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Effect of extrusion conditions and hydrolysis with fiber-degrading enzymes on the production of C5 and C6 sugars from brewers' spent grain for bioethanol production
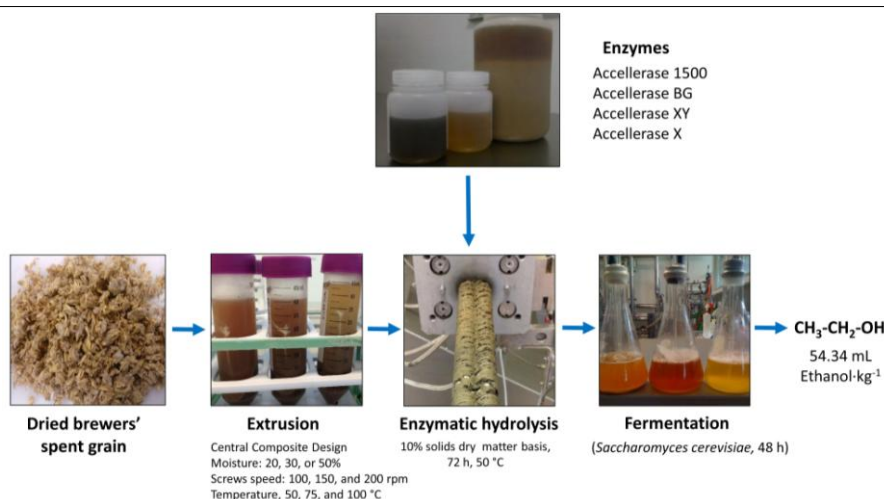
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HIGHLIGHTS

➢ Thermoplastic extrusion improved the enzymatic hydrolysis of brewers' spent grain.

➢ The extruder barrel temperature and screw speed affected the sugars yield.

➢ No enzymatic and yeast inhibitors were detected in all enzymatic hydrolyzates.

➢ Despite the high protein content after hydrolysis, low levels of FAN were achieved.

➢ *S. cerevisiae* fermented glucose into ethanol with a maximum yield after 48 h.

GRAPHICAL ABSTRACT

Enzymes
Accellerase 1500
Accellerase BG
Accellerase XY
Accellerase X

Dried brewers' spent grain → **Extrusion** (Central Composite Design Moisture: 20, 30, or 50% Screws speed: 100, 150, and 200 rpm Temperature, 50, 75, and 100 °C) → **Enzymatic hydrolysis** (10% solids dry matter basis, 72 h, 50 °C) → **Fermentation** (*Saccharomyces cerevisiae*, 48 h) → CH_3-CH_2-OH 54.34 mL Ethanol·kg^{-1}

ARTICLE INFO

ABSTRACT

The bioconversion of brewers' spent grain into bioethanol was investigated in the present study using thermoplastic extrusion and the use of fiber degrading enzymes. The extrusion conditions i.e. tempering moisture, screws speed, and temperature of last zone of the barrel were taken into account in order to optimize the yield of C5 and C6 sugars during the subsequent enzymatic hydrolysis step of the fibers. The most important variable that affected the sugar yield was the extrusion temperature, followed by the screws speed. The best extrusion conditions were 20% tempering moisture, 200 rpm and 50 °C. No enzymatic and yeast inhibitors were detected in any of the enzymatically-treated fiber hydrolyzates. The fermentation resulted in 5.43 mL bioethanol per 100g of extruded brewers' spent grain (dry weight basis). The only sugar consumed was glucose. The free amino nitrogen amount quantified in the hydrolyzates was as low as >20 mg L^{-1}, negatively affecting sugars consumption during the fermentation and consequently the ethanol yield.

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1. Introduction

Brewers' spent grain (BSG) is the most abundant byproduct generated in the brewing industry comprising approximately 85% of the total waste materials (Aliyu and Bala, 2011). BSG is separated by lautering after mashing or starch extraction. This byproduct is composed primarily of protein and lignocellulose. The main use of BSG is for animal feed, particularly for ruminants. Moreover, since BSG is rich in protein and fibers, it has been used successfully as an ingredient for production of high-fiber snacks and breads and as a feedstock for protein hydrolysis and fractionation. It has also been used for the extraction of prebiotics rich in xylo-oligosaccharides (Forssell et al., 2008).

