Improving Sugar Extraction from Brewers' Spent Grain Using Sequential Deproteinization and Acid-Catalyzed Steam Explosion in a Biorefinery Context

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ABSTRACT

Brewers' spent grain (BSG) is a complex biomass composed of sugars and lignin, with a high protein content. In this work, a BSG fractionation process was evaluated to improve sugar extraction. The developed process involved a sequential combination of enzymatic deproteinization and acid-catalyzed steam explosion (SE) pretreatment. Temperature and time effects on the SE pretreatment of deproteinized BSG (BSG-D) were experimentally studied. The deproteinization yield of BSG was 63.9 % and, in the liquid after SE, up to 49.8 % of xylose was recovered using BSG-D. The conditions of the SE were optimized at 173.5 °C for 15.5 min with acid-catalyzed H_2SO_4 (0.5 % w/v). Under these optimized conditions, 30 % and 93 % of xylose and arabinose, respectively, were recovered as monomeric sugars in the liquid; from the solid, up to 72.2 % glucose was recovered using enzymatic hydrolysis (EH).

Abbreviations[1]

[1] AIL: Acid Insoluble Lignin, ASL: Acid Soluble Lignin, BSG: Brewers' Spent Grain, BSG-D: Deproteinized BSG, EH: Enzymatic Hydrolysis, EHY: Enzymatic Hydrolysis Yield, FPU: Filter Paper Units, HTP: Hydrothermal Pretreatment, OSY: Overall Sugar Yield, SE: Steam Explosion, SE-OP: Steam Explosion Optimum Conditions, WIS: Water Insoluble Solids, XOS: Xylo-oligosaccharides

Keywords: brewers' spent grain, acid-catalyzed steam explosion, protein, xylose, glucose, biorefinery.

1. INTRODUCTION

The valorization of different components present in the biomass other than fermentable carbohydrates constitutes a significant advance that could give a boost in the industrial implementation of lignocellulosic biomass-based biorefineries. Among them, proteins, acetic acid, and lignin are the most promising candidates for use in the production of valuable bio-based products [1]. Some new technologies, such as hydrothermal pretreatment (HTP), are very promising for lignocellulose biomass fractionation. HTP can be employed in industrial-scale second-generation biorefineries by applying circular bioeconomy concepts because it does not require chemical inputs other than water, liquid, steam, and/or heat [2]. Additionally, HTP is an attractive pretreatment technology for biomass because corrosion problems and the use of hazardous chemical compounds can be effectively reduced or, ideally, eliminated. One of the most widely studied HTP technologies is steam explosion (SE) with or without acid catalysts. Acid-catalyzed SE was introduced to improve the hemicellulose solubilization and enzymatic hydrolysis yield (EHY) of cellulose. Acids are an essential addition when softwood (with a low potential for autohydrolysis) has to be pretreated, and they can help to reduce the severity of pretreatment with other biomasses [3]. The impregnation of lignocellulose biomass with diluted or concentrated acids also reduces the pretreatment time, temperature, and, simultaneously, the production of inhibiting compounds, leading to the complete removal of hemicellulose [4].

Brewers' spent grain (BSG) is a low-value byproduct of the brewing process. Spent grain is the insoluble part of barley grain, separated during the mashing process before fermentation of the soluble liquid wort [5]. BSG constitutes up to 85 % of the total residue from the brewing process [6, 7, 8]. The structure of BSG is exceptionally heterogeneous, since BSG is composed of cellulose (13–21 %) and non-cellulosic polysaccharides—mainly hemicellulose in arabinoxylan form (19–42 %) and lignin (12–16 %), with a high protein content (19–30 %), reported by many authors [9, 7, 10, 8, 11]. Since the composition of the biomass has been shown to have a negative influence on the efficacy of SE pretreatment [3], the relatively high protein content and residual starch in BSG make the extraction of xylo-oligosaccharides (XOS) from the liquid fraction more difficult. Moreover, the high protein content can also affect the degradation of sugars during SE, considering that Maillard reactions can occur because of the simultaneous presence of proteins and carbohydrates at high temperatures, changing the solubility of the components both in the liquid and in the pretreated solid [9]. Reactions of sugars degradation are usually fostered by high temperature and long residence time. These reactions occur easily for reducing sugar pentoses (xylose and arabinose).

Indeed, among the different biomass types, softwoods are especially recalcitrant to SE due to the lower acetyl group content of their hemicelluloses, which catalyzes the autohydrolysis of the biomass [3]. Protein gelation, gelatinization, and retrogradation of starch during SE of fresh BSG make it challenging to achieve a high yield of xylose recovery in the liquid fraction using SE. Kemppainen et al. [9] found at a maximum of approximately 18.1 % of xylan solubilized after SE at 200 °C for 10 min with and without acid (1 % w/w H_2SO_4). They reported that xylan suffered significantly from the combined

effect of high temperature and prolonged treatment time. They concluded that, in general, the effect of temperature is more important than the effect of contact time on extraction carbohydrates. Swart et al. [12] also found xylose recovery using SE pretreatment of BSG. An increment of sugar yield to 20–30 % using an SO_2-like catalyst at 25 % of dry mass (wt. %) at 180 °C for 10 min of pretreatment. Kemppainen et al. [9] used an epifluorescence microscope to observe the changes that took place in the matrix during SE processing. They found that the tissues from the aleurone layer, pericarp, and husk were recognizable. Additionally, the sample contained large agglomerates contained released protein and small pieces of cell wall. These protein agglomerates seemed to disintegrate into smaller particles that tended to attach to the surfaces of the grain structures present, and the presence of cutin in the raw material had a similar effect. All these effects of the recovery of sugars after SE can be explained by the generation of physical and chemical barriers to the heat and mass transfer of steam to the BSG. Rommi et al. [13] found that up to 29 % of the total Klason lignin consisted of protein and ash in untreated BSG, and this proportion increased to 40 % in steam-exploded BSG.

