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Bioethanol production from organosolv treated beech wood chips obtained at pilot scale

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ABSTRACT

Second generation bioethanol represents an important biofuel, but innovations on biomass pre-treatment and fermentation are needed to improve efficiency and cost-effectiveness in its production. In this work, beech wood chips were treated using an acetone-based organosolv fractionation process at pilot scale, resulting in the isolation of lignin, a high-purity cellulosic pulp and a liquid stream containing hemicellulose sugars. The hemicellulose stream (C5 stream) contained xylose as major sugar and also contained fermentation inhibitors such as furanics, organic acids and phenolics. The cellulosic pulp was enzymatically hydrolysed producing a glucose-rich stream (C6 stream). These streams were used as fermentation substrates by the yeasts *Saccharomyces cerevisiae* and *Spathaspora passalidarum*, in separate cultures. Addition of ammonium phosphate or of yeast extract and peptone (YP) as nitrogen sources improved yeast growth. The C6 stream was readily fermentable. When at a sugar concentration of 192 g L^{-1} and supplemented with YP, the ethanol titre reached 91 g L^{-1}, with a yield of 0.49 g ethanol per g consumed sugars. The C5 stream required detoxification to achieve fermentability. In detoxified C5 stream, *S. passalidarum* produced 0.46 g ethanol per gram consumed sugars. Fermentation of the resulting streams at 10-L scale confirmed the results obtained at laboratory scale. As alternative approach, sequential fermentation of the C5 stream by *S. passalidarum* followed by C6 stream addition and inoculation with *S. cerevisiae* resulted in almost complete sugar utilization and an ethanol yield of 0.41 g per gram consumed sugar albeit with a lower ethanol productivity.

1. Introduction

Renewable liquid fuels such as biobased alcohols are of crucial importance to support the ambitious targets for the (heavy) transport sector decarbonization [1]. Mature technologies for biobased alcohols production are mostly based on edible crops (first generation biomasses, 1G) involving several sustainability issues such as food vs fuel competition. The use of non-food biomass feedstocks composed mostly of (hemi-)cellulose and lignin, also called lignocellulosic or second generation (2G) biomasses, provides an opportunity to improve sustainability of these biofuels. The total bioethanol production in the European Union in 2022 was estimated to be 5.7 billion litres. By far, the major part of the bioethanol was produced from 1G biomasses such as corn (47.8 %), wheat (22.3%) and sugars (13.9 %), with lignocellulosic bioethanol representing 14 % of the total [2]. One of the reasons for the low implementation of 2G bioethanol production is the additional requirements in pre-treatment and fermentation innovations needed to improve conversion efficiency as well as the economics of the lignocellulosic-based production processes [3].

For the solubilisation of sugars from 2G biomasses, pre-treatment methods are implemented with the objective to (1) improve the susceptibility of lignocellulose to enzymatic hydrolysis and therefore reduce enzyme dose requirements and increase the yield of solubilised sugars; and (2) recover other components for co-valorisation (e.g., hemicellulose sugars and lignin). Examples of 2G bioethanol biorefineries at demonstration to commercial stage (1,000–50,000 tonnes ethanol per year) include Clariant's Sunliquid® and IFP's Futurol™ technologies based on mechanical and thermal (aqueous) pre-treatment and Chempolis' Fomicobio™ process based on organosolv fractionation [4]. Thermal (aqueous) pre-treatment partly relies on lignin relocation

Abbreviations: GAC, granular activated carbon; YP, yeast extract and peptone.

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to make the (hemi-)cellulose more accessible for enzymatic saccharification to produce a hydrolysate that contains both C6 and C5 sugars. Organosolv fractionation, on the other hand, relies on solvents that can actively dissolve lignin upon depolymerisation. Mild acetone organosolv fractionation (Fabiola™) has been shown to produce digestible cellulose-rich pulps, as well as hemicellulosic sugars and lignin as co-products from hardwood and herbaceous biomasses [5]. Recently, this process was scaled up using industrial-size beech and birch wood chips at industrially-relevant conditions [6].

For efficient 2G bioethanol processes, the sugars (both C6 and C5 sugars) in the biomass must be fully utilized. In the current 1G bioethanol industrial processes, optimized strains of the yeast *Saccharomyces* are widely used. These strains utilize glucose at high efficiency, but challenges remain with the fermentation of xylose or other sugars in lignocellulosic biomasses [7–10]. To address these challenges, recombinant strains have been created where metabolic routes for xylose or arabinose have been inserted [8,11]. Current recombinant xylose-fermenting yeasts produce lower yields of ethanol from xylose compared to the yields obtained on glucose, and issues about poor strain stability and limited co-utilization of sugars in media simulating lignocellulosic hydrolysates remain to be solved [9].

