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Efficient Acetone-Butanol-Ethanol production by *Clostridium beijerinckii* from sugar beet pulp

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Abstract

Sugar beet pulp (SBP) has been investigated as a promising feedstock for ABE fermentation by *Clostridium beijerinckii*. Although lignin content in SBP is low, a pretreatment is needed to enhance enzymatic hydrolysis and fermentation yields. Autohydrolysis at pH 4 has been selected as the best pretreatment for SBP in terms of sugars release and acetone and butanol production. The best overall sugars release yields from raw SBP ranged from 66.2% to 70.6% for this pretreatment. The highest ABE yield achieved was 0.4 g/g (5.1 g/L of acetone and 6.6 g/L butanol) and 143.2g ABE/kg SBP (62.3 g acetone and 80.9 g butanol) were obtained when pretreated SBP was enzymatically hydrolyzed at 7.5% (w/w) solid loading. Higher solid loadings (10%) offered higher acetone and butanol titers (5.8 g/L of acetone and 7.8 g/l butanol). All the experiments were carried out under not-controlling pH conditions reaching about 5.3 in the final samples.

Keywords: Sugar-beet pulp, autohydrolysis pretreatment, ABE fermentation, *Clostridium beijerinckii*.

1. Introduction

Sugar beet pulp (SBP) is a major by-product of sugar industry. More than 1 million dry tons of sugar beet pulp is produced in US (Zheng et al, 2013) and near 5 million tons are produced in EU countries (Ziemínski et al, 2014). Moreover, the fast depletion of fossil fuels, the rising demand of crude oil and its unstable price in addition to increasing

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concerns over global warming, have renewed interest in the bioproduction of butanol as a chemical and alternative liquid biofuel (Bellido et al, 2014).

Sugar beet pulp contains 20-25% cellulose, 25-36% hemicellulose (mainly arabinans). 20-25% pectin, 10-15% protein and 1-2% lignin (dry weight basis). It has been employed mainly as a feed formulation (animal feed) and also in paper industry due its low lignin content. Commonly dehydration and pelletizing of sugar beet represent more than 30% of the overall energy cost in sugar beet production process.

Due to the high content in carbohydrates, sugar beet pulp has been used for pectin extraction (Chen et al, 2015), enzymatic hydrolysis to galacturonic acid and arabinose (Leijdekkers et al, 2013), anaerobic digestion to methane production (Ziemínski et al, 2014) and fermentation to ethanol (Zheng et al, 2013),

Butanol is a liquid fuel that offers numerous advantages over ethanol: an energy value (29 MJ/L) similar to gasoline (32 MJ/L) and much higher than ethanol (16 MJ/L), is potentially less corrosive; it has a low vapor pressure, lower miscibility with water, a higher flash point and can be transported in existing pipelines. Moreover, it can reduce hydrocarbon emissions by 95%; and oxides of nitrogen by 37%. Butanol application as a replacement for gasoline will outpace ethanol, biodiesel and hydrogen because of its safety and simplicity of use (Huang et al., 2003).

The transformation of sugar beet pulp to biobutanol requires pretreatment and hydrolysis techniques to alter the structure and to release simple sugars, which can be further used as substrate for the production of biofuels through fermentation. Due to the low lignin content of sugar beet pulp, conventional lignocellulosic pretreatments (LHW, steam explosion) are not required and autohydrolysis at low temperature are preferred. Moreover, the use of cocktail enzymes to release monomeric sugars is necessary in order to obtain a high sugar content fermentation media.

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Fermentation of hydrolysates with anaerobic *Clostridia* can produce acetone, butanol and ethanol during the so-called ABE fermentation (the typical ratio of ABE is 3:6:1, where butanol is a major product). Among *Clostridia*, *Clostridium beijerinckii* are recognized as high butanol producers (Bellido et al, 2014) that utilize both hexose and pentose sugars, which are released from lignocellulosic residues after pretreatment and hydrolysis, to produce ABE. The efficient use of the sugar content of lignocellulosic biomass is the key for the economic feasibility of biofuel production (Bellido *et al.*, 2011).

The aim of this study was to determine the viability of using sugar beet pulp as efficient feedstock for ABE fermentation with *C.beijerinckii* DSM 6422. Pretreatments based on autohydrolysis, autohydrolysis modifying pH conditions and acid dilute pretreatment have been explored. For enzymatic hydrolysis, the use of pectinases as supplement to conventional enzymes cocktail has been studied.

The study is focused on comparing pretreatment technologies, enzymatic hydrolysis yields and ABE production by *C.beijerinckii* DSM 6422 which is able to use sugars from cellulosic and hemicellulosic fractions in raw material.

2. Materials and Methods

2.1. Raw material

Sugar beet pulp (SBP) was kindly donated by a Sugar Company located in Castilla y León, Spain. It was freeze stored until use and dried in an oven at 37 ^{o}C before the experiments.

