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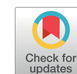
Microwave assisted hydrothermal as greener pretreatment of brewer's spent grains for biobutanol production



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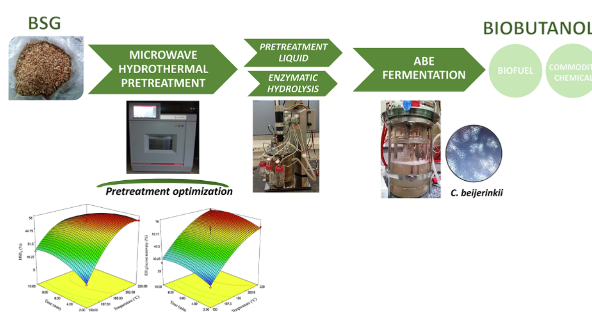
HIGHLIGHTS

- Effective fractionation of BSG by microwave hydrothermal pretreatment.
- No consumption of corrosive chemicals (acid or alkali) as catalysts.
- Total recovery of fermentable sugars of 82% (43 g fermentable sugars/100 g BSG)
- Overall production of 62 kg ABE/t BSG, of which 46 kg/t are butanol.

GRAPHICAL ABSTRACT



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ABSTRACT

A microwave assisted hydrothermal pretreatment technology has been developed to recover fermentable sugars from brewer's spent grain. Microwave hydrothermal pretreatment is considered as a greener pretreatment, as no acid or alkali are used as catalysts. An experimental design was planned to analyze the effect of pretreatment conditions (temperature and time). The objective was to maximize hemicellulosic sugar recovery in the liquid fraction and glucose recovery in enzymatic hydrolysis (referred to untreated BSG), as well as to minimize inhibitors in the liquid fraction to ensure ABE (acetone, butanol and ethanol) fermentability. Optimal conditions were 192.7 °C and 5.4 min, resulting in 64% hemicellulosic sugar recovery, 70% glucose recovery in enzymatic hydrolysate and 2.4 g/L total inhibitors. The liquid fraction obtained under optimal conditions was fermented with *Clostridium beijerinckii*, reaching a butanol concentration as low as 1 g/L. The butanol concentration could be improved by operating at higher solid loadings in pretreatment, which would increase fermentable sugar concentration. Enzymatic hydrolysis of pretreated BSG yielded a sugar solution, which was also fermented, resulting in a butanol concentration and overall yield of 8.3 g/L and 46 kg/t BSG, respectively.

1. Introduction

The Europe 2020 Strategy calls for Bioeconomy as a key element for innovative and green growth in Europe. Advancements in Bioeconomy will improve the management of renewable biological resources and the conversion of these resources into chemicals and biofuels [1]. Turning waste into a resource is an essential part of closing the loop in a circular economy.

Brewer's spent grain (BSG) is an abundant lignocellulosic industrial waste obtained from the brewing industry. In 2014, 37.4 and 180.3 million tonnes of beer from barley were produced in the European

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Union and in the world, respectively [2]. BSG accounts for 85% of total waste generated in breweries [3]. In addition, it is estimated that 20 kg of wet BSG/100 L beer are produced. BSG is available throughout the year at low cost. Moreover, it presents a high content in carbohydrates and proteins, which is why it has conventionally been used as animal feed. However, it is mostly employed by local farms. It presents high moisture content (about 80%) and, if not used within five days after being produced, microbial growth causes a fast spoilage. Therefore, if there is no commercial outlet for BSG, it necessarily has to go to landfill [4].

Due to its high content in carbohydrates, BSG has been used for biofuel production, such as biogas [5], biohydrogen [6] and ethanol [7]. Comparing with ethanol, butanol has such better characteristics as less volatility, being less explosive and hygroscopic, and also a bigger energy density [8]. Therefore, butanol can be considered as an advanced biofuel, which might be generated as a result of a fermentation process using *Clostridia* strains under anaerobic conditions [9]. The fermentation products are acetone, butanol and ethanol, the ratio in which they are usually generated being 3:6:1 [10]. Butanol is also a relevant commodity chemical with a wide range of chemical applications. Worldwide butanol production is estimated about 5 million tons with a current price around 0.9–1.4 USD/kg [11].

One of the main drawbacks in the production of biobutanol is the cost of the feedstock. The use of low-cost, abundant, renewable, non-food use lignocellulosic waste is an opportunity to improve the economic viability of butanol production. Pretreatment, enzymatic hydrolysis and ABE fermentation are the main stages in the biological process of butanol production from lignocellulosic residues, the pretreatment being the most important stage. Its most essential aims are to disturb the recalcitrant structure of the lignocellulosic biomass, separate it into the main components and improve the enzymatic hydrolysis of cellulose [12]. Numerous types of pretreatment have been applied to lignocellulosic biomass to produce fermentable sugars, such as dilute acid, alkaline, organosolv, chemical oxidation, liquid hot water, microwave, steam explosion, or biological pretreatment with fungi [13].

However, the development of greener, more efficient pretreatments, with lower costs and avoiding the use of chemicals, is essential. The microwave assisted pretreatment is considered as an emerging technology. The microwave has currently gained increasing interest in comparison with conventional heating. When conventional heating is used, energy is transferred from the outside surface of the material inwards to the core of the material, so that the outside surface can be overheated remaining a cooler inside region. However, when microwave is employed, energy is uniformly dissipated throughout the material, since heat is induced at the molecular level by direct conversion of the electromagnetic energy into heat [13]. The dipole rotation and ionic conduction are the two more important mechanisms which are responsible of the microwave dielectric heating. Through the dipole rotation interaction, polar molecules try to align themselves with the rapidly changing electric field of the microwave. On the other hand, an instantaneous superheating of the ionic substance takes place by ionic conduction mechanism, which is due to the ionic motion generated by the electric field. In this way, a more efficient energy transfer is achieved when temperature increases [14,15].

Therefore, lignocellulosic biomass can be penetrated by microwave, so that the heat can be generated throughout all the materials rather than an external source. Moreover, water containing in lignocellulosic biomass absorb well the microwave irradiation due to the ionic character of microwave [14]. In this way, microwave heating generates fibre swelling and fragmentation as a result of the internal uniform and rapid heating of large biomass particles [13]. So, it disturbs the recalcitrant structure of the lignocellulosic biomass, separates it into the main components and improves the enzymatic hydrolysis of cellulose [14]. For that reason, microwave pretreatment offers a great number of advantages respect to those under conventional heating, such as short reaction times, uniform and direct heating, simplicity of the process, higher removal of acetyl groups in hemicellulose and lower generation of inhibitory compounds (such as acetic and formic acids, furfural, 5-hydroxymethylfurfural and phenolic compounds). Moreover, the most important aspect is that microwave assisted pretreatment is an energetically efficient method, which does not cause environmental problems while also having a small capital cost [13]. Most studies on lignocellulosic biomass pretreatment by microwave are based on the use of domestic microwave ovens assisted by acid or alkaline catalysts [16,17]. In this work, a hydrothermal pretreatment was used, which is less expensive in comparison with acid or alkaline catalysts. The use of water as a solvent eliminates the requirements of corrosive chemicals, therefore, microwave assisted hydrothermal pretreatment is considered as an environmental friendly pretreatment. A closed microwave reactor, which allows higher temperatures to be reached, was used to perform the hydrothermal pretreatment. The use of high temperatures (150–250 °C) reduces reaction times and improves pretreatment performance.

The purpose of this study was to investigate the efficiency of microwave assisted hydrothermal as technology for efficient BSG pretreatment. Process conditions were optimized by maximizing both hemicellulosic sugar recovery in the liquid fraction and glucose recovery in enzymatic hydrolysis, as well as by minimizing, at the same time, the total inhibitor concentration in the liquid fraction. Moreover, an assessment was carried out of the fermentability to butanol of the liquid fraction and the enzymatic hydrolysate of BSG obtained, at the optimal pretreatment conditions, by fermentation with *Clostridium beijerinckii* DSM 6422. To the best of our knowledge, this is the first work on microwave assisted hydrothermal pretreatment of lignocellulosic biomass for butanol production.

