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Enzymatic hydrolysis of brewer's spent grain to obtain fermentable sugars

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Abstract

Lignocellulosic biomass is a feedstock with the potential to be converted into value-added bioproducts. The use of enzymatic hydrolysis allows the cleavage of lignocellulose into their monomeric units, but there are drawbacks that make its use in industrial biocatalysis unfeasible. In the present study, we describe the hydrolysis of brewer's spent grain with an enzymatic cocktail produced by *Aspergillus niger* CECT 2700 and the comparison with commercial enzymes. In addition, it was determined whether pretreating the material (non-pressurized alkaline hydrolysis or treatment with cholinium glycinate ionic liquid) is necessary. Results show that both pretreatments have a positive effect on the xylose released (10.55 ± 0.07 g/L and 8.14 ± 0.13 g/L respectively), meanwhile the hydrolysis of raw BSG with the enzymatic cocktail produced solutions containing high levels of glucose (18.45 ± 1.66 g/L) and xylose (6.38 ± 0.26 g/L).

Keywords: brewer's spent grain, biorefinery, enzymatic hydrolysis, pretreatments

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1. Introduction

Lignocellulosic wastes are the most abundant repository of carbon around the world. It is estimated that each year the global agricultural sector produces 5×10^9 tons of biomass residues (Naidu et al., 2018), and that total lignocellulosic wastes amount to approximately 200×10^9 tons per year (De Bhowmick et al., 2018). The recent development of new technologies has meant that these biomass residues, rather than being an environmental disposal problem, have become a substrate for several industrial processes due to their renewability, recyclability, and sustainability. They are especially valuable as a feedstock in the biorefinery sector, which is considered an emergent industry with the potential to convert lignocellulose materials into value-added bioproducts, biofuels, and chemicals (Arevalo-Gallegos et al., 2017; Chen et al., 2017).

Within the biorefining processes, enzymatic hydrolysis plays an important role (Chandra et al., 2018). Enzymes are involved in bioprocesses that allow the transformation of biomass into their monomeric units. Such bioprocesses are considered fast, effective and ecofriendly; and the reactions involved are stereoselective, regioselective and chemoselective, and hence they produce enantiomerically pure products (Choi et al., 2015; Patel et al., 2016). However, only 5% of the known enzymes are available on the market, because natural enzymes from bacteria and fungi are often unsuitable for use in industrial biocatalysis and require genetic modifications (Patel et al., 2016; Sanchez and Demain, 2016). The main difficulties of enzymatic hydrolysis are the high cost of enzymes and their thermo-stability, but also the low yields of conversion due to the characteristics of the lignocellulose biomass (Arevalo-Gallegos et al., 2017; Choi et al., 2015; Guo et al., 2018). Despite this, the global market for industrial enzymes is expected to increase from nearly $5.0 billion in 2016 to $6.3 billion in 2021

(Research, 2017). All these facts serve to illustrate the importance of the development of commercial enzymes through screening for new strains that produce interesting enzymes, and also that are able to be used in scale-up processes.

Lignocellulosic material is constituted by a complex structure of cellulose, hemicellulose and lignin fibers. To enhance the accessibility of the enzyme and to release the monomers of this network, certain pretreatments are necessary to change the microstructure, macrostructure, and chemical composition of lignocellulose (Jönsson and Martín, 2016). There are several physical, chemical, physical-chemical and biological methods to pretreat the biomass, depending on the fraction to be released (Arevalo-Gallegos et al., 2017; Chen et al., 2017; De Bhowmick et al., 2018). In the present study, we focus on hemicellulose due to the variety of its applications as biofuel, high value-added compounds for the food and pharmaceutical industries, biopolymers, biomedical applications and so on (Chen et al., 2017; Naidu et al., 2018; Peng et al., 2012). This fraction is a heteropolymer with high heterogeneity depending on the nature of the biomass (softwood or hardwood). In general, there are three main sub-groups of components: mannans, xylans, and xyloglucans. These constituents can be hydrolyzed into pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose), but it is first necessary to break the structure through pretreatment processes. One of the most frequently used is alkali pretreatment, which cleaves the α-ether linkages between lignin and hemicelluloses, as well as the ester bonds between lignin and/or hemicelluloses and hydroxycinnamic acids, creating pores in the structure and allowing the expansion of lignocellulose (Chen et al., 2017; Gírio et al., 2010; Naidu et al., 2018; Peng et al., 2012).

On the other hand, there are novel procedures based on ionic liquid (IL) pretreatment for the dissolution of biomass, also called "green solvents". Some conventional solvents cause the inhibition of enzymes, the advantage of this technology being that it avoids the loss of enzymatic activity of the subsequent hydrolysis (Elgharbawy et al., 2016).

This study presents the results of the hydrolysis of brewer's spent grain (BSG) using an enzymatic cocktail produced by *Aspergillus niger* CECT 2700. The main goals are to determine the efficacy of these enzymes, and if necessary, the pretreatment of the material. As a comparison, commercial enzymes were also assayed.

2. Materials and Methods

2.1 Lignocellulose Biomass: composition and pretreatments

Moist BSG was kindly provided by Letra (Vila Verde, Braga, Portugal) and was dried at 50°C to avoid microbial degradation during storage. The composition of raw BSG was measured by hydrolysis acid quantitative (HAC) following the methodology described by Paz et al. (2018). The moisture content was determined by oven-drying the BSG until a constant weight at 105 °C. The ash content was obtained using a muffle furnace for 6 h at 550 °C. Extractives were quantified using a Soxhlet extractor and ethanol as a solvent recirculating for 24h. After this, the ethanol was vacuum-evaporated in a Büchi rotavapor R-215 (Frankfurt, Germany) at 60 ºC and the remaining extractives were dried at 105 °C until a constant weight was achieved.