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2.1. Pretreatment methods

2.1.1. Dilute sulfuric acid pretreatment

The pretreatment was performed in 500 mL screw cap flasks at optimum conditions for SBP found in the literature: 120 ^{o}C, 0.66% sulfuric acid concentration, 6% solid loading and 5 minutes of reaction time (Zheng et al., 2013).

Solid phase was separated via vacuum filtration and washed with distillate water until the pH of the filtrate reached about 4-5. The solid recovery was measured for mass balance calculation, the solid was characterized and the filtrate was collected for sugar and degradation products analysis (i.e. acetic acid, furfural and 5-hydroxymethylfurfural (HMF)).

2.1.2. Autohydrolysis pretreatment at pH 4

The initial pH of SBP solutions before pretreatment was 4.8-5. In this case, some drops of concentrated sulfuric acid (96%) were added to carry out the pretreatment at pH 4. The pretreatment was performed as the same conditions as above (500 mL screw cap flasks, 120 ºC, 6% solid loading, and 5 minutes of reaction time). Solid phase was separated via vacuum filtration and there was no need of washing. The mass balance and the analysis of solid and liquid fraction were carried out.

2.1.3. Autohydrolysis pretreatment

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The pretreatment was performed following the same procedure as above without the addition of sulfuric acid. Solid and liquid phase were also separated via vacuum filtration to be used in the subsequent enzymatic hydrolysis step. Mass balance calculations, the solid characterization and HPLC analysis of the filtrate were carried out.

Hereinafter, the dilute sulfuric acid, autohydrolysis at pH 4 and autohydrolysis will be denoted as A, B and C pretreatment, respectively.

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated SBP was performed in 100 mL Erlenmeyer flasks containing 5%, 7.5% and 10% (w/w) of dry solid. Operating conditions for enzymatic hydrolysis were: 175 rpm mechanical agitation, 50 ºC, pH 4.8 and 72 hours of reaction time (Bellido et al. 2011). pH was adjusted by the addition of NaOH 3M. Commercial enzyme mixtures were used for saccharification of sugar beet pulp. Celluclast 1.5L (cellulase) and Novozym 188 (β-glucosidase) were purchased from Novozymes (Denmark) containing about 97 filter paper units (FPU) activity/mL and 370 cellobiose units (CBU)/mL, respectively. Pectinase from *Aspergillus aculeatus* (Pectinex® Ultra SPL) was purchased from Sigma-Aldrich (Spain) and containing approximately 9500 polygalacturonase units of activity (PGU)/mL. Enzymes loadings were adjusted to 15 FPU, 12 CBU and 60 PGU/g dry matter (DM).

After hydrolysis, samples were withdrawn, centrifuged, filtered (0.22 μm) and stored for HPLC analyses.

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2.3. ABE fermentation

2.3.1 Microorganism and preparation of inoculum

The microorganism used in this study was *Clostridium beijerinckii* DSM 6422, obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The strain was maintained on Reinforced Clostridial Medium, RCM, (Fluka, Sigma-Aldrich, Spain) in Hungate tubes (18 x 150 mm), in spore form and conserved at -20ºC under anaerobic conditions.

The microorganism was transferred from the stock solution to the growth medium in order to prepare the inoculum. The growth medium was composed of (I) sugar solution: 30 g/L glucose and 1g/L yeast extract; (II) vitamin solution: 0.001g/L PABA, 0.001g/L thiamine and 0.00001g/L biotin; (III) salt solution: 0.20 g/L $MgSO_47H_2O$, 0.01 g/L $MnSO4H_2O$, 0.01 g/L $FeSO_47H_2O$ and 0.01 g/L NaCl and (IV) acetate buffer solution composed of: 0.50 g/L KH_2PO_4, 0.50 g/L K_2HPO_4 and 2.20 g/L ammonium acetate. The sugar solution was autoclaved while solutions II, III and IV were transferred to the medium after the sterilization using a sterilized filter (0.22 μm).

The inoculum was grown in 150 mL serum bottles with rubber septum under anaerobic conditions. The serum bottles were filled with preboiled growth medium and flushed with free O_2 nitrogen. Before inoculating the preculture with the first post-sporal Hungate-tube culture, a heat shock was performed at 80ºC for 3.5 min to stimulate the germination of spores. The preculture was then inoculated (10% v/v) and incubated at 35ºC without agitation for 24 h.

2.3.2 Fermentation assays

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Filtered SBP hydrolysates were used for ABE fermentation assays using *C.beijerinckii*. The solid fraction of hydrolysates was separated by vacuum filtration. Fermentation experiments were carried out in 150 mL serum bottles with rubber septum under anaerobic conditions flushing nitrogen into the liquid fraction. Prior to the addition of the inoculum, the hydrolysates were adjusted to pH 6.2 using NaOH 3M, and then autoclaved at 120 ºC for 20 min. After sterilization, II, III and IV solutions were added to the medium and the preculture was then inoculated at 10% (v/v). All the experiments were carried out in duplicate.