Shindo and Tachibana (2006) and White et al. (2008) investigated the conversion of BSG into bioethanol with steam explosion and acid hydrolysis, respectively. Both investigations were able to convert the pretreated feedstock into C5 and C6 sugars after hydrolysis with fiber-degrading enzymes. They proved the possibility of using the BSG as a potential feedstock for the production of second-generation bioethanol. However, most of the pretreatment technologies reported have also resulted in the production of enzyme and yeast inhibitors due to the harsh process conditions applied (Karunanithy and Muthukumarappan, 2010). Extrusion has been reported to provide a continuous thermophysical pretreatment for lignocellulose bioethanol production without leading to hazardous materials generation (Lin et al., 2012). In addition, there is no published report regarding the use of extruded BSG for bioethanol production. The objective of this research was to study the extrusion conditions (i.e. temperature of the last zone of the barrel, screws speed, and feedstock moisture content) for preparation of BSG for its subsequent enzymatic hydrolysis with fiber-degrading enzymes for second-generation bioethanol production.

2. Material and method

2.1. Materials

BSG was procured from Grupo Cuauhtémoc Moctezuma (Monterrey, México). The wet BSG was transported to the Tecnológico de Monterrey campus Monterrey and dried at 50-60 °C for 24 h.

2.2. Physical-Chemical characterization

Moisture and protein contents were determined using the AACC standard assays 44-15 and 46-13, respectively. For the structural carbohydrates assay, the non-structural material was removed from the BSG to prevent any interferences. More specifically, a two-step extraction process was used to remove water and ethanol solubles according to the methods recommended by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008a). Then, the insoluble fibers were hydrolyzed and filtered for structural sugars quantification with an high-performance liquid chromatography (HPLC) as described by Sluiter et al. (2008b).

2.3. Extrusion pretreatment

A twin-screw co-rotating extruder (BTSM-30, Bühler AG, Uzwil, Switzerland) with a barrel composed of 5 zones, and two independent feeders for the solid raw material and water was used. The temperature of the fifth zone of the barrel was controlled by a heat exchanger device (Tool Temp, Bühler AG, Uzwil, Switzerland). The total length and outer diameter of the screws were 800 mm and 30 mm, respectively, and the L/D ratio was 20. A die with a single 4 mm hole was used. The screws configuration consisted of three different sections: inlet/conveying elements section (for the introduction and transport of the dry feedstock and water), mixing elements section, and the final work elements section consisting of kneading and reverse elements. The solid feed rate was set constantly at 7.3 kg h^{-1} for all the conditions tested. The BSG extrusions were conducted as described in the section 2.3.1. The extruded BSG was dried at 50-60 °C for 6 h and stored in plastic bags.

2.3.1. Experimental design and extrusion conditions

A central composite design was used in the present study. The factors evaluated included the moisture inside the barrel, screws speed and the temperature in the last zone of the barrel. Each factor had a low or high level and a center point with intermediate conditions (Table 1). The center point was repeated four times and all treatments were performed in triplicate. As mentioned arlier, whole dried BSG was fed at a constant rate of 7.3 kg h^{-1} for all the conditions investigated.

Table 1.
Central composite design used for the extrusion conditions using BSG.

Level	Extrusion Variables		
	Moisture [%]	Screws speed [rpm]	Temperature [°C]
Low	20	100	50
High	40	200	100
Center point	30	150	75