BSG was pretreated using two different methodologies. The first was based on non-pressurized alkaline hydrolysis (AH-BSG) following the methodology proposed by Buranov and Mazza (2009). Raw bagasse was treated with a solution of 0.5M NaOH solution and stirred continuously (160 rpm) for 4 h at 50 ºC. After that, the remaining

solid was neutralized and dried at 50 ºC. The second pretreatment was based on the use of cholinium glycinate IL [N_{1112}OH][Gly] (IL-BSG) following the process described by Outeiriño et al. (2019). Prior to this, the IL was synthetized according to the procedure reported by Deive et al. (2015). The mixture of IL-BSG (5% w/w) was placed in a sand bath with vigorous magnetic stirring at 90 ºC for 16 h. After this, the mixture was diluted with acetone/water (1:1, v/v) and stirred (30 min) at room temperature. This caused the precipitation of carbohydrate-rich material (CRM), and also that the lignin-rich material (LRM) remained in the liquid phase.

To determine the effect of carbohydrates arising from the brewing process, and to avoid measurement errors, raw BSG was also washed with water at 50 ºC for 4 h and 160 rpm and dried at 50 ºC. Subsequently, compositional analysis by HAC was carried out to compare the results.

2.2 Fungal culture

Aspergillus niger CECT 2700, acquired from the Spanish Type Culture Collection (CECT, Valencia, Spain), was used as the enzyme cocktail-producing strain. Working cultures of this fungus were grown on PDA medium as agar slants and incubated 7 days at 30 ºC before use. Spores were recollected in a sterile solution of peptone water (0.5 g/L Tween 80 and 1 g/L bacteriological peptone) and countered in a Neubauer chamber.

2.3 Enzymes and activities

An enzymatic cocktail was obtained by solid-state fermentation (SSF) of *Aspergillus niger* CECT 2700 in a rotary drum type bioreactor, meanwhile, the commercial enzyme

preparations Ultraflo L® and Shearzyme® 500 L were gently provided by Novozymes (Bagsvaerd, Denmark).

Before being autoclaved (100 ºC for 60 min), 400 g of raw BSG was slightly moistened. After cooling, the sterilized substrate was loaded into the bioreactor and moisture was corrected with a mineral salts solution (1.3 g/L $(NH_4)_2SO_4$; 5.0 g/L $NaNO_3$; 4.5 g/L KH_2PO_4; 3 g/L yeast extract) at a ratio of 1:2.5 (w/v). The bioreactor was inoculated with 1×10^6 spores/g of dry BSG, and the process was carried out at 30 ºC with an aeration rate of 5 L/min. To prevent solid aggregation and promote the transfer of air and heat, the agitation system was activated for 1 hour at 3 rpm every 12 h. The bioreactor was weighed each day to determine the water losses and bring the fermentation medium to its initial humidity level. After 4 days, the process was finalized and BSG was collected to obtain the crude extract. The procedure consisted of adding distilled water at a ratio of 10 mL/g dry solid substrate and shaking for 1 h at 200 rpm and 30 °C. Finally, the mixture was filtered through a paper filter to remove solids, centrifuged at 2755×g for 15 min to remove the spores, and stored at -20 ºC for further analysis.

Different procedures were used to quantify enzymatic activity. Xylanase activity was determined using the DNS method according to Bailey (1992). Cellulase and cellobiase activities were also quantified by this method but using the procedure described by Ghose (1987). Feruloyl esterase activity was measured following the methodology described by Mastihuba et al. (2002) based on the quantification of ferulic acid released from ethyl ferulate by High-Performance Liquid Chromatography (HPLC). All activities were

expressed as U/ml, being one unit (U) defined as the amount of enzyme required to release 1 μmol of substrate per minute under the assay conditions.

2.4 Enzymatic Hydrolysis

All the enzymes were filter-sterilized using a syringe filter of 0.22 μm pore-size (Sartorius) and diluted in 50 mM Citrate Buffer pH 5 to the concentration under analysis.

To study the effect of ratio BGZ/enzyme, 0.2 U/mL of xylanase preparation was mixed with 5, 10 and 15% (w/v) of dry substrate in 12 ml tubes, incubated at 40 ºC, and shaken at 270 rpm. After this, a range of 5 – 30 U/ml of xylanase preparation was assayed to determine the maximum load of enzyme necessary to hydrolyze the BSG. In addition, the effects of Ultraflo L® and the enzymatic cocktail on the pure maltose were also studied to determine maltase activity. Samples were taken after 5 days, heated at 90 °C for 5 min, and filtered using 0.22 μm pore-size cellulose acetate membranes (Millipore) in order to analyze the carbohydrate concentrations by HPLC.

Finally, the kinetics of the enzyme-catalyzed hydrolysis of raw, washed and alkaline pretreated BSG samples were compared.

2.5 HPLC analysis

All samples obtained after enzymatic hydrolysis and HAC were analyzed by liquid chromatography in order to determine sugars, organic acids and alcohols released. The HPLC (Agilent, model 1200, Palo Alto, CA) was equipped with an Aminex HPX-87H ion exclusion column (Bio Rad 300×7.8 mm, 9 μ particles) and a guard column in a refractive

index detector. This configuration, with a method of 0.3 g/L of sulfuric acid at a flow rate of 0.6 mL/min at 50 °C, allowed the injected samples to be extracted. Concentrations were measured using the corresponding calibration curve.

2.6 Statistical analysis

All data were submitted to analyses of variance (ANOVA) with the Statistica Software 13.0 using Tukey's test at a significance level of $P < 0.05$ to determine statistically significant differences between them.