2.4. Analytical methods

The characterization of SBP was performed according to the Laboratory Analytical Procedures of the National Renewable Energy Laboratory (LAP - 002, LAP - 003, LAP - 004, LAP - 017, LAP - 019) for the determination of total solids, extractives, structural carbohydrates, lignin and ash in biomass. Crude protein was calculated as N x 6.25, being N the Kjeldahl nitrogen. The pectin content was determined via colorimetric method using galacturonic acid as a standard (Melton and Smith, 2001).

The concentration of sugars, acids, solvents and potential inhibitors were determined by HPLC. The detector was based on the refractive index measurement. An Aminex HPX-87H column was used, enabling the quantification of glucose, xylose, galactose, arabinose, mannose, galacturonic acid, acetic acid, lactic acid, butyric acid, furfural, HMF, ethanol, acetone and butanol. Operational conditions were 0.01N H_2SO_4 as the mobile phase, at a flow rate of 0.6 mL/min and 30 ºC (solvents) or 60 ºC (sugars,

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organic acids and inhibitors). All the samples were centrifuged at 13000 rpm for 10 min and filtered before being analysed.

The FPU and CBU activities of cellulase and b-glucosidase were measured according to the literature (Ghose, 1987).

3. Results and discussion

3.1. Sugar beet pulp composition and effect of pretreatments

After the characterization process of SBP, approximately 20.3% cellulose, 33.7% hemicellulose, 27.3% pectin and 7.7% protein were found as major components of the biomass (Table 1). However, in sugar beet pulp, lignin is one of the minor constituents (<2%) compared to other lignocellulosic biomass such straw (>20%), which are commonly investigated as biofuel sources. Lignin is considered as a barrier for the enzymatic attack and the digestibility of the biomass and a pretreatment is necessary to disrupt the structure of the biomass (Hendriks and Zeeman, 2009). In this case, pretreatment severity was expected to be lower than other pretreatments applied to straw or woody biomass (Sun and Cheng, 2002; Alvira et al., 2010, Bellido et al., 2014).

An effective pretreatment must accomplish the following targets: i) make cellulose more degradable by enzymes, ii) avoid sugars lost to increase the concentration of monomeric sugars in hydrolysates, iii) avoid degradation products formation. In this study, three pretreatments varying the severity in terms of acid addition have been evaluated for an effective butanol production from sugar beet pulp.

Table 1 shows the chemical composition of raw material and pretreated SBP, the solid recovery and the solubilized compounds in the liquid fraction of the pretreatments.

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Three different pretreatments were applied to raw SBP as explained in paragraph 2.1. The aims of dilute acid pretreatment (A) were to solubilize hemicellulose and make cellulose more degradable by enzymes. In order to decrease the severity of the pretreatments and save costs of acid addition, an autohydrolysis pretreatment at pH4 (B) and a pretreatment without acid addition (C) have been evaluated. In terms of solid fraction composition, the main effect of pretreatment A on sugar beet pulp was the increase of cellulose and the decrease of the arabinan fraction due to the solubilization of sugars in the liquid phase. The percentage of cellulosic fraction underwent an increase of 69.1% and the arabinan composition dropped by 72.2% as compared to the raw material. With regard to sugars composition in the liquid phase, 5.1 g glucose/100 g for raw material (RM), 5.4 g fructose/100 g RM and 15.8 g arabinose/100 g RM were found as the main monosaccharides, thus indicates that a big percentage of the arabinose is solubilized into the liquid fraction. Potential inhibitory compounds such hydroxymethylfurfural (HMF) in low concentrations (0.08 g/L; 0.12 g/100 g RM) was also found. Therefore, the pretreatment was efficient in terms of hemicellulose solubilization and cellulose concentration; however, the solid recovery was only 47.9% and a considerable amount of sugars were lost since the liquid phase can not be used in the subsequent enzymatic hydrolysis due to extremely low pH values. Thereby, the aims of the pretreatments B and C were increase the solid recovery and obtain a liquid fraction exploitable for the next process stage using lower or zero acid concentration, respectively. With regard to the pretreatment B, carried out at pH 4, the percentage of cellulose underwent an increase of 16.8% and the hemicellulose fraction was slightly solubilized. The solid recovery was much higher (86.1%) and the compounds concentration in the liquid fraction was lower were compared to the pretreatment A. Similar effects were observed in the pretreatment C, the percentage of cellulose

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increased 10.7% and low amounts of sugars were solubilize in the liquid fraction with a solid recovery of 85.3%.

The pectin in the solid fraction decreased with the acid concentration used in the pretreatment ranging from 22.7 to 53.0%. The percentage of lignin and the protein content in the solid phase increased after every pretreatment probably due to the lost of carbohydrates and other components. Zheng et al. (2013) observed an increase of arabinose solubilization with temperature and acid concentration in the range of 29.9-100% in dilute sulphuric acid pretreatment of SBP. These authors observed the pectin removal was superior, ranging from 64% to 94% compared to our study and there was removal of protein in the range between 12% and 90%.

3.2. Enzymatic hydrolysis of pretreated sugar beet pulp

The enzymatic hydrolysis was carried out at three different solid loadings (5, 7.5 and 10%) in order to test the effect of viscosity, the sugars release and yields. Unpretreated and pretreated SBP have been subjected to enzymatic hydrolysis and glucose, fructose, arabinose and galacturonic acid have been identified as main products.