2.4. Enzymatic hydrolysis

After the extrusion, the pretreated dried BSG samples were enzymatically hydrolyzed. Untreated BSG was also enzymatically hydrolyzed as control. The hydrolysis assays were conducted in triplicate by using a total volume of 100 mL in 500 mL flasks in the presence of 10% solid BSG (dry matter basis). Citrate buffer (50 mM) was adjusted to pH 5 with 10 mM sodium azide and was used for all the hydrolyses. Enzymatic hydrolysis was carried out in an orbital shaking incubator (VWR Model 1575) set at 50 °C and 150 rpm for 72 h. Du Pont food grade enzymes i.e. Accellerase® 1500, Accellerase® BG, Accellerase® XC, and Accellerase® XY dosed at 0.25, 0.9, 0.05, and 0.125 mL g^{-1} loaded solids were used, respectively. These enzymes consist in a fibrolytic mixture of exoglucanase, endoglucanase, beta-glucosidase, hemicellulase, and xylanase. The declared activity of each enzyme was as follows: for Accellerase® 1500: 2,200-2,800 carboxymethylcellulose activity units (CMC U)/g and 450-775 para-nitrophenyl-B-D-glucopyranoside units (pNPG U)/g, for Accellerase® BG: 3,000 para-nitrophenyl-B-D-glucopyranoside units (pNPG U)/g, for Accellerase® XC: 1,000-1,400 carboxymethylcellulose activity units (CMC U)/g and 2,500-3,800 acid birchwod xylanase units (ABXU)/g, and for Accellerase® XY: 20,000-30,000 acid birchwod xylanase units (ABXU)/g. The dosage used was the maximum enzyme concentration as recommended by the manufacturer.

2.5. Fermentation

Saccharomyces cerevisiae ATCC® 20252™ capable of fermenting both C5 and C6 sugars was used. The extruded BSG at 20% tempering moisture, 200 rpm and 50°C was enzymatically hydrolyzed and fermented with the *S. cerevisiae* strain without nutrients supplementation. These hydrolyzates contained the highest sugars concentrations. The fermentations were conducted as recommended by Heredia-Olea et al. (2013). An aliquot of 15 mL at 0, 24 and 48 h was taken and stored for HPLC analysis (sugars and ethanol).

2.6. HPLC-based quantification of sugars and inhibitors

The enzymatically-hydrolyzed samples were treated as described in our previous research (Heredia-Olea et al., 2012). Analytes were separated by a Shodex SH1011 column (300 × 7.8 mm) with a flow rate of 0.6 mL min^{-1} of HPLC-grade water containing 5 mM H$_2$SO$_4$ for the quantification of inhibitors and ethanol. The sugar quantification was performed with a Shodex SP0810 column and a cation/anion deasher (Biorad). The column temperature was set at 60 and 85 °C for inhibitors and sugars, respectively, whereas the detector (refractive index detector Waters 2414) and the autosampler at 50 and 4°C, respectively. Standards of ethanol, cellobiose, D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose, acetic acid, 5-hidroxymethylfurfural, and furfural (Sigma Chemical Co. St. Louis, MO) were used. The run times for sugars an inhibitors quantifications were 20 and 45 min, respectively.

2.6.1. Free amino nitrogen determination

A 100 mL aliquot was taken from each filtered hydrolyzate. Free amino nitrogen was quantified with the ninhydrin reaction assay (Lie, 1973).

