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Efficient biobutanol production by ABE fermentation from spent coffee grounds with microwave assisted dilute sulfuric acid pretreatment

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Corresponding Author:	Susana Lucas, Ph. D. Universidad de Valladolid Escuela de Ingenierias Industriales Valladolid, Valladolid SPAIN
First Author:	Juan Carlos López-Linares
Order of Authors:	Juan Carlos López-Linares
	María Teresa García-Cubero
	Mónica Coca
	Susana Lucas, Ph. D.
Abstract:	The integral valorization of potential sugars (cellulosic and hemicellulosic) from spent coffee grounds (SCG), a lignocellulosic residue, is proposed in this work. With this aim, the microwave assisted dilute sulfuric acid pretreatment has been optimized, leading to a hemicellulosic sugar recovery in the pretreatment liquid (HSR L) and an enzymatic hydrolysis yield of 79 and 98%, respectively, at 160.47 ºC and 1.5% H 2 SO 4 . Moreover, the complete digestibility of cellulose (enzymatic hydrolysis yield = 100%) was also discovered for non-pretreated SCG, which is very interesting. Secondly, the production of biobutanol, an advanced biofuel, is also proposed from pretreated SCG enzymatic hydrolysate and pretreatment liquid achieved under optimal conditions. These were fermented by Clostridium beijerinckii , yielding 95 kg butanol/t SCG (dry matter) and 151 kg acetone-butanol-ethanol/t SCG (dry matter).

Efficient biobutanol production by acetone-butanol-ethanol fermentation from spent coffee grounds with microwave assisted dilute sulfuric acid pretreatment

Juan C. López-Linares[a,b], María Teresa García-Cubero[a,b], Mónica Coca[a,b], Susana Lucas[a,b,*]

[a]Institute of Sustainable Processes. Dr. Mergelina s/n, 47011, Valladolid,Spain

[b]Department of Chemical Engineering and Environmental Technology, School of Industrial Engineering, University of Valladolid, Dr. Mergelina, s/n, 47011, Valladolid, Spain

ABSTRACT

The integral valorization of potential sugars (cellulosic and hemicellulosic) from spent coffee grounds (SCG), a lignocellulosic residue, is proposed in this work. With this aim, the microwave assisted dilute sulfuric acid pretreatment has been optimized, leading to a hemicellulosic sugar recovery in the pretreatment liquid (HSR_L) and an enzymatic hydrolysis yield of 79 and 98%, respectively, at 160.47 ºC and 1.5% H_2SO_4. Moreover, the complete digestibility of cellulose (enzymatic hydrolysis yield = 100%) was also discovered for non-pretreated SCG, which is very interesting. Secondly, the production of biobutanol, an advanced biofuel, is also proposed from pretreated SCG enzymatic hydrolysate and pretreatment liquid achieved under optimal conditions. These were fermented by *Clostridium beijerinckii*, yielding 95 kg butanol/t SCG (dry matter) and 151 kg acetone-butanol-ethanol/t SCG (dry matter).

Keywords: spent coffee grounds; microwave assisted dilute acid pretreatment; biobutanol; gas stripping; fed-batch mode; *Clostridium beijerinckii*.

*Corresponding author. Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n, 47011, Valladolid (Spain).
E-mail address: susana@iq.uva.es (Susana Lucas)

1. Introduction

Coffee is a crucial agricultural product, as well as one of the most consumed beverages in the world (Karmee, 2018). According to Ramalakshmi et al.(2009), about 50% of coffee grown across the world is assigned to the production of beverages. In fact, it is the second most commercialized drink worldwide (Buratti et al., 2018), with a production of 152 million/year (Janissen and Huynh, 2018). During the transformation of coffee beans (processing, roasting, and generation of the beverage), huge quantities of residues are generated, such as the husk, pulp, coffee silver skin and spent coffee grounds (SCG) (Karmee, 2018; Kovalcik et al., 2018). In this way, it is worth highlighting the generation of SCG, obtaining 650 kg/t green coffee beans (Murthy and Madhava Naidu, 2012). In addition, when the soluble coffee is prepared, around 2 kg of wet SCG are produced per kg of soluble coffee (Pfluger, 1975). Thus, taking into account the high availability of SCG, as well as its important sugar content, this lignocellulosic residue turns out to be an interesting valuable resource, as it could be used to produce such renewable biofuels as biobutanol (McNutt and He, 2019).

In recent years, biobutanol has become increasingly interesting, since it is considered an important industrial chemical and advanced biofuel. Moreover, it can be generated through biological processes employing lignocellulosic residues as the raw material (Maiti et al., 2018). Furthermore, biobutanol has some excellent properties; for instance, it has a low corrosivity, great energy power (higher than ethanol and similar to gasoline), as well as a low vapor pressure (lower than ethanol), making its handling much safer. In addition, current engines are perfectly able to use this biofuel. Thus, the substitution of gasoline with biobutanol could well be proposed (Lee et al., 2008). On the other hand, biobutanol also has a great many applications as a chemical commodity; for example, in enamels, pharmaceuticals, antibiotics, or the food industry, among

others (Satari et al., 2019).

There are three fundamental steps in the biological production of biobutanol: pretreatment, enzymatic hydrolysis and ABE anaerobic fermentation using *Clostridia* strains. Of these, the pretreatment is the crucial stage, as it breaks down the complex structure of the lignocellulosic biomass, overcoming its recalcitrance, separates the hemicellulose and lignin, and decreases the cellulose crystallinity, thus facilitating the following process of enzymatic hydrolysis. The literature reports a wide variety of pretreatments; for instance, steam explosion, liquid hot water, dilute acid, alkaline or microwave (Kumar et al., 2020). One of the most relevant pretreatments is the dilute acid pretreatment, since it obtains high hemicellulose recoveries (85-95%); pretreated solids with high cellulose content, which can be easily hydrolyzed by enzymes; and it is also viable economically and can be applied profitably on an industrial scale (Kumar et al., 2020; Zheng et al., 2014). On the contrary, one of the main drawbacks of this type of pretreatment is the production of inhibitory compounds, such as acetic and formic acids, furfural, 5-hydroxymethylfurfural (HMF) and phenolic compounds, as a consequence of the high temperatures used in the process (Chaturvedi and Verma, 2013). This type of undesirable compounds for the fermentation could be reduced using different detoxification techniques, such as activated charcoal, ion-exchange resins or overliming, among many others (Kim, 2018).On the other hand, the microwave technique can also assist the dilute acid pretreatment, which is highly beneficial in improving the heating homogeneity and reducing the extraction times; it is also able to enhance the efficiency of the process (Santana et al., 2018).

Nowadays, regarding ABE fermentation, low biobutanol concentrations are generated in this stage, probably due to the end-product cell inhibition (biobutanol), which means that high energy amounts are consumed, thus making the process

economically unprofitable (Pérez-Bibbins et al., 2018). In order to solve this problem, diverse strategies, such as the use of new microorganisms able to put up with high butanol levels (Huang et al., 2014), or the employment of different butanol extraction methods, such as gas stripping, adsorption, pervaporation or liquid-liquid extraction (Pérez-Bibbins et al., 2018), have been studied. Considering butanol extraction methods, gas stripping is considered one of the most suitable for in situ product recovery due to its simple handling, suitability for the cell, low energy requirement and high profitability (Chen et al., 2019; Rochón et al., 2019).

