

# An integrated biorefinery concept for conversion of sugar beet pulp into value-added chemicals and pharmaceutical intermediates

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Over 8 million tonnes of sugar beet are grown annually in the UK. Sugar beet pulp (SBP) is the main by-product of sugar beet processing which is currently dried and sold as a low value animal feed. SBP is a rich source of carbohydrates, mainly in the form of cellulose and pectin, including D-glucose (Glu), L-arabinose (Ara) and D-galacturonic acid (GalAc). This work describes the technical feasibility of an integrated biorefinery concept for the fractionation of SBP and conversion of these monosaccharides into value-added products. SBP fractionation is initially carried out by steam explosion under mild conditions to yield soluble pectin and insoluble cellulose fractions. The cellulose is readily hydrolysed by cellulases to release Glu that can then be fermented by a commercial yeast strain to produce bioethanol at a high yield. The pectin fraction can be either fully hydrolysed, using physico-chemical methods, or selectively hydrolysed, using cloned arabinases and galacturonases, to yield Ara-rich and GalAc-rich streams. These monomers can be separated using either Centrifugal Partition Chromatography (CPC) or ultrafiltration into streams suitable for subsequent enzymatic upgrading. Building on our previous experience with transketolase (TK) and transaminase (TAM) enzymes, the conversion of Ara and GalAc into higher value products was explored. In particular the conversion of Ara into L-gluco-heptulose (GluHep), that has potential therapeutic applications in hypoglycaemia and cancer, using a mutant TK is described.

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Sugar beet pulp is the plant material left after sucrose extraction. It is found in the aqueous by-product stream from the solid–liquid diffuser where the sucrose is extracted. This stream is released at around 70 °C and is currently dried to approximately 88% w/w dry solids (requiring considerable energy input) before being sold as an animal feed (a relatively low value by-product). The focus of this work is on valorisation of the aqueous SBP stream after it leaves the diffuser; the aim is to create a range of value-added products from the SBP while simultaneously reducing the energy costs and greenhouse gas emissions associated with the SBP drying process.

The conceptual design of a biorefinery process that could be integrated within the Wisington facility must seek to utilise all the main components present in the SBP. As shown in Section 2.1, SBP is largely composed of two biopolymers; cellulose and pectin. Unlike citrus pectin, sugar beet pectin has poor gelling properties due to its high degree of esterification hence its applications are limited.<sup>2</sup> The major monosaccharides present in SBP are D-glucose (Glu), L-arabinose (Ara) and D-galacturonic acid (GalAc). Glu is readily fermented by a range of microorganisms and hence provides a route to a range of fermented products. Ara has been shown to be useful for the production of biopolymers after esterification. It can also be reduced to arabinitol, highlighted as one of the 12 top value-added chemicals from biomass, which can be used as a building block for unsaturated polyester resins.<sup>3</sup> Moreover, chemical methods have been developed for converting Ara into potentially useful starting materials for medicinal chemistry.<sup>4</sup> Oxidised sugars, such as GalAc, have also been highlighted as important building blocks for the production of hyperbranched polyesters and plasticisers.<sup>3</sup>

A particular focus of our previous work has been the enzymatic, and chemo-enzymatic, synthesis of chiral aminoalcohols. These are an important class of pharmaceutically-relevant compounds, and their motif is present in antibiotics, anti-viral glycosidase inhibitors as well as sphingolipids.<sup>5</sup> Enzymes such as transketolase (TK), which catalyses asymmetric carbon–carbon bond formation,<sup>6</sup> and transaminase (TAM), which catalyses selective amine group transfer, have been particularly useful in synthesising a range of such products.<sup>7–10</sup> Chiral aminoalcohols therefore represent an interesting class of higher value products that could be accessed from renewable feedstocks such as SBP. The challenge however is to identify variants of the TK and TAM enzymes that are able to convert SBP monosaccharides in biorefinery process streams with adequate productivity.