2.7. Statistical analysis

The analysis of data generated by the central composite design and the response surfaces were performed with the software Statgraphics Centurion XVI with a statistical significance of a=0.05.
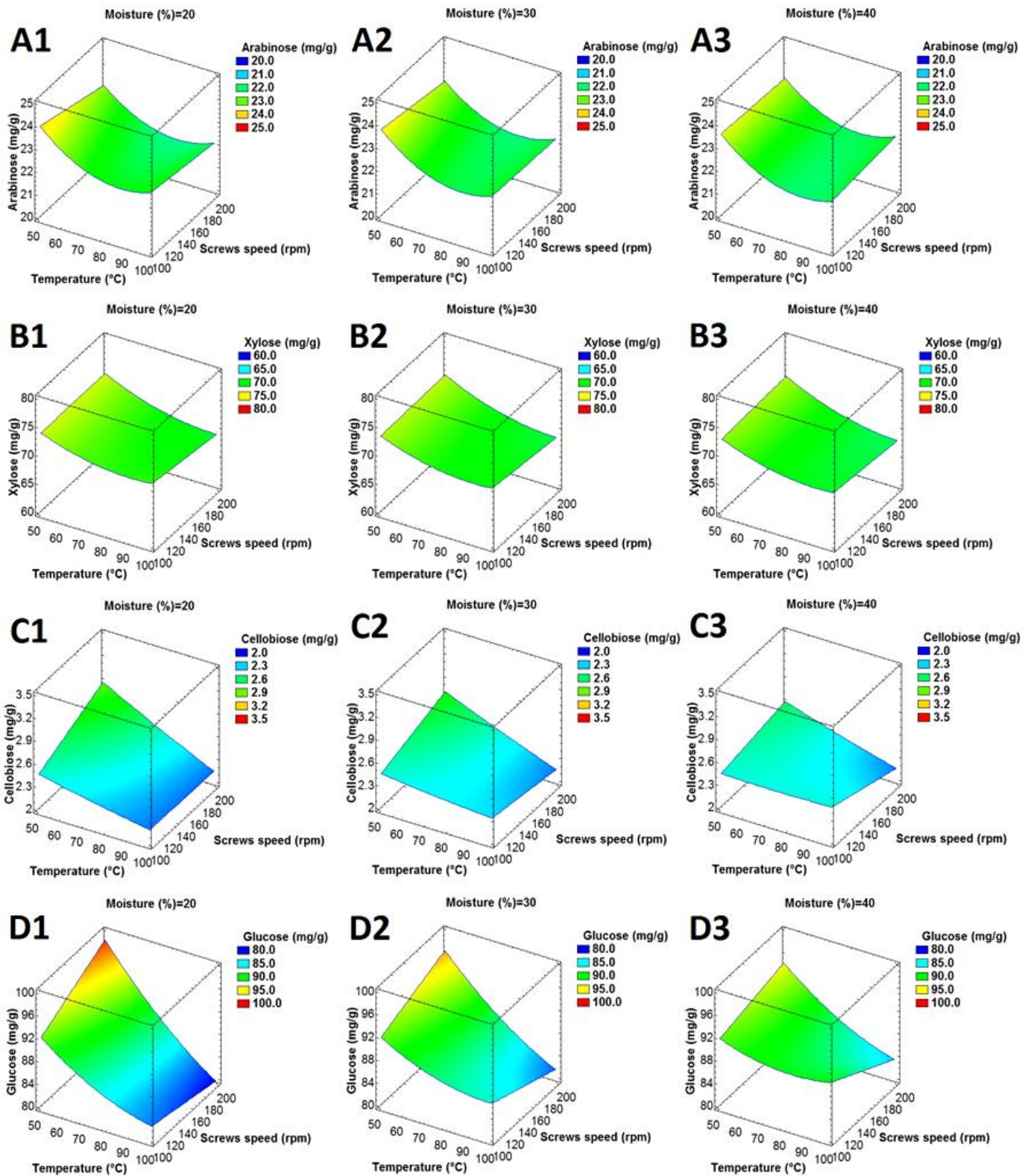

Fig.1. Response surfaces for arabinose (A1, A2, and A3), xylose (B1, B2, and B3), cellobiose (C1, C2, and C3), and glucose (D1, D2, and D3) generated from brewers' spent grain extruded at different temperatures (50, 75, and 100 °C), screws speeds (100, 150, and 200 rpm) and tempering moistures (20, 30, or 40 %) ad was further hydrolyzed with fiber-degrading enzymes. Sugars are expressed in mg g^{-1} BSG (dry weight basis).

3. Results and discussion

3.1. Chemical characterization

The physical-chemical characteristics of the BSG are tabulated in Table 2. BSG contained barley husk, pericarp and seed coat remains rich in polysaccharides and was not starchy. The BSG contained 36.59% structural sugars, out of which 18.03% was glucans and 18.56 C5 sugars (arabinans+xylans). Mannose and galactose were not detected. Such sugar composition indicated that both cellulose and hemicellulose were present in approximately equal amounts. Forssell et al. (2008) reported 45% carbohydrates and 21.5% protein in BSG. The BSG tested herein contained 41.15% carbohydrates (as sugars) and 22.77% protein. In a previously work, White et al. (2008) found a similar sugars content proportion in BSG. However, they reported higher xylan and lignin contents. The composition differences could be ascribed to differences in barley genotype, harvest time, and the malting and mashing conditions employed during brewing (Mussatto et al., 2006; Forssell et al., 2008).