The objective of this work was to evaluate the carbohydrate recovery from SCG using a microwave assisted dilute sulfuric acid pretreatment, as well as the biological transformation of these into biobutanol. With this aim, firstly, the effect of two different factors (temperature and sulfuric acid concentration) on the acid pretreatment were studied through a response surface methodology, the optimization criteria used being the simultaneous maximization of the total sugar recovery from hemicellulose (liquid fraction) and cellulose (solid fraction). Secondly, the raw SCG and the SCG achieved under optimal pretreatment conditions were enzymatically hydrolyzed at a high biomass loading (15% w/v) in order to obtain a solution rich in sugars, which could be butanol fermented by ABE fermentation using *Clostridium beijerinckii* DSM 6422. Likewise, the pretreatment liquid obtained under optimal pretreatment conditions was also fermented to butanol. Finally, in order to remove, in situ, the fermentation end-product generated and then to further improve butanol production, a fermentation process with the enzymatic hydrolysate of SCG achieved under optimal pretreatment conditions was performed in a bioreactor coupled to a gas stripping system, the pretreatment liquid obtained under optimal pretreatment conditions was used as the feed. Although only a few references to butanol production from coffee silverskin (Hijosa-Valsero et al., 2018;

Niglio et al., 2019; Procentese et al., 2018) were found, there were none which used SCG to generate butanol. Moreover, to the best of our knowledge, this is the first work on butanol production from SCG using ABE fermentation in fed-batch mode.

2. Materials and methods

2.1. Raw material

Spent coffee grounds (SCG) were kindly supplied by the coffee industry (Productos Solubles S.A., Palencia). They were dried at 50 °C (moisture content< 1%), milled with a coffee grinder (Moulinex, A505, France) (particle size < 1 mm) and homogenized, being stored at 4 °C until use. The composition was (% w/w): cellulose, 16.3 ± 0.1; hemicellulose, 27.7 ± 0.7 (mannose, 31.3 ± 0.8; galactose and arabinose monomers were not detected); acid-insoluble lignin (AIL), 38.5 ± 0.7; acid-soluble lignin (ASL), 0.7 ± 0.1; extractives, 12.4 ± 0.4 (mannose in extractives, 0.2 ± 0.0); ash, 0.1 ± 0.0; acetyl groups, 0.4 ± 0.0; fat, 9.9 ± 0.16; and protein, 9.4 ± 0.7.

2.2. Microwave assisted dilute sulfuric acid pretreatment

The SCG were pretreated in a Multiwave PRO SOLV reactor 50 Hz with Rotor type 16HF100 (Anton Paar GmbH, Austria, Europe). A total of 16 sample vessels can be used, its capacity volume being 100 mL. The temperature monitoring was carried out with a pressure/internal temperature sensor. Although this monitoring can only be done in one of the vessels, the reactor contains an IR sensor, which is able to measure the temperature of 16 vessels along the runs (for more details, see López-Linares et al. (2019)).

Five grams dry weight SCG and 50 mL sulfuric acid solution was used (10% w/v solid-liquid ratio). The pretreatment time was initiated when each run attained the required temperature, later being cooled to 50 ºC.

Once the pretreatment had finished, the solid and liquid fractions were separated by vacuum filtration. Then, the solid fraction was washed, dried at 40 ºC and weighed, as well as being used in enzymatic hydrolysis essays. The solid recovery (g solid fraction/100 g SCG) was calculated for each experimental run. In addition, the optimal pretreated solid was analyzed for its composition in structural carbohydrates, lignin, ash, and protein. The liquid fractions were also measured, considering the content in monosaccharides and degradation products (formic acid, acetic acid, furfural, hydroxymethylfurfural (HMF) and total phenols). In order to evaluate the effect of the microwave assisted dilute sulfuric acid pretreatment, the recovery of carbohydrates in liquid fractions was calculated as a percentage of the sugar content in the unpretreated SCG.

2.3. Experimental design

In order to choose the optimum conditions for the microwave assisted dilute sulfuric acid pretreatment of SCG, a central composite experimental design was proposed (α = 1.414). The factors selected were temperature (150-190 ºC) and sulfuric acid concentration (0.5-1.5% w/v), while the pretreatment time was fixed at 5 min, all of which were chosen on the basis of previous results (data not shown). In this way, a design with 13 experiments was proposed, with one point and four replicates at the center of the domain selected for each factor under study. Table 1 displays the values of each factor, in both coded and uncoded terms. The commercial software Statgraphics Centurion XVIII was the tool used for design and statistical assesment of experiments.

In order to study the strictness of the pretreatment, the Severity Factor (SF) and the Combined Severity Factor (CSF) were calculated, according to MacAskill et al. (2018) (Eqs. (1) and (2)).

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

$$\text{Combined Severity Factor (CSF)} = \text{SF} - \text{pH} \quad (2)$$

where t is time (min), T is temperature (ºC) and the pH is that of the initial sulfuric acid solution used in each run.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis (EH) tests were carried out in triplicate in an orbital shaker (Comecta Optic Ivymen system) at 50 ºC, 150 rpm, 72 h, pH 4.8 and enzyme load of 15 Filter Paper Units (FPU)/g solid, using the pretreated solids attained in each experimental run as substrate (including the non-pretreated and optimal pretreated SCG). 1.25 g of pretreated SCG and 25 mL enzymatic solution were used (5% w/v substrate loading), employing 100 mL Erlenmeyer flasks. A cellulolytic complex, called Cellic CTec2, was the enzyme used, kindly provided by Novozymes A/S (Denmark), and 0.05 M sodium citrate as buffer.

Samples were withdrawn, centrifuged and analyzed for their monosaccharide content. Furthermore, enzyme blanks were also included to discount the monosaccharides contained in the commercial enzymes. The EH yield was determined as the quotient of the grams of glucose obtained by EH and the glucose content in the untreated SCG.

On the other hand, in order to achieve a solution rich in sugars, the unpretreated and optimal pretreated SCG were enzymatically hydrolyzed in a 2 L Labfors 5 BioEtOH

(Biogen Scientific, Spain). The EH conditions used were the same as those described above, but at 80 rpm, with 15% w/v substrate loading (150 g substrate and 1000 mL enzymatic solution) and using water as the solvent (adjusted to pH 4.8 with solid NaOH) instead of sodium citrate buffer. Once the EH had finished, the enzymatic hydrolysate was separated from the residual SCG by vacuum filtration, analyzed for its monosaccharide and degradation products content, and then employed as fermentation medium in ABE fermentation with *C. beijerinckii*.

2.5. Microorganism

C. beijerinckii DSM 6422, obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany), was used in the ABE fermentation. It was maintained and grown according to Plaza et al. (2017). However, 100 mL penicillin flasks with rubber septum and 50 mL Reinforced Clostridial Medium (RCM) were used in this case. Two thermal shocks (for 2 min) were also performed to stimulate the germination of the spores. The inoculum was grown in an orbital shaker (Comecta Optic Ivymen system) at 35 ºC, 135 rpm over 48 h.

2.6. ABE fermentation

The enzymatic hydrolysates (from both unpretreated and optimal pretreated SCG), as well as the liquid fraction of the optimal pretreatment, were subjected to ABE fermentation with *C. beijerinckii*. The ABE fermentation tests were performed in 100 mL serum bottles with rubber septum under anaerobic conditions (flushing O_2 free nitrogen into the liquid), at 35 ºC and 135 rpm. Vitamin, salt and acetate buffer solutions were used under the same conditions as described by López-Linares et al.