## 2. Results and discussion

### 2.1 Feedstream compositions

**2.1.1 Sugar beet pulp composition.** Valorisation of each of the main components present in the SBP stream is the primary goal of this conceptual biorefinery process design. SBP is a rich source of carbohydrates that consists mainly of cellulose (a polymer of D-glucose) and pectin (a co-polymer of various hexose and pentose sugars) as well as other components such as lignin (<3% w/w) and protein (11% w/w). Sugar beet pectin is formed of a linear backbone, from chains of polymeric D-galacturonic acid with intermittent blocks of alternating L-rhamnose (Rha) and D-galacturonic acid residues, and two different types of side chains. These are linear galactans (polymeric D-galactose) and highly branched arabinans (polymeric L-arabinose).<sup>11,12</sup>



The carbohydrate composition of the washed and pressed SBP used in this work was determined as a fraction of the total dry weight after full acid hydrolysis as shown in Fig. 2. These values are similar to those previously reported for other sources of SBP.<sup>12,13</sup> Ara and GalAc, both from pectin, along with Glu, from cellulose, are the main components accounting for 85% of the total monosaccharides. The utilisation of these three monosaccharides is therefore a focus of the subsequent SBP fractionation and bioconversion studies.

**2.1.2 Vinasse composition.** The cost of enzymes that can be used for biomass solubilisation (Section 2.3.2) or biocatalytic synthesis (Section 2.4) usually has a significant impact on the overall process economics.<sup>14</sup> Within an integrated biorefinery context it is therefore of interest to look for further by-product streams that can be used to support enzyme production by microbial fermentation. This will help reduce the cost of process enzymes if produced on site.

Within the Wisington biorefinery (Fig. 1), vinasse is the by-product of the existing yeast-based bioethanol fermentation. It is the spent fermentation supernatant that is released from the bottom of the first ethanol distillation column. Consequently it will have been concentrated and the thermal treatment promotes side reactions, *i.e.* Maillard reactions between yeast proteins and reducing sugars, as well as precipitation or degradation of heat labile components.

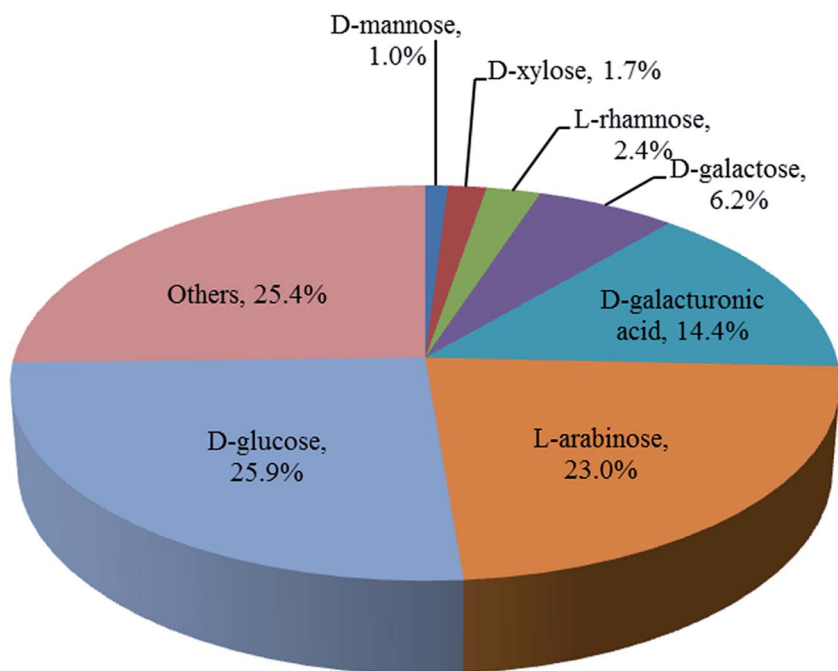


Fig. 2 Composition of the sugar beet pulp used in this work (obtained from the British Sugar biorefinery, Wisington, UK). Monosaccharide concentrations determined using Ion Chromatography (Dionex ICS 5000+, equipped with an electrochemical detector and fitted with a  $4 \times 250$  mm analytical CarboPac PA1 column) after full acid hydrolysis with  $\text{H}_2\text{SO}_4$  2.5% (v/v) at  $121^\circ\text{C}$  for 1 hour.



The vinasse stream used here appears as a dark liquid containing fine suspended solids and has a pH of approximately 5. It has high levels of glycerol, corresponding to about 35% of the total vinasse (on a dry mass basis), at a concentration of 188 g L<sup>-1</sup>. As described in Table 1, the vinasse also contains several monosaccharides, such as D-fructose and Gal, as well as low concentrations of sugar alcohols including mannitol, xylitol and dulcitol. The total protein in the vinasse was quantified at 5 g L<sup>-1</sup>, and the presence of acetate and polyphenols was also determined, which is in agreement with previous publications.<sup>15,16</sup> The high concentration of glycerol, a good carbon and energy source for microbial fermentation, makes the vinasse an interesting fermentation feedstock for enzyme production (Section 2.2) provided simple pre-treatment methods can be established.

## 2.2 Biorefinery enzyme production (L-arabinofuranosidase)

Enzymes are widely used as industrial biocatalysts due to their high specificity and selectivity. Enzymatic processes are considered environmentally friendly as enzymes are biodegradable and tend to produce less waste.<sup>17</sup> In a biorefinery context, enzymes can be used to breakdown polymers released from complex feedstocks, the classic example being depolymerisation of cellulose into D-glucose monomers by cellulases, or for synthetic purposes, e.g. the production of chiral aminoalcohols as described in Section 1.

A range of effective cellulases are already commercially available and we have previously described the cloning and production of TK and TAM enzymes used in chiral aminoalcohol synthesis.<sup>8,10</sup> L-Arabinofuranosidases (AF, EC 3.2.1.55) are exo-type glycosidases that catalyse the successive removal of L-arabinose residues from the non-reducing termini of  $\alpha$ -1,2-,  $\alpha$ -1,3-,  $\alpha$ -1,5- and  $\alpha$ -4,6-linked arabinofuranosyl residues.<sup>18</sup> In order to release Ara monomers from the arabinan side chains of SBP pectin we have identified and cloned a thermostable AF from *Geobacillus thermoglucosidasius*. This is a homodimeric enzyme with a MW of 130 kDa that is able to hydrolyse natural arabinans from different sources, such as sugar beet pectin, yielding monomeric Ara and an intact D-galacturonic acid backbone (GABB).

**Table 1** Concentration of main components present in the vinasse stream. Sugar and sugar alcohols were determined using ion chromatography, glycerol and acetate using HPLC-RI and polyphenols using spectrophotometry with galic acid as reference. The sugar beet vinasse used in this work was provided by British Sugar (Wissington, UK)

Component	Concentration (g L <sup>-1</sup> )
Glycerol	187.6 ± 2.8
Mannitol	8.0 ± 0.03
Galactose	5.5 ± 0.02
Xylitol	5.4 ± 0.09
Fructose	2.0 ± 0.03
Dulcitol	2.0 ± 0.02
Acetate	1.7 ± 0.02
Total protein	5.1 ± 0.08
Polyphenols	6.0 ± 0.02









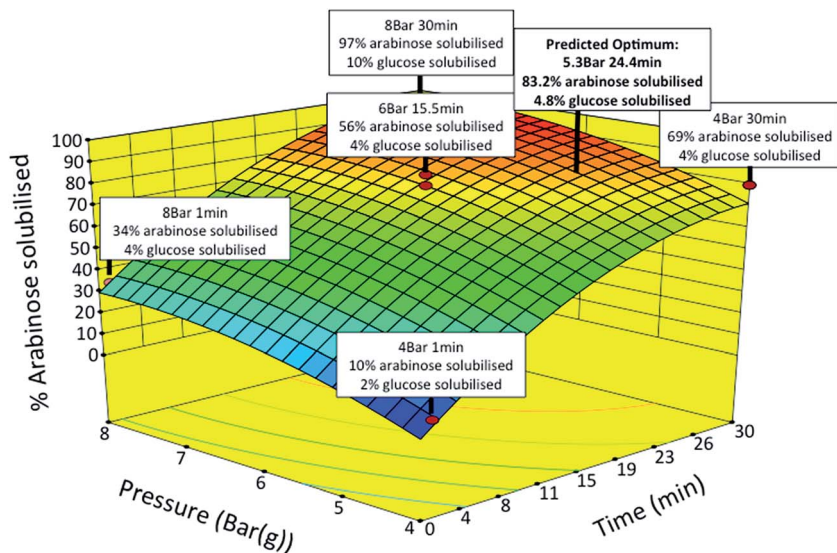


Fig. 4 Surface response diagram showing percentage of L-arabinose solubilised as a function of pressure and time during steam explosion (SE). The red data points represent actual experimental design points. 11 SE pretreatments over various times and operating pressures were performed using a central composite design. SE experiments were carried out in a stirred Parr pressure reactor (1 L capacity, Boston Instruments) with 50 g of whole and washed SBP at the specified pressure and time with agitation fixed at 150 rpm.

**2.3.2 Enzymatic hydrolysis of SBP and separation of L-arabinose by ultrafiltration.** Following SE, the sugar beet pectin is recovered in the soluble fraction as a largely intact polymer. This requires further hydrolysis to release the main pectin monosaccharides: Ara and GalAc (Fig. 2). The initial focus was on Ara since it is the most abundant. Selective depolymerisation of the arabinan side chains of the pectin using L-arabinofuranosidase (AF) was attempted. This would yield soluble monomeric Ara leaving the polymeric GalAc backbone (GABB) intact.

His-tagged AF was produced by *E. coli* fermentation using vinasse as a carbon and energy source (Section 2.2) and then purified with Ni-NTA agarose beads. The soluble sugar beet pectin following SE (Section 2.3.1) was used as a substrate. After incubation for 8 h (100 mg of pure AF per mL of reaction in 0.1 M bis-Tris buffer at pH 7 and at 60 °C), 87% of the polymeric Ara was released as monomer. No other sugar monomers were detected in solution confirming AF selectivity.

Separation of the Ara monomers from the polymeric GABB was then explored. Membrane filtration is a well established unit operation in the food and (bio) pharmaceutical industries.<sup>22,23</sup> In tangential flow filtration (TFF) processes, the feed flow is directed parallel to the membrane surface creating shear and preventing membrane fouling by retained high MW species. Ultrafiltration (UF) uses membranes with a molecular weight cut-off (MWCO) between  $10^3$  to  $10^5$  Da (pore size 1–20 nm) and can be used to separate molecules with at least one order of magnitude difference in size.<sup>23</sup> In this case the GABB would be retained while the Ara monomers (MW = 150 Da) will pass through the membrane.







column *via* centrifugal forces, while the mobile phase is pumped past it through a series of interconnected chambers where the two phases mix and solute partitioning occurs.<sup>24</sup> The lack of a solid stationary phase allows crude process streams to be handled with little pre-treatment which is important in a biorefinery context.

Following steam explosion, the solubilised pectin fraction was fully hydrolysed by adding 2.5% (v/v) sulphuric acid and heating to 121 °C for 1 hour in an autoclave before adjustment to pH 6 with NaOH. This crude hydrolysed material had a total dissolved solids content of approximately 100 g L<sup>-1</sup> and a total sugar concentration around 20 g L<sup>-1</sup> as well as containing unknown coloured impurities giving the material a black colour.

This crude hydrolysed SBP mixture was then separated by CPC. An ethanol : aqueous ammonium sulfate (300 g L<sup>-1</sup>) (0.8 : 1.8 v/v) two-phase system was established that could isolate the monosaccharides as well as remove coloured impurities in the crude sample during phase system formation.<sup>25,26</sup> The lower phase (LP) was utilised as the stationary phase and the upper phase (UP) as the mobile phase with a 950 mL preparative CPC column (Kromaton FCPC-A, Rousset Robatel Kromaton) operated in ascending mode. The crude sample was prepared in the stationary phase. Intermittent extrusion of the liquids in the CPC column is possible by pumping just the LP (stationary phase).<sup>26</sup>

Fig. 5, shows the separation of 152 mL of crude hydrolysed SBP pectin. Coloured impurities were fully eluted before the monosaccharides emerge in the order Rha, Ara, Gal and GalAc. Extrusion after 72 minutes ensured the rapid elution of GalAc from the column; this can be seen from the sharp GalAc peak at



Fig. 5 Centrifugal Partition Chromatography (CPC) of crude hydrolysed sugar beet pectin to separate the main monosaccharides: (Rha) L-rhamnose, (Ara) L-arabinose, (Gal) D-galactose and (GA) D-galacturonic acid. Experiments were performed using a 250 mL semi-preparative CPC column (Kromaton), using an ethanol : ammonium sulphate (300 g L<sup>-1</sup>) (0.8 : 1.8 v/v) phase system at 1000 rpm in ascending mode, with a mobile phase flow rate of 8 mL min<sup>-1</sup>. Stationary phase retention was 50%.



100 min. Extrusion reduced the total run time, increased the GalAc fraction concentration and regenerated the column with a fresh stationary phase for subsequent injections. During biorefinery processing three main fractions could be collected from the CPC. Rha could be collected in a fraction between 33–45 min with 93% recovery and 92% purity. Ara could be collected between 46–66 min with 97% recovery and 84% purity due to the co-elution of Gal. Finally, GalAc was isolated between 78–100 min with over 95% recovery and 96% purity.

CPC appears to be a promising separation technique for biorefinery applications. In this case it allowed the processing of crude, hydrolysed SBP facilitating impurity removal and monosaccharide fractionation. Specifically, it was possible to isolate the two main monosaccharides present in SBP, *i.e.* Ara and GalAc, with high yields and adequate purity. The monosaccharide throughput achieved, operating in elution–extrusion mode, was  $1.9 \text{ g h}^{-1} \text{ L}^{-1}$  of column volume.

#### 2.4 Enzymatic upgrading of L-arabinose catalysed by transketolase

Ara is the main monosaccharide present in sugar beet pectin (Fig. 2) hence it was the initial focus for catalytic upgrading. Within our biorefinery concept it can be recovered by both enzymatic fractionation (Section 2.3.2) and acid hydrolysis followed by CPC (Section 2.3.3). Ara can be the starting material for production of arabinol, one of the top commodity biochemicals from biomass,<sup>3</sup> however our focus is on enzymatic upgrading to higher added value compounds.

TK is a thiamine diphosphate (ThDP) dependent enzyme that has been used in stereospecific carbon–carbon bond formation.<sup>27</sup> The ability of TK to perform the reaction irreversibly with the loss of  $\text{CO}_2$ , when using  $\beta$ -hydroxypruvate (HPA) as the ketol donor, makes it an attractive biocatalyst in industrial synthesis.<sup>6</sup> Utilisation of Ara as an aldehyde acceptor with TK, as shown in Fig. 6 would generate the ketoheptose *L*-gluco-heptulose (GluHep), which has applications in cancer and hypoglycaemia.<sup>28</sup> Natural aldoses accepted by wild-type (WT) TK are phosphorylated, such as D-ribose-5-phosphate and erythrose-4-phosphate, however non-phosphorylated sugars have lower affinities.<sup>29</sup> To achieve a commercially viable conversion at large-scale with the non-phosphorylated substrate Ara, variants of TK with greater specific activity are required.

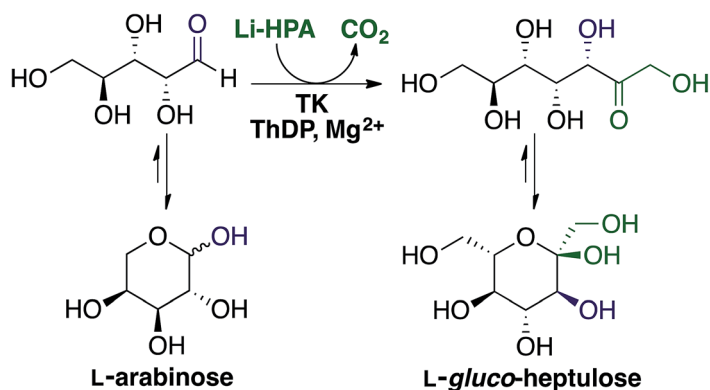


Fig. 6 Transketolase mediated synthesis of *L*-gluco-heptulose from *L*-arabinose using lithium hydroxypyruvate (Li-HPA) as the ketol donor.



Screening of existing TK libraries previously prepared by saturation mutagenesis of conserved residues in the TK active site identified a range of *E. coli* TK mutants that would readily accept Ara.<sup>30</sup> Docking calculations using Ara as a substrate were also performed which highlighted three potentially important residues that could interact with Ara (R358, H461, and R520). The three libraries were screened using a colorimetric plate-based assay developed for high throughput applications with sugar substrates. All *E. coli* TK enzyme variants identified in the screen were then expressed and activities towards Ara compared to the WT transketolase. As shown in Fig. 7, the greatest improvements in specific activities identified during screening were a 2.1-fold increase for R520Y and a 1.5-fold increase for R520P. Small scale conversions of Ara to GluHep were subsequently performed for all 6 variants. The highest conversion yields were obtained for R520Y and H461Y as these had the highest specific activity and the highest expression levels.

