLOGICAL HYDROLYSIS

POTENCIAL BIOQUÍMICO DE METANO A PARTIR DE LA PAJA DE LA CAÑA DE AZÚCAR EN VERACRUZ, MÉXICO: COMBINANDO LOS PRETRATAMIENTOS DE AGUA CALIENTE E HIDRÓLISIS ENZIMÁTICA O BIOLÓGICA

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Abstract

Few studies have analyzed the utilization of raw sugarcane straw (SCS) for biogas generation. It is important to highlight that these studies only report the biomethane potential (BMP) of the SCS, several form of pretreatment obtained low yields of biomethane compared to the highest BMP obtained here. This study presents data on improving Total BMP from SCS (378.80±0.11 ml CH_4/g VS_R), in order to lower the severity of the LHW (Liquid Hot Water) pretreatment and to enhance the efficiency of the enzymatic or biological hydrolysis pretreatment, two-step was employed, as follows: (LHW, 120 °C, 1.5 bars, 31.25 gr SCS dry basis/L of reaction) with enzymatic hydrolysis (commercial cellulose ENMEX® at 20%) and biological hydrolysis (isolated lignocellulolytic bacteria 3.02 * 108 UFC/ml). Finding a higher BMP in the case of the biological hydrolysis treatment in comparison with the enzymatic one, 360.58 and 344.97 ml CH_4/g VS_R respectively. The alteration of the physicochemical composition and physical structure of SCS fibre was higher in the option combining LHW pretreatment and biological hydrolysis. This study can be useful as a future reference in Mexico for a scalable system, in order to assess its feasibility for biomethane generation as a sustainable source.

Keywords: Sugarcane Straw, liquid hot water, biomethane potential, enzymatic hydrolysis, biological hydrolysis.

Resumen

Pocos estudios han analizado la utilización de paja de caña de azúcar (SCS) para la generación de biogás. De estos estudios solo se reporta el potencial de biometano (BMP) del SCS, con pretratamientos que obtuvieron bajos rendimientos en comparación con el BMP más alto obtenido aquí. Este estudio presenta datos sobre la mejora del BMP total de SCS (378.80±0.11 ml CH_4/g VS_R), disminuyendo la severidad del pretratamiento LHW (agua caliente) y mejorando el pretratamiento enzimático o biológico; dos etapas se emplearon de la siguiente manera: (LHW, 120 °C, 1,5 bares, 31,25 gr SCS base seca / L reacción) con hidrólisis enzimática (celulosa comercial ENMEX® 20%) e hidrólisis biológica (bacterias lignocelulolíticas aisladas 3,02 * 108 UFC / ml). Encontrando un BMP más alto para tratamiento de hidrólisis biológica en comparación con la enzimática, 360.58 y 344.97 ml CH_4 / g VS_R respectivamente. La alteración de la composición fisicoquímica y la estructura física de la fibra SCS fue mayor en la opción que combina LHW e hidrólisis biológica. Este estudio puede ser una referencia futura en México para un sistema escalable, con el fin de evaluar su viabilidad como fuente sostenible.

Palabras clave: Paja de caña de azúcar, agua caliente, potencial bioquímico de metano, hidrólisis enzimática, hidrólisis biológica.

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1 Introduction

The composition of the SCS consists mainly of cellulose and hemicellulose (45-52% and 25-75%, respectively) rather than lignin (12-20%). The availability of this lignocellulosic residue is one of the main characteristics that makes it attractive for biogas generation (Janke *et al*., 2015; Sahito *et al*., 2013; Nzila *et al*., 2015; Rossell., 2006; Singh *et al*., 2008; Aguilar Rivera 2010). According to Statista© worldwide sugar production is around 172.5 million metric tons per year, taking into account their projections for 2014 through the year 2015. SCS represents around 15% of the leftover material after sugar production, which generates an estimated 25.88 million metric tons per year of SCS (Aguilar Rivera., 2010). In Mexico, the total area of cultivated sugarcane is close to 800 thousand hectares, and there are around 57 sugar mills, of which 38.6% are located in the state of Veracruz, with an average sugarcane yield of 64.5 tons per hectare (Sentíes-Herrera *et al*., 2014). In the case of Veracruz State the harvests are also the largest in comparison with the rest of the Mexican states that cultivate sugarcane, with around 41.56% (323,650 hectares) of the country's total, according to data reported by the National Commission for the Sustainable Development of Sugarcane (CONADESUCA) for the period 2015-2016. This represents a production of 3.1×10^6 tons of SCS, which guarantees the availability of this residue for use during the year.

Nevertheless, the normal treatment in the country is the traditional one which is based on burning. On the other hand, the current tendency in other countries (like Brazil) has been to promote a green harvest instead and it is even being used as a combustion carrier to generate local energy for the production of alcohol and sugar (Leal *et al*., 2013). Therefore, the current trend in its handling is the implementation of alternative options to combustion. This has been promoted not only due to health problems generated by the emission of the greenhouse gases and ashes (França *et al*., 2012), but also for the ecological and economic implications of soil erosion(Leal *et al*., 2013; França *et al*., 2012). Also it is important to remark that the negative energy balance for its burning in the field can be quite significant, if we consider that the SCS produced per hectare has the equivalent of 29 barrels of oil, 9600 L of ethanol or 3600 kcal per kg of burned SCS (Holanda *et al*., 2016). This new approach will provide a more sustainable and environmentally friendly alternative handling.

It is important to emphasize that the utilization of pretreated sugar cane residues (mostly sugarcane bagasse or press mud) has been widely analysed and previously studied solely for the production of bioethanol (Singh *et al*., 2008; Pereira *et al*., 2011; Canilha *et al*., 2012; Medina *et al*., 2008; Moutta *et al*., 2014; Ferreira *et al*., 2013; Rabelo *et al*., 2011; Saad *et al*., 2008; da Silva *et al*., 2010; Martín *et al*., 2007). Only a few studies have analysed the utilization of raw SCS for biogas generation (Janke *et al*., 2015; Sahito *et al*., 2013; Nzila *et al*., 2015). However, theses authors only report the BMP of the SCS without the implementation of any physicochemical or biological pretreatments.