Some of the advantages that have been identified in the mild acetone organosolv process are related to the optimal conditions applied that lead to high cellulose recovery, high yields of hemicellulose and isolation of a less-degraded lignin in comparison to ethanol-based organosolv performed at similar severity conditions [5]. Delignified cellulosic pulp can thus be hydrolysed and further fermented readily with yeast strains optimized for 1G ethanol production. On the other hand, hemicellulose sugars, including C5 and minor amounts of C6 sugars, can be fermented using alternative strains to maximize ethanol productivity. In view of these developments, alternative strains able to utilize xylose efficiently while being more tolerant to inhibitors than current commercial strains are of interest for 2G processes, even if they show lower performance on 1G substrates. Ethanol production from xylose by native yeast strains, such as *Scheffersomyces stipitis*, *Candida shehatae* and *Candida tenuis*, *Kluyveromyces marxianus*, and *Spathaspora* spp. has been shown [11–13]. Amongst those, *Spathaspora passalidarum* is of special interest because it has produced ethanol at yields of up to 0.48 g ethanol per gram of xylose [11] and at high titres of up to 58 g L^{-1} ethanol from a 12 % xylose, 3 % glucose mixture [14]. Furthermore, it utilized xylose rapidly under anaerobic or microaerobic conditions [15,14]. *S. passalidarum* was found to simultaneously ferment xylose, glucose, and cellobiose [14,16] and several strains have been reported to grow on hydrolysates from lignocellulosic biomasses [12,15,17].

Another challenge refers to the toxic components present in aqueous hemicellulose sugar streams after fractionation. Such components include organic acids (acetic, formic, levulinic acid), furanics (furfural, 5-hydroxymethylfurfural), and lignin-derived compounds, produced or released during pre-treatment which could inhibit the growth of microorganisms [17]. Detoxification refers to the selective removal of these inhibitory compounds from aqueous sugar solutions prior to fermentation. Detoxification approaches have included overliming, ion exchange resins [15,18], activated carbon [19], enzymes [20], membrane filtration [21,13] and liquid-liquid extraction [22]. Each of these methods have advantages and disadvantages in coping with cost-effective removal of a broad range of inhibitory compounds, for instance, partial selectivity to specific functional groups and wide range of operational costs added to the process [9]. Collaborative development of biomass pre-treatment and fermentation technologies is necessary to find process combinations for optimal feedstock conversion into ethanol [9].

In this manuscript we describe fermentation processes based on conventional and alternative strains, in mono- and co-culture for bioethanol production from beech wood sugars. These sugars were made available via mild acetone organosolv fractionation of beech wood chips at pilot scale, which produced separate fractions of delignified cellulose-rich pulp, C5 sugars and lignin for separate valorisation [6]. Downstream hydrolysis of the cellulosic pulp with commercial enzymes was performed to produce C6 sugars. The C5 sugar stream was detoxified to improve its fermentability in a single step using an upscaled flow-through unit, packed with granulated activated carbon. The fermentation results for both C6 and C5 streams have been validated at a 10-L scale. In addition, co-fermentation of the sugar streams in a single culture has been developed to reduce process steps.

2. Materials and methods

2.1. Biomass source and fractionation

Industrial beech wood chips were obtained from Abalon Hardwood GmbH (Schwalmstadt, Germany) and used without further drying. Mild acetone fractionation was performed at pilot scale under previously described conditions [6]. In short, 70 kg dry weight (d.w.) of beech wood chips were treated in a 460 L percolation reactor for 103 min at 140 °C with a final acetone concentration of approx. 60 wt %, pH adjusted to 1.7 with sulphuric acid (1.97 kg per 100 kg d. w. wood) and a liquid-to-solid ratio of 3 kg liquid per kg d.w. biomass. The resulting slurry was processed for liquid/solid separation and pulp washing, disintegration and dewatering as reported previously (for P-BEC-3) [6]. This included several washing steps of the pulp using in total 5 kg 50 wt % acetone and 19 kg water per every kg d.w. beech wood. The washed pulp was pressed to increase the dry matter content to ca. 25 % d.w. After this, the pulp was hydrolysed for 48 h with 0.15 g Cellic® CTec2 and 0.025 g Cellic® HTec2 enzyme mixture per g d.w. pulp (corresponding to enzyme mixture over glucan ratios of 0.22 and 0.037 g g^{-1}, respectively). The hydrolysis was performed with a consistency of 10 wt % and pH = 5 (adjusted with sodium hydroxide). The filtered hydrolysate was evaporated to produce a C6 stream with 54 % total dry matter. The composition of the beech wood chips and the C6 pulp is shown in Table S1.

The liquid obtained after pulp separation was treated through the LigniSep process developed by the Max Planck Institute for Dynamics of Complex Technical Systems- and the Fraunhofer Center for Chemical-Biotechnological Processes. This process is performed in a dispersion tank connected to a falling film evaporator that allows for acetone recovery and isolation of a solid lignin by controlled lignin particle agglomeration [23]. The aqueous C5 sugar stream, obtained after lignin isolation, was detoxified using a fixed bed of granular carbon (Hydraffin CC 1240 Spezial, 12–40 mesh, Donau Carbon GmbH) with a C5 stream to carbon ratio of approx. 25 kg kg^{-1} and a filtrate flow of 1.4 kg min^{-1}. The filtrate was flown through the carbon in two batches to assess two different residence times (35 and 18 min, respectively). Spent carbon was ultimately washed with 2 parts of water to elute any remaining sugars from the bed in each batch and samples were collected from each detoxification batch. The total collected detoxified C5 filtrate and water washings were evaporated in a falling film evaporator (300 mbar, 90 °C) by a factor of 45 in weight basis to produce a concentrated C5 stream of 52 % total dry weight. Analyses of monomeric sugars was performed by HPAEC-PAD and organic acids and furanics by HPLAC-RI/UV as previously described [24].

Concentration of phenolics was determined by HPLC using a Thermo Scientific Vanquish setup equipped with a Diode Array Detector at 210, 254 and 280 nm and an Accucore C30 column and guard. An eluent flow of 1.0 mL min^{-1} was used with a gradient of 10 %–25 % acetonitrile over 35 min where the remaining eluent was a 0.05 wt% trifluoroacetic acid solution.

2.2. Fermentation of sugar streams

2.2.1. Strains

Saccharomyces cerevisiae Ethanol Red® (Leaf - Lesaffre, France) and *Spathaspora passalidarum* CBS 10155 (Westerdijk Fungal Biodiversity

Institute, the Netherlands) were stored as 20 % glycerol stocks at −80 °C.

2.2.2. Laboratory scale fermentations

Small scale batch fermentations (25 mL medium in 100 mL Erlenmeyer flasks) were performed in duplicate. The C5 and C6 sugar streams were diluted in demineralized water and the pH of the solutions was adjusted to 5.5 before sterilisation by autoclaving (125 °C, 15 min). Concentrated solutions of nutrients were autoclaved separately prior to mixing with the sugar streams. Final concentrations per L were yeast extract 10 g, peptone 20 g (YP), diammonium phosphate 5 g (DAP). Dry yeast extract and peptone were added to the detoxified, diluted C5 sugar streams. The pH was adjusted to 5.5 using 5 M NaOH and 5 M H_3PO_4 and solids were removed by centrifugation. The supernatant was sterilised by filtration (0.2 μm filter). Cultivations were started by inoculation of washed and concentrated precultures in saline buffer (0.9 wt% NaCl) to an optical density at 600 nm (OD_{600}) of 0.5 and performed at 25 °C with an agitation speed of 150 rpm.

The fermentability of the sugar streams produced at pilot scale was tested in small scale batch fermentations (30 mL medium, in duplicate). C5 and C6 substrates were diluted in demineralized water and the pH was adjusted to 5.5 and 5.0, respectively (5 M NaOH or 5 M H_3PO_4) prior to sterilisation by autoclaving. Filter-sterilised (0.2 μm filter) nutrients stock solutions were added to concentrations of 10 g L^{-1} yeast extract, 1 g L^{-1} peptone, 1 g L^{-1} KH_2PO_4, 2 g L^{-1} NH_4Cl and 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$ (YP + salts). The reference medium contained YP + salts and 170 g L^{-1} glucose (C6) or 60 g L^{-1} xylose (C5), respectively. Cultivations were performed at 30 °C, inoculation and cultivation conditions were as described above.