3.2. Extrusion effect

3.2.1. C5 sugars hydrolysis

The enzymatic release of both C5 sugars i.e. arabinose and xylose, was only temperature dependent (**Figure 1 A and B series**). The temperature was the only factor that affected the release of the C5 sugars embedded in the hemicellulose matrix. The yield of arabinose was nearly described by a quadratic modeling with a temperature dependent correlation coefficient of R^2=58.09% (**Fig. 1 A1, A2, and A3**). On the other hand, xylose had a linear modeling (**Fig. 1 B1, B2, and B3**) with a correlation coefficient of R^2=41.48%. In both cases, the maximum sugar amounts were released at 50 °C. At this temperature, 30.6 and 57.1% of the total arabinose and xylose were released, respectively. Normally, high pretreatment temperatures lead to higher hemicellulose hydrolysis rates making cellulose more accessible to enzymes (White et al., 2008). Karunanithy et al. (2013) found that extrusion temperatures of around 100°C had a positive effect over hemicellulose degradation into monomeric sugars by the following enzymatic hydrolysis,

but when the extrusion temperature was increased less arabinose and xylose were enzymatically hydrolyzed. Although both sugars were generated after the enzymatic hydrolysis, apparently they were not significantly affected by the screws speed and tempering moisture. Nontheless, these extrusion parameters must be controlled because the temperature in the last zone of the barrel is affected by these factors (Moscicki, 2011).

3.2.2. C6 sugars hydrolysis

Based on the BSG composition (**Table 2**), glucose and the dimer cellobiose were the C6 sugars that were released after the enzymatic hydrolysis step. For the cellobiose hydrolysis, the temperature (P value=0.000), the interactions between temperature-screws speed (P value=0.001), temperature-moisture (P value=0.024), and screws speed-moisture (P value=0.044) were statistically significant. The cellobiose modeling was fitted as a linear regression (R^2=75.89%). The cellobiose released did not exceed 2.8 mg g^{-1} of BSG (dwb). Beside the temperature, the interaction with the tempering moisture also had a negative effect over the release of cellobiose (**Fig. 1 C1, C2, and C3**). For the glucose, the modeling was fitted like a linear correlation (R^2=78.22%). The significant variables were the temperature (P value=0.000) and the interactions between temperature-screws speed (P value=0.003) and temperature-moisture (P value=0.011). Similar to cellobiose, the interaction between temperature-moisture significantly affected the generation of glucose. Interestingly, the application of higher tempering moistures decreased the glucose enzymatic recovery (**Fig. 1 D1, D2, and D3**).