Different types of substrate pretreatment are often suggested as alternatives to enhance the anaerobic digestion process by increasing the accessible surface area, modifying the crystalline structure or partially depolymerizing cellulose, solubilizing hemicellulose and/or lignin, or modifying lignin structure. The pretreated substrate is intended to make anaerobic digestion faster, potentially increase biogas yields and prevent operational constraints, such as excessive electricity requirements for mixing or the formation of floating layers (Janke *et al*., 2015).

The advantages of LHW pretreatment include: limited corrosion problems, no sludge generation, low capital and operational costs and negligible loss of cellulose under normal operating conditions. Furthemore, this processing technology does not require the addition of chemicals such as sulphuric acid, lime, or ammonia that add cost to the process. These chemicals must be neutralized or recovered, thus adding to the expense of the chemicals themselves. Likewise, an optimized controlled pH, liquid hot water pretreatment process will maximize the solubilization of the hemicellulose fraction as liquid soluble oligosaccharides while minimizing the formation of monomeric sugars, these last formations lower the yield of anaerobic digestion (Mosier *et al*., 2005).

Also, microbial pretreatment, as an environmental friendly and low cost pretreatment approach for enhancing enzymatic saccharification and fermentation of lignocellulosic biomass to biofuel, is attracting increasing attention in recent years (Wang *et al*., 2012; Simas-Dias *et al*., 2018). In order to lower the severity requirements of the LHW pretreatment and to enhance the efficiency of the enzymatic or biological hydrolysis pretreatment, a

two-step pretreatment was employed. Therefore, this study aims to contribute innovatively to the subject by analysing and presenting new data on the alteration of the physicochemical composition of the SCS fibre due to the implementation of LHW and enzymatic or biological hydrolysis, as well as for the BMP obtained due to the implementation of pretreatments not previously reported.

2 Materials and methods

2.1 Raw materials

According to Sahito *et al*., 2013 a 10 kg sub-sample of SCS was taken from a sugar cane plantation within the Cordoba region in the state of Veracruz (Mexico). The sub-samples were dried at 45 °C (48 hrs) and kept dry in storage with Ziploc® sealed bags and silica gel. For utilization in the characterization and pretreatment process, samples were milled and passed through sieves with 1.41×10^3 mm y 5 mm screens, respectively. The chemical characterization of the fibres was carried out as described in the TAPPI methods.

Preparation of lignocellulosic residue for the physic chemical analysis: 2.5 g of dried raw biomass was loaded into the cellulose thimble. With the Soxhlet extractor set up, 150 mL of acetone was used as solvent for extraction. Residence times for the boiling and rising stages was carefully adjusted to 70 ºC and 25 min respectively on the heating mantle for a 4 h run period. After extraction, the sample was air dried at room temperature for few minutes. Constant weight of the extracted material was achieved in a convection oven at 105 ºC. The % (w/w) of the extractives content was evaluated as the difference in weight between the raw extractive-laden biomass and extractive-free biomass.

Hemicellulose: 1 g of extracted dried biomass was transferred into a 250 mL Erlenmeyer flask. 150 mL of 500 mol/m^3 NaOH was added. The mixture was boiled for 3.5 h with distilled water. It was filtered after cooling through vacuum filtration and washed until neutral pH. The residue was dried to a constant weight at 105 ºC in a convection oven. The difference between the sample weight before and after this treatment is the hemicellulose content (%w/w) of dry biomass.

Lignin: 0.3 g of dried extracted raw biomass was weighed in glass test tubes and 3 mL of 72% H_2SO_4 was added. The sample was kept at room temperature for 2 h with shaking at 30 min. After the initial hydrolysis, 84 mL of distilled water was added. The second step was made to occur in an autoclave for 1 h at 121 °C. The slurry was then cooled at room temperature. Hydrolyzates were filtered through vacuum using a filtering crucible. The acid insoluble lignin was determined by drying the residues at 105 oC and accounting for ash by incinerating the hydrolysed samples at 575 °C in a muffle furnace. The acid soluble lignin fraction was determined by measuring the absorbance of the acid hydrolysed samples at 320 nm. The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin.

Cellulose: use a specimen of 5 g previously treated as described above. Place the test specimen in a 300-mL Erlenmeyer flask and add 1.5 g of $NaClO_2$ in 150 ml of water (add 10 drops of CH_3COOH before adding the $NaClO_2$). Carefully keep the flask capped with a watch glass in a hot bat at 75 °C during 1 hour, stir occasionally. After the hour add 10 drops of CH_3COOH and 1.5 g of $NaClO_2$, stir and let stand by 1 hour. Repeat the previous procedure twice. Let the reaction cool down in a reservoir of water with ice. After a vacuum filtration was made with a Buchner funnel and a filter (with a constant weight). The solid fraction is then washed with cold distilled water, and acetone. Finally the sample is dried in vacuum (at 40 °C), until it reaches its constant weight. The content of cellulose is determinate by the following equation: % of cellulose = (Weight of the proceeded sample at a constant weight*100) /Weight of the untreated sample in a dry basis.

Total solids: 1) Preparation of evaporating dish−ignite a clean evaporating dish at 550°C for 1 h in a muffle furnace. Cool in desiccator, weigh, and store in desiccator until ready for use. Place 25 to 50 g of sample in a prepared evaporating dish and weigh. Place in an oven at 103 to 105°C overnight. Cool to balance temperature in a desiccator and weigh. Repeat drying (1 h), cooling, weighing, and desiccating steps until weight change is less than 4% or 50 mg, whichever is less. Analyse at least 10% of all samples in duplicate. Duplicate determinations should agree within 5% of their average weight.

Volatile solids: Transfer the dried residue from 1) to a cool muffle furnace, heat furnace to 550°C, and ignite for 1 h. Cool in desiccator to balance temperature and weigh. Repeat igniting (30 min), cooling, desiccating and weighing steps until the weight change is less than 4% or 50 mg, whichever is less. Analyse at least 10% of all samples in duplicate. Duplicate determinations

should agree within 5% of their average weight. Calculation: $\%total\ solids = (A - B) * 100/C - B$; $\%\ volatile\ solids = (A - D) * 100/A - B$ where: A: weight of dried residue + dish, mg, B: weight of dish, C= weight of wet sample + dish, mg and D: weight of residue + dish after ignition, mg.