Specifically for SE of BSG, Kemppainen et al. [9] studied the effect of SE operating conditions on the solubilization of carbohydrates and protein, and on the composition and enzymatic digestibility of the remaining insoluble solids of BSG. According to their results, SE at 200 °C for 10 min without an acid catalyst significantly improved the enzymatic digestibility of the insoluble carbohydrates. Simultaneously, more than a third of the protein present in BSG was solubilized, and most of it was degraded into peptides. However, most of the lignin and protein remained insoluble, and the potential to further dissolve and fractionate these components was not explored. Later, Rommi et al. [13]

evaluated the impact of thermochemical and enzymatic pre-treatments on the fractionation of protein and lignin from BSG, using SE and carbohydrase and protease treatment. Their results revealed that SE and hydrolysis of cell wall polysaccharides substantially increased lignin solubilization and its recovery through acidic precipitation. Meanwhile, effective protein extraction required the use of protease, which increased protein solubilization from 15 % to nearly 100 % from otherwise untreated BSG. Recently, Swart et al. [12] found a higher XOS yield (>73.1 %) after studying the impact of screw press dewatering on subsequent autocatalytic SE HTP (180 °C for 10 min with 25 % initial dry matter content) using two types of BSG. For the case of BSG, Swart et al. [12] confirmed that proteins were degraded in SE, and the crude proteins in the SE residues were consistently higher than the total amino acids. The solid residue from SE run at 200 °C for 4 min showed a crude protein level of 19.6 %, while the amino acid total was 14.4 %. This showed that almost 20 % of the nitrogen was degraded in the insoluble residue from the SE HTP.

The aim of this research was to design and evaluate a fractionation process to convert BSG into a source of valuable sugars and other value-added compounds. The developed biorefinery scheme was divided into three steps: (1) deproteinization and starch removal of BSG using enzymes, and (2) acid-catalyzed SE pretreatment of deproteinized brewers' spent grain (BSG-D), followed by (3) EH of solids after SE. The deproteinization stage was intended not only to achieve BSG protein separation (by valorizing this fraction) but also to reduce the mass and heat barriers of SE pretreatment caused by the high protein content and the presence of starch in the original BSG. The main purpose of the SE was to improve xylan recovery in the pretreatment liquid while generating a cellulose-enriched solid

fraction that could be used for glucose production using EH. Accordingly, temperature and time effects on the SE were experimentally studied. Regarding the technical approach, the best processing conditions for achieving maximum recovery of the compounds of interest were selected, and an overall mass balance for the biorefinery scheme was calculated.

2. MATERIALS AND METHODS

2.1 Raw material

Fresh BSG was provided by Mahou Brewery (Madrid, Spain), with moisture at 79.10 ± 1.32 %. During this research, BSG was stored at -4 °C in hermetically sealed plastic containers until use. All other chemicals used in the experimental work were of analytical grade.

2.2 Enzymatic deproteinization and starch removal from BSG

Deproteinization of BSG was performed using a serine endopeptidase produced using *Bacillus licheniformis* (commercially known as Alcalase® 2.5 L; Novozymes, Denmark). The enzyme was added in doses of 1.9 % w/w [14]. The following operating conditions were kept constant: 10 % (w/v) solid content, 250 rpm, and 60 °C [15]. [16]. The deproteinization process was performed on a bench scale in a 40 L fully automated Biostat® Bplus reactor (Sartorius Stedim Biotech, Germany) under pH 7.0 ± 0.5 conditions. The pH was measured hourly and set to the test value using NaOH 4 M for up to 4 h of reaction. Simultaneously, the remaining starch in the BSG was removed using Spirizyme® and Termamyil® (Novozymes, Denmark) at doses of 200 mg of each enzyme per kg of the substrate [17, 18]. After the deproteinization stage, the slurry was separated by centrifugation at 3,000 rpm for 20 min in a basket centrifuge (Comteifa, Spain). The BSG-

D was washed with hot distilled water, and the solid residues were collected. One part was characterized, and the other part was stored at -4 °C in hermetically sealed plastic containers until pretreatment. The deproteinization yield was calculated using Equation 1.

$$Deproteinization\ yield = \left(\frac{g\ of\ protein\ in\ BSG - g\ of\ protein\ in\ BSG-D}{g\ of\ protein\ in\ BSG}\right) \times 100 \quad (1)$$

2.3 Steam Explosion pretreatment

SE pretreatment was carried out in a 2 L reactor that was part of a batch pilot plant comprising a steam generator, a reactor, and a reception chamber, as described by [19]. The reactor was filled with 200 g of dry BSG-D per batch. Once the reactor was hermetically sealed, high-pressure water-saturated steam was fed into it until the test temperature was reached. To determine the effects of temperature, reaction time, and the use or absence of an acid catalyst on SE performance, the tests described in Table 1 were conducted.

Table 1. Experiments performed to assess SE effects on BSG-D.

Conditions	**BSG-D with acid (H_2SO_4 0.5 % w/v)**						**BSG-D without acid**	
	SE-1	**SE-2**	**SE-3**	**SE-4**	**SE-5**	**SE-6**	**SE-7**	**SE-8**
T (°C)	160	160	170	170	180	180	170	180
t (min)	15	30	15	30	15	30	30	30

The SE-1 to SE-6 experiments conformed to a factorial experimental design for evaluating the effect of temperature (160 °C, 170 °C, and 180 °C) and SE time (15 and 30 min) on xylose, arabinose, and glucose recovery in the liquid fraction. BSG-D impregnation was carried out by soaking the BSG-D with a solution of H_2SO_4 (5 % w/v) for 12 h at room temperature (25 ºC). The SE-7 and SE-8 tests used BSG-D without acid impregnation. After SE, the obtained slurry was separated into liquids and solids (water-

insoluble solids; WIS) using a basket centrifuge (Comteifa, Spain) at 3,000 rpm for 15–30 min.