(2020). The inoculum loading used was 10% (v/v), the initial pH of the fermentation being 5.5, without control during the fermentation.

On the other hand, the enzymatic hydrolysate from optimal pretreated SCG was also subjected to ABE fermentation in a bioreactor coupled to a gas stripping system, in fed batch mode. The fermentation test was carried out in a Biostat B Plus reactor (Sartorius®) at 35 ºC and 50 rpm, under the conditions described previously for serum bottles, but using 550 mL of fermentation medium. The gas stripping process, as well as the fed-batch mode, was started at 24 h of fermentation to mitigate the butanol inhibition, and it was maintained until 168 h. A flow rate of 0.8 vvm was used in the gas stripping system, recycling the off-gases (such as CO_2 and H_2) through the system. With this objective, a twin-head Masterflex ® peristaltic pump and size 18 Tygon pump tubing (Cole-Parmer,Vernon Hills, U.S.A.) were used, the ABE vapors being cooled in a condenser using a glycerol-water (30% (v/v) solution, which was kept at 0 ºC through a refrigerated circulating bath (Fisher scientific, Pittsburgh, U.S.A.). In order to collect the condensate, a 250 mL flask was submerged in the refrigerated circulating bath. The fed-batch mode was performed using the liquid fraction of the optimal pretreatment, employing a feed flow of 14.5 mL/h, which was introduced using a peristaltic pump (Watson Marlow, Cornwall, UK).

The fermentation tests were performed in triplicate. Samples were taken in triplicate during the fermentation tests, centrifuged and analyzed to determine the sugar consumption (glucose and mannose) and acetone, butanol and ethanol production.

2.7. Analytical methods

The structural carbohydrate, lignin and ash content of the optimal pretreated SCG were analyzed by the analytical methods of the National Renewable Energy Laboratory

(NREL) (Sluiter et al., 2011, 2008); while its total protein content was determined using the Kjeldahl acid digestion method described in AOAC (1990) 955.04, a conversion factor of 6.25 being applied.

High Performance Liquid Chromatography (HPLC) was the analytic technique used to measure sugars (glucose, mannose, arabinose and galactose), degradation products (acetic and formic acids, furfural and HMF), ABE solvents (acetone, butanol and ethanol) and other organic acids (lactic and butyric acids). A refractive index detector (Waters 2414) and an Aminex HPX-87H column (at 30 °C (solvents) or 60 °C (sugars, organic acids, furfural and HMF)) were employed, with 0.01 N H_2SO_4 (0.6 mL/min) as mobile phase. The Aminex HPX-87P column, at 80 °C with ultrapure water (0.6 mL/min) as mobile phase, was used for the determination of galactose. Previously, all samples were centrifuged (at 13400 rpm for 10 min) and filtered (by 0.2 μm nylon filters).

In order to measure the oligomeric sugar contained in the liquid fractions of pretreatment, an acid hydrolysis (120 °C, 3% w/v H_2SO_4, 30 min) was performed. The oligomeric sugar content was calculated as the subtraction of the total free sugars before and after acid hydrolysis.

The Folin-Ciocalteu method (Singleton and Rossi, 1965), using gallic acid as standard, was used to determine the total phenols content. All analytical determinations were carried out in triplicate and the average results are shown.

3. Results and discussion

3.1. Effect of microwave assisted dilute sulfuric acid pretreatment on SCG solubilization

A microwave assisted dilute sulfuric acid pretreatment was proposed to solubilize hemicellulosic sugars from the SCG while the cellulose is remained in the pretreated SCG. The influence of the pretreatment on the SCG was evaluated using the CSF parameter, which is typically used in the study of acid pretreatments (MacAskill et al., 2018) since it looks at the combination of temperature, time and sulfuric acid concentration.

Table 1 displays the solid recoveries (SR) achieved for each experimental run. As can be seen, in general, a decrease in the solid recovery was observed by increasing the CSF parameter. In this way, solid recoveries ranged from 54% (CSF=2.57, run 11) to 90% (CSF=0.83, run 4), corresponding to the highest and lowest severity factors, respectively. Considering the center of the domain (runs 1, 2, 3, 8 and 12), solid recovery rates of 69-73% were attained (170 ºC and 1% H_2SO_4).

pH values measured in the liquid fractions of pretreatment, as well as concentrations and recoveries (GR_L, HSR_L) of carbohydrates achieved in these fractions, are also shown in Table 1. So, the pH ranged between 0.91 (run 13) and 1.5 (run 5), which corresponds to the highest and the lowest sulfuric acid concentrations (1.71 and 0.29%), respectively. Regarding the carbohydrates content, a total sugar concentration ranging between 5.8 g/L (CSF=0.83, run 4) and 29.0 g/L (CSF=1.95, run 13) was achieved, the largest quantity being in monomeric form (oligomeric sugars< 10.8%). In this way, only when essayed pretreatment conditions were soft (runs 4 and 6) was a considerable oligomeric sugar content detected (6.9 and 10.8%, respectively). Glucose was detected in all the obtained liquid fractions of the pretreatment, which increased with the severity factor, reaching values of 9.6 g/L (GR_L = 53.5%) at the highest severity conditions essayed (CSF=2.57, run 11). This glucose content obtained in the liquid fractions of the pretreatment probably came from the solubilization of the amorphous cellulose fraction

contained in the SCG (Romero et al., 2018). Nevertheless, mannose was the main sugar measured in the liquid fractions of the pretreatment (Table 1), with concentrations ranging between 5.3 (CSF=0.83, run 4) and 25.2 g/L (CSF=1.95, run 13). Hemicellulosic sugar recoveries in the liquid fraction (HSR_L) followed the same trend as described above for mannose concentrations, the highest value also being attained for run 13 (HSR_L = 80.7%). On the contrary, for CSF > 1.95 (runs 7, 9 and 11), mannose concentrations and hemicellulosic sugar recoveries reduced because of the hemicellulosic sugar degradation reactions. Likewise, at low pretreatment severity (CSF < 1, runs 4 and 6), very low hemicellulosic sugar recoveries were also obtained (< 20%) due to the use of pretreatment conditions that are too mild to solubilize the hemicellulose from the SCG.

The concentration of inhibitor compounds (acetic and formic acids, furfural, HMF and phenolic compounds) generated for each experimental run as a result of the pretreatment process are shown in Table 2. It is worth mentioning that the type and concentration of inhibitors originated depend on the type of pretreatment as well as its severity, being toxic for subsequent fermentation processes (Rajendran et al., 2018). As can be seen in Table 2, in general, except for the highest pretreatment severity (CSF > 2; runs 7, 9 and 11), the concentration of inhibitor compounds is low (< 0.9 g/L), or even not detected. Low concentrations of inhibitor compounds were also found by Juarez et al. (2018) after dilute acid hydrolysis of this same lignocellulosic biomass (SCG). Regarding acetic acid (originated by hydrolysis of acetyl groups) (Larsson, 2000), its concentration was negligible in all experimental runs carried out (< 0.2 g/L). However, more considerable values of HMF (from the degradation of glucose) and total phenols (from extractives and lignin degradation) (Larsson, 2000) (1.4 and 1.7 g/L, respectively) were reached for the most severe conditions of pretreatment (CSF = 2.57,

run 11). Concentrations up to 3 g/L were also detected for formic acid (from HMF and furfural) (Larsson, 2000), also at the highest severity factor (run 11), this being the inhibitor compound found in higher concentrations. It is worth mentioning that furfural was not detected in any experimental run, as it originates from the degradation of pentoses and these are not contained in SCG, unlike other type of lignocellulosic residues such as brewer's spent grains (López-Linares et al., 2019), rapeseed straw (Romero et al., 2018) or olive tree biomass (Martínez-Patiño et al., 2018).