These two high-performing TK mutants were then used for the efficient stereoselective, one-step synthesis of GluHep from pure Ara at preparative scale. The reaction was performed for 24 h and <sup>1</sup>H NMR spectroscopic analysis showed a 1 : 1 ratio between the remaining Ara and the GluHep formed. A facile isolation procedure was developed whereby the mixture was filtered through Amberlite® IR120 to remove Tris buffer and some Ara to give GluHep: further purification using column chromatography gave isolated yields of 45% (H461Y) or 40% (R520Y).

Product characterisation confirmed that the TK-catalysed reaction showed exquisite diastereoselectivity leading to a single *D-threo* ketose where only the more stable  $\alpha$ -anomer was generated; this was consistent with the

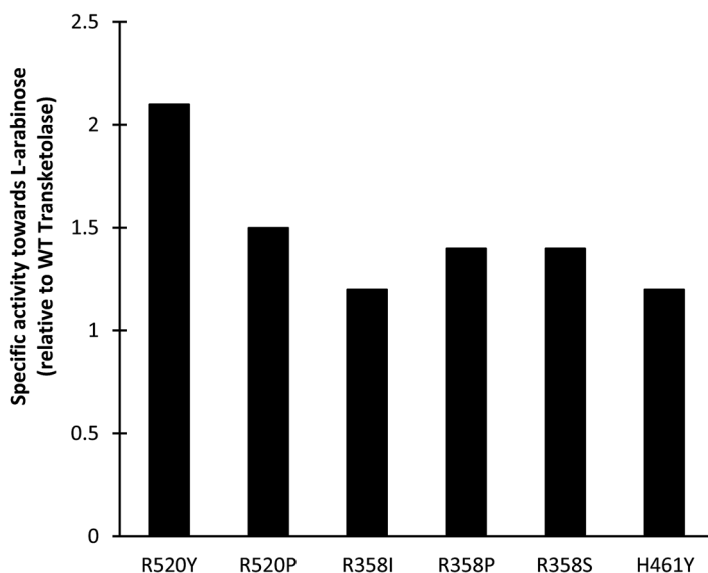


Fig. 7 Specific activities ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) of *E. coli* transketolase mutants toward L-arabinose, relative to the wild-type (WT) enzyme. Experiments performed using clarified lysates with Li-HPA (33 mM), Ara (33 mM), ThDP (1.7 mM) and  $\text{MgCl}_2$  (6 mM) in Tris-HCl (50 mM) at pH 7.0. The specific activity of the WT-TK was  $0.021 \mu\text{mol min}^{-1} \text{mg}^{-1}$ .





of an in-house TAM library indicated that the  $\omega$ -TAM from *Chromobacterium violaceum* can further convert GluHep into the corresponding chiral aminopolyl 2-amino-L-gluco-heptulose.

## 2.5 Cellulose utilisation for bioethanol fermentation

As outlined in Section 1, the conceptual biorefinery design requires the utilisation of all the main carbohydrate fractions present in the SBP. After steam explosion of the SBP under optimised conditions (Section 2.3.1) a relatively pure, insoluble cellulose fraction could be obtained. Since there is already a bioethanol facility at the Wisington site (Fig. 1), it was decided to direct this glucose-rich stream to bioethanol formation.

The solid fraction recovered after SE was therefore subjected to hydrolysis using a commercial cellulase mix: 0.5 mg of cellulase per g of solid at 50 °C (Cellulase 3L-C013L, Biocatalysts Ltd, Cardiff, UK). After incubation for 24 h, the reaction was stopped by centrifugation and the supernatant recovered for monosaccharide analysis. The concentration of soluble Glu was 19 g L<sup>-1</sup> which indicated that virtually all of the SBP cellulose had been monomerised. This also confirmed that the mild SE conditions established in Section 2.3.1 did not lead to the formation of significant cellulase inhibitors. Very low concentrations of other sugars such as Ara, Gal, GalAc and Rha, coming from the remaining pectin after SE, were also detected.