Figure 1 depicts sugars and galacturonic acid (GA) concentration at 5% (a), 7.5% (b) and 10% (c) after 72 hours of saccharification in untreated raw material (SBP hydrolysates), sugar beet pulp from dilute acid pretreatment (SBP_A hydrolysates), sugar beet pulp from autohydrolysis at pH4 (SBP_B hydrolysates) and sugar beet pulp from autohydrolysis pretreatment (SBP_C hydrolysates). The sugars concentration was increasing with the solid loading but not proportionally as a result of the higher viscosity and enzyme inhibition by larger amounts of glucose in the hydrolysate (Tomás-Pejó et al., 2008). With regard to glucose concentration, 27.0, 27.6 and 22.8

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g/L were obtained from SBP_A, SBP_B and SBP_C hydrolysates at 10%, respectively. Although glucose concentration is higher in 10% hydrolysates, the cellulose breakdown is more effective in 5 and 7.5% hydrolysates since larger amounts of glucose are released per 100 g RM (Table 2). The main hemicellulose component in SBP is arabinose and 6.6, 11.9 and 10.4 g/L were found in SBP_A, SBP_B and SBP_C hydrolysates at 10%, respectively. The content of arabinose in hydrolysates from the pretreatment A is logically reduced due to the arabinose lost in the non-utilized liquid phase. The fructose found probably came from the dissociation of the sucrose present in SBP. Concentration values up to 6.7 g/L were obtained in SBP_B hydrolysates. The galacturonic acid appeared as a consequence of pectine activity and higher concentrations were shown in SBP_B hydrolysates with values of 6.4, 8.4 and 11.3 g/L at 5, 7.5 and 10%, respectively (Figure 1).

Depending on the pretreatment and the solid loading employed, reducing sugars yields upon enzymatic hydrolysis (Table 2) ranged from 22.2% (Raw SBP at 7.5%) and 80.3% (SBP_A at 5 and 7.5%). For untreated SBP, the total reducing sugars released at 5% solid loading was slightly favoured (25.1%) compared to 7.5 and 10% (22.2% and 23.0%, respectively). For all the pretreatments, enzymatic hydrolysis yields were lower at 10% solid loading since the mixing and mass transfer is impeded by the higher viscosity in the medium. Similar yields of 80.3% and 79.5% were obtained in hydrolysates SBP_A and SBP_B at 7.5% being the highest yields in all the assays of this study (Table 2).

Overall reducing sugar yield from both pretreatment and enzymatic hydrolysis of pretreated SBP ranged from 29.5% (SBP_A at 10%) to 70.6% (SBP_B at 7.5%). Overall yields were calculated as the ratio between sugars found in hydrolysates and sugars present in RM taking into account the solid recovery (Table 1). The solid

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recovery in pretreatment A was considerably lower (47.9%) compared to pretreatments B and C (86.1 and 85.3%, respectively) due to higher solubilization of SBP into the liquid fraction. In A pretreatment the liquid phase could not be utilized for enzymatic hydrolysis which resulted in carbohydrates lost and lower overall sugars recovery yields. Regarding Table 2, overall sugars yields ranged from 29.5 to 34.5% in pretreatment A, from 66.2 to 70.6% in pretreatment B and from 52.1 to 54.5% in pretreatment C. Minimum and maximum yield values are closed in all pretreatments tested which indicates high efficiency at elevated solid loadings (10%) and the best results obtained in this work in terms of sugars recovery were subjecting SBP to a hydrothermal pretreatment adjusting pH to 4 by sulfuric acid and then carrying out an enzymatic hydrolysis of the pretreated material utilizing also the liquid phase from pretreatment in this stage. Thus, in the best conditions, 70.6% of the sugars present in raw SBP, which corresponds to 79.4% of the sugars present in pretreated SBP_B, are released for bioconversion into solvents in the subsequent fermentation step. These results are in the range of those obtained by Zheng et al. (2013) at similar conditions as in pretreatment A and C, reaching 76.3% and 49.5%, respectively, from SBP enzymatically hydrolyzed at 2% (w/w) solid loading. Kühnel et al. (2011) only achieved 52% of total sugars yield during the enzymatic hydrolysis of pressed sugar beet pulp (5% w/w) pretreated at 120ºC, 1% sulphuric acid (w/w) for 15 minutes.

In all the hydrolysates lactic and acetic acid were detected varying in the range of 0.6-0.9 g/L and 0.3-1.2 g/L (data not shown), respectively, depending on the pretreatment and increasing with the solid loading. The presence of acetic acid can be partly explained by pectin deacetylation since the degree of acetylation in SBP is about 35% (Leijdekkers et al., 2013) and some acid-releasing bacteria in raw SBP could produce some of the acetic and lactic (Kühnel et al., 2011).