2.2 *Liquid hot water pretreatment*

The dry samples were hydrolysed at a ratio of 31.25 g of dry sample/L of water; employing liquid hot water (120 °C, 1.5 bars) in a modified autoclave with a working capacity of 10 L. A 7-hour batch was tested, taking samples each hour in order to determine the required time for the pretreatment in relation to the higher concentration of chemical oxygen demand (COD). Each test was carried out in triplicate. A Schott® bottle (0.75 L) with 15.63 g of SCS and 0.5 L of water (at room temperature 25±2 °C) was used as a control. To determine if there was any significant difference between the tested retention times (1, 2, 3, 4, 5, 6 and 7 h) from the liquid hot water pretreatment, a statistic test by comparison of medians on the Statistic software (v 10) was run.

2.3 *Enzymatic hydrolysis*

The enzymatic activity of commercial cellulase (ENMEX®) was calculated according to Camassola *et al*., 2012 and Ghose., 1987, by the FPA (Filter paper assay) technique. The enzymatic activity of the tested commercial enzyme (ENMEX®) was 0.11 FPU/ml.

Determination of the concentration of cellulase required was estimated by performing three tests varying the concentration between 10, 20 and 30%, proportionate to the LHW treated SCS concentration (31.25 g/L). The operational condition was as follows: Schott® (0.25 L) at a temperature of 50 °C with orbital agitation (180 rpm). The batches were evaluated over 48 hrs, taking into account the COD and total reducing sugars (TRS) at 0, 2, 4, 24 and 48 hrs. Each test was run in triplicate. Once the enzyme concentration was determined, the required time was selected after evaluation of a new 192 h batch, taking readings of the COD and TRS at 0, 24, 48, 72, 96, 120, 144, 168 and 192 hrs.

To evaluate if there were any significant differences between the retention times, an ANOVA test was carried out, followed by a Tukey test in order to determine if there was any significant difference between the retention times and the concentrations of the COD and the TRS (Statistica, version 10).

2.3.1 *Conversion rate of cellulose into sugars (CRCS)*

The conversion percentage of cellulose into sugars was estimated taking into consideration the concentration (31.25 g/L) of the LHW pretreated SCS and its cellulose content (35.8%). Also, a 100% conversion rate of cellulose into sugars (CRCS) was considered to be when the total cellulose content in the enzymatic pretreatment reaction (11.18 g cellulose/L of reaction) was converted into RS by the ENMEX® enzyme in an equivalent way (Rabelo *et al*., 2011; Canilha *et al*., 2012; Vivekanand *et al*., 2014).

2.4 *Biological hydrolysis*

In order to obtain lignin and cellulose degrading bacteria soil samples were taken from a compost pile. These compost piles were elaborated with soil and plant residues, which consist mainly of cloud forest vegetation. The selective isolation of bacteria from the soil samples was carried out as previously described by (Seong *et al*., 2001; Tortora *et al*., 2007; Kutzner 1981). After 24 hrs, several colonies were chosen to grow in selective media in order to determine the presence of cellulases or laccases. The selective media for cellulases was C Dye (Winn 2006). A mineral media with guayacol (0.5 mM v/v) at 99% purity, was used according to Periasamy *et al*., 2010 in order to detect the presence of laccase-like activity.

Once 5 bacteria colonies had been selected (with cellulase- and laccase-like activity) they were used as an inoculum in order to obtain the required suspended bacteria (3.02×10^8 UFC/ml) for the biological pretreatment. Determination of bacterial content was estimated following the McFarland method (Sutton., 2011). The biological hydrolysis was carried out along with the biomethanization of the autohydrolizated sugarcane straw under the same operational condition (0.25 L, at 35 °C and 150 rpm) by the inoculation of a total bacterial concentration of 3.02 * 108 UFC/ml in the final operational volume of the biomethanization test (0.25 L).

Due to the simultaneous process (biological treatment and anaerobic digestion), there was no liquid fraction to analyse at the end of this pretreatment. Therefore, no report on the soluble COD or reducing sugars (RS) on the liquid fraction is made, unlike in the cases of the LHW and enzymatic hydrolysis pretreatment.

2.5 Analysis of the solid fractions of the treated SCS

The determination of lignin, cellulose, hemicellulose, ashes, extractable on water and acetone fractions for the LHW and enzymatic hydrolysis treatment was carried out as previously described by the TAPPI. In the case of the biologically treated SCS, the evaluation on the composition of the fibre was in terms of the removed content of volatile solids (VS_R) according to APHA methods.

2.6 Biomethane potential tests and inoculum

These were carried out to study the biodegradability of the substrates and the methane potential of the combined implementation of LHW pretreatment of SCS along with enzymatic or biological hydrolysis. The BMP tests were carried out in triplicate following the Martínez *et al*., and Rivera adapted protocols. The inoculum was obtained from pilot-scale anaerobic digester processing whey. In accordance with ISO regulation 11734 the inoculum was previously washed in order to eliminate any organic compound that could serve as a carbon source and alter the BMP test.

The BMP tests were performed in triplicate under mesophilic conditions in Schott® glass bottles (0.3 L) with an operational working volume of 0.265 L and 8 g VS_R/L of inoculum. Subsequently, the bottles were flushed with nitrogen and closed with modified screws with one port for samples and another for the methane measurement by the Marriotte flask system. The carbon source was provided with a concentration of 2 g COD/ L and 2 g VS_R/L for the BMP tests of the liquid and solid fractions, respectively.