2.7 Mathematical modeling

In order to reduce experimental error in the data, the experimental concentrations of carbohydrates released (CR) obtained from the enzyme-catalyzed hydrolysis of raw BSG, AH-BSG and washed BSG with the enzymatic cocktail and Ultraflo L® were smoothed by using the bi-logistic (the sum of two logistic models) equation (Guerra et al., 2010):

$$[CR] = \frac{K_1}{1+e^{(c_1 - b_1 \cdot t)}} + \frac{K_2}{1+e^{(c_2 - b_2 \cdot t)}}$$

where K_1 and K_2 are respectively, the maximum concentrations of carbohydrate released (g/L) in the first and second enzyme kinetics phase, b_1 (h^{-1}), b_2 (h^{-1}), c_1 (dimensionless) and c_2 (dimensionless) are logistic parameters (h^{-1}) and t is the time (h). When the kinetics of carbohydrates release displayed only one enzyme kinetics phase, the bi-logistic model takes the form of a simple logistic model.

3. Results and Discussion

3.1 Composition of raw and pretreated lignocellulosic biomass

Table 1 shows the characterization of the raw, washed and the two pretreated BSG samples. Xylan, arabinan and acetyl groups constitute the hemicellulose fraction, whereas glucan units form cellulose. Washed BSG was also characterized because the raw material was collected from the brewery and dried directly, and carbohydrates and other compounds derived from this process could have been attached to the walls of the cereals. The significant differences ($P < 0.05$) found between raw BSG and washed BSG, such as moisture, extractives, ashes, cellulose, and hemicellulose, are shown in **Table 1**. The main cause for such differences is that the sugars from brewery processes interfere with the measures, and for this reason, the comparison with the other pretreatments (AH-BSG and IL-BSG) will be performed with the washed BSG. This was corroborated by HPLC analysis of the water that resulted from washing the material, which showed high amounts of maltose susceptible of being hydrolyzed by the enzymatic cocktail.

In terms of the results of pretreatments, the most notable relates to lignin. The procedure with IL allowed the delignification of the material, causing an increase of the cellulose fraction. IL is considered a suitable solvent for cellulose and lignin, although there are few studies on the solubilization of hemicellulose (Gírio et al., 2010), or the effect on subsequent enzymatic hydrolysis (Yoo et al., 2017). In contrast, the hemicellulosic fraction was not influenced by both pretreatments (AH-BSG and IL-BSG).

3.2 Preparation of enzymatic cocktail and commercial enzymes

The first step evaluated the activity (in U/ml or U/g) of the enzymes produced by *Aspergillus niger* CECT 2700 under the conditions assayed in the rotary drum type bioreactor, as well as the commercial enzymes (**Table 2**). Given that the extraction of

enzymes was carried out with water, stock solutions were prepared with the commercial enzymes with maximal activities of 61.58 U/ml in the same matrix. After that, different enzyme dilutions were prepared with citrate buffers to obtain the corresponding desired concentration.

There are several studies on the solid-state fermentation (SSF) of lignocellulosic biomass to obtain xylanases. For example, Khanahmadi et al. (2018) assayed different agro-industrial wastes (wheat bran, sorghum stover, corn cob, and soybean meal), as well as the effects of different parameters on the production of these enzymes by SSF. Their results showed that the highest xylanase activity (2919 ± 174 U/g) was achieved at 48 h using a wheat bran with a particle size of 0.3–0.6 mm, 70% moisture content (using a 1% (v/v) glycerol solution in distilled water and 1% (w/v) of $(NH_4)_2SO_4$) and with surface aeration. Similarly, Ajijolakewu et al (2017) obtained a xylanase activity of 3246 U/g in 4 days using oil palm empty fruit bunches. In our case, 4060.11 ± 132.46 U/g was obtained, when BSG was used as the substrate.

3.3 Enzymatic hydrolysis under different BSG/volume ratios

Different ratios of raw BSG and BSG pretreated with alkali (AL-BSG) (5, 10 and 15%, w/v) were assayed with 0.2 U/mL of xylanase preparation. The amounts of xylose obtained after the hydrolysis of these substrates are shown in **Figures 1 A** and **B**. On the whole, and independently of the ratio, the fractionation of AL-BSG with the enzymatic cocktail released more pentoses (from 2.27 ± 0.05 to 3.5 ± 0.06 g/L) than raw BSG (from 0.65 ± 0.01 to 2.22 ± 0.04). This confirmed the positive effect of the pretreatment on the material and supported the hypothesis that such a procedure creates pores in the structure instead of removing the lignin (Chen et al., 2017; Gírio et al., 2010; Naidu et al., 2018; Peng et al.,

2012). These results, are in agreement with other studies describing how the alkali pretreatment at low temperatures not only increased the porosity of substrates but also improved the enzymatic hydrolysis of lignocellulose (Dong et al., 2018; Safari et al., 2017; Yan et al., 2015). Regarding the enzymes used (**Fig. 1** and **Tables 1** and **2**), all experiments show that the enzymatic cocktail produced the greatest percentages of conversion ($P < 0.05$), followed by the commercial enzyme Ultraflo L®. **Table 3** shows the percentage of conversion of polysaccharides into their monomeric units. Generally, the conversion of raw BSG was higher at a ratio of 10%, but in the case of AH-BSG, more xylose was liberated at 15% (w/v). However, the statistical analysis determined that these differences were not significant ($P > 0.05$) and thus the ratio of 10% was chosen to continue the study.

Although xylose was obtained as a consequence of xylanase activity, high amounts of glucose were also released (**Figures 1 C** and **D**). **Table 3** shows the percentage of conversion from glucan to glucose. In general, as occurred with xylose, the enzymatic cocktail released more hexoses than commercial enzymes Ultraflo L® and Shearzyme® 500 L (**Figure 1**). Thus, hydrolysis of raw BSG (10 %, w/v) with the enzyme cocktail released 5.71 ± 0.04 g glucose/L, whereas hydrolysis of AH-BSG (10 %, w/v), produced only 2.46 ± 0.23 g glucose /L (**Figures 1 C** and **D**).