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Table 2 also shows overall galacturonic acid yields calculated from SBP hydrolysates as a result of pectinase activity. The sugar beet pulp pectin is a structural heteropolysaccharide contained in the cell wall rich in galacturonic acid. Several distinct polysaccharides have been identified such as homogalacturonans (HG), rhamnogalacturonans I (RG-I), and, to a much lesser extent, rhamnogalacturonan II (RG-II). HG are linear chains of α-(1-4)- linked D-galacturonic acid and linear β-(1-4)-linked galactan and highly branched arabinan, composed of α-(1-5)-linked back bones with α-(1-2) and/or α-(1-3) arabinofuranosyl substitutions, are side chains of RG-I (Leijdekkers at al., 2013; Levigne at al., 2002). Higher concentrations of galacturonic acid were obviously found in 10% hydrolysates from each pretreatment reaching 7.5, 11.3 and 9.7 g/L in SBP_A, SBP_B and SBP_C hydrolysates, respectively (Figure 1c). Nevertheless, the percentage of galacturonic acid released decreased with the dry matter solid loading. Thus, SBP hydrolysates from pretreatment C at 5% solid loading shown the best results in terms of galacturonic acid release yields (66.1%) compared to less favoured conditions (SBP_C at 10%) in which yields decreased to 46.0% (Table 2).

3.3. ABE fermentation from SBP hydrolysates

Pretreated SBP hydrolysates were subjected to ABE fermentation by *C.beijerinckii* at 35ºC for 120h. The fermentation time was selected according to previous results (Bellido et al., 2014) and sugars, acids and solvents were analysed at the beginning and end of the process. All the hydrolysates were centrifuged and sterilized and the liquid fraction was subjected to fermentation. The process was studied taking into account sugars consumption, acids formation, solvents production and ABE and butanol yields.

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Fermentation of hydrolysates with anaerobic *Clostridia* can produce acetone, butanol and ethanol during the so-called ABE fermentation being the typical ratio 3:6:1, respectively, where butanol is a major product (Rajagopalan et al., 2013).In all the assays, *C.beijerinckii* was not able to produce ethanol from SBP hydrolysates which can be positive for further separation processes. The microorganism was able to ferment efficiently the hydrolysates from all pretreatments studied and higher concentrations of acetone and butanol were detected at 10% solid loading hydrolysates which indicated there was no inhibition. Some studies have evaluated the influence of cellulosic sugar degradation products such as furfural and 5-hydroxymethylfurfural (HMF) having a positive effect on ABE fermentation using *C.beijerinckii* (Qureshi et al., 2012). In our hydrolysates, furfural and HMF was not detected, although low concentrations of HMF were found in the liquid fractions of the pretreatments (Table 1); however, acetic acid was found in a larger extent (< 1.2 g/L) but did not exceed inhibition levels (6 g/L) found for other microorganism such *S. cerevisae* (Larsson et al., 1999) and, as a matter of fact, had a positive effect along with lactic acid on the fermentation process by *C.beijerinckii*. Preliminary results showed the presence of acetic and lactic acid in concentrations up to 3.0 g/L stimulated higher acetone and butanol titers in model media by *C.beijerinckii* DSM 6422. In these studies a model solution of 30 g/L of sucrose was used. Butanol concentration increased from 5.5 g/L to 7.8 g/L and acetone concentration increased from 3.0 g/L to 3.5 g/L when lactic acid and acetic acid concentrations were added to the medium increasing from 0.0 g/L to 3.0 g/L and 1.3 g/L to 1.8 g/L, respectively.

Table 3 shows initial sugar after inoculation and total sugar conversion, final acid and ABE concentrations in all the pretreated SBP hydrolysates at the three different solid loadings subjected to fermentation. Fermentation results of SBP hydrolysates at 7.5%

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and 5% were similar to those obtained at 10% but lower acetone and butanol concentrations were achieved due to the reduced availability of monosaccharides. The best results in terms of acetone and butanol concentrations were found in fermentation of hydrolysates from SBP pretreated at pH 4 and enzymatically hydrolyzed at 10% reaching 5.8 g/L acetone and 7.8 g/L of butanol and 0.36g/g of ABE yield, calculated as the ratio between total solvents produced and sugars consumed. These results may be related to a higher concentration of acetic and lactic acid present in this set of experiments, 3.2 and 0.7 g/L, respectively, and higher availability of monomeric sugars. Total sugars conversion is shown in Table 3 and separately, 93.1%, 100.0% and 62.5% of the initial glucose, fructose and arabinose was consumed, respectively, which indicates the C6-carbohydrates preference of *C.beijerinckii*. In a previous work, this microorganism also showed diauxic behaviour, consuming first glucose rather than xylose (Bellido et al., 2014). These results are in accordance with Ezeji et al. (2007), who observed glucose preference over C5-sources on ABE model fermentation by *C.beijerinckii* BA101.