2. Materials and methods

2.1. Raw material

Brewer's spent grain (BSG) was kindly provided by a local brewery and stored at −20 °C until use. Before the experimental runs, the BSG was washed, dried in an oven at 50 °C, milled using a coffee grinder (Moulinex, A505, France) and homogenized. Thus, a BSG was achieved with a 3% moisture content and a particle size lower than 1 mm.

2.2. Microwave assisted hydrothermal pretreatment

Pretreatment was carried out in a Multiwave PRO SOLV reactor 50 Hz with Rotor type 16HF100 (Anton Paar GmbH, Austria, Europe). The multiwave reactor is composed of two standard magnetrons of 1000 W, being 1800 W the maximum microwave power. Pressure vessels, fitted with magnetic stirrers and whose capacity volume was of 100 mL, were made of ceramic and PTFE-TFM. The reactor can be used with up to 16 sample vessels, containing one of them a pressure/internal temperature sensor. Pressure/internal temperature sensor is used in order to permanently control the applied microwave energy by reactor. In addition, an IR sensor was used to register the temperature of all vessels continuously.

BSG and water were mixed at a solid to liquid ratio of 10% w/v (5 g dry weight BSG and 50 mL of water) in each of the pressure vessels of the multiwave reactor. The reactor was heated, being able to reach the selected temperature by adjusting the power input. Different ramps time of microwave heating were used depending on the temperature set in each run (Table 1). After the desired temperature was reached, time counting was initiated. Once the experimental runs (Table 1) finished, the pressure vessels of the microwave reactor were cooled to about 50 °C. The slurry was vacuum filtered and the solid phase was separated from the liquid fraction, washed with distilled water, dried at 40 °C and weighed to determine the solid recovery (g solid fraction/100 g BSG). Structural carbohydrates, lignin and ash content in the solid phase were analyzed, and the pretreated solid was also used as substrate in

Table 1
Experimental design for microwave assisted hydrothermal pretreatment of BSG and Severity Factor (SF).

Run	Temperature (°C)		Time (min)		Ramp time (min)	SF
	Coded	Real	Coded	Real		
1	−1	150	+1	10	22	2.47
2	−1.41	135.5	0	6	20	1.82
3	0	185	0	6	27	3.28
4	0	185	0	6	27	3.28
5	0	185	0	6	27	3.28
6	+1.41	234.5	0	6	35	4.74
7	+1	220	+1	10	33	4.53
8	−1	150	−1	2	22	1.77
9	0	185	0	6	27	3.28
10	0	185	0	6	27	3.28
11	+1	220	−1	2	33	3.83
12	0	185	−1.41	0.34	27	2.03
13	0	185	+1.41	11.66	27	3.57

enzymatic hydrolysis assays. The composition of the pretreatment liquids in terms of monosaccharides and degradation products, such as formic acid, acetic acid, furfural, hydroxymethylfurfural (HMF) and total phenols, was also determined. In order to evaluate the efficiency of the microwave assisted hydrothermal pretreatment, the recoveries of carbohydrates in the pretreatment liquids were calculated as a percentage of the sugar content in the untreated BSG.

2.3. Experimental design

In order to select the optimum conditions for microwave assisted hydrothermal pretreatment of BSG, a central composite experimental design was planned ($\alpha = 1.414$); including one point and four replicates at the center of domain selected for each factor under study and a total of 13 experiments. Experimental runs were carried out in random order. Temperature (150–220 °C) and time (2–10 min) were chosen as independent variables. Experimental intervals were selected from previous results [18]. Table 1 reports the coded and uncoded values of factors in the experimental design. Experimental data were analyzed by the commercial software Statgraphics Centurion XVIII. In order to measure the harshness of the pretreatment, the Severity Factor (SF) was calculated according to MacAskill et al. [19] (Eq. (1)), where t is time (min) and T is temperature (°C).

$$\text{Severity Factor (SF)} = \text{Log}\left[t \times exp\left(\frac{T-100}{14.75}\right)\right] \quad (1)$$

2.4. Enzymatic hydrolysis

The enzymatic hydrolysis tests were carried out in 100 mL erlenmeyer flasks in an orbital shaker (Comecta Optic Ivymen system), the working volume and the solid loading being, respectively, 25 mL and 5% (w/v). The enzymes employed were Cellic CTec2, which is a cellulolytic complex kindly provided by Novozymes A/S (Denmark). In addition, to adjust the pH to 4.8, 0.05 M sodium citrate was used as buffer. The experimental conditions employed were 50 °C, 150 rpm, 48 h, and a Cellic CTec2 enzyme load of 15 Filter Paper Units (FPU)/g solid. Samples were withdrawn at 24 and 48 h, centrifuged and analyzed for monosaccharides and degradation products. The enzymatic hydrolysis tests were performed in triplicate. Moreover, enzyme blanks were used to take into account the monosaccharides content of the commercial enzymes. What is more, raw BSG was submitted to enzymatic hydrolysis to compare the results with those determined for the pretreated solid fractions. Glucose recoveries in enzymatic hydrolysis, EH glucose recovery (referred to pretreated or untreated BSG) were calculated from the grams of glucose released in the enzymatic hydrolysates divided by the structural glucose (as cellulose) content in the pretreated or untreated BSG, respectively.

On the other hand, the solid fraction obtained under the optimal pretreatment conditions was also used as substrate in enzymatic hydrolysis tests at 5% (w/v) of solid load to validate optimization results, under the same experimental conditions described before. For ABE fermentation, the enzymatic hydrolysis of the pretreated BSG obtained under optimal conditions was carried out at a solid loading of 10% (w/v), without sodium citrate buffer, using water as solvent at pH 4.8, to obtain enough concentration of fermentable sugars for ABE fermentation tests. After saccharification, slurries were vacuum filtered and liquid hydrolysates fermented by *C. beijerinckii*.

2.5. Microorganism

The microorganism, *C. beijerinckii* DSM 6422, was obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). It was preserved and grown according to Plaza et al. [20], but using 250 mL serum bottle with rubber septum and 170 mL Reinforced Clostridial Medium (RCM), and carrying out only two thermal shocks for 2 min to stimulate the germination of the spores. The inoculum was grown in an orbital shaker (Comecta Optic Ivymen system) at 35 °C and 135 rpm for 48 h.

2.6. ABE fermentation

The enzymatic hydrolysate and the liquid fraction of BSG obtained at optimal microwave conditions were fermented with *C. beijerinckii*. The liquid fraction was detoxified with activated charcoal. In this case, the pretreatment liquid was mixed with powder activated charcoal in a 2% (w/v) ratio in an orbital shaker (Comecta Optic Ivymen system) at 35 °C, 135 rpm and 1.5 h. After detoxification, the mixture was vacuum filtered and the hydrolysates were measured for their content in sugars and inhibitors. ABE fermentation was carried out at 35 °C and 135 rpm for 120 h under the same conditions as described by Plaza et al. [20]. All experiments were performed in triplicate.

2.7. Analytical methods

Structural carbohydrates, lignin and ash content of the BSG and pretreated BSG were measured using the National Renewable Energy Laboratory (NREL) analytical methodology [21,22]. The extractives composition of the BSG was also determined according to the NREL methodology, NREL/TP-510-42619 [23]. The total starch content was measured using the “Total Starch Assay Kit” method (Megazyme, Ireland) on the raw material previously extracted with water.