Another carbohydrate released during the hydrolytic process was arabinose (**Figures 1 E** and **F**). As observed for xylose, hydrolysis of AH-BSG at a ratio of 15% with both the cocktail or Ultraflo L® offered the best results (**Figure 1 F**). However, both enzymes showed a similar hydrolysis pattern and the maximum levels of arabinose released by the enzymatic activity of the cocktail (2.23 ± 0.06 g/L) or Ultraflo L® (2.48 ± 0.30 g/L) were not significantly different ($P > 0.05$).

3.4 Influence of BSG washing

Initially, the different results obtained with raw BSG and AH-BSG were thought to be due to their different cellulose content (**Table 1**). However, subsequent assays indicated that these variations (32.84 ± 0.08 % in case of raw BSG and 25.99 ± 0.35 % in case of AH-BSG) did not explain the notable differences observed in terms of glucose released. Considering that raw BSG was obtained directly from the brewery, and that it was recovered from the wort, constituted mainly by carbohydrates such as maltose and other peptic sources (Lynch et al., 2016), the BSG was washed with water for 4 h. The resulting wash water was further analyzed by HPLC to quantify the amounts of carbohydrates attached to the material. In order to determine the maltase activity of both the enzymatic cocktail and Ultraflo L®, an hydrolysis of maltose at a concentration of 3.71 ± 0.01 g/L was carried out. Results showed that all the maltose was transformed into 5.21 ± 0.02 g/L and 5.17 ± 0.03 g/L of glucose, respectively. This finding suggests that the majority of this glucose derives from the maltose attached to the cell walls but not from the cellulosic or hemicellulosic fraction. The lower amounts of glucose released from the enzymatic hydrolysis of AH-BSG with the cocktail and Ultraflo L® samples (**Figures 1C** and **D**) supports this hypothesis. The latter could be explained taking into account that during and after treatment with the alkali, the BSG was washed to remove all the hydroxide, and consequently, the maltose present on the walls could be also removed.

3.5 Influence of enzyme load

The next step was to determine the most appropriate enzyme load for hydrolyzing the substrates. Considering that, Shearzyme® 500 L enzyme did not produce satisfactory results (**Figure 1**), the following study was carried out with Ultraflo L® and the enzymatic

cocktail. **Figure 2 A** shows the effect of increasing concentrations of enzymes on the hydrolysis of BSG and AH-BSG. The results indicate that the suitable enzyme concentration for maximizing xylose release was 25 U/mL. As before, pretreated BSG achieved the best results for released xylose (9.03 ± 0.71 g/L xylose with the cocktail and 8.90 ± 0.06 g/L xylose with Ultraflo L®), in comparison with the hydrolysis of BSG with the cocktail and Ultraflo L®, which provided values of 6.23 ± 0.09 g/L and 5.43 ± 0.24 g/L xylose, respectively. With regard to the glucose released (**Figure 2 B)**, and due to the above results, hydrolysis of AH-BSG substrate produced lower levels of this carbohydrate (4.19 ± 0.49 g/L with the cocktail and 9.16 ± 0.16 g/L with Ultraflo L®) than raw BSG (16.85 ± 1.02 g/L with the cocktail and 12.91 ± 1.68 g/L with Ultraflo L®). However, the most appropriate enzyme load for releasing high amounts of glucose was achieved at 15 U/ml enzymes, and there was no variation for increasing the enzyme load (**Figure 2 B**).

In the case of the arabinose extracted from the arabinan (**Figure 2 C**), similar values ($P > 0.05$) between the materials were obtaineed. In this sense, Ultraflo L® at 10 U/ml achieved the highest amounts of arabinose released independently of whether BSG was pretreated (7.50 ± 0.02 g/l) or not (6.88 ± 0.06 g/L). Ultraflo L® enzyme load higher than 10 U/ml did not produce arabinose liberation. When raw BSG and AH-BSG substrates were hydrolyzed with the cocktail, the amounts of arabinose obtained were lower but remained constant independently of the enzyme concentration.

The percentages of conversion of glucan, xylan, and arabinan into glucose, xylose, and arabinose under different loads of enzyme are shown in **Table 4**. As stated above, hemicellulose is not a homogeneous fraction, on the contrary, it is a heteropolymer constituted by different types of xylan, mannans and xyloglucans. Therefore, it is impossible to obtain 100% hydrolysis using only one type of enzyme (Gírio et al., 2010).

Some researchers argue that the use of a consortium or co-culture is a suitable way of increasing the saccharification of biomass (de Oliveira Rodrigues et al., 2017; Uçkun Kiran et al., 2013), while others have suggested that supplementation with additives such as surfactants (Li et al., 2015) or the co-utilization of xylans from different origins (Uçkun Kiran et al., 2013) are good strategies.

In the present study, hydrolysis of pretreated BSG with the cocktail and Ultraflo L® provided similar xylan conversion percentages, 37.55 ± 2.94 and 37.05 ± 0.24 %, respectively. However, with raw BSG substrate, the use of the cocktail provided a significantly ($P < 0.05$) better result (34.18 ± 0.52 %) than the commercial enzyme (29.78 ± 1.30 %). The hydrolysis of untreated BSG with the cocktail has the additional advantage of producing hydrolysates with a high glucose content. These products, rich in glucose and xylose, could ultimately be used as substrates in other biorefinery or biotechnological processes. In this sense, **Figure 3** shows the kinetics of carbohydrates released from the hydrolysis of raw, pretreated and washed BSG substrates with the cocktail and Ultraflo L® (25 U/mL) for 144 h. In this study, kinetics of arabinose liberation were not considered because this sugar was not liberated when 25 U/ml of Ultraflo L® was used, and the amount released by the enzymatic cocktail remained constant through the time with the independence of enzyme load (**Figure 2 C**).