In SBP_A and SBP_C fermentation assays, glucose and fructose were almost exhausted, but only 19.5% and 19.1% of the arabinose was utilized. This effect could be related to a lower concentration of lactic (0.1 and 0.3 g/L, respectively) and acetic acid (2.5 and 2.7 g/L, respectively). Lower concentrations of acid in hydrolysates from pretreatment A could be explained since many compounds are lost in the non-utilized liquid fraction. In the case of pretreatment C, less severe conditions without addition of sulfuric acid could result in minor acid release. However, lower concentrations of solvents are primarily related to lower glucose and fructose availability. As regards acetone and butanol concentration, 3.4 and 5.6 g/L and 3.4 and 4.8 g/L were achieved for SBP_A and SBP_C experiments. Compared to SBP_B experiments, ABE yields are

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slightly lower in the case of SBP_C set (0.34 g/g) and lower for SBP_A set (0.30 g/g) due to the acetone levels decrease maintaining butanol yields almost constant for all the assays (0.19-0.20 g/g). At the end of all the assays, some butyric acid appeared which indicated the fermentation process was not finished since butyric acid is the butanol precursor (Lütke-Eversloh and Bahl, 2011). This could be explained since some arabinose was remained in all the experiments and more time is needed to complete the process in the conditions carried out in the present work. In our study, the pH was not controlled reaching about 5.3 in the final samples and some studies suggested controlling the pH at 5.5 increases solvents concentration and productivity in model medium containing 60 g/L of glucose by *C.beijerinckii* IB4 which probably could improve sugars consumption rate (Jiang et al., 2014). Galacturonic acid was maintained constant during the fermentation process since *C.beijerinckii* was not able to metabolize it. Final concentrations of galacturonic acid (Table 3) are analogous to those observed at the end of the enzymatic hydrolysis step (Figure 1). From a biorefinery point of view, arabinose and galacturonic acid are value-added products which can be utilise as intermediate raw material in the pharmaceutical and fine organic synthesis industries and to synthesize vitamin C, respectively (Nahar and Pryor, 2013).

Analogously to the enzymatic hydrolysis step, the best results in terms of overall and ABE yields were found in SBP_B at 7.5% in which 0.40 ABE/g RM and 0.22 g butanol/g RM were obtained. In this case, the overall production of ABE was 143.2g ABE/kg SBP (62.3 g acetone and 80.9 g butanol) thus 177 liters of solvents (77 liters acetone and 100 liters butanol) could be obtained per ton of SBP. SBP_B at 7.5%. Table 3 also summarizes solvents concentration, butanol and ABE yields and overall yields for each experiment. The pretreatment B with mild acid conditions at pH 4 have shown the best results in terms of acetone and butanol overall yields since more sugars are

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released in the previous enzymatic step compared to pretreatment C, and the solid recovery is higher and there was no need of washing compared to pretreatment A. Relating to Butanol:Acetone ratio, in the literature the conventional ratio for Acetone:Butanol:Ethanol is 3:6:1, since *C.beijerinckii* is not able to produce ethanol from sugar beet pulp in this study, the ratio in the best treatment (SBP_B at 7.5%) is Acetone (4.4): Butanol (5.6) which indicates the acetone production is favored but butanol production is almost maintained at the same value.

To the best of our knowledge, ABE fermentation results from pretreated SBP by *C.beijerinckii* have not been found in the literature, so these results can be compared to other biofuels, raw materials and microorganisms. In a previous study (Bellido et al., 2014), slightly lower overall yields were obtained from wheat straw using the same microorganism (127.7 g ABE/kg of wheat straw). Amiri et al. (2014) achieved 123.9 g ABE/kg rice straw organosolv pretreated and enzymatically hydrolyzed using *C. acetobutylicum* NRRL B-591. ABE yields presented in this study are in the range of those obtained by Li et al. (2014) who reached 0.34 g/g from gelatinized cassava flour fermentation medium with a mutant strain of C.acetobutylicum SE36. Qureshi et al. (2008) obtained 9.4 g/L of ABE and 0.37 g/g in separate hydrolysis and fermentation of dilute acid pretreated wheat straw by *C.beijerinckii* P260. Concerning other biofuels such as ethanol, Zheng et al. (2013) achieved a maximum ethanol yield of 0.4 g/g from pretreated SBP in a simultaneous saccharification and fermentation employing E.coli KO11. Rorick et al. (2009) obtained 92g ethanol/kg of SBP by *S.cerevisae* (Type II-YSC2).

4. Conclusions

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Acetone and butanol can be obtained efficiently from pretreated SBP using *C.beijerinckii* DSM 6422 reaching 0.4 g solvents/g sugars consumed from both hexoses and pentoses. Autohydrolysis at pH 4 (120 ºC, 6% SBP (w/w), 5 minutes) has been established as the best pretreatment improving overall sugars release yields in enzymatic hydrolysis and acetone and butanol yields in the fermentation process. This pretreatment offered advantages over conventional dilute acid pretreatment such as higher solid recoveries, no washing needs and lower use of chemicals. In terms of overall yields, 143.2g ABE/kg SBP (62.3 g acetone and 80.9 g butanol) can be obtained.