2.3.1 Analysis of extracted sugars in the liquid

The extracted sugars in the liquid were analyzed in two ways. First, the sugars released as monomers were quantified immediately using high-performance liquid chromatography (HPLC). Second, for quantification of the oligomers, a portion of the liquid was hydrolyzed using a mild acid (4 % [v/v] H_2SO_4, 120 °C, 30 min) until monomeric sugars were obtained. Thus, the sugar recovery yield of the liquid (Eq. 2) was defined as the sugar release (glucose, xylose, or arabinose) in the liquid, considering the contributions of both the extracted monomeric sugars and oligomers (expressed as monomeric sugars). The monomeric sugars were measured as described in section 2.5.

$$Sugar\ recovery\ yield\ = \left(\frac{g\ of\ sugar\ extracted\ in\ the\ liquid\ after\ SE}{g\ of\ sugar\ in\ BSG-D}\right) \times 100\ \% \qquad (2)$$

After assessment of the SE pretreatment, a multiple optimization response surface analysis was performed, maximizing xylose recovery (% yield) and xylose concentration (g/l) in the liquid fraction, using StatGraphics Plus 5.0 Enterprise Edition (Statistical Graphics Corporation, Princeton, NJ). An additional experiment was performed in quintuplicate under optimum conditions (SE-OP) to validate the optimization process.

2.3.2 Enzymatic digestibility of the WIS after SE pretreatment

The WIS were submitted to an EH test under laboratory conditions. A cellulolytic and xylanolytic cocktail (Cellic® CTec2; Novozymes, Denmark) was added in doses of 15 FPU/g substrate. EH was performed in a 100 ml Erlenmeyer flask with a 5 % (w/v) dry material load in 0.05 M sodium citrate buffer (pH 4.8). Experiments were performed in an

orbital shaker (Certomat-R B-Braun, Germany) at 50 °C and 150 rpm for 72 h. The suspension was then separated by centrifugation (10,000 rpm for 10 min), and the sugar content in the liquid phase was determined as will be described in section 2.5. The results reported are the averages of three tests. The EHY was calculated as follows (Eq. 3).

$$EHY = (g\ of\ sugar\ in\ EH / g\ of\ sugar\ in\ WIS) \times 100 \quad (3)$$

2.4 Chemical analysis of BSG, BSG-D, and WIS

The chemical compositions of the BSG, BSG-D, and WIS were determined using the Laboratory Analytical Procedures (LAP) for biomass analysis provided by the National Renewable Energies Laboratory (NREL).

2.5 Analytical methods

The crude protein content in the samples was determined following a standardized Kjeldahl method (AOAC 984.13). The total starch content was measured using a total starch assay kit from Megazyme International (Bray, County Wicklow, Ireland).

The sugar concentrations of xylose, arabinose, and glucose were quantified using HPLC in a Waters Chromatograph 2695 equipped with a refractive index detector (Waters, Mildford, MA). A CarboSep CHO-682 lead carbohydrate analysis column (Transgenomic, Omaha, NE) operated at 75 °C with an ultrapure water mobile phase (0.5 mL/min) was employed for the separation.

Acetic acid was also quantified using HPLC (Waters, Mildford, MA) with a 410 refractive index detector (Waters, Mildford, MA) and a Bio-Rad Aminex HPX-87H (Bio-Rad Labs, Hercules, CA) column maintained at 65 °C with a mobile phase (5 mM H_2SO_4) at a flow rate of 0.6 mL/min.

3. RESULTS AND DISCUSSION

3.1 Chemical composition comparison of BSG and BSG-D

The first step in the biorefinery design was the deproteinization of BSG using an Alcalase 2.5 L enzyme. Therefore, the raw material considered in the next steps of the process was BSG-D. Fig. 1 shows the characterization of BSG before and after the deproteinization process, for comparison.

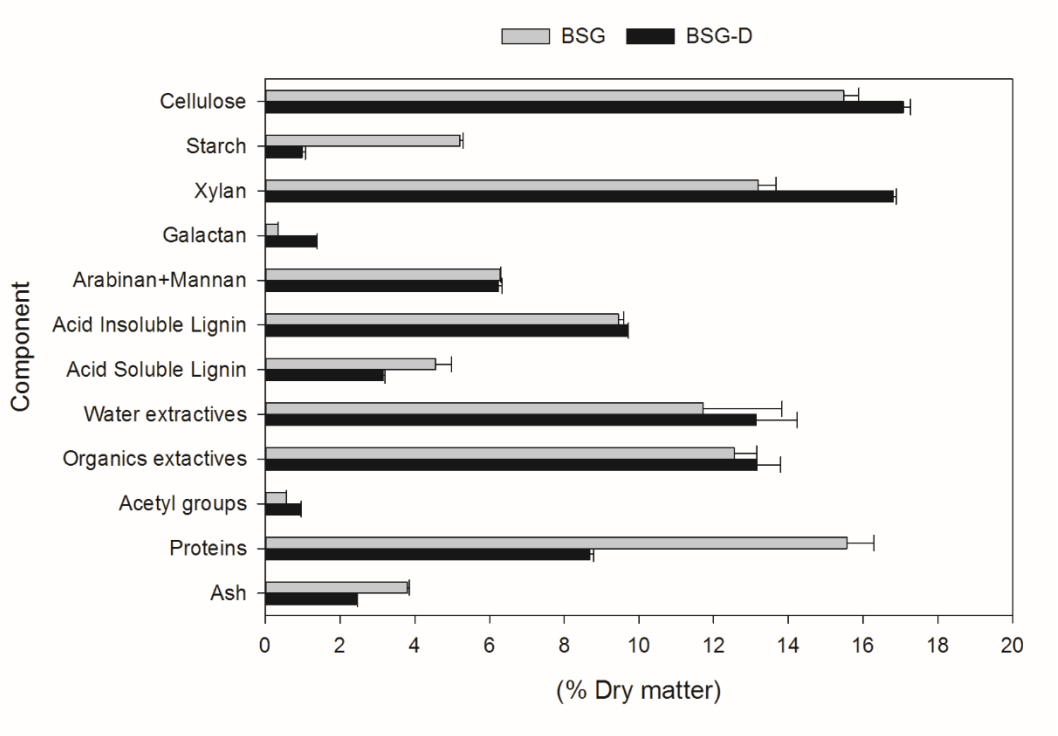

Fig. 1 BSG and BSG-D compositions. Data represent the averages of triplicate tests, and error bars correspond to the standard deviations (SD).