The kinetics of xylose liberation with the two enzyme preparations from the corresponding substrate exhibited a similar behavior (**Figure 3 A** and **3 B)**. However, the kinetics of hydrolysis of raw and washed BSG substrates were different to that of AH-BSG substrate. Thus, meanwhile hydrolysis of the first two substrates with the two enzyme preparations revealed monophasic enzyme kinetics, enzymatic hydrolysis of AH-BSG substrate displayed biphasic kinetics (0-72 h and 72-144 h). Surprisingly, although the

maximum xylose concentration obtained with Ultraflo L® in the first phase was significantly higher ($P < 0.05$) than that obtained with the cocktail, the latter enzyme preparation provided a sugar level (10.55 ± 0.07 g/l) significantly ($P < 0.05$) higher than that (9.42 ± 0.52 g/l) obtained with the commercial preparation (**Figures 3 A** and **B**). With regard to the other two substrates, the maximum final xylose concentrations obtained with the enzymatic cocktail were 5.44 ± 0.04 g/L and 6.38 ± 0.26 g/L from washed and raw BSG, respectively. In contrast, with Ultraflo L®, the released sugar levels were 5.4 ± 0.10 g/L from washed BSG and 4.78 ± 0.04 g/L from raw BSG.

From the detailed observation of glucose liberation kinetics (**Figures 3 C** and **D),** the following features were observed: i) the raw BSG always provided the highest glucose levels (18.45 ± 1.66 g/L with the cocktail and 5.4 ± 0.10 g/l with Ultraflo L®), probably due to the conversion of the maltose attached to the cell walls of the untreated BSG, ii) more glucose was released from the cocktail-catalyzed hydrolysis of washed BSG in comparison to AH-BSG, iii) in the case of Ultraflo L®, the washed substrate provided the lowest glucose level and, iv) hydrolysis of washed BSG and AH-BSG exhibited a biphasic kinetics with cocktail but not with Ultraflo L® preparation.

3.6. Influence of pretreatment with IL

Finally, due to the complexity of the treatments with IL, the CRM samples obtained after the pretreatments were hydrolyzed under optimal conditions (25 U/mL of the enzymatic cocktail for 144 h at a ratio of 10 %). In this case, 8.14 ± 0.13 g/L of xylose and 2.48 ± 0.03 g/L of arabinose were released, which represent conversion percentages of 29.62 ± 0.49 and 30.83 ± 0.42 %, respectively. It can be noted that CRM only retained 5.01

± 1.02 % of lignin, this being purer than raw BSG. Thus, the amount of hemicellulose to be converted is greater than those of the raw or washed BSG substrates.

In addition, the mixture of CRM into xylanase solution became a mucilaginous suspension, that makes difficult the appropriate transference and action of the enzymes. Although there are significant differences between these results and those obtained with the other materials, more studies are necessary to improve the results obtained with CRM and thus to optimize the process and allow the whole conversion of material. For this, Heggset et al. (2016) suggested that a combination of pretreatment with IL and alkali improves the enzymatic digestibility of the biomass, due to each treatment allows the solubilization of a specific component. Thus, IL showed a pronounced effect on digestibility glucan, and alkali pretreatment had a positive effect on the hydrolysis of mannan.

Conclusions

Biorefineries from lignocellulose is a strategy of sustainability that helps to reduce dependence on petroleum-based, non-renewable resources. However, an essential stage in making this technology cost-effective is the suitable conversion of lignocellulose into monomeric units for use in biotechnological processes. In this study, an enzymatic cocktail produced by solid-state fermentation of BSG with *Aspergillus niger* CECT 2700, was used to hydrolyze lignocellulosic biomass. The use of this mixture of enzymes had important advantages with respect to the use of the commercial enzymes Ultraflo L® and Shearzyme® 500 L. First, the cocktail-catalyzed hydrolysis of raw BSG and AH-BSG substrates provided hydrolysates with higher amounts of sugars (xylose and glucose) than the commercial enzyme preparations. Second, cut down the price of the enzymes due to purification processes are not necessary. Since these hydrolysates could be used for

productions of high value-added products (bacteriocins, single cell proteins, biogas, etc.), production of this enzymatic cocktail could provide a profitable enzyme preparation to obtain high levels of fermentable sugars.

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Table 1. Composition of BSG (raw and after different pretreatments)

Components	Raw BSG	Washed BSG	AH-BSG	IL-BSG
Moisture	4.56 ± 0.10*	6.50 ± 0.01 A	6.97 ± 0.01 A	7.99 ± 0.00 A
Ashes	3.26 ± 0.06*	2.46 ± 0.09 A	5.45 ± 0.05 B	3.45 ± 0.02 C
Extractives	14.39 ± 0.10*	12.76 ± 0.14 A	11.57 ± 0.61 A	-
Klason Lignin	17.57 ± 0.36	15.14 ± 0.80 A	19.26 ± 0.28 B	5.01 ± 1.02 B
Cellulose	32.84 ± 0.08*	22.45 ± 0.80 A	25.99 ± 0.35 B	32.24 ± 0.55 C
Hemicellulose	25.85 ± 1.55*	33.78 ± 0.36 A	32.86 ± 0.11 A	31.24 ± 0.98 A

*Shows significant differences between raw BSG and washed BSG. AH-BSG: BSG after pretreatment based on non-pressurized alkaline hydrolysis; IL-BSG: BSG after pretreatment with ionic liquids. Different letters show significant differences between washed BSG and the pretreatments.

