



Green biorefinery for sugar beet pulp valorisation: Microwave hydrothermal processing for pectooligosaccharides recovery and biobutanol production

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ABSTRACT

The complete valorisation of sugar beet pulp (SBP) through pectooligosaccharides (POS) extraction and biobutanol production was studied. A central composite experimental design was planned to optimise the time and temperature of the hydrothermal microwave-assisted extraction. 59.7% of the POS present in the SBP were recovered, and a prehydrolysate with 31.1 g POS/L was obtained under optimal conditions (165 °C and 12 min). The prehydrolysate was further precipitated by ethanol. One-step and stepwise alcohol precipitation were evaluated. The prehydrolysate:ethanol ratio was increased from 1:1–1:3 in the stepwise precipitation, whereas a ratio of 1:2 was used in the one-step precipitation. The pectin fractions differed from each other in their chemical composition. The molecular weight of the pectin fractions ranged from 474 to 523 kDa, and the degree of esterification of all of them was higher than 50%, so they can be considered high methoxyl pectins. On the other hand, the pretreated solid enriched in glucan was subjected to enzymatic hydrolysis with cellulases and fermented by *Clostridium beijerinckii*, producing 8.3 g butanol/L after 48 h of fermentation (53 kg butanol/t SBP). As this research has demonstrated, SBP is a suitable feedstock for a green biorefinery approach. The fractionation of SBP by hydrothermal treatment avoids using acid and alkaline solvents and allows high POS and biobutanol production yields.

1. Introduction

One of the main building blocks of the European Green Deal is the Circular Economy Action Plan for sustainable growth (European Commission, 2020). Although the Europe 2020 Strategy called for a circular economy to reduce pressure on natural resources, a model based on linear economy prevails. Only 12% of secondary materials and resources remain in the economic cycle (European Commission, 2020). In this way, the concept of biorefinery can address this issue by substituting current fossil resources with biomass, making the process feasible and competitive.

Sugar beet pulp (SBP) is an abundant and renewable lignocellulosic waste produced in the sugar beet industry after sugar extraction. Every year, Europe produces around 13 million tonnes of SBP (BBI JU, 2019). SBP has traditionally been used for animal feed, as it is mainly composed of carbohydrates (20–25% cellulose, 15–25% pectin, 20–30%

hemicellulose) and protein (10–15%) (Concha Olmos and Zúñiga Hansen, 2012; Martínez et al., 2018). Carbohydrates can represent a source of valuable compounds (Cabas Candama et al., 2020). Thus, SBP can be valorised in a biorefinery framework by extracting such bioactive molecules as pectin and oligosaccharides or they can be converted into biofuels like biobutanol.

Pectin is a heteropolysaccharide made up of 1,4-linked α -D-galacturonic acid with side chains of neutral sugars such as arabinose (1,5-linked α -L-arabinose) and galactose (1,4-linked β -D-galactose) (Adiletta et al., 2020). Oligosaccharides derived from pectin, known as pectooligosaccharides (POS), have health-promoting effects due to their prebiotic properties. It has been revealed that POS can improve several antibacterial, antioxidant and immunological properties (Wandee et al., 2021). Among the wide range of sugars that can be found in POS, arabinooligosaccharides (AraOS) and galactooligosaccharides (GalOS) give pectin prebiotic properties (Onumpai et al., 2011; Lin et al., 2020a).

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Thus, POS are considered as promising prebiotic products (Gómez et al., 2014). POS can also be used as a gelling, thickening agent and stabiliser. Moreover, POS from SBP has a superior emulsifying activity and can be used in the food industry (Lin et al., 2020b). Lastly, it has been used as a fat replacer, encapsulating agent, and carrier molecule for antimicrobial and antioxidant compounds in active packaging. Moreover, it can be applied as a natural additive in foods and cosmetics. Since more and more consumers are concerned about health, the demand for pectin and POS is growing while the global market remains short (Zhang et al., 2018).

To obtain POS from SBP, a solid-liquid extraction is usually applied. Conventional acid extraction has been extensively studied for pectin recovery (Marić et al., 2018). The use of mineral acids (HCl, HNO₃, H₂SO₄) and organic acids such as citric acid and acetic acid as solvents in a temperature range of 50 – 100 °C for 5 – 240 min has been widely reported by numerous research works (Morales-Contreras et al., 2017; Oliveira et al., 2016; Yang et al., 2018). However, acid extraction has some drawbacks compared to water extraction, such as requiring a subsequent neutralisation step of pectin, equipment deterioration, environmental pollution, and reducing the pectin molecular weight which affects its properties (Abou-Elseoud et al., 2021; Khedmat et al., 2020). On the other hand, emerging extraction technologies, such as pulse electrified extraction (Miklavčič, 2017), subcritical water extraction (Muñoz-Almagro et al., 2019), supercritical fluid extraction (Montañés et al., 2012), enzyme-assisted extraction (Cheng et al., 2017), ultrasound-assisted extraction (Jovanovic-Malinovska et al., 2015), or microwave-assisted extraction (MAE) (Thu Dao et al., 2021), can minimise the use of harsh solvents, provide better yield and quality of extract, and are considered more environmentally friendly (Azmir et al., 2013). Among these technologies, MAE has the advantages of shortening reaction time, improving extraction yield, reducing organic solvent usage, being inexpensive and environmentally friendly (Okolie et al., 2019) and, contrary to conventional heating, energy is homogeneously dispersed through the material. Moreover, MAE has shown higher extraction yields (Cheng et al., 2017) and higher pectin quality recovered (Rodsamran and Sothornvit, 2019) when compared to conventional heating extraction. MAE has been studied for pectin extraction from different feedstocks. Li et al. (2012) extracted pectin from SBP by MAE using H₂SO₄ as solvent (5 – 50 mM) and heated for 2 – 4 min with a power range from 150 to 250 W. Swamy and Muthukumarappan (2017) studied the extraction of pectin from banana peel by MAE using acidified water by HCl as solvent, varying the pH (1 – 3), microwave power (300 – 900 W) and processing time (100 – 200 s). The optimisation of hydrothermal MAE in SBP has not, to the best of our knowledge, been reported yet. Hydrothermal MAE avoids the acid extraction problems mentioned above. Moreover, it is considered a green technology that allows hemicellulose solubilisation, while cellulose remains in the solid for subsequent applications in a biorefinery approach.

After extraction, alcohol precipitation has been reported as an effective method to recover POS from aqueous solutions (Ai et al., 2020; Jacquemin et al., 2012; López-Linares et al., 2020). POS precipitation is based on the compatibility between low molecular-weight oligosaccharides and alcohol. Based on the different properties of polysaccharides, different fractions can be obtained by stepwise alcohol precipitation. Since higher neutral sugar contents and lower yields are obtained at higher ethanol concentrations (Guo et al., 2016), stepwise precipitation could be an alternative to obtaining oligosaccharide fractions with different properties and bioactivities (Ai et al., 2020).

After POS extraction from SBP, a pretreated solid is still mainly composed of cellulose. The “cascade biorefinery approach” requires the development of sequential processes to recover added value compounds and later for biofuel production (Battista et al., 2020). Based on this, the pretreated solid could be valorised through biobutanol production by acetone-butanol-ethanol (ABE) fermentation. Biobutanol is considered an advanced biofuel (Veza et al., 2021), and it has an energy value (29 MJ/L) close to that of gasoline (32 MJ/L). Moreover, biobutanol can be

used as a transportation fuel blending with gasoline. Furthermore, it is usually used as a solvent, plasticiser, chemical commodity, in the cosmetic industry, and as a gasoline additive (Jin et al., 2011). Furthermore, due to the fluctuation in oil prices, interest in biofuels has become stronger. In 2020, the global market size of n-Butanol was estimated at 3.1 billion USD, and it is projected to increase up to 4.0 billion USD by 2025 (Markets and Markets, 2021).

SBP has been studied as a feedstock to produce oligomeric compounds (Li et al., 2012; Martínez et al., 2009) and biofuels such as biobutanol (Bellido et al., 2015) and biohydrogen (Cieciora-Wioch et al., 2020). The present work aims to analyse the integrated use of carbohydrates in SBP in a biorefinery framework to obtain added value bioproducts and advanced biofuels. With this aim, the main step was to optimise POS extraction from SBP by hydrothermal MAE. The effects of temperature and time on POS extraction were evaluated. The recovery of POS from hydrolysate was assessed by one-step and stepwise ethanol precipitation to improve POS purity and recovery. Functional groups on the surface, degree of esterification, and molecular weight distribution of the recovered POS were studied to evaluate the quality of the precipitates and to select a suitable recovery strategy. Furthermore, the pretreated solid was valorised by biobutanol production due to its high cellulose concentration. In this way, the carbohydrates in the SBP can be fully converted to bioproducts (POS) and biofuels (biobutanol) in an integrated concept. To the best of our knowledge, the use of hydrothermal MAE for POS recovery from SBP and the further valorisation of the pretreated solid for biofuel production in a “cascade biorefinery approach” has not yet been reported.

2. Materials and methods

2.1. Raw material

Sugar beet pulp (SBP) from beet was kindly supplied by a sugar factory and stored frozen at – 20 °C until use. The SBP was washed, dried at 60 °C and ground (particle size < 1 mm) before use.

2.2. Microwave-assisted extraction

MAE was conducted in a closed microwave-assisted reaction system (Multiwave PRO SOLV reactor 50 Hz with a Rotor type 16HF100, Anton Paar GmbH, Austria, Europe). SBP and water were mixed in vessels made of PTFE-TFM (volume capacity of 100 mL) fitted with magnetic stirrers. Ceramic pressure vessels contained these PTFE-TFM vessels, one of which includes a pressure/internal temperature sensor. An Infrared (IR) detector continuously registered the temperatures of all the vessels. Moreover, the sensor and pressure/internal temperature sensor permanently controlled the microwave energy applied by the reactor.

The SBP and water were mixed in a solid to liquid ratio of 10% (w/v) (5 g of dry SBP and 50 mL of distilled water). The temperature of each experiment was reached in a heating ratio of 6 °C/min. After the desired temperature was reached, the time started and, once finished, the pressure vessels were cooled to 55 °C.

Solid and liquid fractions were separated by a vacuum filter. The solid was dried at 50 °C and weighed to calculate the solid recovery (g solid fraction after pretreatment/g SBP). The liquid was stored at 4 °C for further analysis (monomeric and oligomeric content and degradation compounds).

2.3. Experimental design for POS extraction

In order to optimise the conditions of POS extraction by MAE, a central composite experimental design was planned. Based on previous experiments (data not shown), the factors selected were temperature (130 – 180 °C) and time (4 – 12 min). Thus, 11 experiments were carried out, including one point and three replicates at the centre of the temperature and time range, as well as two experiments that were under and

over the temperature and time range. The severity factor (SF) was calculated according to MacAskill et al. (2018) (Eq. 1), where t is the time in minutes and T is the temperature in degrees Celsius.

$$\text{Severity Factor (SF)} = \text{Log} \left[t \times \exp \left(\frac{T - 11}{14.75} \right) \right] \quad (1)$$

Finally, a confirmatory run was done in triplicate under the optimal conditions obtained in the central composite experimental design to verify the results obtained by the statistical software.

2.4. POS recovery by ethanol precipitation

To recover POS from SBP prehydrolysates, the POS precipitation experiments with ethanol were developed using the prehydrolysate obtained under optimal conditions. One-step and stepwise precipitation experiments were performed (Fig. 1). The procedure was similar to Ai et al. (2020) but with some modifications. First, the prehydrolysate was precipitated with two volumes of ethanol 96% (v/v) in one-step precipitation, obtaining pectin A and supernatant A. On the other hand, in the stepwise process, the prehydrolysate was firstly precipitated with one volume of ethanol 96% (v/v). After the solid-liquid separation, the supernatant (B1) was precipitated again with two volumes of ethanol and, after that, the supernatant (B2) was precipitated with three volumes of ethanol.

100 mL of prehydrolysate were mixed with 100 and 200 mL of 96% (v/v) ethanol in one-step and stepwise precipitation, respectively, using 500 mL Erlenmeyer flasks. POS precipitation experiments were carried out in an orbital shaker (Comecta Optic Ivymen system) at 25 °C and 100 rpm for 60 min (López-Linares et al., 2020) to homogenise the

mixture and allow it to settle at 4 °C overnight (Dervilly et al., 2000). The liquid and solid phases were separated by centrifugation (3000 g for 10 min). The solid was washed with ethanol 96% (v/v), dried at 60 °C for 48 h and weighed to determine the mass recovered. Solids were stored for further analysis: Fourier transform infrared spectroscopy (FTIR) and degree of esterification.

The solids rich in POS were suspended in water (1 g/L) in an orbital shaker at 35 °C and 200 rpm for 24 h. The POS suspension was analysed for its monosaccharides, oligosaccharides and molecular weight distribution.

2.5. Biobutanol production from pretreated SBP

The pretreated solid obtained under optimal pretreatment conditions was subjected to enzymatic hydrolysis (EH) in an orbital shaker (Optic Ivymen system, Comecta, Spain) to release the structural sugars from the pretreated solid and to use the enzymatic hydrolysate in further acetone-butanol-ethanol (ABE) fermentation. The EH was carried out in triplicate. For each EH experiment, 10 g of pretreated solid was mixed with 100 mL of distilled water in a 250 mL Erlenmeyer flask. Cellic CTec2, a cellulolytic complex supplied by Novozymes A/S (Denmark), was used at 15 Filter Paper Units (FPU)/g solid of Cellic Ctec2 enzyme. The pH was adjusted to 4.8 with 0.1 M NaOH. The experimental conditions were 150 rpm and 50 °C for 48 h. After the EH step, the solid and liquid were separated by centrifugation (3000 g, 10 min). The sugar content in the liquid was analysed. Enzyme blanks were carried out to determine the sugar content in the commercial enzymes. The EH hydrolysis yield was determined by dividing the grams of glucose, arabinose and galactose on the enzymatic hydrolysate by the grams of sugar in the pretreated solid.

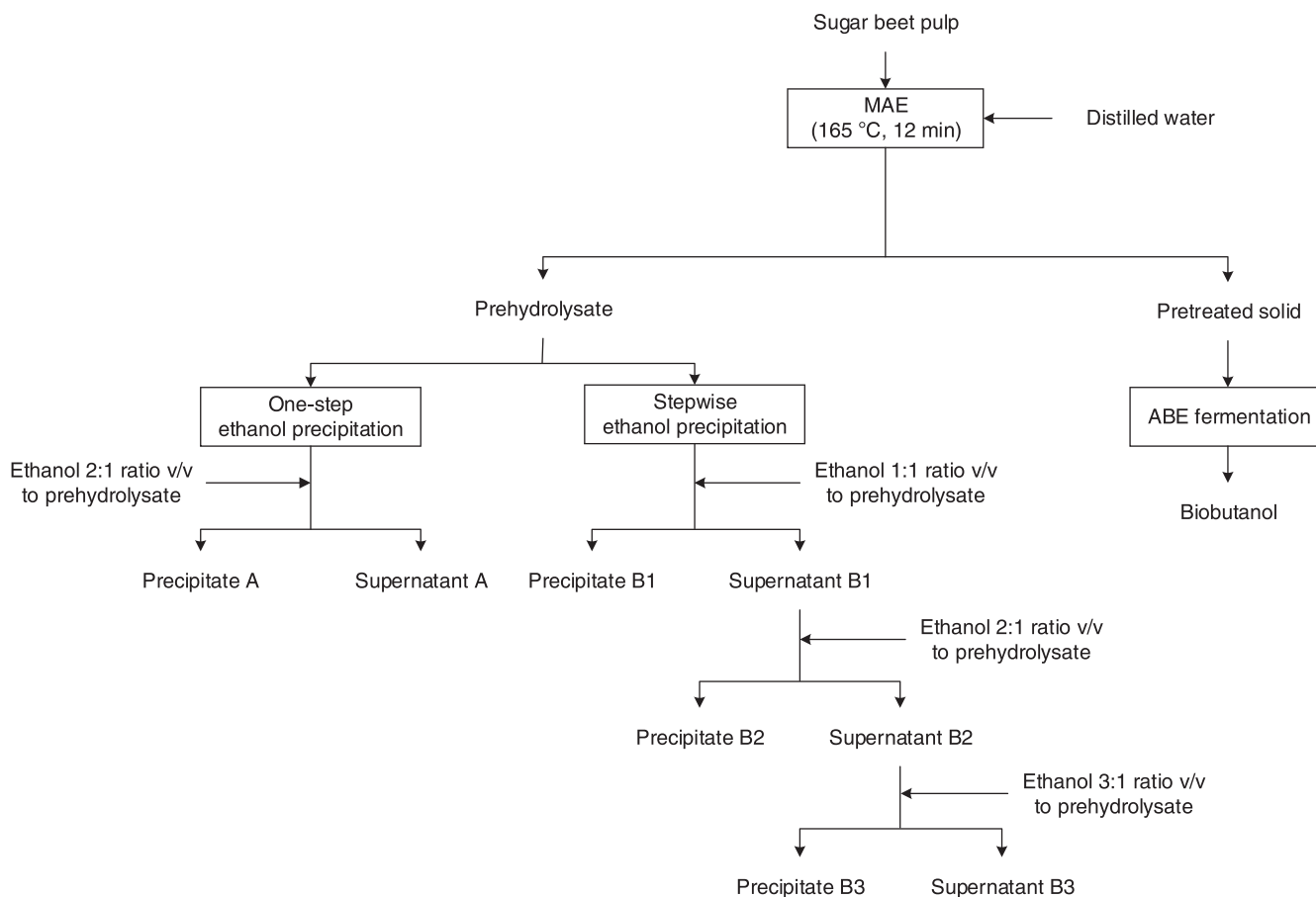


Fig. 1. Scheme of the global process for SBP valorisation.

The microorganism, *Clostridium beijerinckii* DSM 6422, was obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The strain was maintained on Reinforced Clostridial Medium in Hungate tubes (18 × 150 mm) in spore form and cold stored at 4 °C under anaerobic conditions. Inoculum was grown according to Plaza et al. (2017) with some modifications. Briefly, the inoculum was grown in a 250 mL serum bottle with a rubber septum under anaerobic conditions, 100 mL of growth medium (Reinforced Clostridial Medium (RCM) 33 g/L, glucose 5 g/L and xylose 10 g/L) and flushed with nitrogen. A thermal shock was performed at 80 °C for 3 min and at 4 °C for 3 min three times to simulate the germination of the spores. The enzymatic hydrolysate was used for ABE fermentation with *C. beijerinckii* at 35 °C and 135 rpm, sampling at 24, 48, 72, and 120 h. Fermentation experiments were carried out according to Plaza et al. (2017), but in a 100 mL Erlenmeyer flask. The pH of the hydrolysate was adjusted to 5.5 with 0.1 M NaOH. All the experiments were carried out in duplicate.

2.6. Analytical methods

2.6.1. Biomass characterisation

Structural carbohydrates and ash content of SBP and SBP pretreated under optimal conditions were analysed according to the Laboratory Analytical Procedures of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008b, 2008a). The extractive composition of SBP was also determined according to the NREL methods (Sluiter et al., 2008c). The pectin content in SBP was determined via the colourimetric method using galacturonic acid as a standard (Melton and Smith, 2001). Absorbance was measured at 525 nm in a UV-Vis spectrophotometer (Uvmini-1240, Shimadzu Suzhou Instruments Wfg. Shimadzu Corporation, Kyoto, Japan). Protein content was calculated as $N \times 6.25$, N being the Total Kjeldahl Nitrogen (TKN) measured by acid digestion with H₂SO₄ and distillation (KjelFlex K-360 distillatory, BUCHI, Mexico).

2.6.2. High-Performance Liquid Chromatography (HPLC) analysis

HPLC with an Aminex HPX-87 H column was used to analyse the concentration of sugars (glucose, arabinose and galactose), galacturonic acid, potential inhibitors (acetic and formic acids, HMF and furfural), ABE products (acetone, butanol and ethanol) and an ABE co-product (butyric acid). A refractive index detector (Waters 2414, USA) was used. The operational conditions were 0.01 N H₂SO₄ as the mobile phase, at a flow rate of 0.6 mL/min and 60 °C for determining sugars and 30 °C for ABE fermentation analysis. Oligomeric sugars in the pre-hydrolysates were determined by acid hydrolysis (121 °C, 30 min and in a 3% (v/v) H₂SO₄ 96%/sample ratio). Oligomeric sugars were calculated as the difference between the total free sugars after and before acid hydrolysis.

The molecular weight distribution of POS suspensions was determined by High-Performance Size Exclusion Chromatography (HPSEC) using a refractive index detector (Waters 2414, USA) and an Ultra-hydrogel 250 column (Waters, Japan). The operational conditions were

ultrapure water as the mobile phase, at a 0.7 mL/min flow rate and 35 °C (López-Linares et al., 2020). Dextran standards (Sigma-Aldrich, USA) were used with molecular weights of 1, 5, 12, 50, and 670 kDa.

All samples were filtered through 0.22 µm nylon filters before being analysed. All analyses were carried out in duplicate. The average results and standard deviations are shown.

2.6.3. Degree of esterification and FTIR analysis

The pectin degree of esterification was determined according to Gharibzadeh et al. (2019). Briefly, 3 mL of ethanol 96% was added to 0.2 g of precipitated solid and dissolved in 20 mL of distilled water. Two drops of phenolphthalein were added, and then the solution was titrated with 0.1 M NaOH (V₁). 10 mL of 0.1 M NaOH were added, and after 15 min, 10 mL of 0.1 M HCl were also added. The samples were titrated with 0.1 M NaOH (V₂). The pectin degree of esterification was calculated as follows (Eq. 2):

$$\text{Degree of esterification (\%)} = \frac{V_2}{V_1 + V_2} \times 100 \quad (2)$$

The functional-group composition of the pectin was analysed by FTIR (Alpha model, with a Platinum ATR single reflection diamond module, Bruker, USA). Absorbance spectra were obtained in the wavenumber range from 4000 to 400 cm⁻¹.

2.7. Statistical analysis

ANOVA test and the central composite experimental design were carried out by the software Statgraphics Centurion XVIII. Central composite design is a routable array that maximise the response variable selected. ANOVA test was used to conclude the statistical differences at a confidence level of 95% (p < 0.05).

3. Results and discussion

3.1. Characterization of SBP

Table 1 compares the raw material composition with other previously published data. The main components of SBP are hemicellulose (32.5%), glucan (19.9%) and protein (10.6%). Arabinan (25.8%) and galactan (6.7%) are the major carbohydrates in the hemicellulose fraction. This agrees with the data provided by Bellido et al. (2015) and Martínez et al. (2009). Besides carbohydrates, galacturonic acid was also detected (10.0% as galacturonan). Pectin content, analysed by the Melton and Smith (2001) method, was 26.9 ± 0.0% (dry basis), which is in accordance with Bellido et al. (2015), who reported a content of 27.34% (dry basis). Pectin is mainly made up of homogalacturonan (HG), known as smooth pectin, and rhamnogalacturonans I (RG-I), known as hairy pectin (Mao et al., 2019). HG is a linear galacturonic acid polymer methylesterified and acetylated. In contrast, RG-I is made up of chains with galacturonic acid and rhamnose, having many branched arabinan, galactan or arabinogalactan side chains (Martínez et al.,

Table 1
Sugar beet pulp composition (% dry weight).

	This study	Martínez et al. (2009)	Bellido et al. (2015)
Glucan	19.9 ± 0.5	20.1	20.3 ± 1.9
Arabinan	25.8 ± 1.4	17.5	21.8 ± 2.1
Galactan	6.7 ± 0.2	5.3	9.5 ± 0.8
Galacturonan	10.0 ± 0.2	21.0	N.P.
Extractives in water and ethanol	9.1 ± 1.0	N.P.	N.P.
Glucose in water extractives	0.3 ± 0.0	N.P.	N.P.
Arabinose in water extractives	2.8 ± 0.2	N.P.	N.P.
Galactose in water extractives	0.3 ± 0.0	N.P.	N.P.
Galacturonic acid in water extractives	0.9 ± 0.0	N.P.	N.P.
Ash content	10.0 ± 0.3	4.9	3.4 ± 0.2
Protein	10.6 ± 0.6	10.8	7.4 ± 0.01

N.P.: Not provided

2009). SBP is mainly composed of RG-I pectin (Mao et al., 2019). The RG-I structure supports the high hemicellulose content detected in SBP.

Water and ethanol soluble extractives constituted 9.1% of the dried SBP. Additionally, the composition of the water extractives was analysed. Contents in sugars (3.4%) and galacturonic acid (0.9%) were very low, and they were found mainly in oligomeric form. Almost all free sugar content in SBP was removed in previous washing. This may explain the low content of monomeric sugars found in the extractives.

Table 1 verifies the high amount of total carbohydrates (52.4%) and galacturonic acid content. Moreover, pectin analysis confirmed the presence of this polysaccharide in SBP. Therefore, a complete valorisation of carbohydrates in SBP can be approached. Firstly, the POS content (arabinose, galactose and galacturonic acid) can be extracted by hydrothermal MAE. After extraction, a pretreated solid richer in glucan is expected to be obtained (López-Linares et al., 2019). Thus, the pretreated solid rich in glucan can be valorised through biobutanol production by ABE fermentation.

3.2. Recovery of POS from SBP by hydrothermal MAE

The objective of the hydrothermal MAE was to obtain a POS-rich solution by the solubilisation of the hemicellulose content in SBP. A central composite experimental design was planned to assess the influence of temperature and time on POS extraction (Table 2). The SF was calculated (Eq. 1) to analyse the combined effect of the operational parameters (time and temperature) on POS recovery.

Solid recovery is also shown in Table 2. The effect of SF on the biomass solubilisation is noticeable. The lowest solid recoveries (51.0%, 51.7% and 54.2%) were found when the severity of pretreatment was higher (SF = 3.0, 3.6 and 3.4, respectively). Between 65.4% and 67% of the solid was recovered at the central point (155 °C, 8 min). This effect was also reported by López-Linares et al. (2020) and Romero et al. (2018). Hemicellulose is thermally degraded between 150 and 180 °C, while cellulose degradation occurs at more than 200 °C (Patel and Parsania, 2018). Thus, hemicelluloses were the main fraction solubilised during hydrothermal MAE.

The POS concentration corresponds to the sum of galacturonic acid, galactose and arabinose concentration in oligomeric form. The POS concentration (Table 3) ranged from 9.0 g/L (run 11) to 29.8 g/L (run 5). Glucose, galactose and galacturonic acid were mostly found in oligomeric form (Table 3). Contrary to alkali or acid extraction, which might degrade the oligomeric sugars, hydrothermal extraction maximises the oligomeric yield while minimising the content of monomers and degradation compounds (López-Linares et al., 2020). Nevertheless, the percentage of arabinose in oligomeric form ranged from 44.4% (run 3) to 95.9% (run 7). This change is clearly affected by SF. Higher SF (runs 2, 3 and 8) led to a lower ratio of the oligomer to total arabinose (47.2%, 44.4% and 79.1%, respectively). When the SF was lower than 3.0, between 91.2% (run 5, SF = 2.8) and 95.9% (run 7, SF = -2.0) of the arabinose was found as oligomer. So, arabinose was degraded to its

Table 2

Experimental design for pectooligosaccharides recovery by microwave-assisted extraction: severity factor (SF), solid recovery and pH of the prehydrolysates.

Run	T (°C)	t (min)	SF	Solid Recovery (%)	pH
1	155	2.3	2.0	71.3	4.2
2	190	8	3.6	51.7	3.8
3	180	4	3.0	51.0	3.9
4	155	8	2.5	67.0	4.0
5	155	13.7	2.8	63.8	4.1
6	155	8	2.5	65.7	4.1
7	130	4	-2.0	88.4	4.4
8	180	12	3.4	54.2	3.9
9	130	12	2.0	85.1	4.3
10	155	8	2.5	65.4	4.1
11	120	8	1.5	90.5	4.4

monomeric form when exposed to high temperatures for a longer time.

Results in Table 3 show an average concentration of 29.2 g/L and 56.1% of POS recovery at the central point (155 °C and 8 min). Maximum POS recovery corresponded to run 5: under these conditions (155 °C, 13.7 min), 57.2% of the POS present in SBP were recovered. This yield is in accordance with previously published data on hydrothermal MAE of hemicellulose. López-Linares et al. (2019) recovered 57.62% of the hemicellulose from brewer's spent grain by hydrothermal MAE at 185 °C for 6 min Luo et al. (2017) recovered 41% of the hemicellulose present in *Phyllostachys heterocycle* cv. Pubesvens by MAE at 216 °C for 5 min

Among the degradation compounds (formic acid, acetic acid, HMF and furfural), only formic acid was detected in the pretreatment liquids (data not shown). It was found in all experimental runs except runs 7, 9, and 11, where SF was lower (-2.0, 2.0 and 1.5, respectively). The formic acid concentration ranged from 0.1 g/L (run 8) to 0.9 g/L (run 2). Higher concentrations of formic acid were found in runs 2 (0.9 g/L; SF=3.6) and 3 (0.8 g/L; SF=3.0), which corresponded to the runs in which the galacturonic acid concentration was the lowest (2.9 and 2.6 g/L, respectively). This might indicate that galacturonic acid degrades to HMF, and later HMF degrades to formic acid under more severe MAE conditions (Mateos et al., 2012).

3.3. Optimization of hydrothermal MAE conditions

As mentioned above, one of the aims of this work was the recovery of POS from SBP. The prebiotic properties of POS are related to its neutral sugar content (Mao et al., 2019). Thus, the response chosen to optimise the extraction process was POS concentration in the prehydrolysate, which was maximised. A second-order polynomial equation (Eq. 3) was proposed to calculate the response (POS concentration) from the experimental data.

$$\text{POS} \left(\frac{\text{g}}{\text{L}} \right) = -317.363 + 4.342 \cdot T - 0.906 \cdot t - 0.014 \cdot T^2 \quad (3)$$

$$R^2 = 0.9544; \quad R^2_{\text{adjust}} = 0.9090$$

where T is the temperature (°C), and t is the time (min). The POS concentration was predictive with a confidence level of 95% ($p < 0.05$). The value of R^2 was found to be 0.9544, while the value of the adjusted R^2 was 0.9090. R^2 considers all the variables studied, while the adjusted R^2 only evaluates significant variables for the model ($p < 0.05$).

Based on this equation, temperature exerted a positive effect while time slightly negatively affected the POS concentration. This behaviour can be observed in Fig. 2, which plots the response surface for the POS concentration considering temperature and time operation. The stepper curve on the temperature axis shows the influence of this factor on POS concentration optimisation.

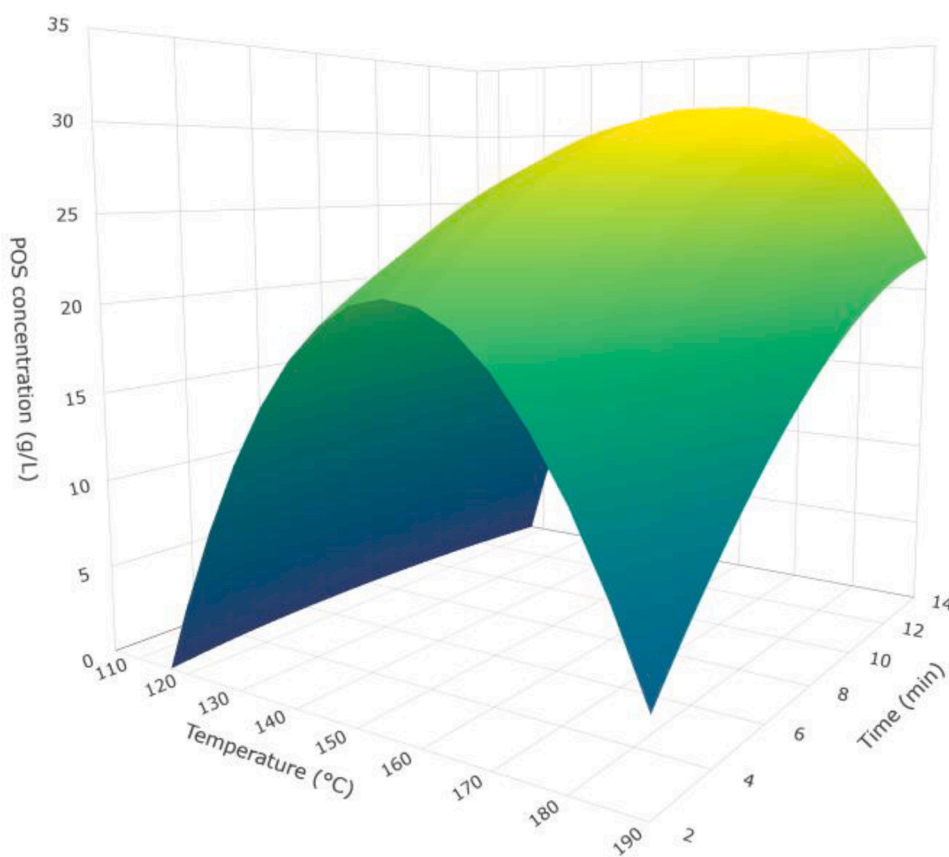
As a result of optimisation, 165 °C and 12 min were the optimal conditions predicted by the model. Under these conditions, the model estimated an optimum POS concentration of 31.4 g/L. To validate the optimisation results, three experimental runs were carried out under the optimal conditions for hydrothermal MAE (SF = 3.0). The composition of the prehydrolysate and pretreated solid are shown in Table 4. A concentration of 31.1 g POS/L was found, and 59.7% of the POS present in SBP were recovered in the prehydrolysate. No degradation compounds were detected. Thus, a good agreement was found between the predicted (31.4 g/L) and the experimental (31.1 g/L) results.

The POS extraction yield was in agreement with previously published data using SBP as the raw material. Leijdekkers et al. (2013) reported a yield of 50% (soluble oligo- and polysaccharides on dry matter) after enzymatic saccharification with Viscozyme L at 45 °C for 12 h. Zheng et al. (2013) recovered 66.9% of arabinose from SBP by conventional acid pretreatment at 140 °C using H₂SO₄ 0.5% (w/w) as solvent at 140 °C for 5 min. Thus, hydrothermal MAE might have a higher extraction yield than enzymatic saccharification, but slightly lower than

Table 3

Pectooligosaccharides (POS) recovery from sugar beet pulp by hydrothermal microwave-assisted extraction: sugar composition of prehydrolysates.

Run	Glucose		Arabinose		Galactose		Galacturonic Acid		POS (g/L)	POS recovery (%)
	g/L	% as oligomer	g/L	% as oligomer	g/L	% as oligomer	g/L	% as oligomer		
1	0.5 ± 0.0	88.1 ± 0.3	13.8 ± 0.0	94.5 ± 0.0	2.2 ± 0.0	98.3 ± 0.0	10.0 ± 0.2	98.3 ± 0.0	25.0 ± 0.2	48.1 ± 0.5
2	0.7 ± 0.0	89.6 ± 0.1	19.2 ± 0.7	47.2 ± 2.0	5.4 ± 0.0	88.6 ± 0.1	2.9 ± 0.0	99.2 ± 0.0	16.8 ± 0.8	32.2 ± 1.5
3	0.6 ± 0.0	89.0 ± 0.1	19.1 ± 0.6	44.4 ± 1.6	5.3 ± 0.0	87.6 ± 0.0	2.6 ± 0.0	99.3 ± 0.0	15.7 ± 0.6	30.1 ± 1.1
4	0.5 ± 0.0	86.0 ± 0.2	18.4 ± 0.0	92.0 ± 0.0	3.4 ± 0.0	97.6 ± 0.0	9.6 ± 0.0	97.5 ± 0.0	29.6 ± 0.0	56.8 ± 0.0
5	0.4 ± 0.0	87.4 ± 0.2	18.9 ± 0.2	91.2 ± 0.1	3.5 ± 0.0	97.8 ± 0.0	9.4 ± 0.0	97.3 ± 0.0	29.8 ± 0.2	57.2 ± 0.5
6	0.4 ± 0.0	86.9 ± 0.1	18.0 ± 0.3	92.2 ± 0.2	3.4 ± 0.0	98.0 ± 0.0	9.5 ± 0.0	97.6 ± 0.0	29.2 ± 0.3	56.0 ± 0.5
7	0.4 ± 0.0	87.4 ± 0.0	4.5 ± 0.2	95.9 ± 0.2	0.9 ± 0.0	91.5 ± 0.0	6.2 ± 0.0	98.6 ± 0.0	11.3 ± 0.2	21.7 ± 0.4
8	0.5 ± 0.0	96.1 ± 0.2	21.4 ± 0.2	79.1 ± 0.2	5.2 ± 0.0	94.3 ± 0.0	6.1 ± 0.0	99.8 ± 0.2	27.9 ± 0.2	53.6 ± 0.4
9	0.4 ± 0.0	86.8 ± 0.1	6.9 ± 0.0	95.5 ± 0.0	1.1 ± 0.0	99.4 ± 0.1	7.8 ± 0.0	98.6 ± 0.1	15.4 ± 0.0	29.5 ± 0.0
10	0.4 ± 0.0	87.4 ± 0.0	17.8 ± 0.6	91.9 ± 0.3	3.3 ± 0.0	97.8 ± 0.0	9.6 ± 0.0	97.6 ± 0.0	28.9 ± 0.6	55.5 ± 1.1
11	0.4 ± 0.0	85.6 ± 0.0	3.3 ± 0.3	95.5 ± 0.4	0.7 ± 0.0	88.7 ± 0.0	5.3 ± 0.0	98.4 ± 0.0	9.0 ± 0.3	17.4 ± 0.6

**Fig. 2.** Response surface: influence of temperature and time on POS concentration in the prehydrolysate.

that of acid extraction. However, besides the advantages of using water instead of acid as a solvent in terms of cost, corrosion and environmental issues, hydrothermal extraction minimises the concentration of degradation compounds. Zheng et al. (2013) pointed out the presence of degradation compounds at ratios of 29.9 mg acetic, 6.2 mg HMF and 1.9 mg furfural per g of SBP. Using water as the solvent, no degradation compounds were detected in the prehydrolysates under the optimal conditions found in our study. As far as we know, POS extraction studies from SBP by hydrothermal MAE have not been previously published.

On the other hand, pretreated solid rich in glucan was obtained, as expected. The glucan content increased from 19.9% in raw SBP (Tables 1) to 35.0% in the pretreated solid (Table 4). Due to its high carbohydrate content (54.4%), the pretreated solid can be valorised by producing an advanced biofuel such as biobutanol by ABE fermentation.

3.4. POS recovery by ethanol precipitation

In order to recover the POS contained in the prehydrolysate, an ethanol precipitation process was assessed. The prehydrolysate obtained under optimal MAE conditions was precipitated with two volumes (one-step precipitation) and one volume (stepwise precipitation) of ethanol. The supernatant obtained in the stepwise precipitation process was further precipitated with two and three volumes of ethanol (Fig. 1). Thus, four precipitates (A, B1, B2, and B3) were obtained and characterised. It should be noted that precipitate B3 could not be completely characterised due to the small amount recovered.

Table 5 summarises the process parameters, such as yield, composition, molecular weight and degree of esterification, of the precipitates obtained. As can be appreciated, the yield of the one-step precipitation (20.0%) is similar to the total yield obtained in the stepwise precipitation process (20.6%). This fact is in accordance with previous studies.

Table 4

Pretreated solid and prehydrolysate composition after microwave-assisted extraction under optimal conditions (165 °C, 12 min).

Component	Value
Pretreated solid (% w/w)	
Glucan	35.0 ± 0.8
Arabinan	13.6 ± 0.5
Galactan	5.8 ± 0.2
Galacturonan	2.1 ± 0.1
Prehydrolysate	
pH	4.0
Glucose (g/L)	0.6 ± 0.0
Arabinose (g/L)	21.2 ± 0.3
Galactose (g/L)	4.6 ± 0.0
Galacturonic Acid (g/L)	7.9 ± 0.1
POS (g/L) ^a	31.1 ± 0.4
POS recovery (%) ^a	59.7 ± 0.7

^a POS: pectooligosaccharides

Guo et al. (2016) precipitated the hydrolysate obtained by acid extraction (pH = 1.5) from SBP, reaching a yield (g precipitate/g SBP) of 9.6% in one-step precipitation (3:1 (v/v) ethanol:prehydrolysate ratio) and a total yield by stepwise precipitation of 9.6%.

Compared to previously published data, the yields obtained in this study are slightly lower. Abou-Elseoud et al. (2021) precipitated pectin from the SBP prehydrolysates obtained by enzymatic extraction (yield of 28.84%) and ultrasonic-assisted enzymatic extraction (yield of 33.5%) using 3 ethanol volumes. Comparing the yields corresponding to precipitates A and B1, it can be shown that an increase in ethanol ratio from 1:1 (precipitate B1) to 1:2 (precipitate A) led to a two-fold increase in yield. Mao et al. (2019) reported a yield of 10.8% by isopropanol precipitation (1:1 (v/v) prehydrolysate:isopropanol ratio) from the prehydrolysate obtained after hydrothermal MAE of SBP at 120 °C for 10 min. This yield is similar to precipitate B1 (10.4%), obtained under a similar prehydrolysate:alcohol ratio.

The precipitate composition is also shown in Table 5. Some impurities of glucan were detected in all precipitates, these being lower in precipitate B2 (11.5 mg/g) and higher in precipitate A (27.7 mg/g). Stepwise precipitation showed a decrease in galacturonan content from B1 (267.1 mg/g) to B2 (202.5 mg/g). This may be caused by the high polarity of the galacturonic acid that precipitates easily with lower ethanol concentrations (Ai et al., 2020). The neutral sugar content increased from 217.4 mg/g (precipitate B1) to 268.8 mg/g (precipitate B2). This tendency is in accordance with previous results (Ai et al., 2020; Guo et al., 2016) in which the neutral sugar content increased; while the galacturonan content decreased when ethanol dosage increased using the stepwise precipitation and prehydrolysate:ethanol ratios from 1:1–4:1 (Guo et al., 2016) and from 1:1–3:1 (Ai et al., 2020). The high yield of galactose with the low yield of arabinose in the precipitated solids is worth mentioning. While concentrations of galactose and

Table 5

Ethanol precipitation: process yield (Y), composition (mg/g precipitate), molecular weight (Mw), and degree of esterification (DE) of the pectin samples obtained in one-step (A) and stepwise (B1, B2, and B3) precipitation.

	Precipitate A	Precipitate B1 ^a	Precipitate B2 ^b	Precipitate B3 ^c
Y (g precipitate/100 g SBP)	20.0	10.4	8.6	1.6
Galacturonan	231.6 ± 2.3	267.1 ± 4.0	202.5 ± 2.1	N.D.
Galactan	165.2 ± 6.7	140.7 ± 3.8	183.6 ± 0.5	N.D.
Arabinan	79.4 ± 0.8	51.6 ± 2.2	73.7 ± 1.0	N.D.
Glucan	27.7 ± 1.0	25.2 ± 1.0	11.5 ± 0.6	N.D.
Neutral sugars	272.3 ± 6.6	217.4 ± 7.0	268.8 ± 1.0	N.D.
Mw (kDa)	488 ± 4	474 ± 9	523 ± 4	N.D.
DE (%)	65.8 ± 1.2	58.3 ± 0.2	59.0 ± 0.3	N.D.

N.D.: not determined

^a Precipitate B1 obtained by ethanol precipitation of the prehydrolysate

^b Precipitate B2 by ethanol precipitation of the supernatant B1

^c Precipitate B3 by ethanol precipitation of the supernatant B2

Table 6

Acetone-Butanol-Ethanol (ABE) fermentation of the pretreated solid: concentration of acetic and butyric acids and butanol and ABE yields (g obtained/g sugars consumed).

Time (h)	Acetic acid (g/L)	Butyric acid (g/L)	Y _{Butanol/sugars} (g/g)	Y _{ABE/sugars} (g/g)
24	0.9 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
48	0.9 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.0
72	0.9 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.0
120	0.7 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.0

arabinose in the prehydrolysate were 4.6 g/L and 21.2 g/L, respectively (Table 4, ratio galactose/arabinose of 0.22), the ratio of galactan/arabinan in the precipitates varied from 2.08 (precipitate A) to 2.73 (precipitate B1). Due to the high content in neutral sugars compared to galacturonan in all precipitates, the pectin extracted may correspond to the RG-I region with neutral sugar side chains, such as galactan and arabinan.

The molecular weight distribution and degree of esterification of pectin are shown in Table 5. The molecular weight is related to the gelling and rheological properties of pectin (Liew et al., 2019). The molecular weight of the compounds in the precipitates ranged from 474 kDa (pectin B1) to 523 kDa (pectin B2). The high molecular weight values can be attributed to the presence of pectin. As reported in previous research (Ai et al., 2020; Dervilly et al., 2000), it seems that the ethanol concentration did not influence the molecular weight of the recovered pectin. Lower molecular weights were found when an acid- or enzymatic-assisted extraction of pectin was carried out (Lim et al., 2012; Abou-Elseoud et al., 2021). A reduction in the molecular weight can also be obtained by enzymatic hydrolysis of the recovered pectin (López-Linares et al., 2020). The degree of esterification of all the precipitates was higher than 50%, ranging from 58.3% (pectin B1) to 65.8% (pectin A). The degree of esterification determines the grade of the pectin, and also affects its gelling and thickening properties (Marić et al., 2018). Pectin can be classified by degree of esterification as high in methoxyl (HM) (degree of esterification > 50%) and low in methoxyl (LW) (degree of esterification < 50%). All precipitates of this study can be classified as HM pectin, so they could form gels when treated at low pH in the presence of high sugar concentrations (Marić et al., 2018).

The chemical structure of the precipitates was analysed by FTIR (Fig. 3). Since the FTIR pattern of all the samples was similar to that reported for commercial pectin by Jiang et al. (2012), it can be confirmed that precipitated solids are mainly composed of pectin. The broad band at 3400 cm⁻¹ was due to the stretching of the hydroxyl groups (Jiang et al., 2012); whereas the absorption at around 2930 cm⁻¹ can be attributed to the O–CH₃ stretching from methyl esters of galacturonic acid (Lim et al., 2012). The strong absorption observed in 1700–1760 cm⁻¹ and 1600–1630 cm⁻¹ were caused by methyl-esterified and non-methyl-esterified carboxyl groups,

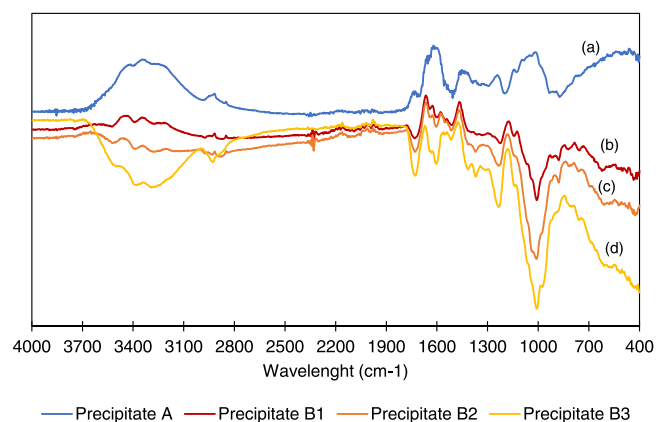


Fig. 3. FTIR spectra of the precipitated solids. (a) Precipitate A; (b) Precipitate B1; (c) Precipitate B2; (d) Precipitate B3.

respectively (Liew et al., 2019). The higher absorbance of pectin A in the 1700–1760 cm^{-1} band corroborates its higher degree of esterification over pectin B1 and B2. The absorption patterns from 800 to 1300 cm^{-1} correspond to C–O stretching, –OH bending and CH_3 deformation (Jiang et al., 2012).

In short, pectin obtained by one-step (pectin A) and stepwise (pectin B1, B2 and B3) precipitation showed similar compositions and properties.

3.5. Biobutanol production from pretreated SBP

The pretreated solid obtained under optimal MAE conditions (165 °C, 12 min) rich in glucan (Table 3) was first hydrolysed enzymatically. The 84.3%, 42.6% and 23.6% of glucose, galactose, and arabinose content, respectively, in the pretreated solid was recovered in the enzymatic hydrolysis. Thus, the concentration of monosaccharides in the liquor was 39.6 g/L, glucose being the main fermentable sugar (glucose: 35.9 g/L, galactose: 1.4 g/L, and arabinose: 2.3 g/L).

The enzymatic hydrolysate was subjected to ABE fermentation with *C. beijerinckii*. As shown in Fig. 4, butanol and ABE were produced mainly within the first 24 h of fermentation obtaining 6.9 and 13.5 g/L, respectively. Concentrations of butanol and ABE after 48 h of fermentation (8.3 and 15.2 g/L, respectively) were significantly higher ($p < 0.05$) from those obtained after 24 h. Butanol and ABE concentrations did not increase significantly after this time, while the yields of butanol and ABE based on sugar consumed remained constant from 48 h to 120 h of fermentation (Table 6). Thus, only 48 h of fermentation were needed for the sugar uptake to reach 90.3%. The productivities of butanol and ABE at 48 h were 0.17 g/(L·h) and 0.32 g/(L·h), respectively. A sample of pretreated SBP was fermented without previous enzymatic hydrolysis, but no butanol was produced (data not shown). The global yields corresponding to 48 h fermentation were 53 kg butanol/t SBP and 61 kg ABE/t SBP. The fermentation results obtained in this study were favourable compared to previous research with *C. beijerinckii* DSM 6422. Bellido et al. (2015) applied an autohydrolysis to SBP (120 °C, 5 min). Pretreated SBP was firstly enzymatically hydrolysed, and then the liquor was fermented, obtaining 42.9 g butanol/kg SBP, a maximum concentration of 4.8 g butanol/L and a yield of 0.20 g butanol/g sugar consumed. López-Linares et al. (2019) analysed the production of butanol from brewer's spent grain after a hydrothermal microwave pretreatment (193 °C, 5.4 min), followed by EH and fermentation of the liquor. They reported global yields of 46 kg butanol/t brewer's spent grain, a concentration of 8.3 g butanol/L and a yield of 0.26 g butanol/g sugar consumed. The higher sugar consumed yield (Table 6) found in this study can be explained by the MAE optimal conditions. Phenolic compounds were not found in the prehydrolysate under optimal conditions (data not shown). It should be noted that

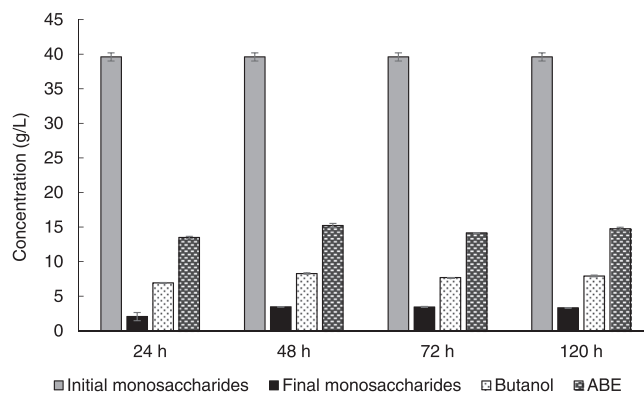


Fig. 4. ABE fermentation of the pretreated solid: monosaccharides, butanol and ABE concentration at different times.

butyric acid was not detected, while the acetic acid concentration was lower than 1 g/L in all samples (Table 6). The absence of butyric acid, a fermentation intermediate, suggests that all sugars were converted into butanol.

4. Conclusions

Carbohydrates contained in SBP were valorised by a cascade biorefinery approach, obtaining highly valuable bioproducts such as POS and biobutanol. Hydrothermal MAE can be considered a suitable technology for SBP fractionation. Under optimal hydrothermal MAE conditions (165 °C and 12 min), 59.7% of the POS were recovered from SBP, obtaining a concentration of 31.1 g POS/L. One-step and stepwise ethanol precipitation were evaluated for POS recovery. High methoxyl pectin fractions with high molecular weight were obtained. Pectin fractions differed from each other regarding their chemical composition. Furthermore, hydrothermal MAE has been demonstrated to be a proper pretreatment for ABE fermentation. The pretreated solid, rich in glucan, was subjected to ABE fermentation, obtaining a global yield of 61 kg ABE/t SBP. Thus, the carbohydrates in the SBP were fully valorised, and it can be considered a suitable raw material for a green biorefinery. Further work might be focused on separation methods by membranes before the precipitation step to reduce ethanol consumption.

CRedit authorship contribution statement

Esther del Amo-Mateos: Methodology, Investigation, Writing – original draft. **Juan Carlos López-Linares:** Methodology, Investigation, **Susana Lucas:** Conceptualization, Supervision, Writing – review & editing; Funding acquisition; **M.Teresa García-Cubero:** Methodology, Funding acquisition, Project administration. **Mónica Coca:** Conceptualization, Supervision, Writing – review & editing; Project administration, Funding acquisition.

Declaration of Competing Interest

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.

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