The concentrations of sugars (glucose, xylose and arabinose), inhibitor compounds (acetic and formic acids, furfural and HMF), ABE solvents (acetone, butanol and ethanol) and other organic acids (lactic and butyric acids) were measured by High Performance Liquid Chromatography (HPLC) with an Aminex HPX-87H column. It is worth mentioning that this column is not able to separate the xylose, galactose and mannose sugars [24]. A refractive index detector (Waters 2414) was used to measure the sugars, while the furfural and HMF were analyzed with a photodiode array detector (DAD) at 280 nm (Waters 996). The mobile phase was 0.01 N H_2SO_4, at a flow rate of 0.6 mL/min and 30 °C (solvents) or 60 °C (sugars, organic acids, furfural and HMF). In order to calculate oligomeric sugars in the liquid fractions obtained in the BSG pretreatment, an acid hydrolysis step (120 °C, 3% w/v H_2SO_4, 30 min) was carried out. Oligomeric sugars were determined as the difference between total free sugars in the liquid fractions before and after acid hydrolysis. Prior to its analysis by HPLC, all samples were centrifuged at 13400 rpm for 10 min, and filtered through 0.2 µm nylon filters. In order to determine the total content of phenolic compounds, the Folin-Ciocalteu method [25] was used, employing gallic acid as standard. Analytical determinations were performed in triplicate and

the average results are shown. Relative standard deviations were below 2%.

3. Results and discussion

3.1. Characterization of BSG

The composition of the raw BSG was as follows (%w/w, dry matter): cellulose, 17.9 ± 0.3; hemicellulose, 28.7 ± 0.8 (xylan, 20.7 ± 0.4; arabinan, 8.0 ± 0.4); acid-insoluble lignin (AIL), 19.4 ± 1.2; acid-soluble lignin (ASL), 6.4 ± 0.1; extractives, 2.3 ± 0.1 (glucose in extractives, 1.3 ± 0.1); ash, 2.7 ± 0.1; acetyl groups, 1.0 ± 0.1; and starch, 2.1 ± 0.0.

Thus, this lignocellulosic residue includes 50% total carbohydrates (46.6 and 3.4% of structural and non-structural carbohydrates, respectively), mainly glucose and xylose. Glucose is found free in extractives (1.3%), as starch (2.1%) and as cellulose (19.7%), so the content of glucose as cellulose represents 85%. However, depending on the type of barley, the time in which the barley harvesting takes place, as well as the processes used in the breweries, the starch composition of BSG can be different [4,26]. It is worth mentioning that the galactose and mannose content in BSG is very low (< 2%), xylose being the major hemicellulosic sugar [7]. Moreover, raw BSG presents a high content of arabinan. So, an arabinose-xylose ratio of 0.38 can be calculated for raw BSG, which is in accordance with the values reported in other studies on BSG [26].

On the other hand, the lignin content in this lignocellulosic residue (25.8%), considering both AIL and ASL, is higher than those reported for typical lignocellulosic biomass such as rapeseed straw [27], olive tree pruning [28], corn cob, wheat straw, coastal bermuda grass or cotton seed hairs [12]. If we compare it with the composition of BSG determined in previous works [7,20,29,30], the lignocellulosic residue employed in this study has a relatively higher total lignin content, considerably lower extractives, and a very similar carbohydrate content as well as other minor components, such as acetyl groups and ash. According to Ivanova et al. [31], BSG contains phenolic compounds (130–160 mg gallic acid/100 g), which can interfere in fermentation processes.

3.2. Effect of the microwave assisted hydrothermal pretreatment conditions on BSG

In order to get the solubilization of hemicellulosic sugars, as well as maintaining the cellulose in the pretreated solid fraction, a microwave assisted hydrothermal pretreatment was employed. The SF parameter was used to assess the impact of the pretreatment on BSG. It is worth remarking that SF is a parameter that takes into account the combination of temperature and time.

Table 2 shows the solid recoveries obtained in the experimental runs. The solid recovery was affected by the pretreatment severity, temperature and time, as these factors influence the non-structural and labile fractions (extractives and hemicellulose fraction, respectively) which are solubilized as a consequence of the pretreatment [32]. As can be appreciated, an increase in the pretreatment severity led to a reduction in the solid recovery. Solid recoveries ranged from 51% to 90%, corresponding to the highest (SF = 4.74, run 6) and lowest severity factors (SF = 1.82, run 2), respectively. At the center of the domain (runs 3, 4, 5, 9, 10), a solid recovery of 61–65% was obtained (185 °C, 6 min).

As can be seen in Table 2, pretreated solid fractions were obtained with a cellulose content of between 16 and 26%. Except for runs 1, 2 and 8, a cellulose enrichment of the pretreated solid was obtained, which is due to the solubilization of extractives and hemicellulose during the pretreatment. The BSG increased its cellulose content from 17.9% (raw material) to 26% (runs 7 and 6), with SF = 4.53 and 4.74, respectively. Nevertheless, no cellulose enrichment (cellulose content < 17.9%) was observed at a temperature of 150 °C or lower, which can be due to the limited hemicellulose solubilization obtained under these experimental conditions (runs 1, 2 and 8). Glucose recoveries in the pretreated solids (GR_s) are also shown in Table 2, obtaining low values (GRs = 74%) for the highest pretreatment severities (runs 6 and 7).

An increase in the lignin content in the pretreated solids was also observed, probably due to condensation reactions between lignin, extractives and inhibitor compounds [33]. Although the complete solubilization of the hemicellulose fraction was not possible for SF < 2 (runs 2 and 8) with pretreated solids containing about 26% hemicellulose, when SF was higher than 4 (runs 6 and 7) this fraction was able to be solubilized almost completely. According to Jørgensen and Pinelo [34], the enzymatic hydrolysis process may be adversely affected due to the hemicellulose content of the pretreated solid, since the enzyme finds it more difficult to access the cellulose.

The pH of the liquid fractions obtained from the pretreatment (data not shown) ranged from 3.42 (run 7, SF = 4.53) to 4.13 (run 2, SF = 1.82), corresponding to the higher and lower severity conditions of pretreatment, respectively. This drop in the pH can be due to the solubilization of acetyl groups from the hemicellulose fraction of the raw material, which occurs in the most severe conditions of pretreatment [12]. Table 2 shows the carbohydrate and inhibitor composition of the liquid fractions released in the pretreatment, as well as the carbohydrate recovery (GR_L, HSR_L) in these fractions. The sugar concentrations ranged from 4.4 g/L (run 2) up to 25.7 g/L (run 13). As can be seen, sugars were mainly detected in oligomeric form (between 58% and 88%), which may be due to the use of water as the catalyst. Despite obtaining mostly xylose and arabinose in the liquid fractions, as can be seen in Table 2, an important glucose content (concentration < 4.9 g/L and GR_L < 25%) was also detected, even in the softest pretreatment conditions (runs 2 and 8, SF < 2). This could come from the non-structural glucose present in the BSG (glucose in extractives and starch) as well as the amorphous cellulose being easily solubilizable [7]. Regarding the xylose, concentrations ranging from 0.8 to 12.1 g/L were measured, reaching the highest values for SF > 3. This fact is also reflected in the hemicellulosic sugar recoveries, yielding values as high as 51–58% for 3.28 ≤ SF ≤ 3.83. It is worth noting that a decrease in xylose and hemicellulosic sugar recoveries in the liquid fractions was observed for the highest SF (runs 6 and 7, SF > 4), as hemicellulosic sugar degradation reactions took place. What is more, as expected, the hemicellulosic sugar recoveries in the liquid fractions were very low (< 10%) when the pretreatment was carried out under mild conditions (SF < 2, runs 2 and 8), because the severity of pretreatment was not sufficient to hydrolyze the hemicellulose remaining in the pretreated solid (HSR_S > 80%).

Inhibitor compounds (acetic and formic acids, furfural, HMF and phenolic compounds), generated as a consequence of the pretreatment, have concentrations that vary depending on the severity of the pretreatment [35]. As can be seen in Table 2, inhibitor compounds (except total phenols) were not detected at low pretreatment severities (SF < 2). The highest concentrations were reached for the most severe conditions of pretreatment (runs 6 and 7, SF > 4). The hydrolysis process of the acetyl groups leads to the formation of acetic acid, obtaining liquid fractions with concentrations lower than 1 g/L. Furthermore, in general, the content in HMF (originated by the degradation of glucose) and formic acid (generated from HMF and furfural) was negligible (< 0.3 g/L). However, higher concentrations (up to 2.2 g/L) were measured of furfural (coming from the degradation of pentoses) and total phenols (originating from extractives and lignin degradation, according to Larsson [36]). Therefore, it is worth remarking that these compounds are considerably toxic for the fermentation process, both individually and synergistically [37]. The concentrations of furfural and phenolic compounds are, in general, higher than those reported after the acid pretreatment of BSG by other authors [3,7,20]. This is probably due to the use of higher pretreatment temperatures in this work.