The results showed that the protein content in the biomass decreased by nearly 45 %. The starch fraction in the BSG used in this work (5.2 %) was higher than that reported in other works. Xiros et al. [20] reported a value of 2.7 %, Steiner et al. [8] a range from 0.6 % to 4.0 %, and 3.1 % [9]. However, a range from 2 % to 13 % was also reported [21, 22]. Starch fraction hydrolysis was conducted simultaneously with deproteinization, considering

the high value determined for the BSG used in this research. The starch fraction was reduced to 0.98 % in BSG-D by the action of α-amylase (Termamyil®) and glucoamylase (Spirizyme®) enzymes, causing a reduction of 81.2 % compared to BSG. In a further work protein and starch removal should be optimized to increase the protein and starch remotion of the BSG prior to SE pretreatment.

After the protein and starch removal, the glucan content in the BSG-D decreased to 18.1 %, slightly increasing the cellulose content to 17.1 % and greatly decreasing the starch content to 1 %. The hemicellulose content increased slightly to 24.4 %, but the arabinan+mannan content remained stable in both biomasses. The acid soluble lignin (ASL) was solubilized slightly by the deproteinization step. In contrast, the percentage of AIL remained almost constant, as did percentages of the extractives (both organic and aqueous), and their fractions did not show significant differences. The value of AIL (9.45 %) was corrected to account for ash and protein content (based on total nitrogen) to obtain an accurate estimation, producing a percentage (10 %) analogous to that reported by [9]. They also adjusted this value due to the high protein content of BSG. Total ash in BSG was 3.8 %, with approximately 66 % being an inorganic material that could be removed by washing or aqueous extraction of the biomass. That extraction was evidenced by the reduction of the ash content in the BSG-D. Finally, the acetyl group percentage was 0.6 % a relatively low value compared to other residual biomasses, such as olive stone (5.9 %) [23], olive tree pruning biomass (2.3 %) [24], barley straw (1.8 %) [25] and cardoon (3.8 %) [26]. Despite the augmentation of acetyl groups in the BSG-D (0.93 %), this low value

indicated a need to impregnate the biomass with acid prior to SE to achieve better hemicellulose solubilization, as will be discussed later.

3.2 Effect of enzymatic deproteinization of BSG before SE pretreatment

A deproteinization stage for BSG was considered desirable to increase the xylose yield, partially eliminating the negative wall effect that this fraction had on the mass and heat transfer of the SE. Additionally, deproteinization of previous SE would permit valorization of one of the most representative components of this biomass—the protein—affording the advantages associated with obtaining bio-based products from this fraction in a biorefinery and circular economic context. Additionally, the use of enzymes to deproteinize the BSG had advantages for obtaining compounds derived from the protein in the liquid, such as protein hydrolysates, oligopeptides, peptides, and amino acids, which were not expected to suffer further degradation or loss of their functional properties, which have more commercial value than protein itself. Characterization of protein hydrolysates has been well documented by [27] and their thecnofunctional properties has already done by [28, 29, 30, 31, 32, 33].

The protein extraction yield for the tests carried out after 4 h in the 40 L reactor was 63.9 %. It should be noted that this occurred without any prior grinding stage that would decrease the particle size of the BSG and with wet BSG as starting material considering the prohibitive additional costs associated with drying BSG on an industrial scale [34] and biorefinery approach. The results obtained in this stage of the process were lower than those reported by [28, 35, 36]. However, was comparable with Laine et al. [37], who recovered 60 % of BSG protein when the concentration of the NaOH was increased to 1 N. Celus et al. [28], reported protein contents to 68-74 % using Alcalase as an extraction aid.

Treimo et al. [36] evaluated the enzymatic solubilization of BSG proteins using various commercial peptidase mixtures and found that Alcalase® was the most effective peptidase to solubilize BSG proteins, with an ability to extract up to 77 % of total protein. Niemi et al. [35] found that the pre-hydrolysis of BSG with carbohydrase facilitated the protein solubilization to 76 % using an Alcalase®, too. However, they conducted their experiments on a laboratory scale (50 mL) and with milled BSG. Untreated BSG, steam-exploded BSG, and steam-exploded and carbohydrase-treated BSG were exposed to an alkaline protease at pH 10 [13]. They concluded that for all raw materials, considerably more protein could be solubilized by the protease treatment than without it. SE reduced the enzymatic protein solubilization but enhanced the extract recovery in the centrifugation step, presumably due to the lower water-binding capacity of steam-exploded BSG compared to untreated BSG. In contrast to the enzymatic protein solubilization, which decreased after SE, the non-enzymatic protein solubilization increased from 15 % for untreated BSG to almost 40 % for steam exploded BSG. This was probably due to a decrease in the protein molecular size caused by the degradation of proteins during SE, as detected by SDS-Page analysis [13]. Rommi et al. [13] also concluded that, in terms of preventing lignin co-extraction, untreated BSG may be the preferred raw material for enzymatic recovery of protein hydrolysates. An optimization process to evaluate the yield in pilot scale is desirable, to increase the protein recovery prior the pretreatment and to evaluate the application industrial.

3.3 Impact of sugar extraction from BSG-D after acid-catalyzed SE pretreatment

3.3.1 Sugar recovery yield

SE was studied mainly to improve the extraction of xylan in the liquid fraction after the pretreatment, with the objective of valorizing this fermentable sugar, especially in the

hydrolysis and fermentation stages. The study confirmed the impact of prior deproteinization of BSG on reducing the heat and mass transfer barrier of the pretreatment and thereby increasing the sugar recovery in the liquid. Fig. 2 shows the results of SE pretreatment for the xylose, arabinose, and glucose recovery yields from BSG-D. Each yield was divided into three parts: (1) potential sugar solubilized in the liquid; (2) remaining sugars in the WIS; and (3) losses due to degradation of the components to other substances, loss of fine material during the washing of the solids, or losses that occurred through vessel transfer.