3.2. Enzymatic hydrolysis of pretreated SCG

In order to evaluate the influence of the microwave assisted dilute sulfuric acid pretreatment on SCG, the solids resulting from the pretreatment in each experimental run were enzymatically hydrolyzed.

In this way, the enzymatic hydrolysis of pretreated SCG resulted in hydrolysates with glucose and mannose concentrations ranging from 4.5 to 12 g/L and 0 to 1 g/L, respectively (Table 3). As can be seen, except for the highest pretreatment severity assayed (CSF > 2.01; runs 9 and 11), very similar glucose concentrations (10-12 g/L) were achieved in all experimental runs carried out. In addition, no mannose was detected for these high pretreatment severities. Table 3 also shows the values of the EH yield attained for each experimental run. As can be appreciated, a complete or almost complete digestibility of cellulose (99-100%) was achieved for CSF < 1.3 (runs 4, 6, 5 and 10), which were the lowest pretreatment severities assayed. In this context, it is worth highlighting that, as can be seen in Table 3, the enzymatic hydrolysis test was also performed directly using the non-pretreated SCG, the complete digestibility of the cellulose being achieved (EH yield = 100%). This behavior could be due to the fact that the SCG has a sufficiently broken and disordered structure and cellulose with a

sufficiently low crystallinity to allow the access of the enzymes without requiring a previous pretreatment step, unlike what happens with most lignocellulosic residues reported in the literature, with such materials as brewer's spent grains (López-Linares et al., 2019), sugarcane bagasse (Gomes et al., 2020), hybrid *Pennisetum* (Wang et al., 2020) or poplar (Chu et al., 2019). The morphological changes obtained by SEM analysis on SCG before and after microwave assisted dilute sulfuric acid pretreatment, supports this hypothesis, since the same morphological structure can be observed on the SCG before and after pretreatment (supplementary data). On the other hand, the EH yield decreased when the most severe pretreatment conditions were used (CSF > 1.74), reaching EH yields as low as 27% for the highest pretreatment severity assayed (CSF = 2.57; run 11), which could be because the degradation of glucose takes place simultaneously with the hydrolysis of polysaccharides (Mussatto et al., 2011; Díaz-Blanco et al., 2018). Therefore, the main objective of using the microwave assisted dilute sulfuric acid pretreatment in this work was the recovery of the hemicellulosic sugars contained in SCG (27.7%) in the resulting pretreatment liquid, which cannot be recovered effectively by enzymatic hydrolysis.

Concerning *overall sugar recovery* (Table 3), which is a parameter that considers both sugars hydrolyzed in the pretreatment liquids, as well as glucose and mannose contained in the enzymatic hydrolysates (referred to total sugars contained in unpretreated SCG), ranged between 48.9% (run 11, CSF = 2.57) and 90.4% (run 13, CSF = 1.95). It is worth mentioning that this parameter was proposed in order to assess the global efficiency of the microwave assisted dilute sulfuric acid pretreatment. As can be appreciated, even though the lowest overall sugar recovery (52.3%) was achieved for the lowest pretreatment severity (CSF = 0.83, run 4), the highest value (90.4%) was obtained at 170 ºC and 1.71% H_2SO_4 (run 13, CSF = 1.95). High overall sugar

recoveries (83-86.6%) were also attained at the central point (170 ºC and 1% H_2SO_4). Thus, in conclusion, a percentage as high as 90% of the potential sugars contained in SCG can be recovered using the microwave assisted dilute sulfuric acid pretreatment.

3.3. Optimization of the microwave assisted dilute sulfuric acid pretreatment

The microwave assisted dilute sulfuric acid pretreatment of SCG was assessed considering the generation of sugars from hemicellulose and cellulose fractions, with two independent variables (temperature and acid concentration) being varied for this purpose. Therefore, the hemicellulosic sugar recovery in the pretreatment liquid (HSR_L) and the EH yield were selected as responses. From the experimental results of the HSR_L (Table 1) and the EH yield (Table 3), quadratic models with interaction between the factors were obtained in terms of coded factors (Eqs. (3) and (4), respectively):

$$HSR_L = 71.50 + 5.44\,T + 6.24C - 20.05\,T\,C - 23.03\,T^2 \quad (3)$$

(R^2 = 0.9928; R^2 adjust = 0.9880)

$$\text{EH yield} = 92.35 - 25.50\,T - 4.90C - 7.70\,T\,C - 14.42\,T^2 \quad (4)$$

(R^2 = 0.9989; R^2 adjust = 0.9982)

where T is the temperature (°C) and C is the sulfuric acid concentration (%, w/v). As the values of R^2 and adjusted R^2 (Eqs. (3) and (4)) are displayed, as well as the confidence level (95%, $p < 0.05$); a good agreement between the experimental and predicted values was achieved for both responses.

Regarding the HSR_L, as can be seen in Eq. (3), both temperature and sulfuric acid concentration factors have a positive influence, the effect of the sulfuric acid concentration being higher. On the other hand, a significant negative interaction between the temperature and acid concentration was also appreciated, demonstrating that the combined effect of both temperature and acid concentration can lead to a reduction in the HSR_L. This could be due to an incomplete hemicellulose hydrolysis or

sugar degradation at very soft or severe pretreatment conditions, respectively. This same trend can also be seen in Fig. 1a, which is the response surface plot for the HSR_L response, considering the temperature and sulfuric acid concentration factors.

Concerning the EH yield response (Eq. (4)), both temperature and sulfuric acid concentration factors negatively affected this response, the influence of the temperature being much higher than the acid concentration. In addition, a considerable negative interaction between temperature and acid concentration was also discovered. This behavior, which can also be seen in Fig. 1b, is in agreement with the complete digestibility of cellulose (99-100%) described previously (*Section 3.2*) for unpretreated SCG and for pretreated SCG with soft pretreatment conditions (CSF < 1.3, runs 4, 6, 5 and 10).

On the other hand, as the purpose of the microwave assisted dilute sulfuric acid pretreatment of SCG was the recovery of the highest possible amount of sugars contained in this lignocellulosic residue, both the HSR_L and EH yield responses were maximized simultaneously using the so-called desirability function (Bukzem et al., 2016). In this way, the optimal conditions found by the model were 160.47 ºC and 1.5% H_2SO_4, predicting values for the HSR_L and EH yield of 79.5 and 100%, respectively. Then, in order to validate the model, the optimal conditions were experimentally reproduced, yielding experimental values for the two responses studied (HSR_L and EH yield) of 79 and 98%, respectively. So, by comparing the experimental and predicted values, differences lower than 1% were detected for both responses, which demonstrates the high reliability of the optimization process carried out in this study. What is more, the pretreatment carried out under optimal conditions led to a cellulose-enriched solid (20.5 vs 16.3% in unpretreated SCG), which still contained a considerable mannans content (9%). Likewise, a pretreatment liquid with a concentration of monomeric sugars

as high as figure g/L was achieved (glucose, 2.9 g/L; mannose, 24.7 g/L), its level of inhibitor compounds being low at 1.1 g/L (HMF, 0.4 g/L; formic acid, 0.2 g/L; acetic acid, 0.2 g/L; total phenols, 0.3 g/L).