Using the combination of 50 °C, 200 rpm, and 20% feedstock moisture generated the maximum glucose (98.51 mg·g^{-1}) which represented a total recovery of about 47.8%. In fact, the high hemicellulose and lignin contents (especially the latter for it absorbs cellulosic enzymes) negatively affected the enzymatic hydrolysis of cellulose and the removal of these components could result in higher glucose release (Mussatto et al., 2008). Forrssell et al. (2008) reached the same conclusion, suggesting an acid or alkaline treatment to enhance de hemicellulose hydrolysis or lignin removal. It is important to mention that the chemical hydrolysis could also lead to the production of inhibitory compounds which need to be removed before enzymatic hydrolysis and fermentation.
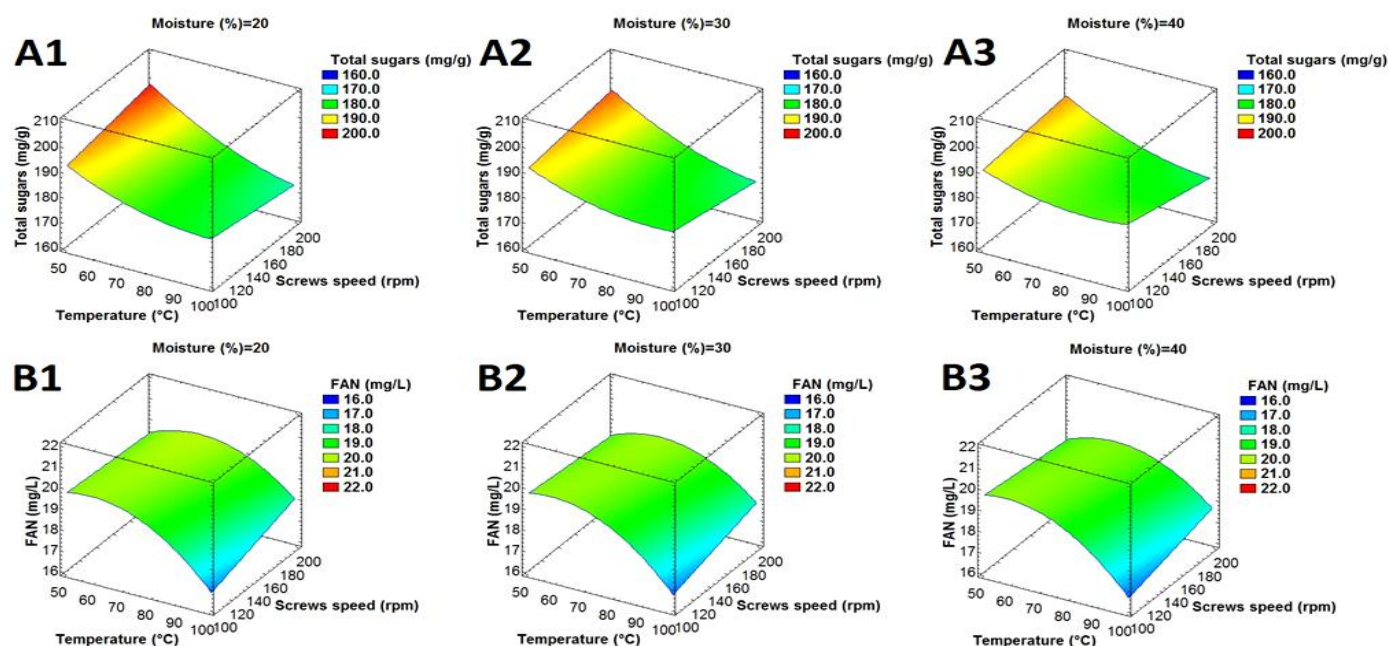

Fig.2. Response surfaces for total sugars (A1, A2, and A3) and free amino nitrogen (B1, B2, and B3) generated from brewers' spent grain extruded at different temperatures (50, 75, or 100 °C), screws speeds (100, 150, or 200 rpm) and operating moistures (20, 30, or 40%) that were further hydrolyzed with fiber degrading enzymes. Sugars are expressed in milligrams g^{-1} of BSG and FAN is expressed in milligrams L^{-1} (dry weight).

Table 2.
Physical-chemical characteristics of brewer's spent grain (proximate, fiber and carbohydrates composition analyses).

Component	Amount (%) ± SD[1]
Protein	22.77 ±0.02
Water extractives	7.61 ±0.51
Ethanol extractives	12.63 ±0.36
Glucan	18.56 ±0.11
Xylan	11.44 ±0.07
Arabinan	6.59 ±0.09
Acetyl groups	0.22 ± 0.01
Acid insoluble lignin	11.05 ±0.78
Acid soluble lignin	8.00 ±1.41

[1] Mean values are expressed on dry matter basis.

3.2.3. Total sugars hydrolysis

For total sugars hydrolysis, a linear regression with a regression coefficient of R^2=70.07% was the best model. The statistically significant extrusion variables were temperature (P value=0.000) and the interaction between temperature-screws speed (P value=0.036). The feedstock moisture was relevant for cellobiose and glucose yields. However, the effect of extrusion temperature was minimal in terms of total sugar production (**Fig. 2 A1, A2, and A3**).

