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Short Communication

Effect of pretreatment and enzymatic hydrolysis on the physical-chemical composition and morphologic structure of sugarcane bagasse and sugarcane straw

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HIGHLIGHTS

- Physical and chemical changes were detected in the pretreated biomass.
- Microwave/acid glycerol solution pretreatment led to biomass morphologic change.
- Treated lignocellulosic material was more easily hydrolyzed by the enzymes.

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ABSTRACT

The present work aimed to study the effect of the pretreatment of sugarcane bagasse and straw with microwave irradiation in aqueous and acid glycerol solutions on their chemical composition, fiber structure and the efficiency of subsequent enzymatic hydrolysis. Thermogravimetric analysis showed that the pretreatment acted mainly on the lignin and hemicellulose fractions of the bagasse, whereas, in the straw, lesser structural and chemical changes were observed. The images from transmission electron microscopy (TEM) revealed that treating bagasse and straw with acid glycerol solution loosened the cell walls and there was a breakdown in the pit membrane. The treated material was submitted to hydrolysis for 72 h and higher yields of reducing sugars were observed compared to the untreated material (250.9 mg/g from straw and 197.4 mg/g from bagasse). TEM images after hydrolysis confirmed the possible points of access of the enzymes to the secondary cell wall region of the pretreated biomass.

1. Introduction

Brazilian production of sugarcane during the last harvest (2014/2015) was around 632 million tons, producing 28 billion liters of ethanol (anhydrous + hydrated). Around 30% of the sugarcane produced was stored as dry biomass in plants and was burned for energy co-generation (Perrone et al., 2016). The sugarcane bagasse and straw are the main by-products of the sugar and ethanol industry and can be an important source of sugar for use in biotechnological processes for obtaining high added value products (Wanderley et al., 2013).

The conversion of lignocellulosic residues to cellulosic ethanol is currently a topic of great interest around the world. This process consists of three steps: (***i***) pretreatment of the raw material to reduce the lignin content and to increase the polysaccharide exposure; (***ii***) enzyme hydrolysis to convert the polysaccharides into the glucose and xylose monomers; and (***iii***) fermentation of the sugars to ethanol. The main technical and economic challenges in this process are the development of inexpensive pretreatments that improves the accessibility of the enzymes to cellulose without the formation of compounds toxic to the fermentation processes, especially phenolics, and without loss of reducing sugar in the pretreatment step (Mesa et al., 2011).

Organosolv treatment is an effective technique in which lignin is extracted from lignocellulosic biomass through the use of an organic solvent such as ethanol, ethylene glycol or glycerol. The polar structure of glycerol can easily penetrate the lignocellulosic material providing an effective reaction medium for the delignification. As it can reach high temperatures at atmospheric pressure, this reduces the energy consumption (Novo et al., 2011). Micro-

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wave radiation is uniformly absorbed by the solution and provides an intense rotational movement of the water molecules generating heat, useful in promoting the disintegration of lignocellulosic complex (Chen et al., 2011). The pretreatment of rice straw with microwaves in a basic medium reduced the hydrolysis time by 50% to produce the same amount of reducing sugar compared to the conventional process (Zhu et al., 2006).

In a previous paper, Moretti et al. (2014) observed that, for sugarcane bagasse immersed in concentrated glycerol and pretreated with microwave irradiation, the chemical composition was altered (the lignin was reduced and the cellulose increased), when compared to samples immersed in distilled water or diluted phosphoric acid (pH 3.0). Structural modifications and improved hydrolysis were also seen. However, some issues remained like the possible use of aqueous glycerol solution instead of concentrated glycerol and the association of glycerol with sulfuric acid and also the effect of such treatment on sugar cane straw.

Microwave irradiation of sugar cane bagasse and straw in neutral and acid aqueous glycerol media at atmospheric pressure is a safe and economically feasible pretreatment and, in fact, this is the first study performed to evaluate the effects of an extract of *Myceliophthora thermophila* M.7.7 on the morphological changes in the fibers in this pretreatment and on the subsequent hydrolysis.

2. Material and methods

2.1. Materials

Sugarcane bagasse and straw were kindly provided by Guarani from their mill in Olimpia/SP-Brazil. The biomass was washed with distilled water until sugar-free, dried at 60 °C, ground to 1–3 mm sieve size and kept at room temperature protected from the light. The sugarcane bagasse and straw contain 47% and 43% de cellulose, 16% and 15% hemicellulose and 27% and 23% de lignin, respectively.