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Tables and Figures

Table 1

Pretreatment	Raw SBP	SBP_A	SBP_B	SBP_C
Composition of the solid fraction (%, dry weight)				
Glucan	20.34 ± 1.93	34.39 ± 2.56	23.76 ± 1.89	22.50 ± 1.74
Xylan	1.73 ± 0.03	3.01 ± 0.65	0.31 ± 0.02	0.46 ± 0.05
Arabinan	21.81 ± 2.12	6.07 ± 0.92	21.57 ± 2.21	21.20 ± 2.08
Galactan	9.54 ± 0.81	3.58 ± 0.32	9.02 ± 0.97	8.47 ± 0.85
Mannan	0.62 ± 0.06	1.45 ± 0.12	1.09 ± 0.08	0.68 ± 0.09
Pectin	27.34 ± 1.75	12.85 ± 1.21	21.12 ± 1.64	18.94 ± 1.37
Acid insoluble lignin	1.93 ± 0.33	9.59 ± 0.65	4.24 ± 0.63	6.25 ± 0.74
Acid soluble lignin	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Crude protein	7.36 ± 0.01	9.80 ± 0.05	8.14 ± 0.08	8.22 ± 0.11
Ash	3.41 ± 0.22	7.51 ± 0.34	4.22 ± 0.21	7.15 ± 0.40
Solid recovery	N.A.	47.92 ± 9.29	86.14 ± 2.60	85.32 ± 2.32
Composition of liquid fraction/ washing liquid (g/100 g RM)				
Glucose	N.A.	5.09 ± 0.52	1.05 ± 0.15	0.31 ± 0.06
Fructose	N.A.	5.37 ± 0.44	1.02 ± 0.24	0.31 ± 0.05
Arabinose	N.A.	15.77 ± 0.71	0.36 ± 0.01	0.29 ± 0.08
Acetic acid	N.A.	1.69 ± 0.05	0.08 ± 0.01	0.07 ± 0.02
Galacturonic acid	N.A.	3.25 ± 0.04	0.25 ± 0.03	0.13 ± 0.05
HMF	N.A.	0.12 ± 0.01	0.08 ± 0.01	0.03 ± 0.01
Furfural	N.A.	N.D.	N.D.	N.D.

N.A.: Not applicable, N.D.: Not detectable

Table 2

EH yield reducing sugars (% of sugars in pretreated RM)

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Solid loading/Experiment	SBP	SBP_A	SBP_B	SBP_C
5%	25.1	80.3	78.8	64.7
7.5%	22.2	80.3	79.5	66.4
10%	23.0	68.6	74.5	59.4
Overall yield reducing sugar (% of sugars in RM)				
Solid loading/Experiment	SBP	SBP_A	SBP_B	SBP_C
5%	25.1	34.5	70.0	54.5
7.5%	22.2	34.5	70.6	52.1
10%	23.0	29.5	66.2	52.0
Overall yield GA (% of GA in RM)				
Solid loading/Experiment	SBP	SBP_A	SBP_B	SBP_C
5%	0.0	60.9	57.6	66.1
7.5%	0.0	53.9	49.2	52.4
10%	0.0	52.3	48.1	46.0

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Table 3

Solid loading 5%														
	Glucose (g/L)	Fructose (g/L)	Arabinose (g/L)	Sugar Conv. (%)	HAc (g/L)	HLa (g/L)	HBu (g/L)	GA (g/L)	Acetone (g/L)	Butanol (g/L)	Y But (g/g)	Y ABE (g/g)	Butanol (g/kgSBP)	ABE (g/kgSBP)
SBP_A	15.6	1.7	2.8	90.6	0.9	0.0	1.5	3.9	2.0	4.2	0.23	0.34	79.2	116.7
SBP_B	15.0	3.1	4.4	90.7	1.5	0.0	2.0	5.8	2.6	4.5	0.21	0.34	85.6	134.7
SBP_C	11.9	2.2	4.5	86.2	2.6	0.0	2.8	6.1	2.2	3.2	0.20	0.34	60.9	102.5
Solid loading 7.5%														
	Glucose (g/L)	Fructose (g/L)	Arabinose (g/L)	Sugar Conv. (%)	HAc (g/L)	HLa (g/L)	HBu (g/L)	GA (g/L)	Acetone (g/L)	Butanol (g/L)	Y But (g/g)	Y ABE (g/g)	Butanol (g/kgSBP)	ABE (g/kgSBP)
SBP_A	24.2	2.9	4.5	89.6	1.4	0.0	0.7	5.0	3.1	5.2	0.18	0.29	64.0	101.8
SBP_B	21.8	4.6	7.0	87.9	1.7	0.0	1.0	8.2	5.1	6.6	0.22	0.40	80.9	143.2
SBP_C	15.6	3.1	6.1	86.0	2.2	0.0	2.3	8.0	2.8	4.3	0.20	0.33	53.6	88.5
Solid loading 10%														
	Glucose (g/L)	Fructose (g/L)	Arabinose (g/L)	Sugar Conv. (%)	HAc (g/L)	HLa (g/L)	HBu (g/L)	GA (g/L)	Acetone (g/L)	Butanol (g/L)	Y But (g/g)	Y ABE (g/g)	Butanol (g/kgSBP)	ABE (g/kgSBP)
SBP_A	26.8	3.3	5.3	84.7	1.8	0.0	0.8	6.9	3.4	5.6	0.19	0.30	50.0	80.7
SBP_B	27.5	6.2	9.3	87.5	1.7	0.0	0.7	10.5	5.8	7.8	0.21	0.36	70.2	122.6
SBP_C	19.4	4.1	8.5	75.7	2.2	0.0	1.0	9.0	3.4	4.8	0.20	0.34	42.9	73.5