The temperature had a negative effect over the total sugar generation. According to Kurananithy and Muthukumarappan (2010), the shear stress during extrusion disrupts the lignocellulose structure of BSG. The use of low temperatures and low tempering moisture caused a better shear stress inside the extruder barrel. The average maximum value was obtained using 50 °C, 200 screws rpm, and 20% moisture. These conditions generated an extruded feedstock that after enzyme hydrolysis resulted in 48.4% sugars based on the total fiber. The control treatment released 33.8% of the total sugars (139.03±2.26 mg total sugars g^{-1} BSG). By using the extrusion pretreatment, the total sugars yield was improved by 14.6%. Forssell et al. (2008) tested various commercial fibrolytic cocktails on BSG and achieved 28% recovery of the total sugars. Likewise, White et al. (2008) treated the BSG with hot water at 121°C and after the hydrolysis with fiber-degrading enzymes achieved 184 mg total sugars g^{-1}. This yield was similar to the sugar amount reported in the present research, proving that physical pretreatments had a positive effect towards releasing sugars from BSG cellular walls during the hydrolysis step. The same authors obtained 150, 140 and 70 mg g^{-1} of glucose, xylose and arabinose, respectively, by treating the BSG with diluted HNO_3 acid hydrolysis and fiber-degrading enzymes. Although they achieved 1.8 times more sugars, the proportion between C5 and C6 sugars was approximately similar to the ratio of sugars recorded herein.

The complex cell wall structure (cellulose, hemicellulose, lignin, and protein), is the most relevant barrier diminishing enzyme performance and sugar yields (Pandey et al., 2011). Amomng all, lignin is the most important barrier which reduces the releasing of sugars. The B contained about 18% lignin (**Table 2**). In fact, compared to other lignocellulosic byproducts such as corn stover (7%), wheat straw (17%), rice straw (12%), and sorghum bagasse (11%) (Reddy and Yang, 2005), the investigated BSG contained higher lignin content. In a previous work on extruded sweet sorghum bagasse hydrolyzed with fibrolytic enzymes, total sugar yield of 67.5% was accomplished (Heredia-Olea et al., 2013). This could be explained by the fact that the bagasse contained 14% lignin and thus the subsequent enzymatic hydrolysis step performed better. After enzymatic hydrolysis, the proteins remain intact or insoluble, acting as an important barrier against the subsequent steps of bioethanol production (Forsell et al., 2008; Faulds et al., 2009).

3.2.4. Free amino nitrogen

The free amino nitrogen (FAN) concentration was fitted as a quadratic model in the temperature axis (**Fig. 2 B1, B2, and B3**). FAN was affected by the temperature (P value=0.000), square temperature (P value=0.005), and the interaction between temperature-screws speed (P value=0.044). The FAN amounts in the hydrolyzates ranged from 17.6 to 19.9 mg L^{-1}. The low FAN yield occurred because of the high lignification degree of BSG. Forrssell et al. (2008) found that after enzymatic hydrolysis of BSG, the protein practically remained intact. During the malt mashing, some of the barley and the adjunct

proteins are hydrolyze into low molecular weight peptides and amino acids (quantified as FAN), leaving the remaining proteins between the cellular walls layers (Celus et al. 2006). Even after the enzymatic degradation of cell walls, these proteins are mainly coated with lignin, preventing the protein release from the matrix. It has also been reported that the disulphide cross-linkings of proteins forms a coat on the surface layers of spent grain (Faulds et al., 2009). The concentration of FAN in the hydrolyzates was low for the key fermentation step and therefore, the hydrolyzates needed to be supplemented with yeast food rich in nitrogenous compounds (Thomas and Ingledew, 1990).

3.2.5. Inhibitory compounds

None of the hydrolysates contained significant amounts of inhibitory compounds such as acetic acid, furfural, or HMF. In fact, the harsh conditions involved in most commercial chemical pretreatments induce the rupture of hemicellulose and its subsequent dehydration (Pandey, 2011). While the extrusion conditions employed in this research did not generate these deleterious hydration compounds (Lin et al., 2012), especially at 50°C when the highest amount of sugars was produced. The null generation of inhibitors is advantageous for the ethanol production and from the environmental viewpoint because the chemical detoxification step of the process can be skipped. White et al. (2008) reported the existence of inhibitors in their BSG test (no amounts were reported), and they proposed adjusting the pH and inclusion of a detoxifying step for the hydrolyzates prior to the subsequent fermentation step. With the extrusion pretreatment, no detoxifying step was needed because no extreme conditions like acid or alkali pretreatments for example were applied.