The BMP tests were set as follow:

a) BMP from the liquid fractions: Liquid fraction from the LHW pretreated SCS (LF_{LHW}) and Liquid fraction from the enzymatic hydrolysis of the LHW pretreated SCS (LF_{EH}).

b) BMP From the solid fractions: Raw SCS (R_{SCS}), Solid fraction of the LHW pretreated SCS (SF_{LHW}), Solid fraction of the enzymatic hydrolysis of the LHW pretreated SCS (SF_{EH}) and Solid fraction of the biological hydrolysis of the LHW pretreated SCS (SF_{BH})

2.7 Determination of BMP of the liquid and solid fractions

Following Nguyen., 2014 the BMP was calculated by the following equation.

$$BMP\,from liquid\,fractions(ml CH_4/gCOD) = CH_4max/COD_R \quad (1)$$

$$BMP\,from solid\,fractions(ml CH_4/gCOD) = CH_4max/VS_R \quad (2)$$

Where CH_{4max}: Maximal experimental methane generation in ml; COD_R removed COD during the BMP test, VS_R: Removed volatile solids during the BMP test.

For the conversion of units from COD to VS_R, the equivalence was calculated by the following equation:

$$1\ g\ of\ COD = VS_R\ removed\ by\ the\ treatment/\ final\ COD\ of\ the\ generated\ liquor\ of\ the\ treatment \quad (3)$$

Following Nguyen., 2014 the percentage of the removed substrate (SR), defined as the percentage of the COD (for the liquid fractions) or VS_R (for the solid fractions) removed due to the biomethanization process, was calculated by the following equation:

For the liquid fractions:

$$SR(\%) = COD_R * 100/COD_I \quad (4)$$

Where COD_R: removed COD during the biomethanization, COD_I: initial COD.
For the solid fractions

$$SR(\%) = VS_R * 100/VS_I \quad (5)$$

Where VS_R: removed volatile solids during the biomethanization, VS_I: initial VS.

2.8 Total BMP

The total BMP yields of the pretreatments were calculated from the summary of the yield of each biomethanization test as follows:

- Blank tests: BMP of the SCS without treatment
- BMP of the pretreated SCS by LHW
- BMP of the LHW plus BMP of the enzymatic hydrolysis (EH).
- BMP of the LHW plus BMP of the biological hydrolysis (BH).

Table 1. Summary of relationships between structural features and digestibility (taken from Zhu *et al*., 2008).

Structural feature	Quoted relationships with digestibility of the lignocellulosic residue	
Physical	Surface area	Favorable
	Crystallinity	Unfavorable/No correlation
	Degree of polymerization	Unfavorable/No correlation
	Pore volume	Favorable
	Particle size	No correlation
Chemical	Lignin content	Unfavorable
	Hemicellulose content	Unfavorable
	Acetyl groups	Unfavorable

To determine significant differences between the BMP yields obtained from the pretreatment (LHW) and treatments (enzymatic or biological hydrolysis) an ANOVA test was carried out. Then a comparison between medians was calculated in order to establish if there was any significant difference between the BMPs generated by the estimated global balances of each biomethanization test. In order to confirm the significant difference between the BMPs of each global balance, a Tukey test of independent samples by variable was carried out following Salinas.

2.9 Structural modifications

The structural modifications in the solid fractions due to the implementation of the pretreatments were observed by electron microscopy. Images were taken using a JSM-6510LV SEM at 20 kV, and processed according to the protocols of the Renewable energy unit of the Yucatan Center for Scientific Research (CICY - its acronym in Spanish) by its technical specialist (Tanit Toledano Thompson).

3 Results and discussion

3.1 Lignocellulosic biomasses

Although, sugarcane straw is used in some places like Brazil as an organic fertilizer on the field, there is no current application for these residua as a basis for the generation of biomethane (Janke *et al*. 2015). This situation can be due to a lack of incentives to produce bioenergy, as well as for the lack of cases of study on the analysis of its biomethane potential. The anaerobic digestion of sugarcane residues can be considered as a useful strategy due its dual application, since the byproduct (digestate) could be used as an additive mineral fertilizer on the field and the biogas could be sold as a new energy byproduct of the sugarcane industry (Janke *et al*. 2015). Nevertheless, it is important to consider the seasonality of the crop and explore alternative lignocellulosic wastes that can be taking as a temporary replacement when this residue is no longer available (Janke et al 2015).

Likewise, the composition of lignocellulosic residues had several structural features that can influence its anaerobic digestion. They can be summarized into physical and chemical features.

Summary of relationships between structural features and digestibility (taken from Zhu *et al*., 2008)

It must be taken in consideration that there are still some disagreements of the effect of some of these features, but the most common results are presented on the following table.

3.2 Chemical composition

The chemical composition of the SCS (dry basis) used for the pretreatment processes is presented in the following Fig. 1.

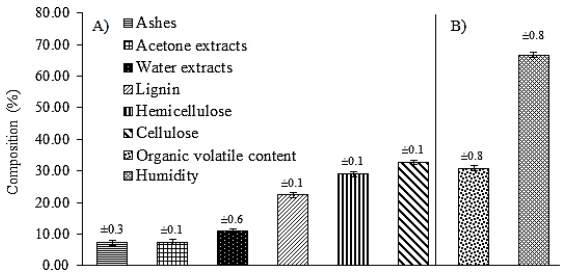

Fig. 1. Chemical composition of the SCS in dry (A) and fresh (B) basis (% wt).

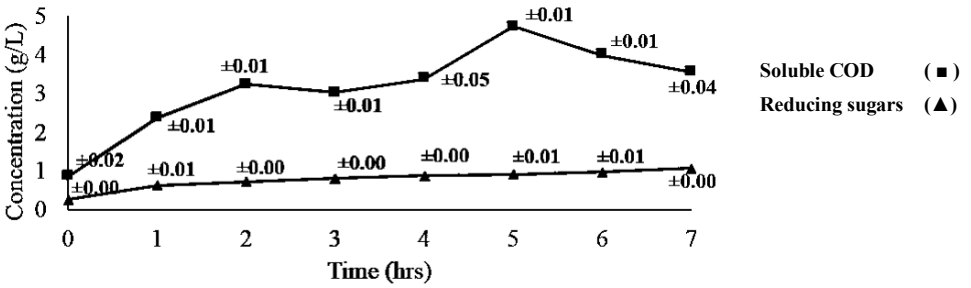

Fig. 2. Averages of soluble COD and total reducing sugars concentrations, generated during the LHW pretreatment of the SCS.

The ashes content of the dry basis (7%) was similar to that previously reported by Rossell., 2006 (8%). This parameter is very important due to its properties as an inorganic fraction of the SCS that make it non-degradable during the bio-hydrolysis and anaerobic digestion processes. The lignin content (22.4%) was similar to that previously reported by Aguilar Rivera., 2010 (20.3%). As for the cellulose content (33%), it was similar to that previously reported by da Silva *et al*., 2010 who found 33.6% of cellulose content in dry basis (Fig.1 A). This fraction is especially important because the by-products (pentoses, hexoses and uronic acids) of its degradation during the pretreatment, can be used through the anaerobic digestion for methane generation.

The content of water extracts (11%) in the dry basis (Fig.1 A) was similar to that reported by Pereira *et al*., 2011 (11.5%). This part represents the tannins, gums, starch and dyes. There was a lower percentage of acetone extracts (7.45%) in comparison with the water extracts (11%). In comparison with the figure previously reported by Waldheim *et al*., 2000 the organic volatile content (OVC) of dry basis (80.6%) was lower than that reported in this study (93%). Finally, as it is shown at the Fig.1 B the SCS presented a high moisture content (65%), similar to the results reported by Waldheim *et al*., 2000 who reported 67.7% of moisture on a fresh basis. Also 30.82% of OVC was found, which is very important due to the estimate of the initial concentration of terpenes, fatty acids and sugar content.

3.3 Liquid hot water pretreatment

3.3.1 Soluble COD and reduced sugars

The averages of the results are presented at the Fig.2, as can be seen, the soluble COD presented its maximum concentration (4.73 g/L) after 5 hours of the pretreatment, while during the following hours the concentration dropped due to the recondensation of previously solubilised components from lignin (González *et al*., 2014) (Fig.2). The RS show a gradual increment in concentration until it reached its maximum after 7 hours (1.07 g/L) (Fig.2).

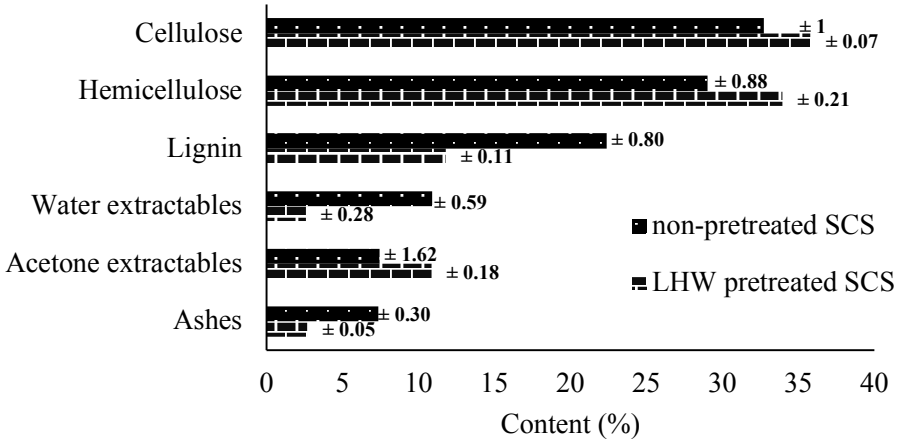

Fig. 3. Results on the composition of the solid fraction (dry basis) of the LHW pretreated SCS.

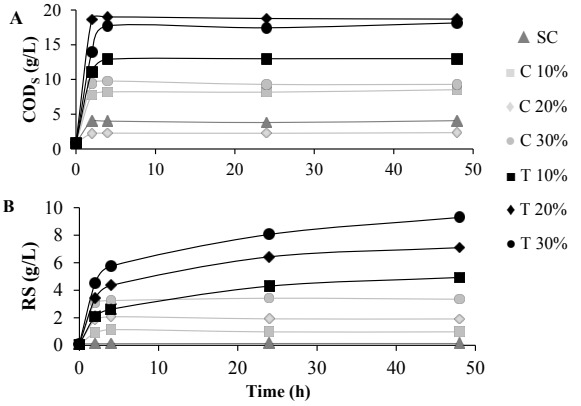

Fig. 4. Average concentrations of CODS (A) and RS (B) of the generated liquid fractions from the enzymatic hydrolysis of the LHW pretreated SCS by the ENMEX® commercial enzyme at 10, 20 and 30%. SC: control (substrate without the enzyme) C: Control enzyme (enzyme without the substrate) T: Test (enzyme + substrate) CODs: Soluble chemical oxygen demand RS: Reducing sugars

Nevertheless, the maximum concentration of soluble COD was lower than that previously reported by González *et al*., 2014 and Canilha *et al*., 2012 who established lower required times (15 - 23 min) but at higher temperatures (160 - 230 °C) to achieve a maximum soluble COD of 69 g/L with sugarcane bagasse as a raw material. It is important to highlight that in this case, the higher severity of the LHW González *et al*., 2014 and Canilha *et al*., 2012 vs this study, improves the concentration of soluble COD in the pretreated SCS, but also requires more energy (approximately 45.35 W-h per kg of treated SCS in comparison with this study) (Zhou *et al*., 2010).

On the other hand, the maximum RS concentration obtained in this experiment (0.13 g/g pretreated SCS) was the same as that previously reported by González *et al*., 2014, who had a concentration of 0.13 g/g of pretreated sugarcane bagasse. However, the use of other chemicals during the LHW pretreatment (like pressurized carbon dioxide) gave better results on the sugar yield concentration like xylose (8.67 g/L) at a lower temperature (115 °C) and required time (1 hour), which has been widely studied by Aguilar *et al*., 2010. Also, the increase in the RS by 94% in the liquid fraction of the LHW pretreated SCS, exceeded that previously reported by Ferreira *et al*., 2013 who achieved an increment of 83% on the treated SCS vs the control test (non-treated). Considering the results of the median comparison of the obtained CODs, the retention time of 5 hrs gave the highest value in comparison with times of 0, 1, 2, 3, 4, 6 and 7 hrs. Therefore, the selected retention time for the LHW treatment was 5 hrs.

3.3.2 Solid fraction

After determining the required time for the LHW pretreatment and the solubilization percentage of the treated SCS, the characterization of the solid fraction was carried out (Fig.3). As shown in the figure, LHW pretreatment has a major impact on the lignin content due to its 10.6% decrease in comparison with the non-pretreated SCS (22.38%±0.8) (Fig.3). These results exceed those reported by Moutta *et al*., 2014, who achieved an increase of the lignin percentage in the pretreated fibre up to 0.65% applying a temperature of 195 °C at 2.5 atm for 10 min and a SCS concentration of 100 g/L (dry basis).

The cellulose percentage increased by 3.09% in comparison with the non-pretreated SCS, exceeding that previously reported by Ferreira *et al*., 2013 who reported an increment of 0.55% in the LHW pretreated SCS. This could be due to the higher severity of the operational conditions (195 °C at 2.5 bar) which directly affects the solid fraction by increasing the solubilization of the lignin content. Previous investigations had reported the tendency to decrease of the lignin content, showing that the LHW pretreatment generates solid fractions with a higher content of cellulose, in comparison with the non-pretreated lignocellulosic residue (Ferreira *et al*., 2013; da Silva *et al*., 2010; Gurgel *et al*., 2014; Castro Gomez., 1985; Allen *et al*., 2001).

The hemicellulose content of the pretreated SCS was 4.94% higher than non-treated SCS, unlike the figures previously reported by Ferreira *et al*., 2013 who reported a decrease by 28.7 and 29.22%, respectively. The severity of the operational conditions in these cases promoted the solubilization on the hemicellulose content of the solid fraction, unlike the case of the present study. On the other hand, extracted water decreased by 8.32%, while in the case of Ferreira *et al*., 2013 this fraction reported an increase of 2.63%.

As for the inorganic fraction of the SCS, the ash content was reduced by a 4.67%, which was higher than that reported by Ferreira *et al*., 2013 who presented an increment of 0.34% on the pretreated SCS.

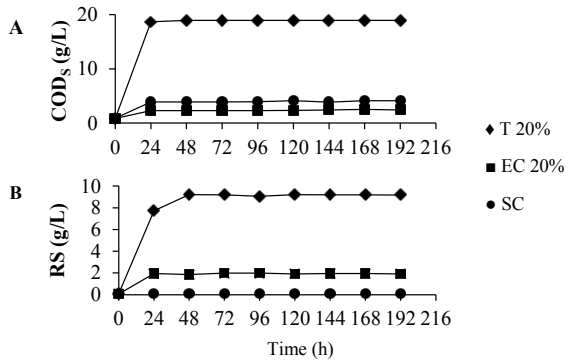

Fig. 5. Average concentrations of CODS (A) and RS (B) generated by the liquid fraction from the enzymatic hydrolysis (ENMEX® at 20%) of the LHW pretreated SCS. EC: Enzyme control, SC: Substrate control, T: Test.

3.4 Enzymatic hydrolysis

3.4.1 Soluble COD and reduced sugars

The soluble COD and reducing sugars (RS) obtained by the use of 10, 20 and 30% of enzyme (raw cellulase, ENMEX®) are presented on the followed graphic (Fig. 4 A and 4 B) The tests with 20 and 30% of enzyme showed a similar increasing tendency in terms of the CODS concentration (Fig.4 A). However, within 4 hours the test at 20% of enzyme exceeded the test at 30% by 1.2 g/L of CODS (Fig.4 A). Nevertheless, the test at 30% of enzyme generated a higher RS concentration (9.29 g/L) in comparison with the 20% test, which reached a RS concentration of 7.09 g/L in 48 hrs (Fig.4 B). Taking into consideration the soluble COD and the amount of required enzyme, a 20% enzyme concentration (6.25 g/L) was selected for the enzymatic hydrolysis (Batalha *et al*., 2015; Saska *et al*., 2006; Ferreira-Leitão *et al*., 2010).

A second test was implemented with the selected 20% concentration of the enzyme (ENMEX®), in order to determine the required time for the treatment, measuring the CODS and RS concentration every 24 hours for 192 hours (Fig. 5 A and B).

According to the results the test reached its maximum CODS concentration (18.96 g/L) in 48 hours (Fig.5 A) with a RS concentration of 9.21 g/L (Fig.5 B), which remained steady until the next 192 hours of the experimentation. The ANOVA and Tukey tests demonstrated a significant difference between the test (enzyme 20%) and the controls of enzyme (EC) and substrate (SC), in the case of the CODs and RS up to 48 hrs.

Table 2. Removal of volatile solids (VS_R) and treated SCS fibre (R_F) on the BMP tests of the solid fractions.

Employed substrate	VS_R (%)	R_F (%)
R_{SCS}	8.70	43.74
SF_{LHW}	8.31	41.33
SF_{EH}	5.92	29.50
SF_{BH}	9.21	45.50

Therefore, this retention time (48 hrs) was the one selected for the implementation of the enzymatic hydrolysis.

3.4.2 Solid fraction

As shown in Fig. 6 there was an increase in the lignin content (13.21%) in comparison with the LHW pretreated SCS, after the enzymatic pretreatment. This could be due to the cellulose solubilization at 6.18% due to the cellulose enzyme action on the pretreated SCS. A similar tendency was previously reported by Ferreira *et al*., 2013 who achieved 18% of solubilization of the cellulose fraction after a LHW pretreatment and an enzymatic pretreatment (Celluclast y Novozym 188) of SCS (Krishnan *et al*., 2010). However, although there was a markedly higher response to pretreatment conditions as shown by its higher lignin extraction during the pretreatment liquid phase, this meant a higher concentration of inhibitors due to the degradation of the extracted sugars (Ferreira *et al*., 2013).

On the other hand, a decrease of 1.96% in the hemicellulose content in the enzymatic treated versus the LHW pretreated SCS was obtained. There was also an increase of 6.84% in water extraction as well as a decrease of 5.26% in acetone extraction. However, the ashes content remained constant (Fig. 6). Solubilization of cellulose and hemicellulose content of the liquid fraction of the enzymatically treated SCS (Fig. 6) can be assessed from the increase in the RS (9.21 g/L) and the CODS (18.96 g/L) of the liquid fraction generated due to the enzymatic treatment versus the liquid fraction of the LHW pretreatment of the SCS (1.07 and 4.73 g/L, respectively).