2.2. Microwave treatment of sugar cane bagasse

Five g of dry sugarcane bagasse or straw with a 1–3 mm sieve size were added to a 250 ml round-bottom flask and impregnated for 20 h with 30 ml of 70% v/v solution of glycerol in water or sulfuric acid (0.02 M). Then it was submitted to microwave irradiation (1300 W – 2450 MHz) for 2 min at 130 °C measured using an infrared thermometer. After microwave irradiation, 30 ml of distilled water was added to the flask, shaken and filtered. The liquid fraction was used for the quantification of reducing sugars (RS) and total phenolic compounds (TPC) released. The solid fraction was dried at 60 °C and used in the fiber analysis and enzymatic hydrolysis.

2.3. Chemical analysis

Total reducing sugars and total phenolic compounds released after pretreatment were quantified according to the methods described by Somogyi-Nelson (Somogyi, 1952) and Folin-Ciocalteu (Singleton et al., 1999), respectively. Cellulose, hemicellulose and lignin content were measured according to the laboratory analytical procedures of the National Renewable Energy Laboratory (NREL) for standard biomass analysis (Sluiter et al., 2011).

2.4. Morphological and physical analysis

The morphological analyses of the biomass were performed in a Philips transmittance electron microscope CM-100, strictly as reported by Moretti et al. (2014).

Thermal degradation was performed in a PerkinElmer TGA-4000 thermogravimetric balance, and the differential scanning calorimetry (DSC) was performed in a PerkinElmer DSC-8000 calorimeter. X-ray diffraction patterns were recorded in a Rigaku Miniflex 300 diffractometer, operating at 30 kV in an angular range of 3–70° at 2θ min^{-1}. The degree of crystallinity (CI) was calculated according to Eq. (1):

$$CI = \frac{H_c}{H_a + H_c} \times 100 \quad (1)$$

where H_a corresponds to the height referring to the amorphous phase (2θ∼18°) and H_c corresponds to the height related to the crystalline phase (2θ∼22°) (Browning, 1967).

2.5. Enzyme hydrolysis

Enzyme hydrolyses of untreated and treated samples were carried out in 50 ml flasks with rubber stoppers containing 2.5% of dry substrates in a final reaction volume of 20 ml of enzyme solution. The samples were incubated for 72 h at 55 °C using a reaction mixture containing an acetate buffer (pH 5.0, 0.1 M) and enzyme solutions obtained from the cultivation of *M. thermophila* M.7.7. Tests were performed by taking, as a reference, the total protein of the enzyme solutions (5 mg/g of substrate). The activities of the enzyme solutions in units per gram of dry substrate were: endoglucanase 825 U/g; xylanase 6050 U/g and β-glucosidase 5 U/g.

3. Results and discussion

The pretreatment under microwave irradiation with acid glycerol solution (MW/H_2SO_4G) provided the highest release of RS (10.4 mg/g from bagasse and 4.3 mg/g from straw) and the highest quantity of TPC (19.0 mg/g from bagasse and 8.0 mg/g from straw). Similar results for sugar released (10.9 mg/g of dry biomass) were obtained by Linde et al. (2008), with wheat straw treated with steam explosion and sulfuric acid as solvent.

When the ratio between the amounts of sugar and phenols released is considered, the data show that the treatments with microwave/aqueous glycerol solution (MW/H_2OG) give an average proportion of 1.1 in contrast to an average of 0.5 from those treated with MW/H_2SO_4G. On the other hand, the solid fraction of all the treated samples showed slight changes in the biomass composition (the maximum observed was a 4% decrease in lignin in the pretreated bagasse sample with MW/H_2SO_4G). These results are significant because they indicate the selective action of the treatment, decreasing the toxicity of the fermentation broth by phenolic compounds.

TGA curves showed that the weight loss onset temperature $T_{(onset)}$ occurs at approximately 320 °C except for bagasse pretreated with MW/H_2SO_4G which started around 350 °C, indicating higher thermal stability for this material (Fig. 1A, B). DTG curves of all bagasse and straw samples showed two defined peaks at around 360 and 405 °C except for bagasse pretreated with MW/H_2OG (Insert Fig. 1A, B). The first peak can be attributed to the decomposition of hemicellulose and lignin whilst the second one corresponds to the cellulose decomposition (Perrone et al., 2016). Bagasse pretreated with MW/H_2SO_4G showed only one peak, which reached its maximum at 405 °C. The absence of the other peak (360 °C) is due to the degradation of lignin and hemicellulose