Table 2. Enzymatic activities of the *Aspergillus niger* CECT 2700 extract and the commercial enzymes used

Enzymes	Enzyme activity			
	Xylanase	**Cellulase**	**Cellobiase**	**Feruloyl esterase**
***A. niger* extract (U/mL)**	61.58 ± 1.77	0.22 ± 0.01	0.43 ± 0.02	0.04 ± 0.00
***A. niger* extract (U/g)**	4060.11 ± 132.46	14.84 ± 0.52	28.22 ± 1.42	2.61 ± 0.00
Ultraflo L® (U/mL)	3360.70 ± 28.78	34.75 ± 0.47	25.47 ± 1.18	n.d.
Shearzyme® (U/mL)	1011.08 ± 8.31	16.72 ± 4.32	17.19 ± 0.27	n.d.

n.d.: not determined

Table 3. Conversion (%) of glucan, xylan, and arabinan into glucose, xylose, and arabinose

			Ratio BGZ/enzyme		
Substrates	**Enzymes**	**Polysaccharides**	**5 % (w:v)**	**10 % (w:v)**	**15 % (w:v)**
Raw BSG	Cocktail	Glucan	11.40 ± 0.16	15.65 ± 0.12	2.95 ± 0.10
		Xylan	6.80 ± 0.09	12.20 ± 0.24	3.55 ± 0.07
		Arabinan	5.69 ± 0.34	10.19 ± 0.19	00.00 ± 0.00
	Ultraflo L®	Glucan	4.95 ± 0.26	7.58 ± 0.79	6.66 ± 0.08
		Xylan	4.21 ± 0.34	7.53 ± 0.92	5.27 ± 0.21
		Arabinan	9.76 ± 0.11	18.60 ± 2.35	14.21 ± 0.11
	Shearzyme®	Glucan	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
		Xylan	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
		Arabinan	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
AH-BSG	Cocktail	Glucan	5,73 ± 0.03	8.51 ± 0.78	11.09 ± 0.76
		Xylan	9.47 ± 0.22	12.73 ± 0.68	14.58 ± 0.23
		Arabinan	6.18 ± 0.43	11.57 ± 0.73	16.75 ± 0.47
	Ultraflo L®	Glucan	3.91 ± 0.02	4.97 ± 0.09	5.32 ± 0.64
		Xylan	3.29 ± 0.16	4.68 ± 0.03	5.35 ± 0.70
		Arabinan	8.79 ± 0.34	14.38 ± 0.62	18.60 ± 2.29
	Shearzyme®	Glucan	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
		Xylan	1.44 ± 0.01	1.89 ± 0.01	2.01 ± 0.14
		Arabinan	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00

Table 4. Conversion (%) of glucan, xylan, and arabinan into glucose, xylose, and arabinose, respectively, under different enzymatic loads.

Substrates	Enzymes/Polysaccharides		Enzyme load 5 U/ml	10 U/ml	15 U/ml	20 U/ml	25 U/ml	30 U/ml
Raw BSG	Cocktail							
		Glucan	33.81 ± 2.28	35.87 ± 0.16	47.30 ± 0.51	48.97 ± 0.11	46.18 ± 2.79	46.21 ± 0.74
		Xylan	24.20 ± 0.08	28.28 ± 0.11	29.56 ± 0.05	32.92 ± 0.89	34.18 ± 0.52	35.52 ± 0.35
		Arabinan	21.78 ± 0.44	25.88 ± 0.32	25.45 ± 0.79	28.35 ± 1.41	29.95 ± 0.07	31.45 ± 0.66
	Ultraflo L®							
		Glucan	19.60 ± 0.80	25.49 ± 0.04	30.98 ± 0.59	32.97 ± 0.22	35.37 ± 4.61	32.80 ± 1.00
		Xylan	18.68 ± 0.20	23.63 ± 0.03	23.41 ± 0.90	26.32 ± 0.72	29.78 ±1.30	25.69 ± 0.43
		Arabinan	51.20 ± 0.23	74.28 ± 0.17	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
AH-BSG	Cocktail							
		Glucan	13.93 ± 1.41	15.75 ± 0.05	13.70 ± 0.18	13.66 ± 0.76	14.53 ± 1.70	4.52 ± 0.13
		Xylan	27.48 ± 0.73	32.18 ± 0.00	31.54 ± 0.56	34.30 ± 0.21	37.55 ± 2.94	3.79 ± 0.33
		Arabinan	16.93 ± 0.98	19.24 ± 0.05	20.15 ± 0.03	19.43 ± 0.68	21.81 ± 1.79	9.52 ± 0.06
	Ultraflo L®							
		Glucan	16.18 ± 1.83	21.87 ± 0.04	29.47 ± 0.21	28.46 ± 0.33	31.73 ± 0.55	26.37 ± 5.12
		Xylan	22.36 ± 0.35	28.25 ± 0.54	29.49 ± 0.70	30.88 ± 1.80	37.05 ± 0.24	15.12 ± 2.25
		Arabinan	37.68 ± 0.63	51.71 ± 0.46	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00

Figure legends

Figure 1. Carbohydrates released from different ratio weights/volume of raw BSG and AH-BSG with Cocktail, Ultraflo L® and Shearzyme® 500 L.

Figure 2. Carbohydrates released under different enzyme loads. Triangles represent raw BSG + Cocktail; Circles represent AH-BSG + Cocktail; Squares represent raw BSG + Ultraflo L® and diamonds Ultraflo L® + AH-BSG.

Figure 3. Kinetics of carbohydrates released in the enzyme-catalyzed hydrolysis of raw BSG (triangles), AH-BSG (circles) and washed BSG (squares) with the enzymatic cocktail and Ultraflo L®. The curves drawn through the experimental concentrations of carbohydrates released were obtained according to the bi-logistic model (1).