3.5 Biological hydrolysis

3.5.1 Solid fraction

The SFLHW BMP test showed a removal of VS content of 8.31%; this indicates that the contribution

of the biological hydrolysis to the degradation of the organic fraction of the SCS was by an extra removal of 0.9% of its VS_R content (Table 1). Also, as shown in Table 1, the SFBH BMP test gave the highest percentage of removed treated SCS fibre (45.5%) in comparison with the SFEH, SFLHW and RSCS BMP tests.

Saad *et al*., 2008 obtained similar results for biological hydrolysis with bacteria in sugarcane bagasse (SB), where the content of the organic matter of the fibre (87.9%) showed a decrease in comparison with the non-treated SB (94.7%). Other studies reported the same trend; leading to the conclusion that biological hydrolysis along with previous chemical pretreatments reduces the organic matter content of the treated lignocellulosic residues, due to its assimilation by the microorganisms present in the biological pretreatment used. (Chandra *et al*., 1991; Deraz *et al*., 2001; Kholif *et al*., 2005).

Table 3. Summary of values from the measured parameters on the BMP tests of the liquid and solid fractions.

Pretreatment	Substrate	Employed fraction for BMP test	BMP (ml CH_4/g COD_R or g VS_R)[*1]	SR (%)	Authors
NaOH	SCS	LF	262.5±1.00	ND	Janke *et al*., 2017
CaO_2H_2			183		
H_2O_2	SCB		152	ND	Rabelo *et al*., 2001
LHW	SCS		316.12±1.83	86.50	This study
EH	SCS		342.68±1.07	92.03	This study
Steam explosion (225 °C, 10 min) EH (Cellic CTec2, Novozymes®)	SCB		124 216	ND	Vivekanand *et al*., 2014
Steam explosion (180 °C, 15 min.)	Weat straw		331	ND	Bauer *et al*., 2009
LHW (120 °C, 60 min, 1 atm)	SCS		220	ND	Bolado-Rodríguez *et al*., 2016
Non	SCS	LF	254	ND	Janke *et al*., 2017
BH (*Pleurotus florida*)	Corn straw		380	ND	Zhou *et al*., 2010
Non	SCS		341.23±0.05	43.67	This study
LHW	SCS		334.93±1.35	41.50	This study
EH	SCS		244.97±0.00	29.62	This study
BH	SCS		360.58±0.00	45.85	This study

BMP: Biomethane potential, SR: Percentage of substrate removed, EH: Enzymatic hydrolysis, BH: Biological hydrolysis, SCB: Sugar cane bagasse, SCS: Sugar cane straw, LF: Liquid fraction, SF: Solid fraction, ND: Not determinate.

[*1] The BMP for the liquid fraction were calculated in terms of ml CH_4/g COD_R, and for the solid fractions in terms of ml CH_4/ g VS_R. Where COD_R is COD removed and VS_R is VS removed.

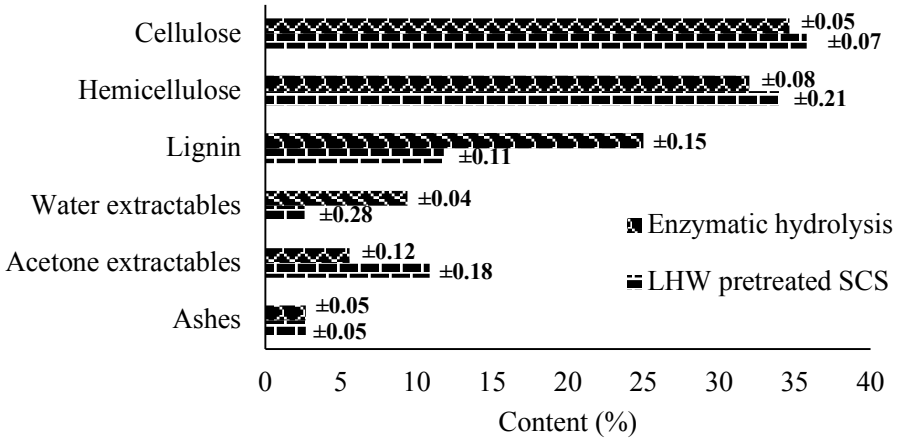

Fig. 6. Results on the composition of the solid fractions of the enzymatic hydrolysis (orange) and LHW (blue) pretreatments.

3.6 Biomethane potential

3.6.1 BMP liquid fraction

Analysing the BMP tests of the liquid fractions, we found that the BMP of the LHW and enzymatic hydrolysis (EH) pretreatments were close to the theoretical results (395 ml CH_4/g COD_R, according to Houbron *et al*., 2012) with 316.12 and 342.68 ml CH_4/g COD_R, respectively, Table 2. These results were higher than those previously reported by Rabelo *et al*., 2011 and Janke *et al*., 2017 who obtained a maximum BMP of 183-152 ml and 262.5 CH_4/g COD_R, respectively. This could be due to the lack of a pretreatment of the liquid fractions prior to the anaerobic digestion, which is required in order to achieve a better assimilation due to the removal of toxic compounds generated by the pretreatment.

3.6.2 BMP solid fraction

Likewise, the solid fraction of the EH pretreatment was the one with the lowest BMP, reaching 244.97 ml CH_4/g VS_R, and removing only 29.62% of the solid fraction (Table 2). This can be attributed to the lignin content (24.99%), which exceeds that preserved after the LHW pretreatment (11.78%). This tendency was previously discussed by Vivekanand *et al*., 2014, who find a correlation between the lignin content and the BMPs generated. This was due to a lower result on the BMP (124 ml CH_4/g VS_R) after the anaerobic digestion of the pretreated SCB (Table 2), in comparison with the one additionally treated by EH (216 ml CH_4/g VS_R). They attribute the decrease on the BMP to the lignin content, which was higher (46%) in the case of the steam explosion pretreatment and lower in the case additionally treated by enzymatic hydrolysis.