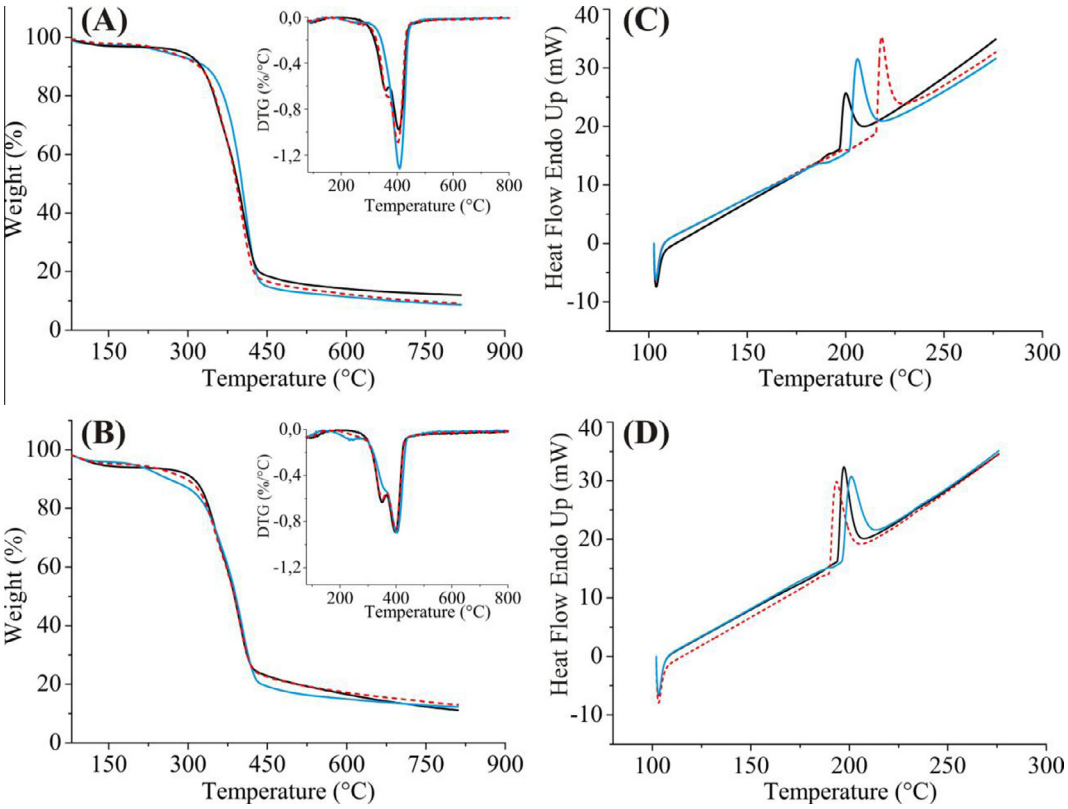

Fig. 1. Sugar cane bagasse and straw characterization: **A-**TGA/DTG$_{(insert)}$, and **C-**DSC present bagasse samples; **B-**TGA/DTG$_{(insert)}$, and **D-**DSC present straw samples. Where: **(Black line)** untreated sample, **(Red dashed line)** MW/H_2OG, **(Blue line)** MW/H_2SO_4G. *TGA/DTG condition: heating rate 50 °C/min; nitrogen atmosphere (20 mL/min); range temperature of 30–600 °C. **DSC condition: heating rate of 25 °C/min; purging gas nitrogen (20 mL/min) range temperature of 100–275 °C.

fractions during the pretreatment. In addition, an increase in the intensity of the peak around 405 °C was attributed to the enrichment of the cellulose fraction. The changes in the DTG straw curves were observed in the samples treated with MW/H_2SO_4G. A decreasing of the peak near to 360 °C was attributed to hemicellulose and lignin degradation. However, the cellulose portion did show not any notable change as can be seen in a peak near 400 °C (Insert Fig. 1B).

DSC thermograms showed endothermic peaks near 100 °C due to water evaporation, while the pyrolysis of lignin, hemicellulose and cellulose was an exothermic process. The curves obtained from the bagasse (Fig. 1C) showed that, after pretreatment, it became resistant to thermal degradation. Untreated bagasse showed an exothermic peak with $T_{(onset)}$ at 197 °C whereas, for the sample treated with MW/H_2OG, $T_{(onset)}$ occurs at 215 °C due to the removal of the less organized structures making it more thermal resistant (Moretti et al., 2014). The sample treated with MW/H_2SO_4G showed a lower $T_{(onset)}$ (202 °C) due to the degradation of lignin.