Finally, it can be concluded that, considering both sugars hydrolysed in the pretreatment liquids as well as glucose and mannose contained in the enzymatic hydrolysates, an overall sugar recovery of 93% was achieved under optimal conditions of pretreatment. This result is also comparable to those reported by other authors using dilute sulfuric acid pretreatment. Thus, for instance, López-Linares et al. (2020) recovered 87% of the carbohydrates contained in brewer's spent grain using this type of pretreatment. Rojas-Chamorro et al. (2020) also reached total sugars recoveries as high as 94% (referred to sugar content in raw biomass) from brewer's spent grain via the dilute sulfuric acid pretreatment.

3.4. Batch fermentation from SCG hydrolysates

In order to obtain a sugar solution with a concentration high enough to be fermented to butanol, the unpretreated and optimal pretreated SCG were enzymatically hydrolyzed using a substrate loading as high as 15% w/v and Cellic CTec2 (a cellulolytic complex). In this way, enzymatic hydrolysates with a sugar concentration as high as 39.1 g/L (glucose, 33.2 g/L; mannose, 5.9 g/L) and 37.5 g/L (glucose, 36.2 g/L; mannose, 1.3 g/L) were achieved from unpretreated and optimal pretreated SCG, respectively. These were used as the initial ABE fermentation broth. Moreover, the optimal pretreatment liquid, which unlike enzymatic hydrolysates was formed mainly by hemicellulosic sugars, was also subjected to ABE fermentation with *C. beijerinckii.* The levels of toxic compounds to fermentation were not high, and nor were the enzymatic hydrolysates or optimal pretreatment liquid (< 0.4 g/L, mainly total phenols).

Regarding the ABE fermentation, Fig. 2 shows the initial and final monosaccharide concentrations, as well as the butanol and ABE concentrations for enzymatic hydrolysates and optimal pretreatment liquid. As can be seen in the three cases, almost all the sugars were consumed by *C. beijerinckii* at 48 h fermentation (sugar uptakes = 99.2-99.7%) (Table 4). As can be seen in Fig. 2, butanol concentrations of 7.7 and 6.7 g/L were achieved for unpretreated and optimal pretreated SCG enzymatic hydrolysates, respectively, which correspond to high butanol yields (0.23 and 0.21g/g sugars consumed, respectively) and productivities (0.160 and 0.140 g/L·h, respectively) (Table 4). High ABE concentrations were also yielded for both enzymatic hydrolysates (11.4 and 10.4 g/L ABE, respectively), resulting in ABE yields of 0.34 and 0.33g/g sugars consumed, respectively, and ABE productivities of 0.238 and 0.216 g/L·h, respectively. On the other hand, the fermentation of the optimal pretreatment liquid also led to high butanol and ABE yields (0.20 and 0.33 g/g sugars consumed, respectively), similar to those obtained for enzymatic hydrolysates. However, productivities obtained in this case were lower (0.074 and 0.121 g/L· h, respectively), probably due to slightly higher inhibitor levels found in the optimal pretreatment liquid (mainly of HMF, increasing the synergistic effect originated in this case). Butanol and ABE concentrations of 3.6 and 5.8 g/L, respectively, were attained in this case. Therefore, considering the enzymatic hydrolysate and pretreatment liquid resulting from the SCG pretreatment under optimal conditions, 95 kg butanol/t $SCG_{(DM)}$ (dry matter) and 151 kg ABE/t $SCG_{(DM)}$ can be attained, thus highlighting the usefulness of pretreating the SCG to recover the hemicellulose fraction contained in the SCG. Furthermore, no detoxification was necessary in any of the fermentations carried out.

Some examples of fermentation of enzymatic hydrolysate and pretreatment liquid reported from different lignocellulosic biomass and by different butanol producing

microorganisms were collected and analyzed (data was shown in supplementary material). As can be observed, the sulfuric pretreatment was the most used, mainly in low concentrations. In this way, as can be seen, the bioconversion of these hydrolysates in ABE fermentation processes resulted in ABE concentrations ranging from 6.7 g/L to 11.8 g/L. Nguyen et al. (2019) reported 8.5 g/L ABE from green macroalgae *Enteromorpha intestinalis* by fermentation with *C. acetobutylicum*, when both cellulosic and hemicellulosic fractions after pretreatment with sulfuric acid (121 ºC, 60 min, 270 mM H_2SO_4) were used. López-Linares et al. (2020), who pretreated brewer's spent grain with dilute sulfuric acid (147 ºC, 2 min, 1.26% H_2SO_4), also using the microwave technique and fermenting with *C. beijerinckii* both cellulosic and hemicellulosic fractions, after detoxification with activated charcoal, were able to achieve butanol and ABE concentrations of 8.2 and 11.8 g/L, respectively. Nimbalkar et al. (2017) reached a butanol production of 4.4 g/L with a total ABE of 6.7 g/L from the batch fermentation with C. *acetobutylicum* of detoxified press mud slurry pretreated with sulfuric acid at 121ºC, 15 min and 1.5% H_2SO_4.

Therefore, the results obtained in this work, considering both enzymatic hydrolysate (6.7 g/L butanol and 10.4 g/L ABE) and pretreatment liquid (3.6 g/L butanol and 5.8 g/L ABE), are comparable to those reported with other biomasses, even with the same microorganism. Furthermore, it is worth highlighting that the results attained in this work were favorable compared to those reported by Hijosa-Valsero et al. (2018) also with a coffee residue (coffee silverskin). These authors attained butanol and ABE concentrations as low as 7.0 and 11.4 g/L, respectively, but a slightly higher butanol yield (0.27 g/g), when fermenting the slurry enzymatic hydrolysate of coffee silverskin pretreated by autohydrolysis (170 °C, 20 min) with *C. beijerinckii*.

On the other hand, in order to increase the butanol production, while mitigating the butanol inhibition at the same time, a fed-batch fermentation system with in situ gas stripping was proposed. The initial fermentation broth used was the enzymatic hydrolysate resulting from SCG pretreated under optimal conditions, while the fed-batch mode was performed using the optimal pretreatment liquid. Fig. 3 displays the (a) sugars (glucose and mannose) and (b) products (butanol, ethanol and acetone) profiles through the fermentation. As can be seen, the total amount of sugars were consumed by *C. beijerinckii* at 22 h of fermentation. However, when the fed-batch mode was started (at 22 h fermentation), although the glucose continued to be consumed in its entirety, a progressive accumulation of mannose was observed during the fermentation. Likewise, the total solvents originated (considering solvents collected in the gas stripping condensate and those remaining in the fermentation broth) (Fig. 3b) also decreased through the fermentation. This can be due to the presence of inhibitor compounds in both the enzymatic hydrolysate and the optimal pretreatment liquid. Although the levels of inhibitors are low in both hydrolysates and were then correctly fermented in the batch mode, in the fed-batch mode these compounds are accumulated progressively, so *C. beijerinckii* could be inhibited. Moreover, other toxic compounds different from those measured in this work (such as caffeine, caffeic acid or chlorogenic acid, among others) could also be found in both hydrolysates (Karmee, 2018), which increases the inhibition of the microorganism. In addition, a possible synergistic effect between them could also take place, significantly increasing the inhibition of *C. beijerinckii* (Domínguez et al., 1999). Furthermore, the flow rate used in the gas stripping system should also be investigated more closely, as it has been found to be one of the main factors affecting the solvent/ABE recovery system (Ezeji et al., 2005). Therefore, further research should be done to look for operating strategies with stripping (for instance, pulse feeding,

intermittent stripping ...) which allows the fermentation productivity and performance to be improved.