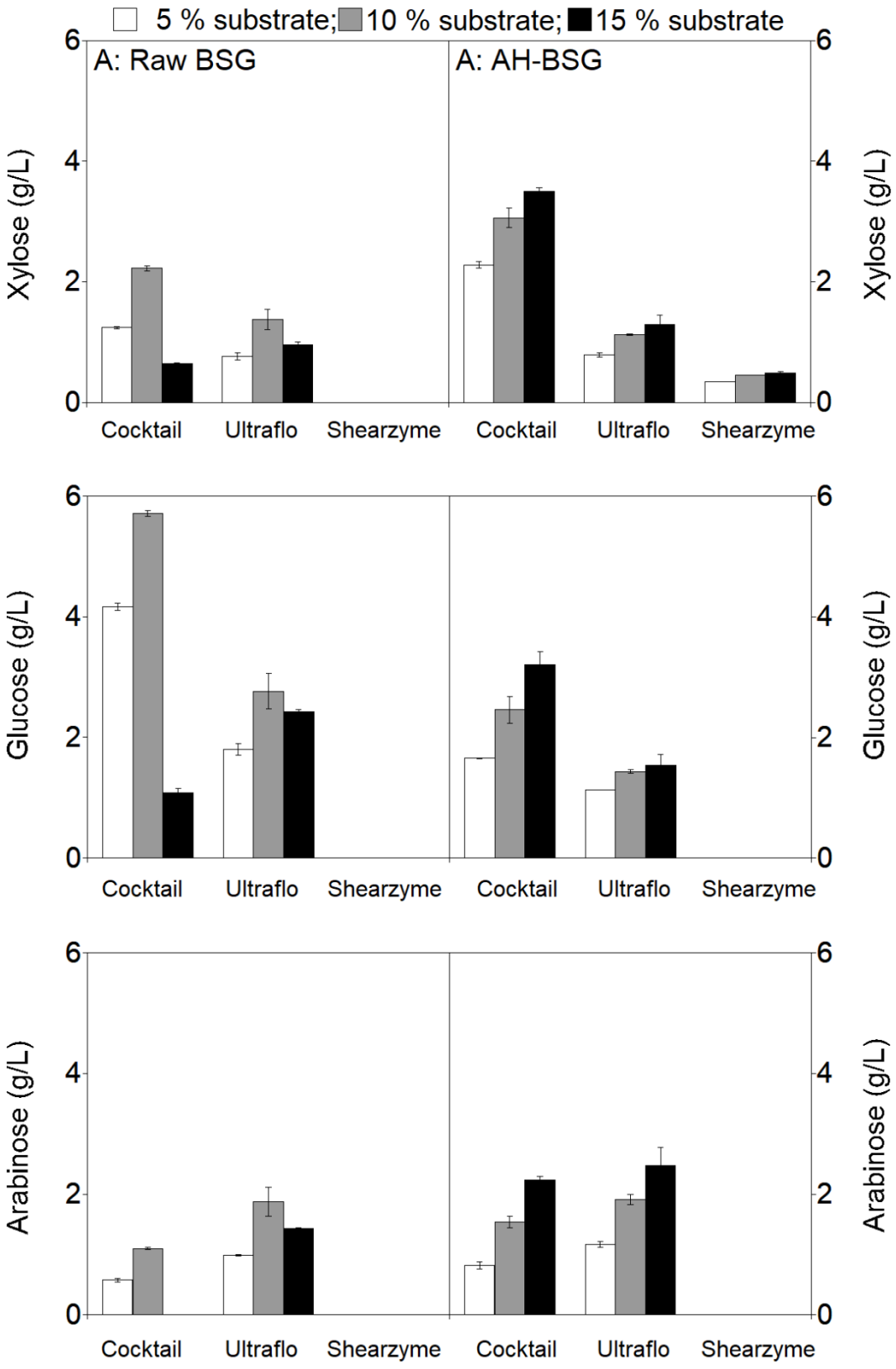
5 % substrate; 10 % substrate; 15 % substrate
A: Raw BSG
A: AH-BSG
Xylose (g/L)
Glucose (g/L)
Arabinose (g/L)
0
2
4
6
Cocktail
Ultraflo
Shearzyme

Figure 1

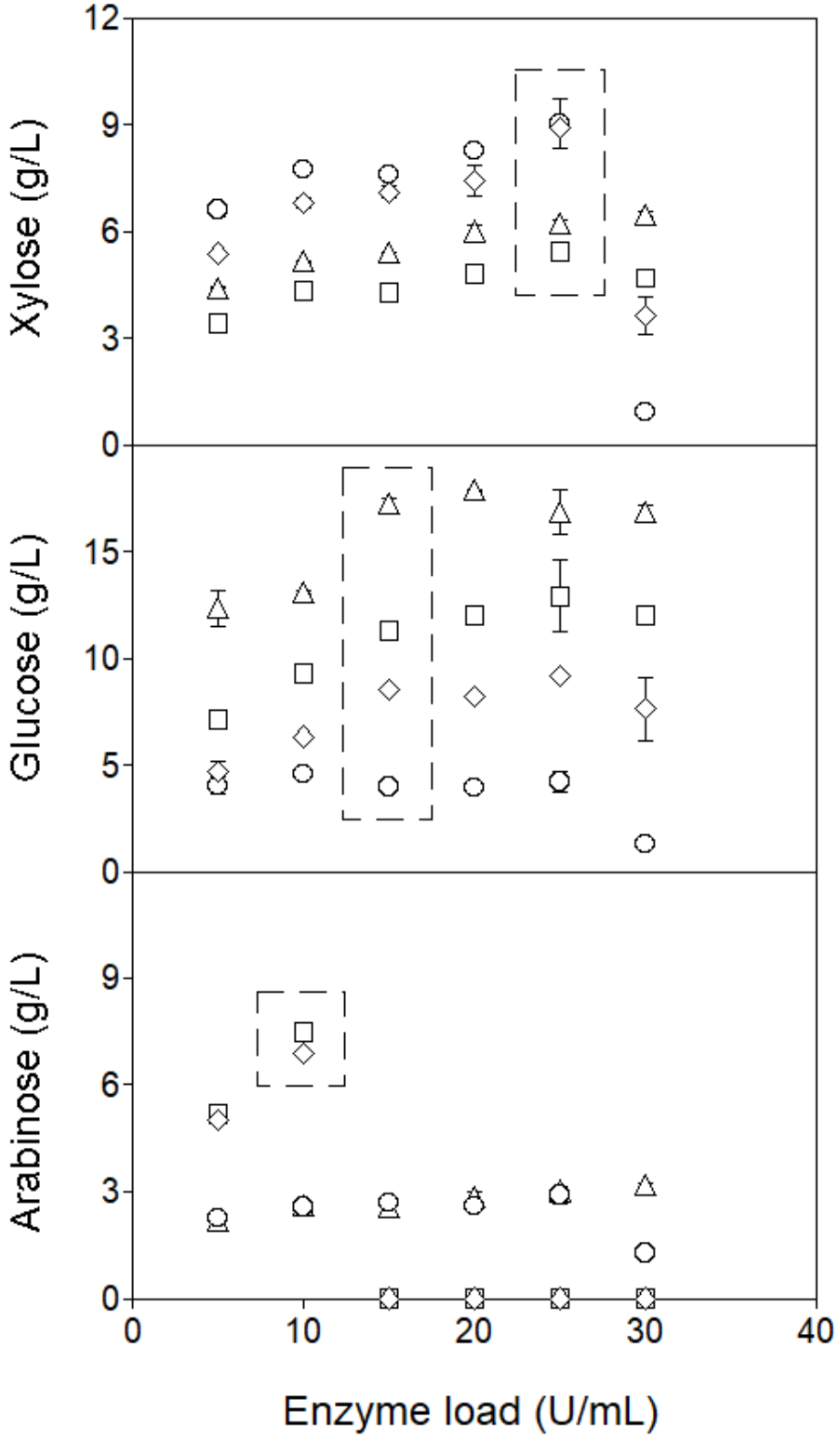
Xylose (g/L)
Glucose (g/L)
Arabinose (g/L)
Enzyme load (U/mL)
0
3
6
9
12
0
5
10
15
0
3
6
9
0
10
20
30
40