On the other hand, the highest BMPs were obtained from the non pretreated and the BH tests exceeding those previously reported by Bauer *et al*., 2009; Bolado-Rodríguez *et al*., 2016 and Janke *et al*., 2015 (Table 2). Nevertheless, the BH pretreatment proved to have the highest BMP. This result was close to that previously reported by Yang *et al*., 2003 who used fungi instead of bacteria for their BH pretreatment. Also, the BH pretreatment increased substrate removal by 4.35% in comparison with the BMP test of the EH, LHW and non pretreated SCS (Table 2), showing the biodegradability contribution of the biological treatment of the pretreated SCS.

Likewise, the time to reach such maximal cumulative volume was lesser in the case of the BMP tests of the liquid fractions (66-69 hrs) than in the case of the solid fractions (300 hrs). This could be due to the bioavailability of the organic fraction in each BMP test, which is higher in the case of the liquid fractions rather than the solid ones (Fig 7). This difference is even more remarkable in the case of high solid anaerobic digestion (AD), which in comparison with the AD of liquid fractions or low solid AD generates and byproduct with higher solid content and requires a longer retention time in order to accomplish a full biomethanization of the provided carbon source (Fagbohungbe *et al*. 2015).

Table 4. Total BMPs due to the implementation of the pretreatments.

BMP tests	Total BMPs (ml CH_4/g SV_R)	Authors
SCS	341.23±0.40	This study
LHW	353.16*±1.36	This study
LHW + Enzymatic hydrolysis	332.62*±0.25	This study
LHW + Biological hydrolysis	378.80*±0.11	This study
Alkaline hydrolysis	291±1.00	Janke *et al*., 2017
Hydrothermal	267±18	

Were SCS: Sugarcane straw

*Taking in consideration the average equivalents of the BMPs in ml CH_4/g SVR for the Liquid fractions of the LHW pretreatment and enzymatic hydrolysis treatment.

3.6.3 Total BMP

In the following Table the summary of the BMPs by the raw SCS, the implementation of the LHW as pretreatment and its application along with the enzymatic and biological hydrolysis, are presented. Following the ANOVA statistical analysis and median comparisons, a significant difference ($p : 0.001$) between the total BMP and the BMP tests were found. The median comparison proves that the LHW plus the BH pretreatment was the best option among the BMP tests (Table 3).

Janke *et al*., 2017 was the only study that made an analysis of the liquid and solid fractions generated by the applied pretreatments. Nevertheless, the results from this study exceed those of Janke *et al*., 2017 who used an alkaline (12 g NaOH/100g of fresh SCS) and hydrothermal (75 °C, stirred by 30 min at 100 rpm) pretreatment for the biomethanization of the SCS. This proves that the implementation of less aggressive and expensive pretreatments (or even non pretreatment) can be applied for the utilization of the SCS in the anaerobic digestion process.

3.7 Structural modifications

After the characterization of the SCS a series of electron microscopies were taken in order to see physical structural modifications in comparison with the original SCS (Fig.8) As can be appreciated in Fig.7, the SCS (8A and B) after the LHW pretreatment presented structural modifications altering the physical integrity of the SCS (Fig. 8C, D and E). This is more evident when we observed the rupture of its surface (Fig. 8C) and within the fibre (Fig. 8D and E). These modifications promote the increase of porosity and accessibility of the pretreated fibre in comparison with the original non-pretreated SCS. After the EH pretreatment, we can observe the rupture of the SCS surface (Fig.8 F and G) and within the fibre (Fig. 8H). Also, these changes promoted an increase of the structure degradation and porosity of the treated fibre in comparison with the LHW pretreated SCS.

After the BH pretreatment (Fig. 8I, J and K) the SCS presented structural modifications altering the physical integrity of the LHW pretreated SCS (Fig. 8C, D and E). This is more evident when we observe the presence of released cellulose fibres (Fig. 8I), which with a magnification of 2500 X show decomposition in their structure by the visualization of multiple holes along the fibre (Fig. 8J). In Fig. 8 K we can see the colonization by microorganisms of the BH pretreated SCS Surface. Also, the special adhesion of the bacteria to the solid fraction by a structure commonly denominated as glycocalyx (Weimer *et al*., 2006) is visualized. This adhesive structure has been previously reported and described by Weimer *et al*., 2006, and is due to the presence of microorganisms able to biodegrade lignocellulosic residues. This biological presence and activity increased the porosity and degradation of the LHW pretreated SCS, in comparison with the SCS treated by enzymatic hydrolysis.

Conclusions

The implementation of the LHW and BH proved to be better than the LHW plus EH (ENMEX®) due to the Total BMPs achieved (378.80 and 332.62 ml CH_4/g SV_R, respectively) and the structural changes in the generated solid fractions. These structural changes increased the porosity and allowed the liberation of the cellulose fibres in the case of the biological hydrolysis treatment.

Likewise, the unification of the BH pretreatment along with anaerobic digestion, versus the enzymatic one, allows the simplification of the process. The compatibility of the operational requirements from the BH pretreatment with anaerobic digestion, presents an attractive opportunity to reduce the costs and adaptations required, which are increased in the case of enzymatic hydrolysis due to the requirement of the separation of the process pretreatment-anaerobic digestion and the special operational needs (pH 7, 50° C, citrate buffer). Finally, this study could be useful as a future reference in Mexico for the development of new studies on a larger scale, in order to assess its feasibility for biomethane generation as sustainable bioenergy source. The energy potential of the generated biomethane can provide an alternative and sustainable energy source for the sugar mills nearest to the greatest sugarcane producers. In this matter the state of Veracruz is one of the most suitable locations on Mexico for the test this technology on large scale.

Nomenclature

SCS	raw sugarcane straw, g/L
BMP	biomethane potential, ml CH_4/g VS_R
LHW	liquid hot water, 120 °C, 1.5 bars
COD	Chemical Oxygen Demand, hrs
TRS	total reducing sugars, g/m^2d^1
CRCS	conversion rate of cellulose into sugars, g cellulose/$L_{reaction}$
RS	reducing sugars, g/L

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