4. Conclusions

A microwave assisted dilute sulfuric acid pretreatment is proposed in this work, which has proven to be a suitable method to recover both cellulosic and hemicellulosic sugars from SCG. The optimal extraction conditions were found to be 160.47 ºC and 1.5% H_2SO_4, recovering 79% of the hemicellulosic sugar contained in the SCG and almost the complete digestibility of cellulose (EH yield = 98%) from the resulting pretreated SCG. Moreover, it was possible to valorize these sugars recovered by ABE fermentation to biobutanol, achieving 95 kg butanol/t $SCG_{(DM)}$ and 151 kg ABE/t $SCG_{(DM)}$.

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Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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TABLES

Table 1. Carbohydrate content (g/L), oligomeric sugar (%) and pH of liquid fractions after microwave assisted dilute sulfuric acid pretreatment of SCG. Recovery (%) of solid (SR), glucose (GR) and hemicellulosic sugars (HSR) in the liquid (subscript L) fractions.

Run	T	%H_2SO_4 (w/v)	CSF	SR (%)	pH	Glucose (g/L)	Mannose (g/L)	Oligomeric sugars (%)	GR_L (%)	HSR_L (%)
1	170	1	1.74	69.5	1.05	2.5 ± 0.0	22.2 ± 0.0	0.1	13.7	70.9
2	170	1	1.74	69.2	1.04	2.5 ± 0.1	22.9 ± 0.1	n.d.	14.2	73.1
3	170	1	1.74	73.0	1.05	2.3 ± 0.0	20.9 ± 0.2	n.d.	13.0	66.9
4	150	0.5	0.83	90.3	1.28	0.5 ± 0.0	5.3 ± 0.0	6.9	2.9	17.0
5	170	0.29	1.24	85.2	1.5	1.0 ± 0.1	10.0 ± 0.3	1.5	5.7	31.9
6	142	1	0.91	90.2	1.05	0.6 ± 0.0	5.6 ± 0.0	10.8	3.5	18.0
7	190	0.5	2.01	62.8	1.32	4.8 ± 0.2	21.9 ± 0.2	n.d.	26.7	69.9
8	170	1	1.74	68.6	1.07	2.5 ± 0.0	22.8 ± 0.1	n.d.	14.1	73.0
9	190	1.5	2.46	55.2	0.97	9.0 ± 0.1	13.1 ± 0.1	n.d.	50.2	42.0
10	150	1.5	1.28	86.0	0.94	1.1 ± 0.0	9.0 ± 0.0	0.6	6.3	28.9
11	198	1	2.57	53.8	1.07	9.6 ± 0.0	9.6 ± 0.3	n.d.	53.5	30.7
12	170	1	1.74	69.5	1.07	2.5 ± 0.0	22.3 ± 0.4	0.2	14.0	71.2
13	170	1.71	1.95	62.9	0.91	3.8 ± 0.2	25.2 ± 0.0	n.d.	21.2	80.7

SR: solid recovery (%).
GR_L (glucose recovery in liquid fractions): g glucose in liquid fractions/100 g glucose in SCG
HSR_L (hemicellulosic sugar recovery in liquid fractions): g hemicellulosic sugars in liquid fractions/100 g hemicellulosic sugars in SCG
n.d.: not detected

Table 2. Inhibitor compounds content (g/L) of liquid fractions after microwave assisted dilute sulfuric acid pretreatment of SCG.

Run	CSF	Acetic acid (g/L)	Formic acid (g/L)	HMF (g/L)	Total phenols (g/L)
1	1.74	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
2	1.74	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.4 ± 0.0
3	1.74	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
4	0.83	0.1 ± 0.0	n.d.	n.d.	0.2 ± 0.0
5	1.24	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
6	0.91	0.1 ± 0.0	n.d.	n.d.	0.2 ± 0.0
7	2.01	0.2 ± 0.0	0.7 ± 0.0	1.9 ± 0.1	0.7 ± 0.0
8	1.74	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
9	2.46	0.2 ± 0.0	2.6 ± 0.2	1.2 ± 0.0	1.3 ± 0.1
10	1.28	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
11	2.57	0.2 ± 0.0	3.0 ± 0.1	1.4 ± 0.1	1.7 ± 0.1
12	1.74	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
13	1.95	0.2 ± 0.0	0.5 ± 0.0	0.9 ± 0.1	0.5 ± 0.0

n.d.: not detected

Table 3. Enzymatic hydrolysis of the pretreated solids after microwave assisted dilute sulfuric acid pretreatment of SCG.

		Carbohydrate concentration (g/L)		EH yield (%)	Overall sugar recovery (%)
Run	CSF	Glucose	Mannose		
1	1.74	12.0 ± 0.0	0.5 ± 0.0	92.9	85.3
2	1.74	11.9 ± 0.0	0.5 ± 0.0	92.3	86.6
3	1.74	11.5 ± 0.0	0.5 ± 0.0	94.0	83.0
4	0.83	10.0 ± 0.0	1.0 ± 0.0	100.0	52.3
5	1.24	10.4 ± 0.0	0.8 ± 0.0	99.0	61.1
6	0.91	10.1 ± 0.0	0.9 ± 0.0	100.0	53.0
7	2.01	11.3 ± 0.0	0.2 ± 0.0	79.4	83.6
8	1.74	11.9 ± 0.1	0.5 ± 0.0	91.4	86.1
9	2.46	6.6 ± 0.0	0.0 ± 0.0	40.5	59.8
10	1.28	10.3 ± 0.1	0.6 ± 0.0	98.6	58.7
11	2.57	4.5 ± 0.0	0.0 ± 0.0	27.0	48.9
12	1.74	11.9 ± 0.0	0.5 ± 0.0	92.6	85.4
13	1.95	12.0 ± 0.1	0.3 ± 0.0	84.2	90.4
Non-pretreated SCG		9.9 ± 0.6	2.3 ± 0.1	100.0	29.1

EH yield, %: g glucose achieved in enzymatic hydrolysis/100 g glucose in unpretreated SCG.
Overall sugar recovery (%):g glucose and mannose achieved in enzymatic hydrolyzates and pretreatment liquid /100 g total sugars in unpretreated SCG.

Table 4. ABE fermentation of the unpretreated and optimal pretreated SCG enzymatic hydrolysates, and optimal pretreatment liquid. Initial monosaccharide concentration (g/L), 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 the time of maximum production of butanol and ABE.