Figure 2

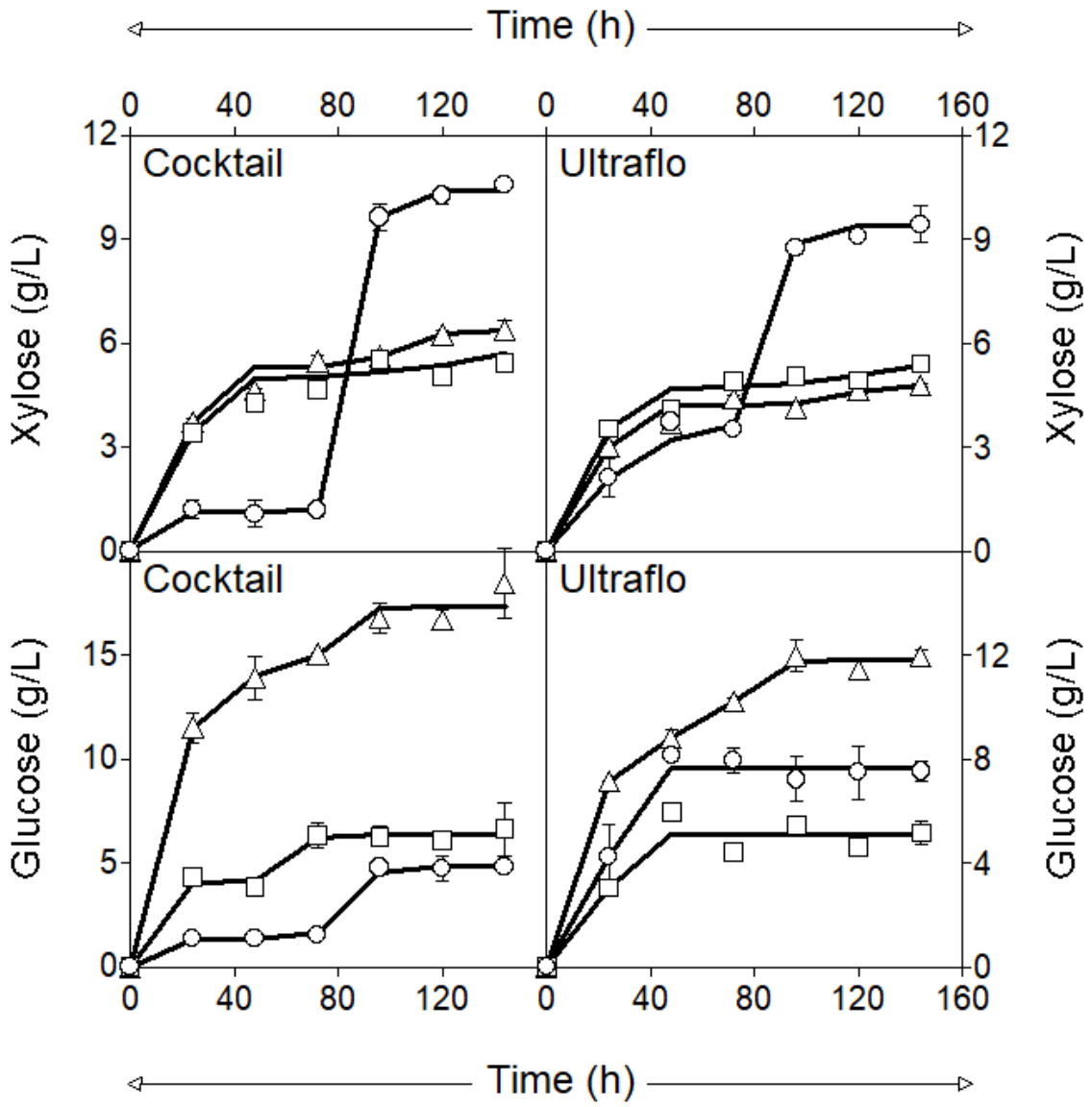
Time (h)
Cocktail
Ultraflo
Xylose (g/L)
Glucose (g/L)
0
40
80
120
160
3
6
9
12
5
10
15
4
8

Figure 3