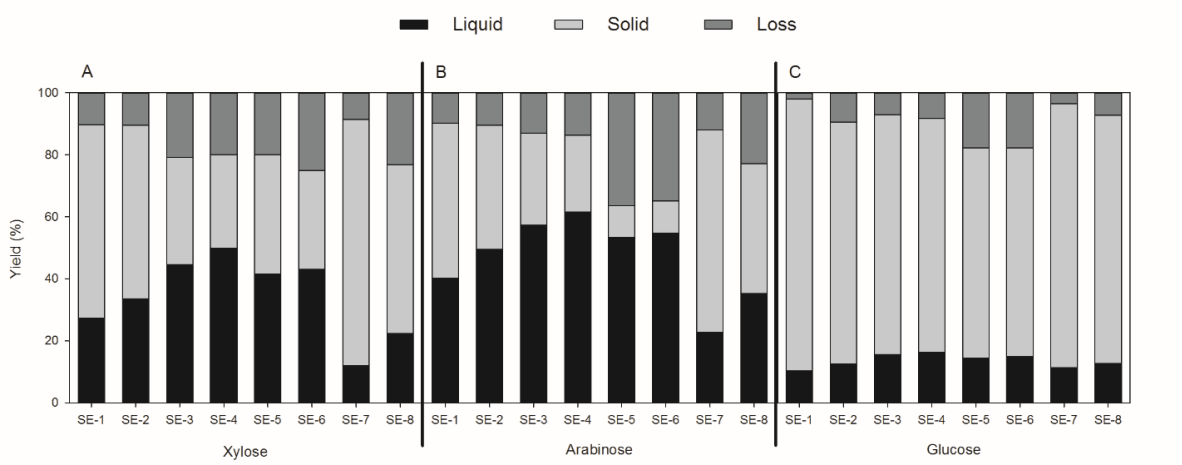

Fig. 2 Yields of xylose, arabinose, and glucose in liquid and solid fractions (insoluble); percentages of losses after the acid-catalyzed pretreatment (SE-1 to SE-6); and controls without acid (SE-7 and SE-8) using BSG-D

Experiments with acid on BSG-D (SE-1 to SE-6) confirmed the positive effect of an acid catalyst in the solubilization of hemicellulose sugars by the SE pretreatment. A noticeable augmentation of xylose and arabinose yields was observed compared to yields without acid (SE-7 and SE-8). The highest xylose (49.8 %) and arabinose (61.5 %) yields were obtained under the SE-4 conditions (170 °C and 30 min). There was no evident

increase of extracted glucose in the liquid after pretreatment with or without acid; only a maximum recovery of 16.2 % was achieved in SE-4. Compared to [9, 12], the prior enzymatic deproteinization step in this research produced increased xylose and arabinose recovery yields in the liquid fraction after SE pretreatment. Xylan and arabinan yields <20 % were reported by [9] at 200 °C for 10 min with or without acid, and a xylose yield <25 % and an arabinose yield <40 % were obtained by [12] at 180 °C for 10 min with 25 mg of SO_2 as the catalyst acid.

Additionally, the sugar recovery yields for xylose, arabinose, and glucose in the liquid fraction showed that temperature had a positive effect when it increased from 160 ºC to 170 ºC, but not from 170 ºC to 180 ºC. Since the temperature increased both the kinetics and the mass and heat transfer rates, the behavior previously described meant that the effect on the transfer phenomena prevailed for the first increment. The loss of arabinose at 180 ºC was approximately 3.5 times that observed at 160 ºC after 15 min or 30 min of contact; the increase confirmed the explanation for losses for the three sugars (Fig. 2).

Regarding the contact time, at the three temperatures assessed, a positive effect was noticed, but it was smaller at the highest temperature; for example, the arabinose yield was augmented from 40.2 % to 49.9 % when contact time increase of 15 to 30 min at 160 ºC but was almost the same 53.5 % (15 min) and 54.8 % (30 min) at 180 ºC. At the highest temperature assessed (180 ºC), the kinetics of the sugar decomposition reaction predominated over the time effect on extraction (mass transfer). In comparison, Swart et al. [12] reported a higher oligosaccharide fraction (75.1 % of XOS yield and 37.0 % of arabinan yield) in total dissolve solids from SE with short residence times (15 min), lower

temperatures (180°C) and high dry matter content (25%) screw over BSG without acid. However, they do not probe temperatures below to 180 °C.

The liquid fraction contained both monomeric and oligomeric solubilized sugars, as found by other authors and in other biomasses [38, 12, 39]. The solubilization percentages of xylose, arabinose, and glucose as monomeric sugars in the liquid are presented in Fig. 3.

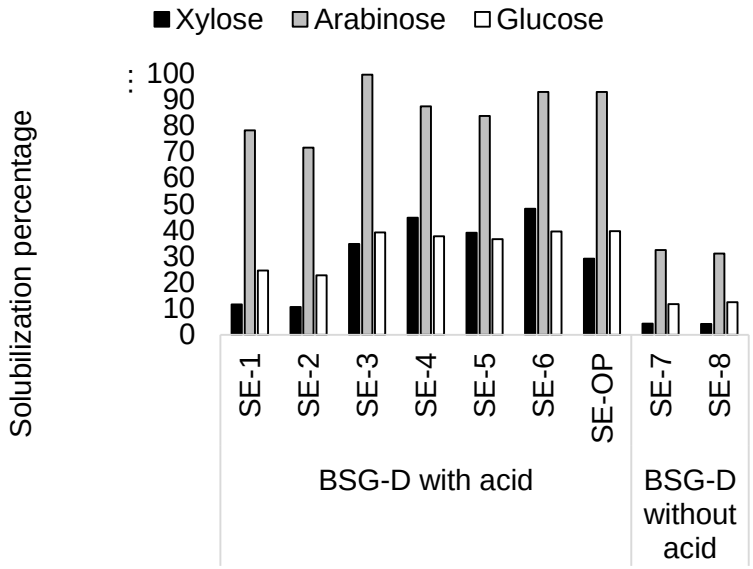

Fig. 3 Percentages of solubilized simple sugars in the liquid fraction after acid-catalyzed SE pretreatment (SE-1 to SE-6), under the optimized condition (SE-OP), and controls without acid (SE-7 and SE-8) using BSG-D