Co-cultures and sequential cultures were performed in 100 mL shake flasks with 30 mL medium. C6 and detoxified C5 substrates were diluted in demineralized water, the pH was adjusted to 5.3, and the substrates were sterilised by autoclaving. Sterile nutrient stock solutions were added to obtain concentrations of 20 g L^{-1} yeast extract, 2 g L^{-1} peptone, 2 g L^{-1} KH_2PO_4, 4 g L^{-1} NH_4Cl, 0.6 g L^{-1} $MgSO_4{\cdot}7H_2O$ (2x concentrated YP + salts). Sequential cultures were started with 20 mL medium. After 42 h, 10 mL of a solution of C6 substrate or pure glucose in demineralized water was added. The temperature of the cultures was 30 °C, inoculation and cultivation conditions were as described above.

Fermentations at 0.2 L scale were performed in Multifors bioreactors (Infors HT, CH) in duplicate. Diluted C6 stream (50 % w/v) was supplemented with YP. YP-medium containing glucose 180 g L^{-1} and xylose 10 g L^{-1} was used as reference. Undiluted C5 sugar stream was used, to which dry YP was added. The reference culture consisted of YP-medium with 3 g L^{-1} glucose and 47 g L^{-1} xylose. The complete media were sterilised by filtration (0.2 μm filter). During cultivation, the pH was controlled at 5.0, with a temperature of 35 °C, for the C6 stream cultures. For the C5 stream cultures, the temperature was 25 °C, and the pH was controlled at 5.5. In all cultures, the agitation speed was 150 rpm. The headspace of C6 stream cultures was flushed with air (0.1 L per min) the first 4 h and subsequently with N_2 gas. The C5 stream cultures were flushed through the medium with 0.02 L per min (0.1 vvm) with air. C6 stream cultures were inoculated with a concentrated culture of *S. cerevisiae* in a saline solution (20 mL, OD_{600} of 1.0). The inoculum of C5 stream cultures consisted of 1 mL of *S. passalidarum* culture in saline to an OD_{600} of 0.05. Struktol antifoam was added when necessary.

2.2.3. Upscaled fermentations

Fermentations with 10-L working volume were performed in a 20 L bioreactor (Applikon Biotechnology, NL and Biostream International, NL). Stock solutions of medium components were autoclaved separately. The composition of the medium used was: 10 g L^{-1} yeast extract, 1 g L^{-1} peptone, 1 g L^{-1} KH_2PO_4, 2 g L^{-1} NH_4Cl and 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$ (YP + salts). Cultures were inoculated with 3 % (v/v) of precultures grown on YPX or YPG medium, for the C5 or C6 stream, respectively.

The C5 fermentation was performed as a fed-batch process where the initial C5 substrate content in the medium in the batch phase was 15 %, w/v. After 42 h, the medium was fed with an additional 18 %, w/v during 14 h. The C5 fermentation was performed at 30 °C, pH = 5.5, stirrer speed of 300 rpm, and an air flow rate of 1 L min^{-1}, interrupted by the period of 30–42 h with a flow rate of 0.17 min^{-1}. The C6 fermentation was operated as a batch process with 50 %, w/v diluted C6 substrate. The fermentation was performed at 30 °C, pH = 5.0, stirrer speed of 200 rpm, with an air flow rate of 1–2 L min^{-1} during the first 21 h, followed by a N_2 flow rate of 1–3 L min^{-1} till the end of the fermentation. Samples were regularly collected from the cultures for analysis of cell growth, sugars and products.

2.2.4. Analysis of metabolites

Sugars, organic acids and ethanol were measured using a Waters HPLC system equipped with a Shodex RSpak KC-811 column as described previously [25]. Additionally, sugars were determined by high-performance anion exchange chromatography (HPAEC) using an ICS-5000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column as previously described [26].

3. Results

3.1. Sugars from upscaled mild acetone fractionation of beech wood chips

The pre-treatment process used in this study (Fabiola™) uses an aqueous acetone solution as solvent and sulphuric acid as catalyst at relatively mild process conditions for obtaining high yields of a cellulose-enriched pulp, a hemicellulose sugar hydrolysate, and isolated lignin (Fig. 1). In previous work, conditions were setup at lab scale in order to maximize the recovery of the components in each of these streams [5]. In the present work, additional enzymatic hydrolysis of fractionated pulp using commercial enzymes and detoxification of the C5 stream were implemented with the purpose of validation of their suitability for ethanol fermentation. Table 1 presents the C5 and C6 sugar stream compositions obtained from upscaling the beech wood fractionation that was used in the present work. The C6 sugar stream resulting from cellulosic pulp hydrolysis did not contain significant amounts of furanic or phenolic inhibitors. Acetic acid was found in a relative ratio of 275:1 g C6 sugars to g acetic acid (1.6 g kg^{-1}).

Validation of the process at pilot scale showed a comparable translation of the process from laboratory to pilot scale regarding the fractionation performance [6]. However, as the operation of downstream processing units is not yet optimized, the inefficient washing of the non-disintegrated fractionated wood pulp required relatively large amounts of washing liquid. This caused unwanted dilution effects in the obtained C5 stream after acetone and lignin removal (Table 1). Furthermore, the C5 sugar stream contained relatively higher concentrations of furanics (furfural and HMF, 1.0 g kg^{-1}), phenolics (0.08 g kg^{-1}) and organic acids (4.5 g kg^{-1}) after lignin precipitation and solvent recovery, corresponding to a relative ratio of 2:1 g C5 sugars to g total inhibitors and 9:1 g C5 sugars to g of phenolics and furanics. As discussed later in Section 3.3, abundancy of these compounds was found to be inhibitory and therefore, a detoxification process was designed to achieve sufficient fermentability. In initial screening tests, different doses of granular activated carbon (GAC) were tested to generate C5 streams with different degrees of toxicity (Table S2). Up to 20 % of acetic acid was removed when using a carbon application dose of 6 g GAC per 100 g sugar stream (Fig. S1). Phenolics were removed readily with small amounts of carbon, while a carbon application dose of at least 2 g per 100 g sugar stream was required to remove 87 % of HMF. Sugar losses were limited to maximum 17 % for most carbohydrates at the highest carbon application dose (Fig. S1). Based on this work, an upscaled continuous detoxification process was adapted at both lab and pilot scale using a commercial granular carbon available in bulk volumes (Fig. S2). In the upscaled test, the granular activated carbon was configured in a column or bed, where washing after elution of the

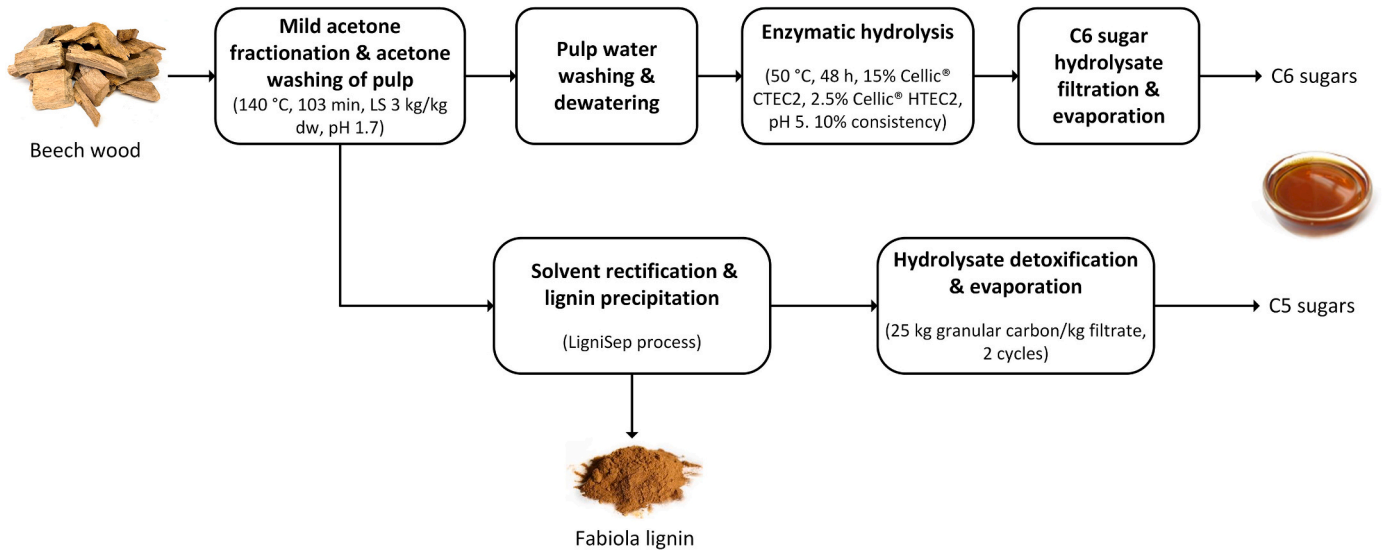

Fig. 1. Mild acetone fractionation process, enzymatic hydrolysis and detoxification for the production of lignin, hemicellulose (C5) and cellulose (C6) sugars for fermentation.

Table 1
Composition of C5 and C6 sugar streams obtained from upscaled fractionation of beech wood. Abbreviations: nm, not measured; bdl, below detection limit.

	C5 stream	C5 detoxified stream		C5 detoxified and concentrated stream	C6 stream	C6 concentrated stream
		Batch 1 (35 min)	Batch 2 (18 min)			
Sugar monomers concentration, g kg^{-1}						
Glucose	0.85	0.76	0.72	25.5	62.2	449.1
Mannose	0.42	0.36	0.35	12.2	0.73	4.56
Galactose	0.52	0.46	0.44	15.8	bdl	1.29
Rhamnose	0.31	0.29	0.27	9.82	bdl	0
Xylose	10.1	9.16	8.61	324.1	4.04	27.3
Arabinose	0.35	0.28	0.28	9.88	bdl	0
Sugar oligomers, g kg^{-1}						
Glucose	0.18	nm	nm	0	nm	0
Mannose	0.07	nm	nm	0	nm	1.35
Galactose	0.14	nm	nm	0	nm	0
Rhamnose	0.07	nm	nm	0	nm	0.28
Xylose	0.22	nm	nm	0	nm	0
Arabinose	0	nm	nm	0	nm	0.24
Fermentation inhibitors, g kg^{-1}						
Formic acid	0.34	0.19	0.19	3.24	nm	bdl
Acetic acid	4.15	3.06	2.72	6.12	nm	1.66
5-hydroxymethyl-furfural (HMF)	0.08	bdl	bdl	0.34	nm	bdl
Furfural	0.96	0.046	0.076	0.062	nm	bdl
Vanillic acid	0.004	bdl	bdl	0.018	nm	bdl
Vanillin	0.020	bdl	bdl	0.018	nm	bdl
Syringaldehyde	0.061	0.008	0.011	0.19	nm	0.029

detoxified C5 stream was also performed to recover sugars retained in the bed. Based on fermentation results and lab flow tests, two different contact times were selected for testing at pilot scale to provide an overall application dose of 4 g GAC per 100 g sugar stream. Minimal effect of contact time was observed for inhibitor removal with complete removal of both HMF and vanillin in both trials (Table 1). A longer contact had a small effect on the removal of furfural (95 % vs 92 %) and syringaldehyde (87 % vs 82 %), respectively for 35 and 18 min contact time. After detoxification, the relative mass ratio of C5 sugars to total inhibitors was ca. 3:1 in both batches. However, the relative mass ratio of C5 sugars to furanics and phenolics was increased substantially to 175:1 and 102:1, for batches 1 and 2 respectively, compared to the starting C5 stream. Sugar concentrations were slightly lower in the two detoxified batches compared to the initial C5 streams. This was mainly due to the dilution caused by the addition of water to wash the carbon bed. Sugar losses were only 2.4 % taking into account the amounts of produced detoxified hydrolysate.

Detoxified C5 sugar stream was further concentrated, up to 324 g L^{-1} of xylose (Table 1). The evaporation process had also an impact on the levels of organic acids and furfural. These compounds were partly removed due to their volatility. For the extent of evaporation performed in the pilot scale experiment, the relative mass ratios of sugar to total inhibitors increased from 3:1 to 33:1, given the significant evaporation of acetic acid and furfural. Due to the relative low volatility of phenolics, these compounds mostly remained in the C5 stream after evaporation.

3.2. *Fermentation of C6 stream by Saccharomyces cerevisiae ethanol red®*

S. cerevisiae Ethanol Red is a commercially available strain used for ethanol production from glucose-rich substrates. Initial fermentability tests on the C6 stream were carried out using a dilution of 10 % (v/v), giving an initial glucose concentration of approximately 32 g L^{-1}. When grown on diluted C6 stream, the strain showed poor growth. Therefore,

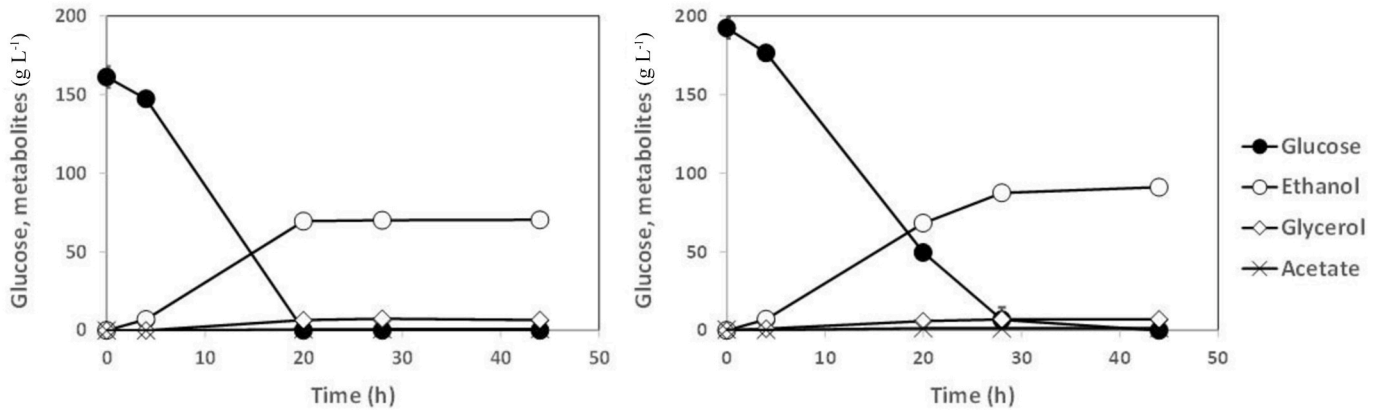

Fig. 2. Ethanol production by *S. cerevisiae* cultures with 0.2-L volume in bioreactors on (right) YP medium with beech C6 sugar stream (C6 + YP) (50 % w/v) and (left) on YPG reference medium.

this stream was supplemented with di-ammonium phosphate (DAP) (5 g L^{-1}) or with a mixture of yeast extract (10 g L^{-1}) and peptone (20 g L^{-1}) (YP). All glucose was consumed within 24 h in the cultures supplemented with yeast extract and peptone (C6 + YP). In those tests, an OD_{600} of 15 was reached after 48 h, indicating good performance. On the cultures supplemented with DAP, the growth was similar to that observed for the diluted C6 stream without supplements, but the sugar consumption reached 94 %, with an ethanol production of 11.3 g L^{-1} (Fig. S3, Table S3).

Good growth and glucose consumption were observed in the C6 stream diluted at 10, 20 and 50 % (v/v) and supplemented with YP (Fig. S4), with ethanol as main product and acetic acid as a minor product (Table S3). On the medium with the lowest dilution (sugar content of 181 g L^{-1}), the ethanol concentration reached 89 g L^{-1} and the acetic acid concentration was 2 g L^{-1} after 48 h of cultivation. The ethanol yield was 0.49 g per g of consumed glucose, which is 96 % of the theoretical value (0.51 g g^{-1} sugar).

Batch fermentations at 0.2 L scale were conducted to further characterise the fermentation of the C6 stream diluted at 50 % (v/v) and supplemented with yeast extract and peptone (C6 + YP). In these fermentations, the first 4 h of the cultivations were kept at low O_2 conditions followed by anaerobic conditions to promote ethanol formation. Glucose in C6 medium was completely consumed in 44 h with a volumetric rate of 6.4 g L^{-1} h^{-1} (Fig. 2, Table 2). In the reference cultivation on YP medium, with a lower content of pure glucose, the consumption of glucose was completed after 20 h and with a higher rate of 7.8 g L^{-1} h^{-1}. However, the ethanol yield in C6 medium was higher than in the reference medium (0.49 vs. 0.45 g per gram of consumed glucose, respectively). Other products were glycerol and acetic acid (0.8 g L^{-1}) (Table 2). The C6 sugar stream from beech wood were deemed suitable for upscaled ethanol fermentation.

3.3. Fermentation of C5 stream by S. passalidarum

The C5 sugar stream used in initial shake flasks tests contained a mixture of xylose (circa 80 % (w/w) of all sugars), with low amounts of arabinose, glucose, galactose and rhamnose (Table S2). The yeast strain *Spathaspora passalidarum*, was selected for production of ethanol from the C5 stream, as this strain utilises xylose efficiently [14,16]. The C5 stream as such did not support yeast growth, and at a dilution of 6 % (v/v), poor growth was observed (Fig. S5).

Supplementation of C5 stream diluted at 10 % (v/v) with diammonium phosphate (DAP, 5 g L^{-1}) or with yeast extract and peptone (YP) both resulted in better growth and ethanol production, with best results with the latter supplements (Fig. S5). Subsequently, the C5 stream supplemented with YP was tested as feedstock for fermentation using dilutions. Poor growth and sugar consumption was observed in cultures with a content of the C5 stream of more than 10 % (v/v) (Fig. S6, Table S4). This indicated that toxic components were present in this stream, and that nutrient supplementation was not sufficient to allow sugar utilization at low stream dilutions.