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Figures

Figure 1a

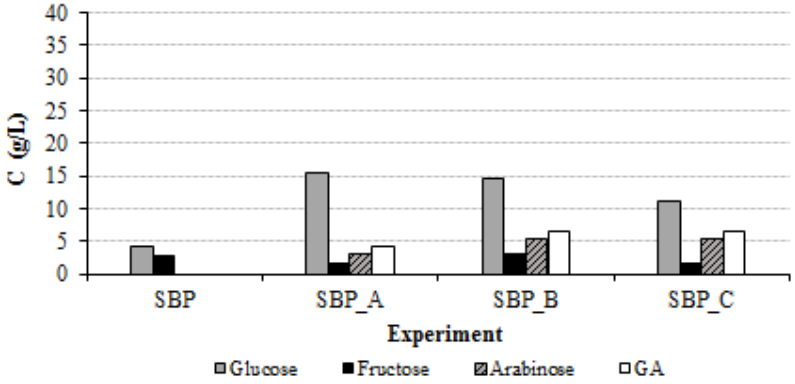


Figure 1b

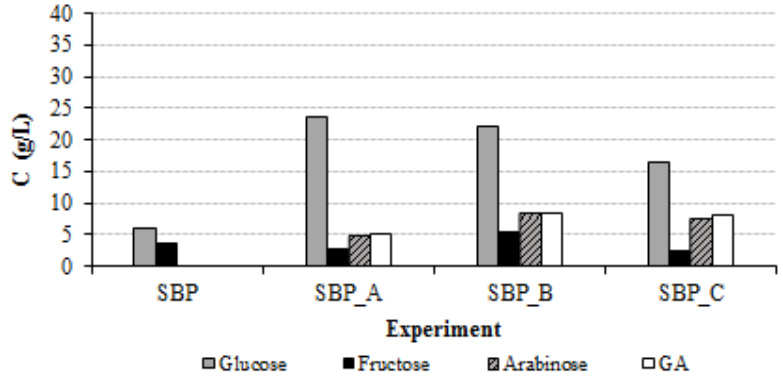
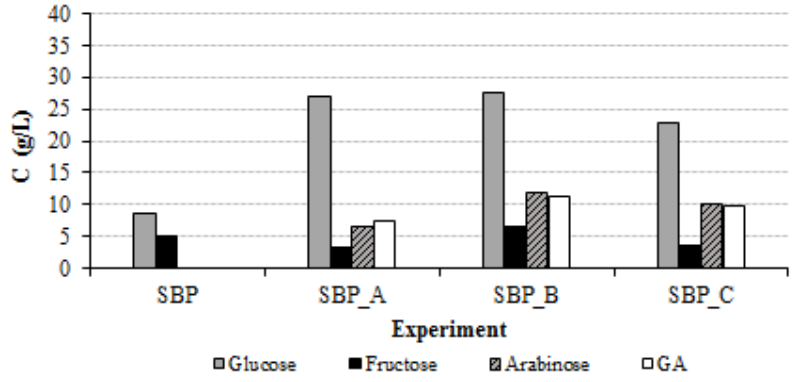


Figure 1c



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Captions

Table 1. Chemical composition of untreated and pretreated SBP and the solubilized compounds in the liquid fraction of A pretreatment (dilute sulfuric acid), B pretreatment (autohydrolysis at pH4) and C pretreatment (autohydrolysis).

Table 2. Enzymatic hydrolysis yields related to sugars in pretreated SBP, overall yields related to sugars in raw SBP and overall galacturonic acid release yield related to raw SBP at different solid loadings.

Table 3. Initial glucose, fructose and arabinose (g/L), total sugar conversion (%), final acetic acid (HAc), final lactic acid (HLa), final butyric acid (HBu) and final galacturonic acid (GA) (g/L), acetone and butanol production (g/L), butanol and ABE yields (g/g sugars consumed) and overall butanol and solvents production (g/kg raw SBP) by *C.beijerinckii* in pretreated SBP hydrolysates at different solid loadings.

Figure 1. Glucose, fructose, arabinose and galacturonic concentration (g/L) at 5% (a), 7.5% (b) and 10% (c) solid loading in enzymatic hydrolysis of raw and pretreated SBP.

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- Sugar beet pulp (SBP) is a promising feedstock for ABE fermentation.
- Autohydrolysis at pH 4 has been selected as the best pretreatment for SBP.
- Pretreated SBP was enzymatically hydrolysed at different solid loadings.
- *C.beijerinckii* could ferment efficiently pretreated SBP hydrolysates.

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