Table 2
Recovery of total solids (%), and composition of the solid (g/100 g pretreated BSG) and liquid (g/L) fractions after microwave assisted hydrothermal pretreatment. Recovery (%) of glucose (GR) and hemicellulosic sugars (HSR) in the solid (subscript S) and liquid (subscript L) fractions.

Solid Fraction

Run	SF	Solid Recovery (%)	Cellulose (g/100 g pretreated BSG)	Hemicellulose (g/100 g pretreated BSG)	Lignin (g/100 g pretreated BSG)	GR_S (%)	HSR_S (%)
1	2.47	82.26	16.25 ± 1.05	23.64 ± 0.59	30.11 ± 0.59	74.74	67.84
2	1.82	90.30	16.28 ± 0.97	26.74 ± 0.54	28.28 ± 0.32	82.16	84.24
3	3.28	61.42	21.27 ± 0.30	11.01 ± 0.14	41.97 ± 0.42	73.03	23.59
4	3.28	63.92	20.40 ± 0.22	13.17 ± 0.13	39.93 ± 0.44	72.89	29.36
5	3.28	65.26	21.21 ± 0.55	13.88 ± 0.27	36.73 ± 0.28	77.36	31.59
6	4.74	51.73	25.87 ± 0.70	2.04 ± 0.13	57.26 ± 1.02	74.81	3.68
7	4.53	51.23	26.02 ± 0.82	1.62 ± 0.13	59.30 ± 0.52	74.49	2.89
8	1.77	88.74	16.35 ± 0.35	26.08 ± 0.50	29.81 ± 0.43	81.12	80.74
9	3.28	62.78	21.29 ± 0.57	11.83 ± 0.33	39.59 ± 0.35	74.73	25.91
10	3.28	64.71	22.65 ± 0.32	14.01 ± 0.33	38.20 ± 0.43	81.91	31.63
11	3.83	54.83	25.02 ± 1.18	6.61 ± 0.18	49.23 ± 0.50	76.67	12.64
12	2.03	70.82	20.27 ± 0.20	17.57 ± 0.13	32.86 ± 0.56	80.22	43.42
13	3.57	60.96	24.14 ± 0.96	11.97 ± 0.36	39.18 ± 0.15	82.23	25.46

Liquid Fraction

Run	SF	Carbohydrates				Inhibitors					GR_L (%)	HSR_L (%)
		Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Oligomeric sugars (%)	Acetic acid (g/L)	Formic acid (g/L)	Furfural (g/L)	HMF (g/L)	Total phenols (g/L)		
1	2.47	3.2 ± 0.0	3.7 ± 0.0	3.6 ± 0.0	84.1 ± 0.0	0.1 ± 0.0	n.d.	n.d.	n.d.	0.3 ± 0.0	16.08	22.35
2	1.82	2.5 ± 0.1	0.8 ± 0.0	1.1 ± 0.0	87.9 ± 0.3	n.d.	n.d.	n.d.	n.d.	0.2 ± 0.0	12.85	5.75
3	3.28	4.3 ± 0.2	12.1 ± 0.1	6.6 ± 0.1	78.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	n.d.	1.2 ± 0.1	21.97	57.62
4	3.28	4.0 ± 0.1	10.8 ± 0.0	6.5 ± 0.0	78.9 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	n.d.	1.1 ± 0.1	20.43	53.46
5	3.28	4.1 ± 0.0	10.2 ± 0.0	6.4 ± 0.0	79.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.	1.0 ± 0.0	20.78	51.14
6	4.74	4.2 ± 0.2	11.2 ± 0.1	3.4 ± 0.1	66.7 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	1.6 ± ± 0.1	0.2 ± 0.0	2.0 ± 0.2	21.18	44.93
7	4.53	4.1 ± 0.1	9.8 ± 0.0	2.7 ± 0.0	58.0 ± 0.0	0.9 ± 0.0	0.3 ± 0.0	2.2 ± 0.1	0.3 ± 0.0	2.2 ± 0.2	21.01	38.53
8	1.77	3.0 ± 0.0	1.3 ± 0.0	1.7 ± 0.0	87.3 ± 0.2	n.d.	n.d.	n.d.	n.d.	0.5 ± 0.0	15.38	9.36
9	3.28	4.2 ± 0.0	10.8 ± 0.1	6.4 ± 0.1	78.7 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	n.d.	0.9 ± 0.1	21.21	53.31
10	3.28	4.1 ± 0.1	10.2 ± 0.1	6.3 ± 0.1	79.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.	0.9 ± 0.0	21.03	50.96
11	3.83	4.9 ± 0.0	12.0 ± 0.1	5.3 ± 0.0	73.8 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	1.7 ± 0.2	24.78	53.35
12	2.03	3.7 ± 0.2	7.7 ± 0.0	5.7 ± 0.0	81.8 ± 0.1	0.1 ± 0.0	n.d.	n.d.	n.d.	0.7 ± 0.1	18.91	41.46
13	3.57	4.5 ± 0.2	11.3 ± 0.2	6.3 ± 0.1	77.8 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	n.d.	1.3 ± 0.1	22.89	54.46

GR_S (glucose recovery in solid fractions): g glucose in solid fraction/100 g glucose in BSG.
HSR_S (hemicellulosic sugar recovery in solid fractions): g hemicellulosic sugars in solid fraction/100 g hemicellulosic sugars in BSG.
GR_L (glucose recovery in liquid fractions): g glucose in liquid fractions/100 g glucose in BSG.
HSR_L (hemicellulosic sugar recovery in liquid fractions): g hemicellulosic sugars in liquid fractions/100 g hemicellulosic sugars in BSG.
n.d.: not detected.

3.3. Enzymatic hydrolysis of pretreated BSG

The solid fractions obtained after the microwave assisted hydrothermal pretreatment of BSG were submitted to enzymatic hydrolysis (5% (w/v) solid load) in order to study the efficiency of the pretreatment. Enzymatic hydrolysates with glucose and xylose concentrations ranging from 2.6 to 14.3 g/L and 1.1 to 3.5 g/L, respectively, were obtained (Table 3). The lowest concentrations of monosaccharides were obtained at one of the softest pretreatment conditions (135.5 °C and 6 min), resulting in a hydrolysate with 2.6 g/L glucose and a glucose recovery in enzymatic hydrolysis, EH glucose recovery (referred to pretreated BSG), of only 29.1%, which suggests that it is necessary to apply more severe pretreatment conditions to facilitate the access of enzymes to the cellulose. However, when the pretreatment severity factor was higher than 4 (run 6 and 7), hydrolysates with more than 14 g/L glucose were measured, corresponding to an EH glucose recovery (referred to pretreated BSG) as high as 99–100%. In fact, these results are four fold higher than those achieved in the enzymatic hydrolysis of raw BSG (25.6%), which evidences the effectiveness of the microwave assisted hydrothermal pretreatment.

High EH glucose recovery (referred to pretreated material) has also been determined for BSG submitted to different pretreatments, such as at 155 °C and 2% H_3PO_4 [7], at 121 °C and pH 1 using H_2SO_4 [20], by 1% (w/v) HCl or 3% (w/v) NaOH [38], or at 121 °C and 0.16 N HNO_3 [39]. However, a much lower cellulose conversion to glucose (76%) was achieved by pretreating BSG with liquid hot water at 190 °C [40], which suggests that the microwave assisted pretreatment is much more effective in comparison with pretreatment methods by conventional heating. Furthermore, values of saccharification as low as 68% have been reported for wheat straw after microwave pretreatment in the presence of 2% NaOH [16]. After microwave pretreatment assisted by deep eutectic solvent (choline chloride and lactic acid) of *Miscanthus*, switchgrass and corn stover, saccharification yields of 40, 75 and 78.5%, respectively, were reported [17]. In these reports, domestic microwave ovens were used, which is indicative of the limitation of domestic microwave in comparison with the multiwave closed reactors.