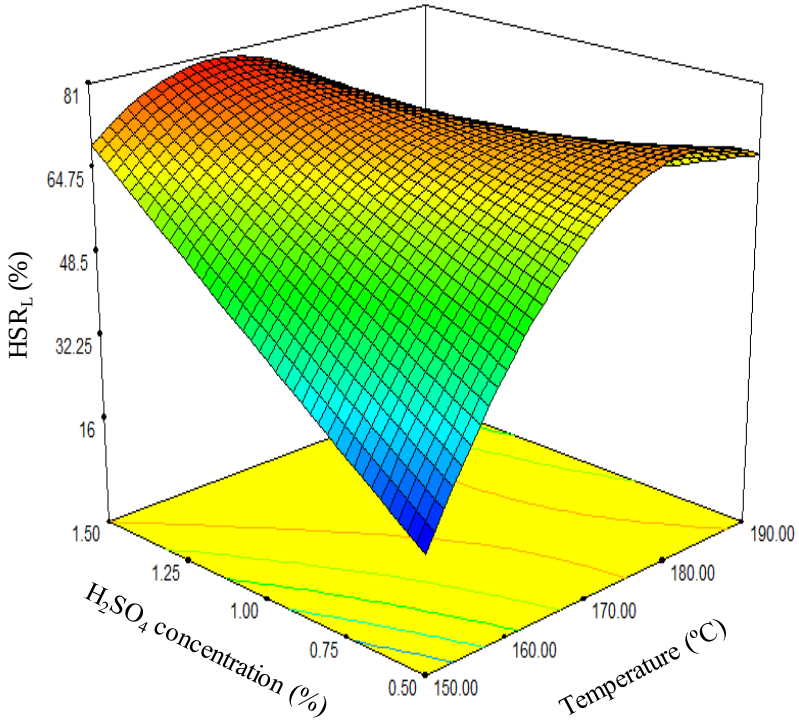
	t (h)	Sugar uptake (%)	Acetic acid (g/L)	Butyric acid (g/L)	$Y_{BUT/sugars}$ (g/g)	$Y_{ABE/sugars}$ (g/g)	P_{BUT} (g/L·h)	P_{ABE} (g/L·h)
Unpretreated SCG enzymatic hydrolysate	48	99.6 ± 0.0	0.4± 0.1	0.4± 0.1	0.23	0.34	0.160	0.238
Optimal pretreated SCG enzymatic hydrolysate	48	99.7 ± 0.0	0.4± 0.0	0.3± 0.0	0.21	0.33	0.140	0.216
Optimal pretreatment liquid	48	99.2 ± 0.1	0.7± 0.1	0.4± 0.1	0.20	0.33	0.074	0.121

Figure captions

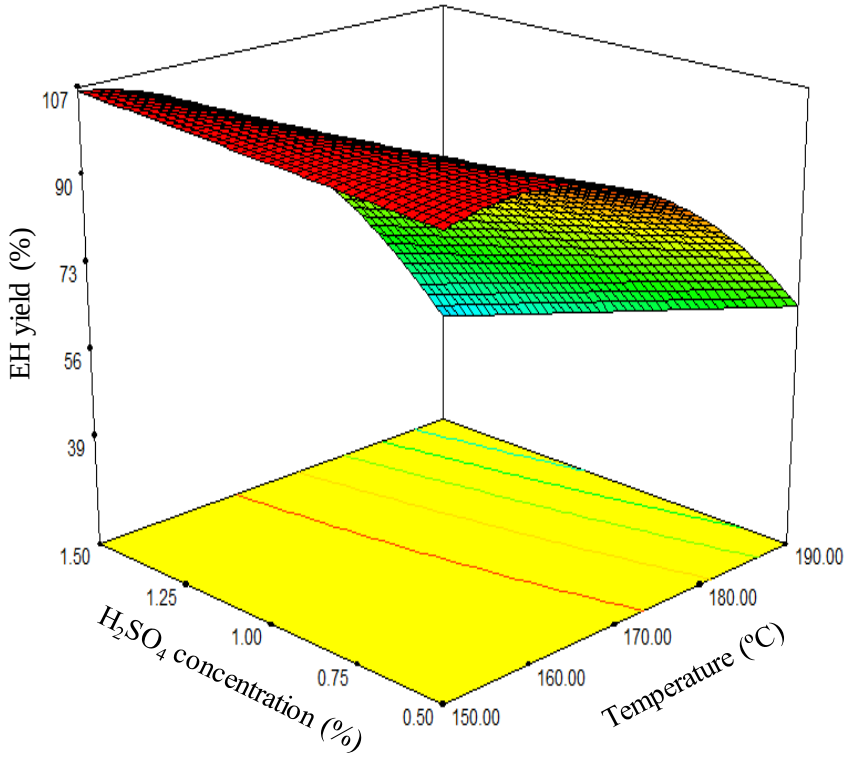
Fig. 1. Response surface plots for (a) hemicellulosic sugar recovery (HSR_L) and (b) EH yield as a function of temperature and sulfuric acid concentration.

Fig. 2. ABE fermentation of unpretreated and optimal pretreated SCG enzymatic hydrolysates, and optimal pretreatment liquid.

Fig. 3. Fermentation fed-batch profiles with in situ gas stripping of optimal pretreated SCG enzymatic hydrolysate. The fed-batch mode was performed using the optimal pretreatment liquid. Dashed lines show the total production considering solvents collected in the gas stripping condensate and those remaining in the fermentation broth. The continuous vertical line in gray colour shows the time in which the gas stripping and feed-batch processes are started (at 22 h of fermentation).


81
64.75
48.5
32.25
16
HSR$_L$ (%)
1.50
1.25
1.00
0.75
0.50
H_2SO_4 concentration (%)
150.00
160.00
170.00
180.00
190.00
Temperature (°C)

a)


107
90
73
56
39
EH yield (%)
1.50
1.25
1.00
0.75
0.50
H_2SO_4 concentration (%)
150.00
160.00
170.00
180.00
190.00
Temperature (°C)

b)

Fig. 1.