The unpurified supernatant from the cellulase hydrolysis was subsequently used as a fermentation medium for the growth of a commercial *Saccharomyces cerevisiae* strain (Alcotec 24 Turbo Yeast, Hambleton Bard Ltd, Chesterfield, UK). This was incubated at 30 °C for 24 h in shake flasks. At the end of the fermentation process, almost all of the Glu was consumed producing 0.48 g of ethanol per g of Glu. For an unoptimised fermentation process this value is very close to the theoretical ethanol/glucose yield of 0.51 g g<sup>-1</sup> confirming the feasibility of this approach. Analysis of the spent fermentation medium showed that the concentrations of the other monosaccharides present in the cellulose hydrolysis stream remained unaltered over the 24 h incubation. These could be directed to anaerobic digestion (Fig. 1) for energy generation. Likewise, the CO<sub>2</sub> formed during fermentation can be captured and pumped into the glasshouse to support British Sugar's horticulture business and it can also be captured and liquefied for commercial sale (Fig. 1). This would have the added benefit of reducing overall emissions and hence the environmental impact.

## 2.6 Process economic modelling and life cycle analysis

Having demonstrated SBP fractionation, separation and conversion, it is necessary to establish a whole process flowsheet underpinned by material and energy balances for each of the unit operations. These are needed for estimating the capital and operating costs of the integrated biorefinery. Models of the standard unit operations are well known and we have previously developed mechanistic models of the TK<sup>32</sup> and TAM<sup>33</sup> reactions needed to simulate bioconversion kinetics. Economic and Life Cycle Analysis (LCA) will ultimately inform the commercial feasibility and environmental sustainability of the biorefinery concept. These analyses will also establish process competitiveness against benchmark products: bioethanol production will be benchmarked against





gasoline, biopolymers against polylactic acid, and speciality chemicals against succinate and ibuprofen (representing low- and higher-value products, respectively).

Fig. 8 shows an indicative process flowsheet for a facility processing 2000 metric tonnes of SBP (on a dry basis) per day and operating seasonally. The flowsheet depicts the main operations of SBP processing, cellulose conversion to bioethanol, Ara release and bioconversion to GluHep and fractionation of the GalAc backbone followed by conversion to 6-amino-2,3,4,5-tetrahydroxyhexanoic acid. Preliminary simulation results obtained using SuperPro Designer® (Intelligence, Inc., N.J., U.S.A.) identified a costly inventory of lithium hydroxypyruvate, which acts as the ketol donor in the synthesis of GluHep from Ara (Section 2.4) and accounts for a significant percentage of raw materials costs. Finding alternative, cheaper donors would therefore be economically favourable. Once finalised, the flowsheet will allow us to identify rate-limiting steps that require further research and development and to study the financial trade-off between equipment scale and operational efficiency.

### 3. Conclusions and future work

This work has demonstrated the technical feasibility of an integrated biorefinery concept in which wet sugar beet pulp can be fractionated by steam explosion into pectin and cellulose-rich streams. The three main monosaccharides present in the two biopolymers (Ara, Glu and GalAc) can then be solubilised, using either enzymatic or chemical hydrolysis, and separated into crude fractions suitable for conversion into higher value products.

It has been shown that the Ara-rich fraction can be enzymatically upgraded by TK into GluHep, which has potential therapeutic applications.<sup>28</sup> Likewise, we have demonstrated that the Glu-rich fraction can be fermented by a Yeast strain to produce bioethanol with high yield (in a facility that already exists at the British Sugar Wisington site). Current work is examining the stereoselective amination of the remaining GalAc fraction using transaminases including the *C. violaceum*  $\omega$ -TAM. Initial studies have suggested that this enzyme can convert GalAc into 6-amino-2,3,4,5-tetrahydroxyhexanoic acid which can be used for the synthesis of biopolymers such as polyhydroxypolyamides<sup>34</sup> and as a precursor for potent antiviral polyhydroxyazepanes.<sup>35</sup> Three of the enzymes used in this study (AF, TK and TAM) have been expressed in *E. coli* which can yield high specific enzyme activities when grown on diluted sugar beet vinasse.

Future studies will address some of the major engineering challenges associated with the integrated SBP biorefinery concept. These are summarised below.

- Increasing the yield and productivity of the Ara and GalAc bioconversions by investigating substrate/product inhibition and fed-batch operation.
- Evaluating the economic and environmental benefits of the proposed biorefinery based on whole process modelling and LCA alongside market analysis of the synthetic products.
- Implementing next generation unit operations, specifically intensified and flow reactor technologies, to further increase space-time yields.
- Exploring the potential of synthetic biology approaches to engineer host cells capable of simultaneous pectin hydrolysis and Ara/GalAc upgrading or cellulose hydrolysis and ethanol fermentation.



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