A decrease in the $T_{(onset)}$ of the exothermic peak from 194 °C for untreated straw to 189 °C after treatment with MW/H_2OG was observed (Fig. 1D). However, it went up to 197 °C with MW/H_2SO_4G with a decrease in the lignin and hemicellulose content due to an increase in the crystallinity of the fiber. The crystallinity indices were calculated according to Eq. (1). After treatment with MW/H_2SO_4G, both bagasse and straw show higher crystallinity (72.4% and 67.3%, respectively). Meanwhile, pretreated samples with aqueous glycerol produced hardly any changes in this parameter when compared to the untreated materials. A better correlation was expected between the XRD and the TGA/DTG data. It is possible that, in the treatment with aqueous glycerol, compounds have been liberated but not inserted into the structure of the material, different from that which occurred in the samples treated in the acid medium. This hypothesis is supported by the data on the released TPC, a decrease in the lignin and hemicellulose content and a slight increase in the proportion of cellulose.

TEM techniques were used to evaluate the microstructural modifications in the material after treatment. For untreated sugarcane bagasse, Fig. S1A and S1B show clear conservation of secondary walls (SW), middle lamella (ML) and plasmodesmata (PD) in the pit membrane (PM). In the samples treated with MW/H_2OG, there was a slight breakdown of the walls around the pit membrane (Fig. S1C), a change in electron density of the material and ruptures in the secondary walls, while the middle lamella was preserved (Fig. S1D). For the bagasse treated with MW/H_2SO_4G, there was a loosening of the secondary cell wall and disruption of the hemicellulose in this region. In some areas, liquefaction of the cell walls (Fig. S1F) and an overall breakdown of the pit membrane (Fig. S1E) were observed. Pores did not form in the outer region of the cell wall after CO_2 and SO_2 pretreatment of the sugar cane as reported by Corrales et al. (2012).

In untreated sugarcane straw, looser secondary cell walls are clearly observed (Fig. S1H). Similar to the bagasse, the pit membrane and plasmodesmata of the straw were well preserved (Fig. S1G). There were no changes in the secondary cell wall from straw treated with MW/H_2OG (Fig. S1J), but there was a slight disruption or rupture of the pit membrane (Fig. S1I). In the treatment using MW/H_2SO_4G very similar effects to those obtained with the pretreatment of sugarcane bagasse were observed. There was a loosening of cell walls, so that it (the secondary wall) even becomes liquefied (Fig. S1L) and, again, there was a breakdown in the pit membrane, as well as the absence of plasmodesmata (Fig. S1K). These observations are in agreement with those from Katahira et al. (2014) for corn straw after organosolv pretreatment in different acid mediums.

Data from TEM revealed the lignin re-localization in both pretreated samples. Globules of a coalesced lignin-rich fraction were displayed on cell wall surfaces and around the pit membrane of the treated materials with MW/H_2SO_4G (Fig. S1F,K,L). Donohoe et al. (2011) observed the same effects on pretreated switchgrass biomass with ammonia fiber expansion and liquid hot water. In the present work, both pretreatments were effective in making structural changes to the bagasse and straw. However, the morphology of biomass treated with MW/H_2OG is more preserved than biomass treated with MW/H_2SO_4G. The action of organosolv treatment combined with acid creates large increases in diffusion pathways for a greater accessibility of the hydrolytic enzymes to the cell walls.

After 72 h of hydrolysis, analysis of RS (Fig. 2) showed that the sugarcane bagasse treated with MW/H_2SO_4G was more susceptible to enzyme attack, since, in these samples, there were 1.4 times the quantity of RS compared to the untreated bagasse (105 mg/g from bagasse). Similarly, the treated straw samples with MW/H_2SO_4G showed a higher yield of reducing sugars (250.9 mg/g from straw) when compared to untreated straw samples (197.4 mg/g from straw). The enhanced glucose yield in the pretreated materials occurred due to the enzymes finding easy ways to penetrate disorganized fibers. It seems clear that the increase in the bagasse crystallinity after the pretreatment with MW/H_2SO_4G did not interfere with the performance of the enzyme.

The enzyme solution obtained from the culture of *M. thermophila* on lignocellulose material (sugar cane bagasse, corn straw and wheat bran) presented a high content of phenolic compounds derived from hydrolysis of the substrate. According to Kim et al. (2011), these phenolic compounds could be inhibiting the enzymes during the hydrolysis. In the present work, the phenolic compound concentrations were near to 3.5 mg/ml which possibly inhibited the action of cellulases and hemicellulases.

Hydrolyzed materials were morphologically analyzed using TEM. Untreated bagasse and straw with zero time of hydrolysis kept their morphologic structures rigid and compact (Fig. S2A,B,I,J) and the material treated with MW/H_2SO_4G disorganized morphology (Fig. S2E,F,M,N).

The untreated bagasse after 72 h of enzyme hydrolysis showed a slight displacement of the plasmodesmata in the pit membrane and a change in the electron density, besides loosening in the cell walls and middle lamella (Fig. S2C,D). In the bagasse samples, pretreated with MW/H_2SO_4G, disorganization of the pit membrane was seen, and also swelling of the secondary cell wall was clearly shown (Fig. S2G,H). The extensive swelling may be an indication of the action of cellulases and hemicellulases over the loosened region caused by pretreatment (Fig. S2F) since the middle and inner layers of the secondary cell wall are constituted of about 80% of cellulose and hemicellulose (Liu, 2015).

Cell walls from hydrolyzed untreated straw showed some slight rupture of the pit membrane and secondary wall region (Fig. S2K,L) in comparison with that of zero time of hydrolysis. On the other hand, structural modifications were not observed in samples previously treated with MW/H_2SO_4G after 72 h of hydrolysis (Fig. S2O,P) when this was compared to the same material at zero time of hydrolysis (Fig. S2M,N).

The lignin-rich middle lamellar regions of the cell walls in all the materials hydrolyzed for 72 h (Fig. S2D,H,L,P) kept their morphologic structures similar to those in samples with zero time hydrolysis (Fig. S2B,F,J,N). This was expected since no significant ligninolytic activities were revealed in the enzymatic complexes (data not shown). Pretreatment with MW/H_2SO_4G was shown to be effective, since the liquid fraction presents few inhibitors, such as RS and TPC, for enzyme hydrolysis. Also, the acid pretreatment did not damage the polysaccharide fraction as shown by the thermal analysis and the crystallinity index. Although this pretreatment was shown to be mild to the biomass, the small changes occurred were crucial to the enzymatic hydrolysis, since it increased the sugar yield. Pretreatment did not destroy the biomass samples, but from morphological analyses changes in the cell wall structure were observed.

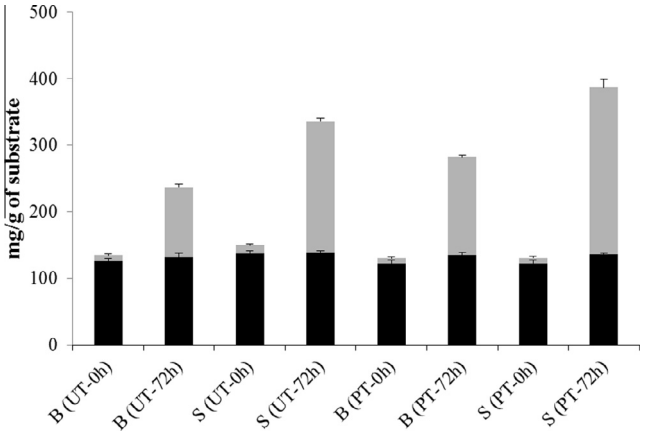

Fig. 2. Enzyme hydrolysis of sugar cane bagasse **(B)** and sugar cane straw **(S)**, untreated (**UT**) and pre-treated (**PT**) in MW/H_2SO_4G, at 55 °C for 72 h, using 5 mg of protein from *M. thermophila* M.7.7. per gram of substrate. Where: (**Gray bar)** = Total reducing sugar, (**Black bar) =** phenolic compounds, (**0 h)** = zero time of enzyme hydrolysis and (**72 h)** = after 72 h of enzyme hydrolysis.

4. Conclusion

Microwave irradiation associated with both aqueous and acid glycerol solutions promotes slight physical changes on sugarcane bagasse and straw fibers as presented in the XRD, DTG, TGA and DSC results. TEM of the cell wall revealed some points of loosening of the structure for pretreated sample which improved the enzyme hydrolysis yield (1.4 times higher than untreated samples). These results confirm the high potential of this pretreatment technology since it did not damage the fibers nor did it release inhibitors compounds that can difficult further ethanol fermentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.08.075.

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