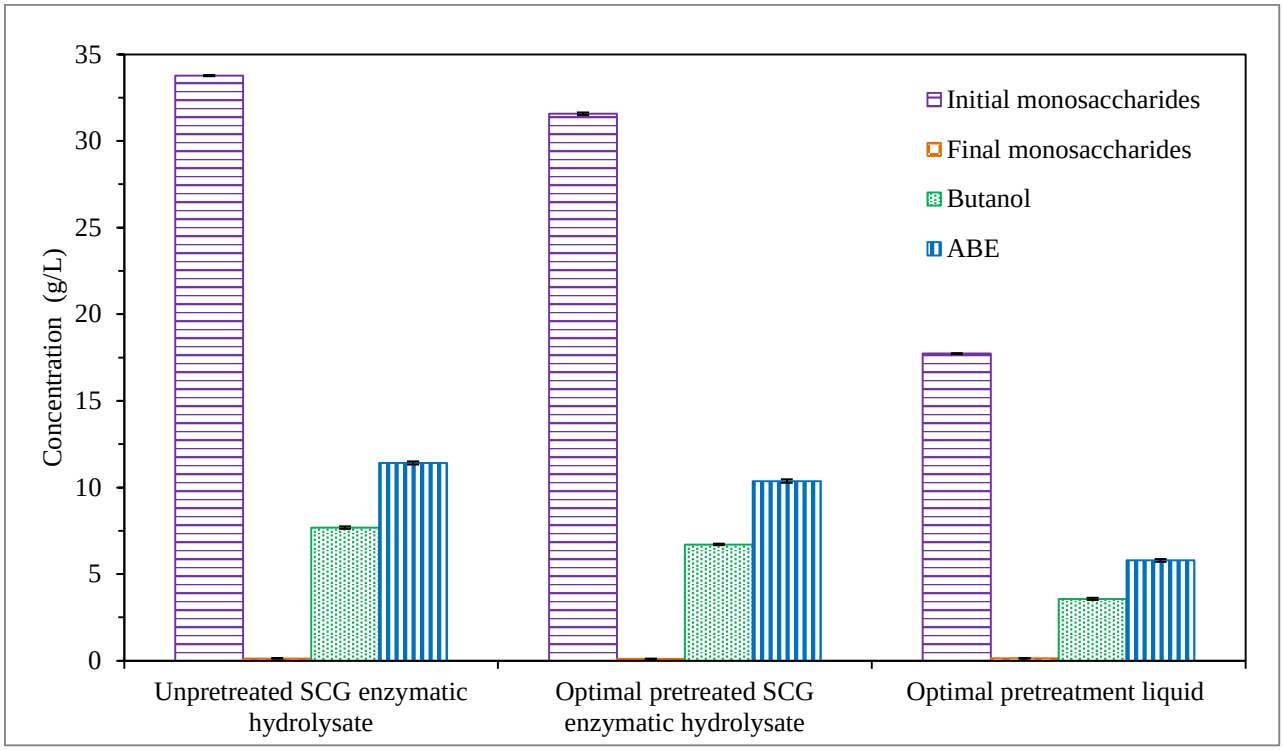
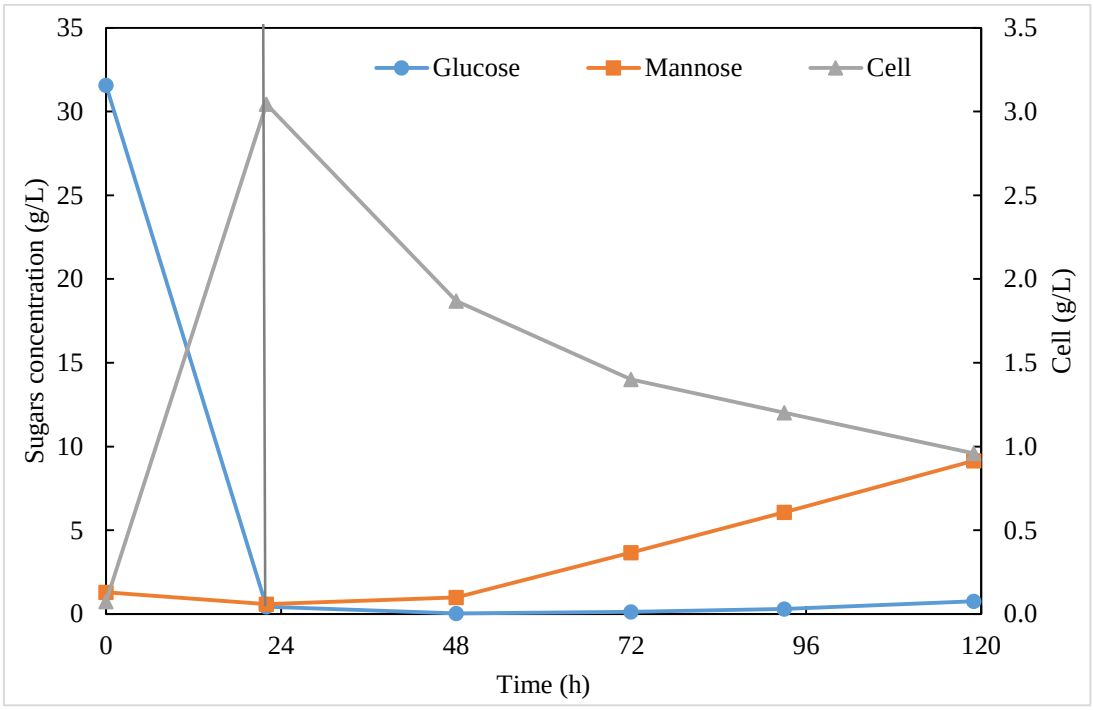
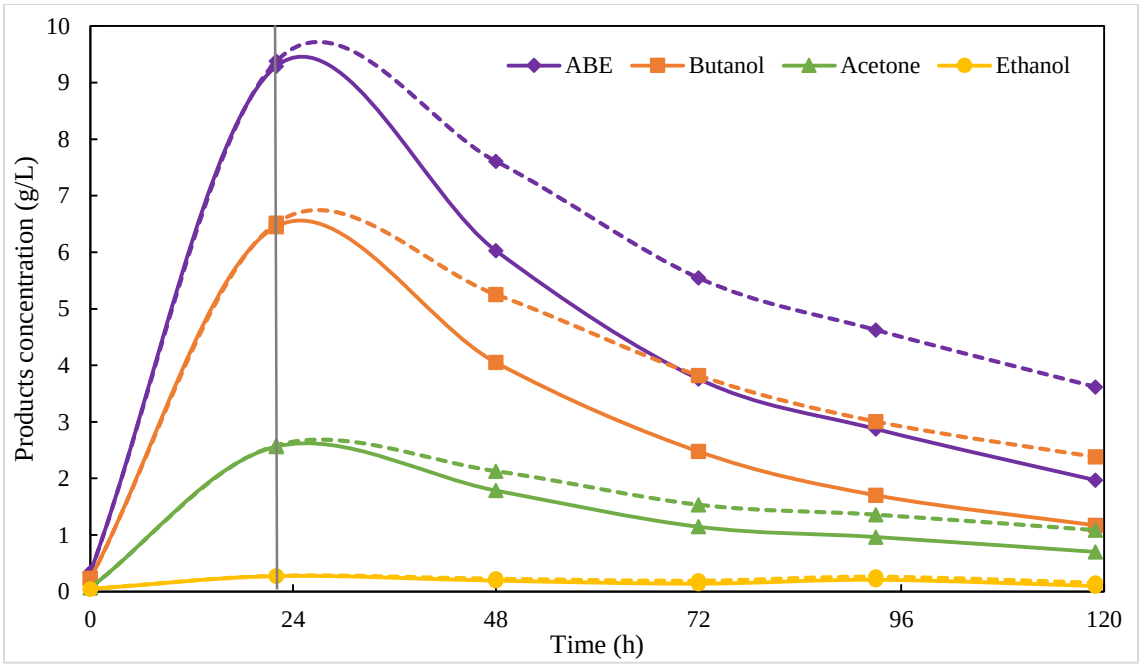

35
30
25
20
15
10
5
0
Concentration (g/L)
Initial monosaccharides
Final monosaccharides
Butanol
ABE
Unpretreated SCG enzymatic hydrolysate
Optimal pretreated SCG enzymatic hydrolysate
Optimal pretreatment liquid

Fig. 2.


Glucose
Mannose
Cell
Sugars concentration (g/L)
Cell (g/L)
Time (h)
0
5
10
15
20
25
30
35
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
24
48
72
96
120

a)


ABE
Butanol
Acetone
Ethanol
Products concentration (g/L)
Time (h)
0
1
2
3
4
5
6
7
8
9
10
0
24
48
72
96
120

b)

Fig. 3.

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Electronic Annex

Supplementary data.docx

Credit Author Statements

Juan C. López-Linares → Investigation, methodology, writing-original draft

María Teresa García-Cubero → Conceptualization, supervision, writing-original draft

Mónica Coca → Conceptualization, formal analysis, supervision

Susana Lucas → Conceptualization, writing-review editing, project administration

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: