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Selective fractionation of Sugar Beet Pulp for release of fermentation and chemical feedstocks; Optimisation of thermo-chemical pre-treatment

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Abstract

The effect of time and pressure on the selective extraction of sugar beet pectin using steam pre-treatment on unprocessed Sugar Beet Pulp was evaluated using a design of experiments approach. This process gave the highest solubilisation of pectin oligomers at a relatively low pressure and longer time (5 Bar, 24 min), whilst leaving the majority of the cellulose fraction intact. This method of steam pre-treatment fits into the concept of a sugar beet biorefinery as it valorises an existing waste stream without requiring any further physical processing such as milling or dilution with water. The residual cellulose fraction was enriched in cellulose and could be effectively fermented into ethanol by yeast after enzymatic digestion, producing 0.48 g ethanol per gram of glucose.

Introduction

Over 17 million tonnes of sugar beet were grown in Europe in the 2012/13 season (statistics from the European commission for agriculture and rural development, http://ec.europa.eu/index_en.htm). The factory processing wastes from sugar beet

manufacture (Sugar Beet Pulp) have a dry-matter content of 18-23% w/w after sugar extraction (Kunel et al 2011) and are mainly converted into a low value animal feed, incurring significant drying and transportation costs (Zheng, 2013). SBP has a very high carbohydrate content (~80% w/w) predominantly made up of glucose (26% of the total w/w) in the form of cellulose, together with arabinose (23%) and galacturonic acid (15%) in the form of sugar beet pectin. Unlike many waste lignocellulosic materials, it is very low in lignin (~1-2%), making it relatively easy to process and the mild conditions under which sucrose extraction is carried out (60 °C, with 75 wt% of water) make SBP a potential raw material for saccharification and subsequent conversion of sugars to value-added products (Olmos and Hansen, 2012). However, the %w/w of cellulose is not high enough to make SBP a cost effective feedstock in hexose based fermentations. Previous studies in this area have focused on either complete saccharification of SBP for bioethanol or biogas production (Kunel et al. 2011, Zheng et al. 2012) or the selective fractionation of pectin by enzymatic treatment (Leijdekkers et al, 2013). Extraction using acidic treatments often results in the loss of cellulose (Sun, 1998), the generation of fermentation inhibitors (Larsson et al, 1999) and products which are enriched in homogalacturonans but low in neutral sugars (Rombouts and Thibault, 1986). Equally, scaling up enzymatic treatments is expensive and requires supplementation with cellulases to increase the yield of pectin, which reduces recovery of the cellulose fraction (Leijdekkers et al, 2013). Hot water treatment (HWT) has been shown to be an effective pre-treatment for SBP resulting in the solubilisation of 40 – 60% of the total biomass, depending on temperature and length of treatment (Hu et al, 2008). These treatments can solubilise all of the hemicellulose, up to 22% of the cellulose and 60% of the lignin. HWT does not require any addition of other reagents and so has the advantage of being lower cost than chemical or

enzymatic treatments and produces only small amounts of the degradation products that could inhibit subsequent fermentation when present at higher concentrations (Ziemiński et al, 2014). Previous studies using HWT have been done at low dry matter concentrations between 1-8% (Zhang and Shahbazi, 2011), with some requiring an additional milling step to reduce particle size before treatment (Kunel et al, 2011). Further size reduction or addition of water to SBP after sucrose extraction is not desirable, due to the additional costs incurred.

In this study we present the optimisation of a pressurised steam pre-treatment for the selective fractionation of SBP, which does not require the addition of water or a reduction in particle size. This selective release of arabinose and galacturonic acid allows cellulose to be recovered as an enriched by-product of the fractionation in a 'Biorefinery' context, making the hydrolysis and utilisation of cellulose for ethanol production cost effective. A statistical Design of Experiments (DoE) approach has been used in order to: (i) obtain a detailed understanding of the factors influencing arabinose and galacturonic acid release and their interactions, and (ii) develop statistical models enabling accurate prediction of the optimum conditions for the solubilisation of arabinose and galacturonic acid while minimising hydrolysis of cellulose.

Materials and Methods

2.1 Pretreatment

2.1.1 Pre-wash

SBP from the 2012 / 2013 UK harvest was supplied by AB sugar (Wissington, Norfolk, UK). The material was received frozen but had not undergone any further processing after sucrose extraction. One kilogramme of SBP was defrosted and washed twice in ultrapure water (18.2M Ω .cm) at room temperature to remove residual sucrose, then pressed in a manual screw press to remove excess moisture. The percentage dry weight of washed and pressed SBP was calculated by drying samples at 105°C for 48hrs and calculating the percentage of the total starting weight. The dry weight of the washed and pressed SBP was 27.76% (\pm 0.08). A small amount of this washed and pressed SBP was dried to ~98% dry solids for the exact carbohydrate composition to be determined.

2.1.2 Steam Pre-treatment

A stirred Parr pressure reactor (1L capacity, Boston Instruments) controlled with a Parr 4843 control module was used for all treatments. Fifty grams of whole, washed SBP was loaded into the pre-heated reaction vessel and the heating jacket was fitted around the reaction vessel to help with heat retention. High-pressure steam (10Bar, 184°C) was allowed into the vessel until the required pressure was achieved (between 4 – 8Bar(g), equivalent to 152°C – 175.5°C). Gauge pressure (Bar(g)) was monitored throughout each experiment and more steam allowed into the vessel as required in order to keep the pressure at a constant level. The reactor was fitted with a three-arm, self centering anchor stirrer with PTFE wiper blades, and set to 150rpm. At the end of each experiment, the pressure was released instantly to achieve explosive decompression ('steam explosion'). The pressure release valve was connected to a collection bottle through ¼" insulated tubing. Upon release of the pressure, some of

the liquefied pectin fraction escaped through the connecting tubing and was collected in a 1L Duran bottle cooled on ice. The release of steam and vapour ensured that no liquid was retained in the tubing during this process. The outside of the reaction vessel was quickly cooled with water until the gauge pressure inside the vessel had reached zero. The vessel contained the insoluble residue and remaining liquefied fraction. This was re-combined with the collected liquid fraction and the solid and liquid fractions separated by straining through a muslin cloth. The solid material was washed in 100ml of ultrapure water and then pressed to remove excess liquid, which was added to the soluble fraction. The volume of the soluble fraction was measured and the insoluble fraction dried at 60°C overnight.

2.1.3 Statistical experimental design

Response surface methodology was used to determine the optimum conditions and effects of two independent variables A: time and B: pressure using Design Expert 9 software (Stat Ease, Minneapolis, USA) for experimental design and analysis. The yields of arabinose, galacturonic acid and glucose released in the soluble fraction were the measured responses. A central composite design consisting of 11 experimental runs was used, including three replicates at the centre point. The pressure ranged from 4Bar to 8Bar and the time from 1min to 30min with the centre point at 6Bar for 15.5min (see table 1). The experiments were performed in a random order. The model was validated with triplicate experiments at the optimum conditions and analysing the resulting fractions.

2.2 Analysis of carbohydrates by Ion Chromatography

Ion Chromatography was performed using a Dionex 5000+ fitted with a 4 x 250mm analytical CarboPac PA1 column. Flow rate was set to 1.0ml / min running 0-15min: 25mM NaOH, 15-20min: linear gradient of 25-75mM NaOH, 20-30min: 75mM NaOH with linear gradient of 0-260mM NaOAc, 32-34min: 75mM NaOH with 260mM NaOAc, 34-42min: 200mM NaOH, 42-52min: 25mM NaOH (Adapted from Kuhnel, 2012).

Calibration was performed with standard sugars obtained from Sigma and made up to the desired concentration in ultrapure H₂O. Monomeric sugar concentrations were calculated directly from the soluble fraction. Oligomeric sugars were first hydrolysed into their constituent monomers by the addition of 106µL of 72% H₂SO₄ to 3ml of each soluble fraction (in triplicate) and autoclaving for 1hr at 121°C. Samples were neutralised with solid CaCO₃ and filtered before analysis. To calculate the carbohydrate composition of the insoluble residue from each pre-treatment; 100-300mg of the washed and dried material was acid hydrolysed by the addition of 3ml 72% H₂SO₄ for 1hr at 30°C, 150rpm. After 1hr, 84ml of ultrapure H₂O was added and the sample autoclaved for 1hr at 121°C. Samples were neutralised with solid CaCO₃ and filtered before analysis. Hydrolysis and analysis of all samples was repeated in triplicate.

2.2.1 Detection of degradation products

Fractions collected from the optimised pre-treatment condition were tested for the presence of furfural, hydroxymethylfurfural (HMF) and acetic acid by HPLC (Agilent 1200 Series HPLC) using a 300 x 7.8mm Rezex ROA-Organic Acid H+ (8%) column (Phenomenex, Cheshire, UK), running at 65°C in 5% H₂SO₄, 0.6ml/min. Samples were not tested for the presence of aldehydes.

2.3 Fermentation of insoluble residue

2.3.1 Enzymatic hydrolysis

The insoluble residue recovered from the optimised pre-treatment was combined with ultrapure H₂O (equivalent to 5% dry solids) in a 250ml Duran bottle and sterilised by autoclaving for 15min at 121°C. This sterilised mixture was incubated with 0.5mg of cellulase 13L – C013L per g of glucan (Biocatalysts Ltd, Cardiff, UK) containing a high proportion of cellulase activity with additional cellobiase, beta glucosidase and beta glucanase side activities for complete cellulose breakdown. The hydrolysis was performed at 50°C with shaking for 24hrs. After 24hrs, the concentration of each sugar released was analysed by Ion Chromatography. The digested sample was centrifuged at 4000 x g for 20min to remove the insoluble residue and the soluble fraction sterilised at 121°C for 15min. The analysis was repeated after this process and the concentration of glucose was unchanged after sterilisation.

2.3.2 Yeast fermentation

A commercially available strain of *Saccharomyces cerevisiae* (Alcotec 24 Turbo Yeast, Hambleton Bard Ltd, Chesterfield, UK) was grown in 1 x YNB (Melford, UK) containing 5g/L glucose. The starter culture then was used to inoculate YNB containing either the digested SBP residue or the equivalent concentration of glucose (~16g/L final concentration). Incubations were carried out in triplicate at 30°C with shaking for 24hrs. After 24hrs, the ethanol and residual glucose concentrations were determined by HPLC (as described for detection of inhibitory compounds) and Ion Chromatography respectively.

2.4 Protein extraction and analysis

Samples of washed and untreated SBP, the insoluble fraction from steam pre-treated SBP and the insoluble residue recovered after cellulase hydrolysis were milled to <4mm particle size in a Waring spice grinder (Waring, USA). Protein extraction was performed by incubating 100-300mg of the sample in 10ml of 0.1M NaOH at 80°C for 3hrs. Extracted protein concentration was determined by Biorad protein assay (Biorad, Hemel Hempstead, UK) using a calibration curve constructed with an albumin standard (ThermoScientific, MA, USA) made up in 0.1M NaOH.

3. Results and discussion

3.1 Carbohydrate composition of SBP

The carbohydrate composition of the washed, pressed SBP was determined as a fraction of the total dry weight after acid hydrolysis as (%w/w): rhamnose (2.4%), arabinose (23.0%), galactose (6.2%), glucose (25.9%), mannose (1.0%), xylose (1.68%) and galacturonic acid (14.4%). The total amount of each sugar available for extraction in 50g SBP could then be calculated.

3.2 Thermo-Chemical Pre-treatment of SBP

3.2.1 Experimental design: identification of factors and ranges

The low lignin content of SBP means that high temperature treatments are not required to solubilise the pectin fraction. Operating at lower temperatures (<190°C,

11Bar(g)) also significantly reduces the decomposition of pentose sugars into furfural and acetic acid and limits the amount of cellulose solubilised (Heitz et al, 1991). Below 4 Bar(g), the pressure was not sufficient to create an explosive decompression and so a pressure range between 4-8 Bar(g) was selected. Treatment times had an upper limit of 30 min to make the process industrially relevant, where long treatment times are avoided due to the additional costs incurred.

The pre-treatments were run at a high percentage dry weight, exploiting the naturally high water content of SBP (~72%). The addition of high pressure steam was sufficient to heat the water in the SBP and effectively solubilise of the pectin fraction, resulting in a concentrated soluble fraction.

3.2.2 Statistical pre-treatment experiments

Eleven steam-explosion pre-treatments over various times and operating pressures (which also determines the temperature) were performed according to the central composite design described above. The total monomeric and oligomeric sugars in the soluble fraction (including the 100ml wash) of the pre-treated samples was determined by ion chromatography. These values were then used to calculate the percentage of each sugar released from the total available in the starting material (table 1). The amount of galacturonic acid released into the soluble fraction was always less than 50% of the starting amount, but the remainder was not present in the insoluble residue with the cellulose fraction. A sample of 1g of pure galacturonic acid was subjected to the 5Bar(g) pressure for 24min and then analysed by IC and HPLC as described. This treatment resulted in a 35% loss of galacturonic acid compared to the starting amount. The loss was not due to the decarboxylation of galacturonic acid to

arabinose or subsequent degradation to furfural as no arabinose or furfural could be detected in the sample post treatment. It is likely that galacturonic acid is being degraded to a volatile compound which is then being lost during the pressure release from the system, in agreement with previous findings (Bornik and Kroh, 2013).

3.2.3 Generation of models to describe total soluble sugar release

3.2.3.1 Identification of significant parameters and suitable models

The experimental data for the total soluble yield (combined monomer and oligomer) of glucose, galacturonic acid and arabinose were fitted to linear, two-factor interaction (2FI), quadratic, and cubic polynomials using Design Expert software. The models were compared to see how well they fitted the data, and the model with the best fit was selected in each case. Tables 2-4 list the analysis of variance (ANOVA) for the fitted quadratic (arabinose and galacturonic acid) or 2FI (glucose) polynomial model representing the yield of each sugar over the range of conditions tested. The ANOVA shows that the models for arabinose and glucose are highly significant as the Fisher F-test values are 31.33 and 10.01 respectively and yield a low probability value in each case (Prob>F less than 0.005). This probability value means that there is less than a 0.5% chance that a "Model F value" this large could occur due to noise. The "Lack of fit F-Value" of 0.84 and 0.37 for arabinose and glucose are not significant relative to pure error. The probability value (Prob>F greater than 0.1) means that there is a high chance that a "Lack of fit F-Value" this large could occur due to noise. The degradation of galacturonic acid with temperature reduces the confidence of the model, but it is still significant with a "Model F value" of 4.57 and a probability value less than 0.05 (Prob>F <0.05). This probability value means that there is less than a 5%

chance that a “Model F value” this large could occur due to noise. The “Lack of fit F-Value” of 2.91 is not significant relative to pure error. The probability value (Prob>F greater than 0.1) means that there is a 27.96% chance that a “Lack of fit F-Value” this large could occur due to noise.

3.2.3.2. Analysis of models

Table 5 summarises the statistical analysis performed on each model. The goodness of fit of each of the models was further checked by the coefficient of determination (R^2). For arabinose the R^2 value is 0.947, indicating that only 5.3% of the total variation is not explained by the model. For glucose and galacturonic acid, the R^2 values are 0.790 and 0.504 respectively, indicating that a greater percentage of the total variation cannot be explained by the model. The individual model terms of time (A) and temperature (B) are also considered. Values of "Prob > F" less than 0.0500 indicate model terms are significant. The significant model terms in each case are; arabinose (A, B, A^2), galacturonic acid (AB) and glucose (A, B). The “Adequate precision” ratio (Adeq) measures the signal to noise ratio should be greater than 4 for the model to be significant. For each of the models (arabinose, glucose and galacturonic acid), the adequate precision ratio is greater than 4, indicating a good fit for the model. From the above analysis, it can be concluded that the models for Arabinose and glucose represented by equations (1) and (3) can be confidently used to navigate the design space.

Eqs. (1)–(3): statistical equations for prediction of arabinose, galacturonic acid and glucose release based on the ANOVA tables shown in [Tables 2–4](#). Constants shown in term of uncoded values: where T = time (min), P = pressure (Bar).

$$\text{Arabinose} = -(81.33) + (4.29 * T) + (28.24 * P) - (0.07 * T^2) - (1.79 * P^2) \quad (1)$$

$$\text{Galacturonic acid} = +(35.9) - (0.06 * T * P) + (9.34E-003 * T^2) \quad (2)$$

$$\text{Glucose} = (+2.13) - (0.11 * T) - (0.02 * P) + (0.04 * T * P) \quad (3)$$

3.2.4 Prediction and verification of optimal Arabinose release

Having established effective models for prediction of arabinose and glucose release, the models were used to predict optimal conditions for maximising the release of arabinose into the soluble fraction with limited hydrolysis of cellulose. The degradation of galacturonic acid with heat resulted in a model of lower confidence compared to those for arabinose and glucose. Optimisation was therefore biased towards maximising the yield of arabinose (>70%) and minimising the yield of glucose (<6%) in the soluble fraction, thereby retaining the cellulose in the insoluble fraction. Time and pressure ranges were defined as 1-30min and 4-6bar as the highest solubilisation of pectin oligomers was seen at a relatively lower pressures and longer time with higher pressure and shorter time combination being less effective due to the degradation of sugars and breakdown of the cellulose fraction. Design Expert software predicted one solution to satisfy these criteria; 5.33Bar for 24.37min predicting a yield of 83.2% of the arabinose, 39.8% of the galacturonic acid but only 4.9% of the glucose in the soluble fraction (fig. 1). The surface response shown in fig. 1 shows that the area around the optimum conditions is broad enough to allow for the optimal values of time and pressure to be rounded to whole numbers to compensate for the limited fine control of the steam explosion technique; the optimal values for time and pressure were therefore adjusted to 24min and 5Bar. These conditions were carried out in triplicate and the yields of each sugar in the soluble and insoluble fraction determined (fig. 2). No furfural or HMF could be detected under these conditions which is important because these compounds are known to inhibit growth of microbial

cultures. The yields of arabinose, glucose and galacturonic acid in the soluble fraction were very close to those predicted; which supports the statistical validity of the model.

3.3 Fermentation of insoluble residue

3.3.1 Enzymatic hydrolysis

The insoluble fraction remaining after SBP pre-treatment under optimal conditions was subjected to hydrolysis with a commercial cellulase as described in Section 2. After 24hrs incubation, all of the available glucose in the sample had been monomerised; suggesting that the steam pre-treatment is sufficient to prepare the cellulose for hydrolysis without the need for any additional processing. The digested sample was centrifuged at 4000 x g for 20min to remove any residual solids and then the soluble fraction sterilised at 121°C for 15min. The carbohydrate composition determined by IC was: rhamnase (0.2g/L), arabinose (0.8g/L), galactose (0.2g/L), glucose (19g/L), xylose (0.6g/L), galacturonic acid (0.1g/L).

3.3.2 Yeast fermentation

The monomeric sugars released by cellulase treatment of the insoluble residue after pectin release was tested as a fermentation substrate for growth of Turbo-yeast, and compared to a control containing the same concentration of pure glucose. The optical density of each culture was measured at 600nm after 24hrs growth and the residual carbohydrate and ethanol concentrations determined (table 6). The YNBG cultures reached a higher optical density after 24hrs but consumed less of the available glucose and produced less ethanol compared to the culture containing the cellulase-treated

SBP fraction. The YNBG cultures produced an average of 0.38g ethanol per gram of glucose consumed and the SBP cultures 0.48g ethanol per gram of glucose. The concentration of other carbohydrates in the SBP media was unchanged after 24hrs. This suggests that the SBP cultures were showing signs of metabolic uncoupling, metabolising glucose more rapidly, which would reduce the dissolved oxygen in the cultures and increase ethanol yield, while producing lower cell densities.

3.4 Protein Extraction

Protein extraction was performed on samples of raw untreated SBP, residual solids after optimised steam pre-treatment and the solid fraction after cellulase hydrolysis. Steam pre-treatment resulted in a loss of around 1% w/w protein, but the final insoluble product from cellulase hydrolysis was enriched in protein as the cellulose fraction had been removed. The percentage of protein per unit dry weight of SBP was almost doubled after cellulase hydrolysis compared to the raw, untreated material from 0.08g protein per g of SBP to 0.15g/g in the cellulase treated sample. This increase in protein and low fibre content could allow the residue from hydrolysis, together with the cells produced during fermentation, to be utilised as poultry feed (Koc et al. 2010; Bovo et al. 2015)

4. Conclusions

The selective fractionation of SBP can be achieved in a single pre-treatment, fitting perfectly into the concept of a sugar beet biorefinery as an existing waste stream is valorised but does not require any further physical processing such as milling, the addition of chemicals or dilution with water. The resulting pectin fraction is largely oligomeric, which could be valuable for its functional properties or converted to

monomers for use as chemical precursors or biological substrates. The residual cellulose can be used as a high quality fermentation feedstock, as inhibitory compounds are not generated during this process.

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