The acid-catalyzed SE achieved the breakdown of free sugars mainly from hemicellulose polymer and to a lesser extent for cellulose. All three sugars were solubilized in higher percentages at 170 °C and 180 °C compared to 160 °C. The arabinose released in the liquid was >70 % for the three temperatures evaluated and reached an even greater percentage compared with other sugars in the assays without acid. For this study, the losses or degradation of arabinose reached a maximum of 35.6 % at 180 °C. The losses of

arabinan were also around 30 % at 180 °C and this percentage increased gradually with the treatment time and temperature [9, 13]. The maximum amount of monomeric arabinose (99.5 %) was obtained under the SE-3 conditions (170 °C and 15 min). Silveira et al. [38] showed that arabinosyl units are the most labile saccharide branches in the hemicelluloses of sugarcane bagasse, which in turn are quickly released from the biomass even under mild SE conditions. More than 97 % of anhydrous arabinose units were hydrolyzed by both auto- and sulfuric acid-catalyzed bagasse pretreatment. The recovered xylose yield in the liquid achieved a maximum solubilization of 48.2 % as a monomeric sugar in the SE-6 test, which meant that 30–50 % of the hemicellulose fraction was solubilized in XOS form in the other tests. The highest XOS yields (>73.1 %) were obtained for both BSGs evaluated [12], under the selected process conditions: 180 °C and 10 min with 25 % initial dry matter content in. However, they founded a xylose yield <10 % at these process conditions. The glucose solubilization reached 30–40 % at 170 °C or 180 °C. An increase in the solubilization percentage of the three sugars was evident with the acid-catalyzed SE pretreatment. Statistically, the time factor ($p < 0.05$) was not significant in this solubilization percentage.

In a biorefinery context, the presence of arabinose and xylose as free sugars in the liquid after acid-catalyzed SE pretreatment has several advantages. Firstly, these sugars can be used to directly obtain value-added bioproducts in other stages of the process. Secondly, if the oligomeric sugars require EH after pretreatment, this stage reduces the amounts of endo-1,4-β-D-xylanases, β-xylosidases, and auxiliary enzymes such as α-L-arabinofuranosidases, feruloyl-xylan esterase, among others. These enzymes are necessary

to break the glycosidic bonds of the complex arabinoxylan structure, constituting both a technical advantage and an economic one in the overall process.

3.3.2 Optimization of the acid-catalyzed SE pretreatment

A second-order mathematical model was adjusted to the experimental design presented in Table 1 using the SE-1 to SE-6 tests. Equation 4 represents the xylose yield (*Y*), and Equation 5 represents the concentration in the liquid fraction (*C*). These equations include the temperature (*T*, ºC) and time (*t*, minutes) effects:

$$Y = -3250.87 + 37.765T + 3.06333t - 0.10825T^2 - 0.016333T \times t \quad (4)$$

$$C = -736.547 + 8.404T + 2.54078t - 0.023375T^2 - 0.0158333T \times t \quad (5)$$

The statistically optimal value for both response variables found using these equations was obtained for the process at 173.5 °C for 15.5 min. The estimated recovery yield of xylose in the liquid fraction was 46.4 %, with a concentration of 14.7 g/L. An acid-catalyzed SE (SE-OP) was performed experimentally in quintuplicate under these conditions to validate the prediction. The yield and concentration obtained were 47.0 ± 1 % and 14.8 ± 1.26 g/L, respectively, which verified the optimization results, demonstrating the repeatability of the experimental procedure, which was chosen as the optimized process to operate the acid-catalyzed SE pretreatment in the designed biorefinery.

3.3.3 Chemical composition of BSG-D after acid-catalyzed SE pretreatment

Table 2 summarizes the compositions of WIS recovered from BSG-D after the SE tests under the conditions presented in section 2.3. The results showed that glucan content values were almost constant, with a slight increase after the pretreatment. This fraction represented

60–85 % of the glucan remaining in the solid fraction (Fig. 2). Due to the high recalcitrance and inherent characteristics of cellulose (i.e. crystallinity and limited accessibility), cellulose is significantly less degraded during pretreatment than hemicelluloses; thus, lower amounts of glucose and cello-oligosaccharides were found in C5-liquors (Fig. 3), as reported by [38], for sugarcane bagasse using acid-catalyzed SE (0.5 g of acid [w/w] per 100 g of dry-matter bagasse). Similarly, Pal et al. [40] observed that >95 % of glucan was retained in a solid fraction of autocatalyzed (180 °C, 10.5 bar, 20 min pretreatment time) pretreated sugarcane bagasse. The increase of the AIL content in the WIS varied from 29 % (SE-7 and SE-8) to 41 % (SE-4 and SE-6), showing a higher value for the test done with acid than the test without acid, and the percentage was higher for 170 °C and 180 °C than for 160 °C. These values can be explained by the presence of acid-insoluble non-lignin compounds, including waxes, protein, and ash [41]. In addition to these compounds, some soluble products that formed during the SE became incorporated into the Klason lignin fraction, forming an insoluble acid component, so-called pseudolignin [13]. In this study, no correction for AIL in the WIS was made, but Rommi et al. [13] study found that up to 29 % of the Klason lignin corresponded to protein and ash for BSG and increased to 40 % for BSG pretreated by SE. This finding was also reported by [9], although these authors did not conduct a deproteinization step prior to SE pretreatment.

In general, the hemicellulose fraction of the lignocellulose complex is mainly solubilized, while cellulose and lignin remain in the fiber fraction during acid-catalyzed SE pretreatment [42]. SE pretreatment preferentially altered the hemicellulose component. In

Table 2. Chemical composition of the WIS after acid-catalyzed SE (SE-1 to SE-6 and SE-OP) and in controls without acid (SE-7 and SE-8) using BSG-D. Data represent averages of the triplicate values and standard deviations.

Component (% dry matter)	BSG-D with acid							BSG-D without acid	
	SE-1	SE-2	SE-3	SE-4	SE-5	SE-6	SE-OP	SE-7	SE-8
Glucan	23.6 ± 1.5	25.6 ± 0.7	26.5 ± 0.4	27.1 ± 0.8	24.4 ± 0.6	25.1 ± 0.4	26.4 ± 1.2	23.2 ± 0.8	25.5 ± 1.1
Hemicellulose	16.9 ± 1,9	18.9 ± 0.1	13.5 ± 0.2	12.3 ± 0.3	13.3 ± 0.2	11.7 ± 0.2	13.5 ± 0.3	24.7 ± 0.5	23.4 ± 0.6
Xylan	12.0 ± 1.8	14.6 ± 0.1	9.4 ± 0.2	8.6 ± 0.3	11.0 ± 0.2	9.4 ± 0.2	12.5 ± 0.8	18.9 ± 0.3	18.3 ± 0.4
Galactan	-	-	0.7 ± 0.08	0.6 ± 0.04	0.8 ± 0.05	0.6 ± 0.02	-	-	-
Arabinan+Mannan	4.9 ± 0.1	4.3 ± 0.06	3.4 ± 0.09	3.1 ± 0.07	1.2 ± 0.08	1.3 ± 0.06	1.5 ± 0.6	5.7 ± 0.2	5.2 ± 0.2
Acid insoluble lignin	32.3 ± 0.5	32.0 ± 0.2	38.4 ± 0.2	41.1 ± 0.4	38.3 ± 2.9	40.6 ± 0.2	37.0 ± 1.4	29.3 ± 0.3	29.1 ± 0.1
Acetyl groups	1.01 ± 0.02	1.01 ± 0.03	0.6 ± 0.01	0.5 ± 0.01	0.6 ± 0.04	0.6 ± 0.03	0.5 ± 0.1	1.0 ± 0.03	1.0 ± 0.01
Proteins	11.1 ± 0.5	9.2 ± 0.3	12.7 ± 0.3	12.8 ± 0.3	12.9 ± 0.3	12.6 ± 0.1	12.7 ± 0.3	9.4 ± 0.3	8.1 ± 0.9
Ash	3.1 ± 0.06	2.7 ± 0.12	3.2 ± 0.06	3.2 ± 0.07	2.9 ± 0.02	3.1 ± 0.04	2.4 ± 0.1	2.9 ± 0.03	2.8 ± 0.01
SUM	88	89.3	94.8	97	92.4	93.6	92.5	90.6	89.9

"-" = not found

the SE-7 and SE-8 tests (without acid impregnation), the hemicellulose fraction was higher than in the tests that were catalyzed by acid (SE-1 to SE-6), confirming the effect of acid on the release of xylan in the liquid fraction and that the cellulose content was characteristic of SE pretreatments using acid.

3.3.4 EH of BSG-D after acid-catalyzed SE pretreatment

To evaluate the effectiveness of SE pretreatment for improving glucose and xylose release by EH and determine the most appropriate pretreatment conditions for the biorefinery approach, EH experiments were conducted under the standard conditions described in Section 2.3.2. Fig. 4 shows the results for the glucose and xylose EHY, calculated using Equation 3. The EHY of glucose varied from 59.7 to 77.8 % in the experiments with acid impregnation, and in the test without acid, a lower value of 18.7 % was obtained. In the case of xylan, for the tests with BSG-D previously impregnated with acid, 35–45 % of xylan EHY was found, and 27.6 % was found for the (SE-6 and SE-7) tests without acid.

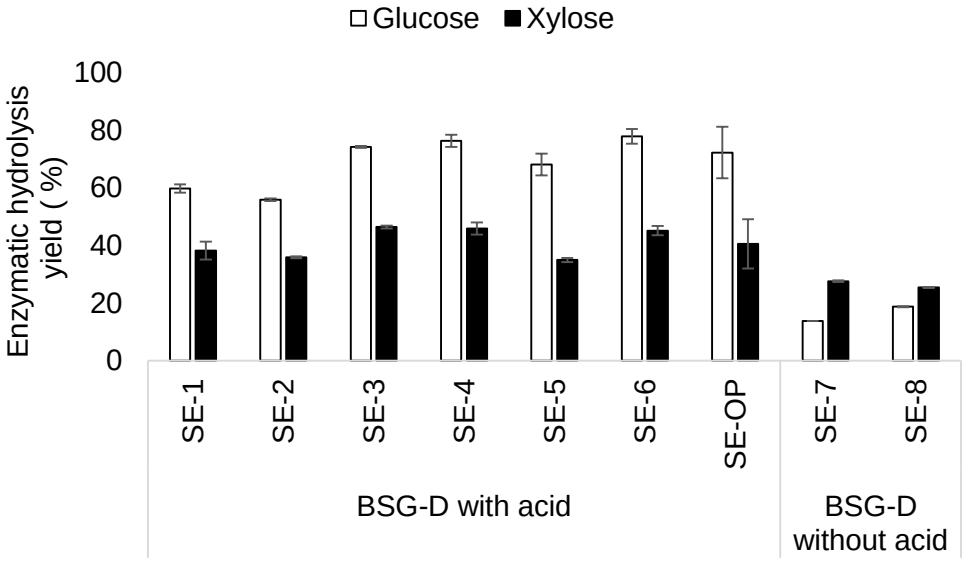

Fig. 4 Yields of EH of WIS using Cellic® Cetec2

Kemppainen et al. [9] reported yields based on their analysis of reducing sugars within a 24 h hydrolysis period. Glucan was hydrolyzed most efficiently (up to 72 % hydrolysis yield), but the xylan hydrolysis yield was lower (up to 35 %), using 3 % solids loading without a deproteinization step. Our experiments were carried out at 5 % w/v and good performance was observed for BSG-D with higher solid loads. Padilla-Rascón et al. [23] reported that almost 90 % of the sugars had already been released after 24 h, following acid pretreatment under optimum conditions (128 °C, 10.5 % acid and 33 % solids) combined with SE at 195 °C for 5 min using olive stone. In general, it was found that a previous deproteinization stage increased the accessibility of enzymes on cellulose and the residual XOS in the biomass. However, to achieve higher yields through xylan hydrolysis, it is necessary to supplement the commercial enzyme cocktails with pure accessories, such as α-L-arabinofuranosidases and esterases [43]. Another strategy to increase this yield could be a milling step. Padilla-Rascón et al. [23] increased the glucose EHY from 32–39 % up to 70–78 % using olive stone after milling the WIS obtained in the SE. In this work, the BSG was not ground at any stage. Although the values shown in Fig. 4 reflect the maximum potential of release sugars after 72 h incubation, it is crucial to analyze the sugar percentage released at 24 h to reduce and optimize the time taken for this stage concerning the scaling of the overall process: all this into account the approach to a biorefinery.