Glucose recovery in enzymatic hydrolysis was also referred to untreated BSG (Table 3), as it is interesting to measure the quantity of glucose in the BSG (raw untreated) which can be recovered by enzymes. In this way, the highest EH glucose recovery rates (in this case, referred to untreated BSG) were reached at the most severe pretreatment conditions (run 6 and 7, SF > 4), obtaining values of about 74%. This recovery is much higher than that reported by Plaza et al. [20] (49.4%) in the pretreatment of BSG at 121 °C and pH 1 using H_2SO_4. As can be observed, in general, the EH glucose recoveries (referred to untreated BSG) are lower than these referred to pretreated BSG, which is because of the solubilization of carbohydrates in the liquid fraction as a consequence of the pretreatment [32]. It is worth mentioning that the EH

Table 3
Enzymatic hydrolysis of the pretreated solids. Carbohydrates composition (g/L) and glucose recoveries (EH glucose recovery, %) referred to pretreated or untreated BSG. Overall sugar recoveries (%) referred to untreated BSG.

Run	SF	Carbohydrates concentration (g/L)		EH glucose recovery (%)		Overall sugar recovery (%)
		Glucose	Xylose	referred to pretreated BSG	referred to untreated BSG	
1	2.47	3.6 ± 0.0	2.2 ± 0.0	40.1	30.0	38.3
2	1.82	2.6 ± 0.1	2.2 ± 0.1	29.1	23.9	25.0
3	3.28	10.9 ± 0.2	3.4 ± 0.1	93.1	68.0	77.9
4	3.28	10.1 ± 0.1	3.5 ± 0.1	90.1	65.7	74.3
5	3.28	8.9 ± 0.2	3.3 ± 0.0	76.3	59.0	70.1
6	4.74	14.1 ± 0.4	1.3 ± 0.1	99.3	74.3	66.7
7	4.53	14.3 ± 0.2	1.1 ± 0.0	100.0	74.5	62.3
8	1.77	2.9 ± 0.1	2.2 ± 0.1	31.8	25.8	28.8
9	3.28	9.5 ± 0.2	3.3 ± 0.0	81.4	60.8	72.2
10	3.28	9.3 ± 0.1	3.3 ± 0.0	74.6	61.1	70.9
11	3.83	12.3 ± 0.2	2.6 ± 0.0	89.4	68.5	74.0
12	2.03	6.8 ± 0.1	2.7 ± 0.1	60.6	48.6	58.7
13	3.57	9.8 ± 0.4	3.2 ± 0.1	73.5	60.5	73.0

EH glucose recovery, % (referred to pretreated BSG): g glucose by enzymatic hydrolysis/100 g glucose (contained as cellulose) in pretreated BSG
EH glucose recovery, % (referred to untreated BSG): g glucose by enzymatic hydrolysis/100 g glucose (contained as cellulose) in untreated BSG.
Overall sugar recovery (%): sum of glucose and xylose grams in enzymatic hydrolyzates and pretreatment liquid/100 g total sugars in untreated BSG.

glucose recovery (referred to untreated BSG) has been calculated considering only the glucose contained as cellulose, because of the content of starch and non-structural glucose of BSG that can be easily solubilized during pretreatment, thus not remaining in the pretreated solid fractions [41].

On the other hand, furfural and HMF were not detected in the enzymatic hydrolysates, while the measured concentrations of acetic and formic acids, and total phenols were very low (< 0.3 g/L) (data not shown). This is due to the low solid load (5% w/v) in enzymatic hydrolysis and because pretreated solids were washed with distilled water after pretreatment.

Overall sugar recoveries are also shown in Table 3. This parameter considers the glucose and xylose released by the enzymatic hydrolysis of pretreated solids, as well as the carbohydrates solubilized in the pretreatment liquid fractions, referred to total sugar content in the untreated BSG. Overall sugar recoveries from 25 to 78% were achieved, these values corresponding to a low SF (SF = 1.82, run 2) and the central severity factor (SF = 3.28, run 3), respectively. However, overall sugar recoveries lower than 78% were obtained at the highest SF (runs 6 and 7, SF > 4), as sugar degradation took place. Then, the microwave assisted hydrothermal pretreatment enables 78% of the potential sugars contained in BSG (41 g of fermentable sugars from 100 g of BSG) to be recovered.

3.4. Optimization of the microwave assisted hydrothermal pretreatment

In this work, the microwave assisted hydrothermal pretreatment of BSG was carried out with the purpose of recovering the maximum amount of sugar while trying to generate the minimum amount of inhibitory compounds. Therefore, using the desirability function, which is a method to simultaneously optimize a series of responses [42,43], the pretreatment was optimized by maximizing simultaneously both hemicellulosic sugar recovery in the liquid fraction (HSR_L) and glucose recovery in enzymatic hydrolysis (referred to untreated BSG) and at the same time minimizing the total inhibitor compound content in the liquid fraction. The responses to HSR_L, EH glucose recovery and total inhibitor in the liquid fraction were predicted through second-order polynomial equations (Eqs. (2), (3) and (4), respectively):

$$HSR_L = 53.30 + 14.45T - 6.95Tt - 14.48T^2 - 6.92t^2 \quad (2)$$

$$\text{EH glucose recovery} = 62.93 + 19.82T + 3.37t - 7.43T^2 - 4.73t^2 \quad (3)$$

$$\text{Total inhibitor in liquid fraction} = 1.40 + 1.63T + 0.34t + 0.38Tt + 0.63T^2 \quad (4)$$

where the independent factors were T (temperature, °C) and t (time, min).

Variance analyses (ANOVA) for HSR_L, EH glucose recovery and total inhibitor content in the liquid fraction are shown in Table 4. HSR_L, EH glucose recovery and total inhibitor content in the liquid fraction models were predictive, as suggested by their values of R^2 and adjusted R^2 and the confidence level (95%, $p < 0.05$). As shown in the Table 4, the mathematical model for HSR_L has a higher F-value (144.06) than EH glucose recovery and total inhibitor content models. In addition, in this model, the linear term (T) was more significant than the interaction effect of temperature (T) and time (t) as well as the quadratic terms T^2 and t^2. Concerning EH glucose recovery, value of R^2 was found to be 0.9728, which indicates that 97.28% of the total variation in EH glucose recovery is attributed to the pretreatment variables studied. Moreover, the value of adjusted $R^2 = 0.9593$ indicates that the model accounts for 95.93% of the variability in the EH glucose recovery. On the other hand, in the EH glucose recovery as well as in the total inhibitor content, the temperature showed a more significant effect than the time due to its higher F-value and its lower p-value (Table 4).

As can be seen in Eq. (2), regarding the HSR_L response, although the time factor did not affect the response, the temperature was significant, with a high positive influence. However, a slight interaction between both factors was perceived on HSR_L, but with a negative influence. Then, a decrease in HSR_L can take place as a consequence of the combined effect of both factors, which can be due to the sugar degradation at high pretreatment severity, or because the severity was not enough to hydrolyze the hemicellulose. In Fig. 1a, which is the response surface plot showing the influence of temperature and time on HSR_L, this trend can also be appreciated. In addition, as can be seen in Fig. 1a, the highest HSR_L was reached close to the central point conditions (SF = 3.28, 185 °C and 6 min).

Regarding EH glucose recovery, as can be observed in Eq. (3), both temperature and time factors have positive effects, while the interaction between both factors was insignificant. However, the influence of temperature was much higher. Therefore, it can be observed that higher values for EH glucose recovery can be reached when temperature and time in the pretreatment are simultaneously higher. This behavior can

Table 4
Analysis of variance for the responses a) HSR_L, b) EH glucose recovery (referred to untreated BSG) and c) total inhibitor compound content in the liquid fraction.

a)

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value (Prob > *F*)	Remarks
Model	3500.30	4	875.07	144.06	< 0.0001	Significant
A-Temperature	1669.81	1	1669.81	274.90	< 0.0001	
AB	193.35	1	193.35	31.83	0.0008	
A^2	1397.71	1	1397.71	230.10	< 0.0001	
B^2	239.43	1	239.43	39.42	0.0004	
Residual	42.52	7	6.07			
Lack of Fit	13.69	3	4.56	0.63	0.6315	Not significant
Pure Error	28.83	4	7.21			
Cor Total	3542.82	11				
R-squared	0.9880		Adj R-squared[a]	0.9811		
Mean	40.18		Pred R-squared[b]	0.9645		
C.V. %[c]	6.13		Adeq Precision[d]	31.046		

b)

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value (Prob > *F*)	Remarks
Model	3716.74	4	929.18	71.64	< 0.0001	Significant
A-Temperature	3141.23	1	3141.23	242.20	< 0.0001	
B-Time	90.83	1	90.83	7.00	0.0294	
A^2	384.41	1	384.41	29.64	0.0006	
B^2	155.88	1	155.88	12.02	0.0085	
Residual	103.76	8	12.97			
Lack of Fit	47.58	4	11.89	0.85	0.5621	Not significant
Pure Error	56.18	4	14.05			
Cor Total	3820.49	12				
R-squared	0.9728		Adj R-squared[a]	0.9593		
Mean	55.44		Pred R-squared[b]	0.9173		
C.V. %[c]	6.50		Adeq Precision[d]	25.094		

c)

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value (Prob > *F*)	Remarks
Model	18.22	4	4.55	132.94	< 0.0001	Significant
A-Temperature	16.14	1	16.14	471.29	< 0.0001	
B-Time	0.69	1	0.69	20.07	0.0029	
A^2	2.69	1	2.69	78.40	< 0.0001	
AB	0.36	1	0.36	10.64	0.0138	
Residual	0.24	7	0.034			
Lack of Fit	0.044	3	0.015	0.30	0.8236	Not significant
Pure Error	0.20	4	0.049			
Cor Total	18.46	11				
R-squared			Adj R-squared[a]	0.9796		
Mean			Pred R-squared[b]	0.9675		
C.V. %[c]			Adeq Precision[d]	38.564		

A: Temperature (°C).
B: Time (min).
[a] Adjusted R^2.
[b] Predicted R^2.
[c] Coefficient of variation.
[d] Adequate precision.

also be observed in Fig. 1b, which is the 3D response surface plot for EH glucose recovery. The lowest and highest values for this response were performed when the pretreatment took place at the lowest and highest values of temperature and time, respectively (that is, at the lower and higher severity conditions of pretreatment, respectively).

Concerning the total inhibitor concentration in the liquid fraction (Eq. (4)), both temperature and time as well as the interaction between both factors have positive effects, being the influence of temperature slightly higher. In this way, higher total inhibitor concentration in the liquid fraction is expected when temperature and time in the pretreatment increase simultaneously (Fig. 1c).

Therefore, the optimal conditions were found to be 192.7 °C and 5.4 min, predicting the model values of HSR_L, glucose recovery in enzymatic hydrolysis (referred to untreated BSG) and total inhibitor concentration in the liquid fraction of 55.8%, 66.3% and 1.7 g/L, respectively, obtaining a desirability value of 0.824. A confirmatory experimental run was carried out under these optimal conditions in order to validate the model. The composition of the pretreated solid fraction was: cellulose, 24.65 ± 2.38%; hemicellulose, 8.77 ± 0.70%; and lignin, 43.43 ± 1.15%. The pretreated solid enriched in cellulose was submitted to enzymatic hydrolysis, leading to an EH glucose recovery (referred to untreated BSG) of 69.5%. Moreover, a liquid fraction with about 26 g/L total sugars (Fig. 2a, Raw PL) (HSR_L = 63.8%) and 2.4 g/L total inhibitor compounds, mainly phenols and furfural (Fig. 2b, Raw PL) was obtained. Therefore, as can be tested, an acceptable adjustment between the predicted and experimental values was achieved. It is

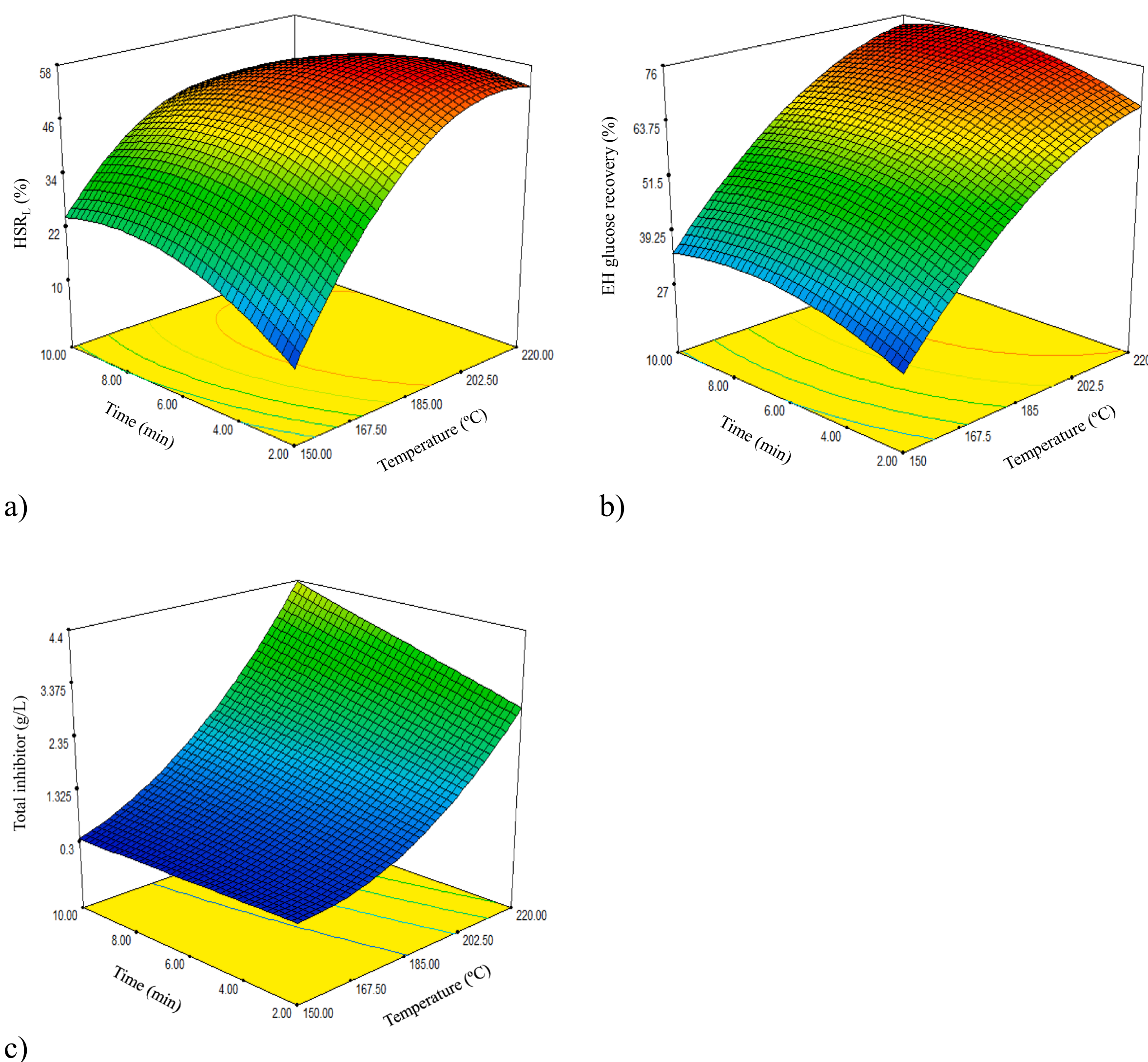

Fig. 1. Response surface plots for a) hemicellulosic sugars recovery (HSR$_L$), b) EH glucose recovery (referred to untreated BSG) and c) total inhibitor concentration in the liquid fraction as a function of pretreatment temperature and time.

worth mentioning that using the microwave assisted hydrothermal pretreatment under optimized conditions (192.7 °C, 5.4 min), along with the subsequent enzymatic hydrolysis, recovered 82% of the potential sugars contained in BSG (43 g of fermentable sugars from 100 g of BSG). Moreover, optimum microwave pretreatment time (5.4 min) is very short, which is one of the most important advantages of microwave in comparison with conventional heating (by convection or conduction) [13]. In this way, a pretreatment time of 30 min was necessary for the pretreatment of BSG with liquid hot water at 190 °C [40], acid hydrolysis at 121 °C and pH 1 using H_2SO_4 [20], or pretreating BSG by 1% (w/v) HCl or 3% (w/v) NaOH [38].