3.3. Fermentation

During the first 24 h of fermentation, only 24.6% of the total sugars were consumed by the yeast generating 24.61 mg of ethanol g^{-1} of the extruded BSG treated with the fiber-degrading enzymes (**Table 3**).

Table 3.
Different sugars and ethanol concentrations obtained from the extruded BSG at 50 °C after 48 h enzymatic saccharification and fermentation with *S. cerevisiae* 20252™ at 10% solids fraction[1,2,3].

Fermentation Time (h)	Compound (mg g^{-1})					Ethanol	Cellulose Conversion (%)
	Arabinose	Cellobiose	Glucose	Xylose	Total Sugars		
0	33.60 ±0.95 a	3.43 ±0.20 a	91.96 ±4.42 a	72.00 ±3.20 a	200.99 ±7.28 a	ND	-
24	33.68 ±0.33 a	3.78 ±0.14 b	39.78 ±2.42 b	74.31 ±1.71 a	151.56 ±2.96 b	24.61 ±0.18 a	50.4%
48	33.92 ±1.46 a	3.94 ±0.06 b	2.93 ±0.13 c	73.77 ±1.92 a	114.55 ±139 c	42.88 ±0.77 b	87.8%

[1] Means with different letter within columns are significantly different.
[2] The compounds not detected by the HPLC were represented by the acronym ND.
[3] Modified from Dowe and McMillan (2008). % Cellulose Conversion =100×(Ethanol amount /(glucose amount+(0.51×(cellobiose amount×1.111))))

The cellulose conversion at this point of fermentation was 50.4%. However, after 48 h of fermentation, 43.0% of the total sugars were consumed and 42.88 mg of ethanol g^{-1} was produced. This means that 87.8% of the glucose contained in the treated BSG was converted. At both fermentation times, only glucose was consumed, fermenting 56.7 and 96.8% of the total glucose at 24 and 48 h, respectively.

White et al. (2008) reported an ethanol yield of 8.3 g L^{-1} after 48 h fermentation with *Pichia stipitis* strain using spent grain pretreated with diluted acid and hydrolyzed with fiber degrading enzymes. However, they also did not ferment the arabinose. In the present research, an ethanol yield of 4.28 g L^{-1} was achieved by fermenting only glucose, reaching similar the fermentation yield reported by Whithe et al. (2008). Heredia-Olea et al. (2013) also used the same *S. cerevisiae* strain and xylose and arabinose were fermented. On the contrary, these sugars were not fermented in the present work,. According to Scheper (2007), in order to ferment C5 sugars into ethanol, right amount of oxygen is needed. This factor and the low FAN

contained in the hydrolyzates (19.38±0.50 mg L^{-1}) could explain the low ethanol yield and the absence of C5 fermentation herein. Thus, nutrient supplementation and initial aeration might be required for a successful fermentation of C5 sugars and to further improve the ethanol yield.

4. Conclusion

The extrusion pretreatment disrupted the BSG structure and made the extruded feedstock more susceptible to the subsequent enzymatic hydrolysis step. The BSG extruded at 50 °C, 200 rpm screws speed, and 20% feedstock moisture, released 48.4% of the total sugars and more importantly did not generate yeast inhibitors. Relatively low and constant amount of FAN was recorded in the extruded and enzymatically-hydrolyzed BSG. Such amounts of FAN were not sufficient for successful fermentations. After 48 h fermentation and using 10% solid loading, the enzymatically-hydrolyzed BSG yielded 5.43 mL ethanol L^{-1}. The yeast only consumed glucose and therefore, low ethanol yields were achieved. The remaining spent material still contained high protein and lignin contents that could be considered for other bioprocesses.

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