3.3.5 Mass balance and overall yield of the fractionation process for BSG—A biorefinery approach

The potential of BSG as renewable feedstock to produce various chemical products

makes it a suitable material for analyzing biorefinery strategies. The mass balance of the different BSG components produced during overall processing under the best conditions of the designed biorefinery is presented in Fig. 5. According to these results, the overall sugar yield (OSY) was 72.7 % for xylan and 70.3 % for glucan. OSY refers to the amount of sugar released in both pretreatment and EH in relation to the amount of sugar in raw BSG, expressed in percentage. It is calculated summing up the production of sugars in EH step (stream 6) and the recovery of sugars in acid-catalyzed SE (stream 4) after deproteinization step.

The prior deproteinization of BSG increased the OSY of xylan by 21 % compared to the solubilization reported in the study of [9] without deproteinization at 200 °C and 10 min of SE pretreatment. This result confirmed the decreased barrier effect in the release of sugars due to the protein and starch content present in the biomass and the impact of using an acid-catalyst. Nevertheless, it was possible to obtain recovery percentages >70 % of xylose with SE, as reported by [23] using olive stone (195 °C, 5 min) and [44] using olive tree pruning biomass (187 °C, 30 min); however, these biomasses had no protein and starch in their compositions. The OSY for glucan (70.3 %) was like the value (72.9 %) reported by [9], who also used a BSG and SE pretreatment (without deproteinization step). They describe that glucan's solubilization was only slightly higher than that of xylan and arabinan. Still, it must be noted that glucan recovery in the residual solids was significantly higher than that of xylan and arabinan due to their degradation during the pretreatment (200 °C and 10 min). However, after the process designed in this study, the increase of xylan recovery proves the influence that had the protein and starch remotion of BSG structural matrix, prior SE pretreatment, and least generation of degraded compounds, thanks to milder

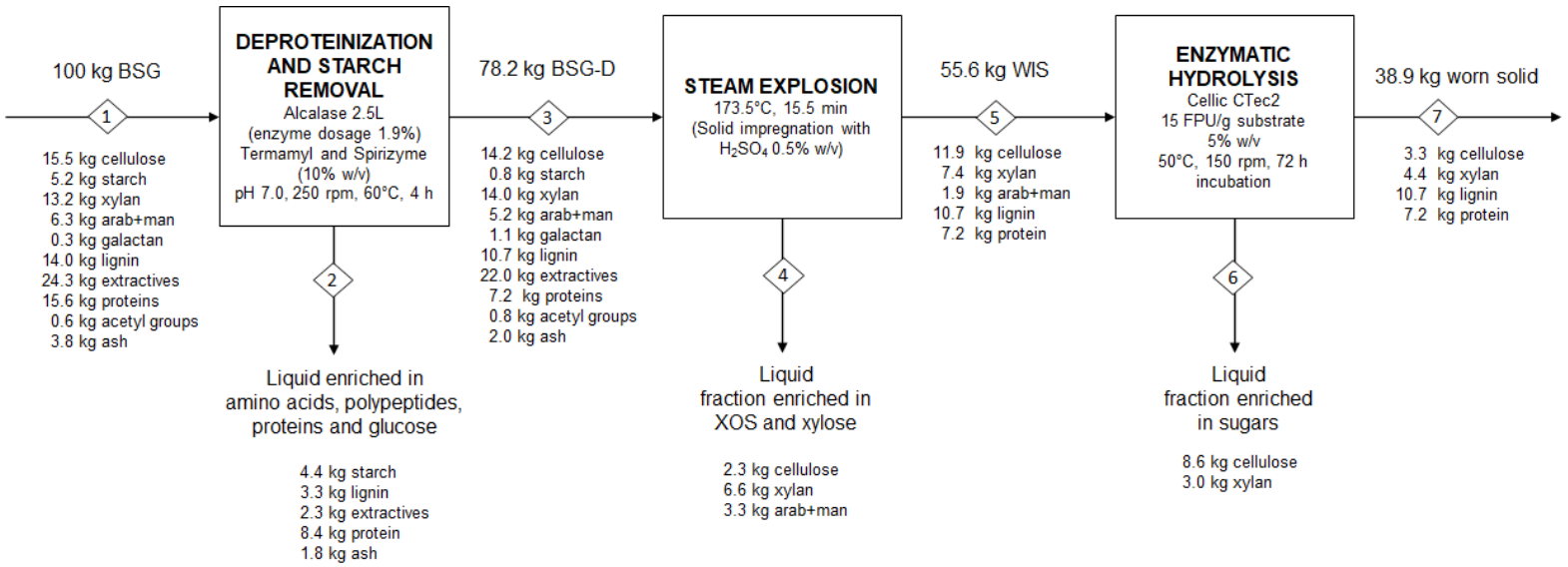

Fig. 5. Mass balance of 100 kg of BSG, submitted to deproteinization by enzymes, acid-catalyzed SE, and EH

process conditions. Finally, it is worth highlighting the optimization of the pretreatment conditions that were carried out in this investigation, which resulted in a decrease in SE pretreatment temperature (173.5°C), that it is essential to reduce the energy consumption in a scalable process into a biorefinery context.

Overall, the mass balance and the different streams with added value obtained in this study can will be estimate the economic feasibility of this subproduct of the beer industry. Further studies should consider the possibility of converting the oligosaccharides or monomeric sugars contained in the different liquid streams generated during the process designed into valuable compounds, such as bioethanol or xylitol. The revalorization of the compounds recovered as the protein hydrolysates and lignin remaining in solids after the process must be strengthened to improve the potential of BSG for use within a biorefinery context. Finally, it is worth mentioning that the mass balance of stream 5 does not include the correction of protein in the lignin fraction and the WIS were not characterized in their extractives content.

4. CONCLUSIONS

The research proved that simultaneous deproteinization and starch removal using enzymes, and acid-catalyzed SE followed by EH of BSG increased sugar production in a fractionation process that was also effective for producing different value-added products in a biorefinery context. The acid-catalyzed SE using BSG-D under selected conditions had a positive effect on the release of hemicellulose compounds, especially xylose and arabinose, as simple sugars into the liquid fraction. This process also had the benefit of using the whole wet BSG and requiring no previous biomass adaptation process (drying or milling).

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