3.5. ABE fermentation of the pretreatment liquid and enzymatic hydrolysate obtained under optimal conditions

The pretreatment liquid and the enzymatic hydrolysate obtained under optimal pretreatment conditions were fermented with *C. beijerinckii* DSM 6422 for 120 h to analyze the fermentability of these streams. The enzymatic hydrolysis was carried out at a 10% (w/v) solid load to increase the fermentable sugars concentration. Although ABE fermentation was set at 120 h, the differences in sugar uptake and ABE production between 96 and 120 h were negligible (data not shown).

3.5.1. Fermentation of the pretreatment liquid

The fermentation of the pretreatment liquid (PL) reached butanol and ABE concentrations as low as 1.04 and 1.23 g/L, respectively (Fig. 2c). The low solvent concentrations could be due to the presence of toxic compounds. In order to reduce inhibitor compounds and so enhance ABE fermentability, the PL was detoxified by activated charcoal, which is a method with high effectiveness, low cost and high ability to remove inhibitors [44,45]. The sugar and inhibitor composition of the PL before and after detoxification are shown in Fig. 2a and b. Thus, as can be observed in Fig. 2b, a high reduction of furfural and phenols (100 and 59%, respectively) was achieved. Although the elimination of acetic acid was more limited (only 22%), suggesting a poor effectiveness of this method for organic acids reduction, the acetic acid concentration in the PL did not exceed the inhibitory limits reported in the literature [46]. This behavior has also been reported by other authors in the detoxification of hydrolysates from palm press fiber [47] and *Agave lechuguilla* [48]. The sugar loss as a consequence of detoxification was 13% (Fig. 2a). Regarding ABE fermentation, activated charcoal detoxified PL was fermented, resulting in 2.3 and 2.7 g/L butanol and ABE, respectively (Fig. 2c). As can be seen, the totality of sugars were not consumed by *C. beijerinckii* in any case (Fig. 2c), with sugar uptakes of 48% and 69% for raw PL and activated charcoal detoxified PL, respectively (Table 5). As a result, low yields of butanol

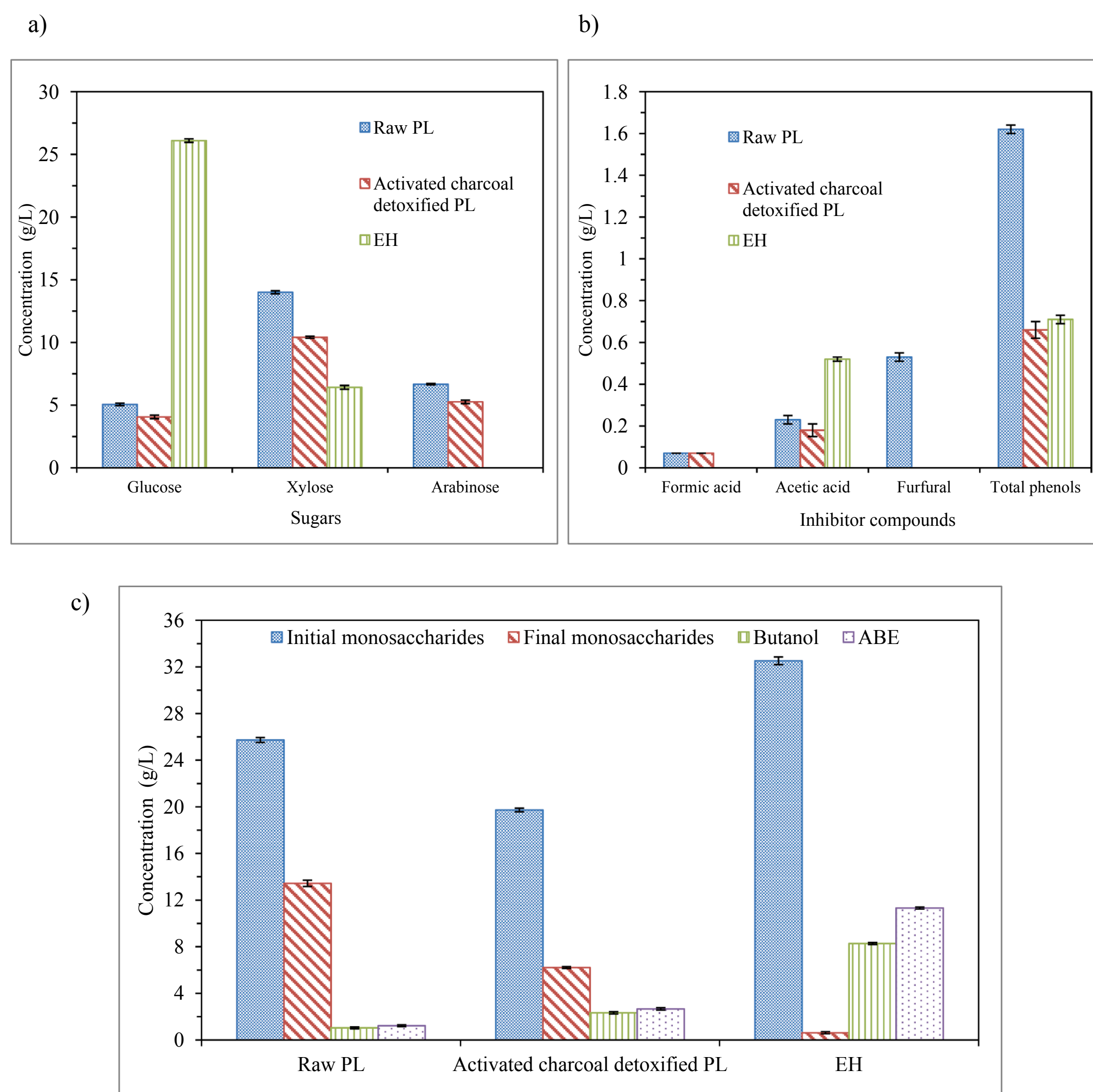

Fig. 2. Sugar (a) and inhibitor (b) concentrations (g/L) in the pretreatment liquid obtained under optimal conditions (PL), before and after activated charcoal detoxification, and in the enzymatic hydrolysate (EH) of the pretreated BSG obtained under optimal conditions. ABE fermentation (c) of PL before and after activated charcoal detoxification and EH.

(0.08 vs 0.17 g/g sugars consumed) and ABE (0.10 vs 0.20 g/g sugars consumed) were achieved for raw PL and activated charcoal detoxified PL, respectively (Table 5). The butanol and ABE productivities calculated were also low in both cases (< 0.037 g/L·h). The poor sugar consumption and butanol and ABE production can be due to the low initial sugar concentrations in hydrolysates (< 26 g/L). According to Survase et al. [49], the lack of sugar in the fermentation medium might lead to the accumulation of acids and, then, in the early ending of fermentation. As shown in Table 5, high concentrations of butyric acid (ranging from 3.1 to 4.2 g/L) were obtained in both cases. Butyric acid is produced during the acidogenic phase and should have been used to a greater extent during the solventogenic phase. Therefore, it is crucial for the fermentation that the medium has an excess of sugars.

This hypothesis was checked experimentally through the fermentation of the two fermentation mediums (raw PL and activated charcoal detoxified PL), but supplemented with 20 g/L glucose in all cases so as to ensure the presence of sugar excess. In this case, the two hydrolysates were successfully fermented, both detoxified and non-detoxified, higher sugar uptakes being achieved (82 and 87% for raw PL and activated charcoal detoxified PL, respectively), as well as higher concentrations

Table 5
ABE fermentation of the enzymatic hydrolysate and pretreatment liquid obtained under optimal conditions. Total sugar uptake (%), final acetic acid concentration (g/L), final butyric acid concentration (g/L), butanol and ABE yields ($Y_{BUT/sugars}$, $Y_{ABE/sugars}$ expressed as g/g sugars consumed), and butanol and ABE productivities (P_{BUT}, P_{ABE} expressed as g/L·h) at 96 h fermentation.

	Sugar uptake (%)	Acetic acid (g/L)	Butyric acid (g/L)	$Y_{BUT/sugars}$ (g/g)	$Y_{ABE/sugars}$ (g/g)	P_{BUT} (g/L·h)	P_{ABE} (g/L·h)
Pretreatment liquids							
Raw liquid	47.8 ± 1.3	1.7 ± 0.1	3.4 ± 0.2	0.08	0.10	0.011	0.013
Activated charcoal detox.	68.5 ± 0.7	1.1 ± 0.1	4.2 ± 0.1	0.17	0.20	0.032	0.037
Enzymatic hydrolysates							
Raw hydrolysate	98.1 ± 0.3	0.7 ± 0.0	0.9 ± 0.1	0.26	0.35	0.086	0.118

of butanol (7.3 and 6.9 g/L for raw PL and activated charcoal detoxified PL, respectively) and ABE (9.8 and 9.5 g/L for raw PL and activated charcoal detoxified PL, respectively). Moreover, butanol and ABE yields of about 0.20–0.21 and 0.27–0.28 g/g sugars consumed, respectively, were obtained. Therefore, in order to get higher butanol concentrations from PL fermentation, it is necessary to increase the concentration of sugars, which could be achieved by increasing the solid loading in pretreatment. The recovery of arabinoxylans could be another alternative for PL valorization. The concentrations of xylose and arabinose in PL are 14 g/L and 6.7 g/L, respectively, 72% of them as oligomers. The co-production of arabinoxylans with several marketable applications in food, feed, pharmaceutical industry could increase the profitability of butanol production from BSG [50].

The success of the non-detoxified PL fermentation after the addition of glucose reveals that the concentrations of organic acids, furfural and HMF in the hydrolysate did not interfere in ABE fermentation. Moreover, according to Rojas-Chamorro et al. [7], BSG has a high protein content (about 21%), which can be hydrolyzed as a consequence of pretreatment and, then, the microorganism might use it as a nitrogen source, enhancing the fermentation process. However, the PL phenols content (1.62 g/L, Fig. 2b) might be higher than the limit established by Bellido et al. [46], who pointed out that ABE production can be reduced by 30% as a consequence of phenol concentrations higher than 0.5 g gallic acid/L. Inhibition by phenolic compounds could explain the slightly better results obtained for activated charcoal detoxified PL, when not supplemented with glucose, as can be seen in Fig. 2c and Table 5 (for instance, butanol concentrations of 1.04 vs 2.34 g/L and butanol yields of 0.08 vs 0.17 g/g sugars consumed for raw PL and activated charcoal detoxified PL, respectively).

It is worth highlighting that, unlike most ethanol-producing microorganisms, a great amount of carbohydrates, such as cellobiose, starch, sucrose, dextrin, fructose, glucose, xylose, galactose, arabinose, mannose and fructose, can be consumed by butanol-producing *Clostridia*. Nevertheless, *C. beijerinckii* was able to consume hexoses more easily (with uptakes ranging from 75 to 96%), while the uptake of pentoses was much lower (< 61%). This behavior was also reported by Sarchami and Rehmann [51] on the ABE fermentation of the sulfuric hydrolysate of Jerusalem artichoke with *C. saccharobutylicum* DSM 13864.

Maiti et al. [52] fermented with *C. beijerinckii* NRRL B-466 brewery liquid waste hydrolysates and starch industry wastewater, determining slightly higher butanol and ABE yields (0.25 and 0.27 g/g sugars consumed, respectively). Higher butanol yields (0.35 g/g sugars consumed) were also reported recently by Kumar and Banerjee [53], who fermented with *C. beijerinckii* (ATCC 55025-E604) hydrolysates of bamboo obtained after pretreatment with laccases (as ligninolytic enzyme) and saccharolytic enzymes (cellulases and xylanases).

3.5.2. Fermentation of the enzymatic hydrolysate

Fig. 2c shows the monosaccharide consumption after ABE fermentation of the enzymatic hydrolysate (EH), highlighting that *C. beijerinckii* was able to consume all sugars in the EH (sugar uptake = 98.1%, Table 5). In addition, as can be seen in Fig. 2c, a butanol concentration of 8.27 g/L was achieved, which led to high butanol yields and productivities (0.26 g/g sugars consumed and 0.086 g/L·h, respectively) (Table 5). Detoxification was not necessary in this case, as the concentrations of toxic compounds (0.52 and 0.71 g/L of acetic acid and total phenols, respectively, Fig. 2b) were lower than the inhibitory limits for this type of microorganism [46]. Moreover, higher concentrations, yields and productivities of ABE (11.32 g/L, 0.35 g/g sugars consumed and 0.118 g/L·h, respectively) were obtained in comparison with the pretreatment liquid (Table 5). It is worth mentioning that the ABE solvents (acetone:butanol:ethanol) are usually generated in a 3:6:1 ratio [54]. On the contrary, no presence of ethanol was observed, which can be considered positively for the downstream. As was observed with the pretreatment liquids, *C. beijerinckii* showed greater preference for the consumption of glucose than xylose, exhausting firstly glucose and later xylose (data not shown). The overall production of ABE, calculated by mass balances, was 62 kg/t BSG, of which 46 kg/t were butanol. The production of butanol was lower than values previously reported [20] because fermentation of the pretreatment liquid produced a low concentration of butanol.

The butanol concentrations and yields obtained from EH were higher than those found recently by Hijosa-Valsero et al. [55], who fermented with *C. saccharobutylicum* DSM 13,864 the EH obtained from potato peel previously pretreated by autohydrolysis (140 °C, 56 min), yielding 7.6 g/L butanol (butanol yield, 0.186 g/g). A slightly higher butanol concentration and a similar butanol yield (9.5 g/L and 0.25 g/g) were achieved by Xing et al. [56] in the ABE fermentation by *C. saccharobutylicum* DSM 13,864 of EH from rice straw, which had previously been pretreated in two-stage (deep eutectic solvent and 1% Na_2CO_3).

4. Conclusions

Microwave assisted hydrothermal pretreatment is effective for fractionating BSG without using acid or alkali catalysts. Pretreatment optimal conditions were 192.7 °C and 5.4 min. Under these conditions, the recovery of potential sugars contained in BSG was 82% (43 g fermentable sugars/100 g BSG). The pretreatment liquid contained 25.7 g/L of sugars, of which 76.7% were in oligomeric form. The fermentation of the liquid fraction barely reached 1 g/L butanol due to the lack of fermentable sugars. For valorization of the liquid fraction by butanol production, higher solid loading in pretreatment should be performed. Other alternative of valorization could be the recovery of arabinoxylans with industrial applications. Butanol concentration from the enzymatic hydrolysate fermentation was 8.3 g/L which corresponds with an overall yield of 46 kg butanol/t BSG. In order to improve process performance, it is necessary to operate at higher solid loadings to achieve concentrated solutions of fermentable sugars and thus reduce the size of equipment. Under such conditions, the development of alternatives for detoxification of hydrolysates and coupled fermentation-separation strategies will be key to increase butanol overall production.

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