The growth inhibition observed on the C5 sugar streams was most likely caused by the presence of lignocellulose by-products, i.e., weak organic acids, furanic and phenolic compounds (Table S2). Beech wood hydrolysates may also contain other inhibitory phenolic compounds typically found in the bark fraction of the biomass, such as catechin, which were not measured in this study [27]. These components, released during chemical pre-treatment of lignocellulose, are all potential fermentation inhibitors [28]. The fermentability of the detoxified C5 streams resulting after treatment with various doses of GAC was tested.

Table 2
Fermentation characteristics of *S. passalidarum* batch cultivations (0.2 L) in medium with detoxified beech C5 sugar stream (C5 + YP) or YPXG medium (reference culture) and *S. cerevisiae* batch cultivations (0.2 L and 10 L) in YP and YP + salts medium, respectively, with beech C6 sugar stream (50 %, w/v) or YPG medium (reference culture). Abbreviation: bdl, below detection limit.

	0.2 L scale fermentations				10 L scale
	C5 + YP	YPXG reference	C6 + YP	YPG reference	C6 + YP and salts
Xylose, other sugars at start, g L^{-1}	49.4 ± 1.5	45.8 ± 0.4			
Glucose at start, g L^{-1}	3.1 ± 0.5	2.2 ± 0.0			
Glucose + xylose at start, g L^{-1}			192.4 ± 6.7	161.2 ± 6.8	225.0
Sugar consumption rate, g L^{-1} h^{-1}	1.1 ± 0.1	1.1 ± 0.1	6.4 ± 0.3	7.8 ± 0.3	7.9
Ethanol production, g L^{-1}	22.6 ± 0.1	20.3 ± 0.1	91.1 ± 0.8	70.1 ± 0.4	103.3
Ethanol yield, g g^{-1} of consumed sugars	0.46 ± 0.03	0.43 ± 0.00	0.49 ± 0.01	0.45 ± 0.02	0.48
Ethanol productivity, g L^{-1} h^{-1}	0.5 ± 0.0	0.5 ± 0.0	3.1 ± 0.1	3.5 ± 0.0	3.9
Glycerol production, g L^{-1}	0.6 ± 0.1	0.4 ± 0.0	7.1 ± 1.1	7.4 ± 0.8	5.6
Xylitol production, g L^{-1}	0.3 ± 0.0	0.5 ± 0.2	bdl	bdl	bdl

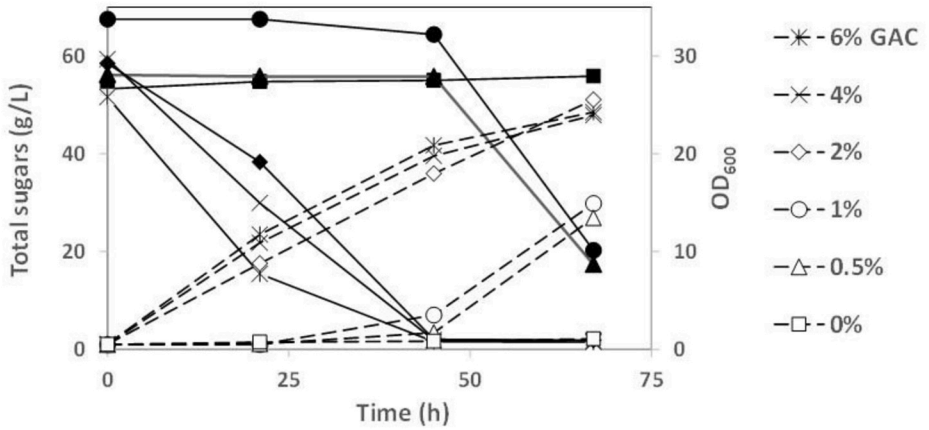

Fig. 3. Growth of *S. passalidarum* in shake flaks on detoxified C5 sugar stream (total sugar content of 51–67 g L^{-1}) in YP medium (25 mL). Detoxification was performed by adsorption on granular activated carbon (GAC, % or g GAC per 100 g C5 stream). Dashed lines represent OD_{600} values, and solid lines represent total sugars values. The different substrate streams are identified in the legend on the right.

In these samples, most of the furanic and phenolic compounds were removed (Table S2). Growth of *S. passalidarum* was observed in medium with circa 50–60 g L^{-1} of total sugars of detoxified C5 streams. A maximum positive effect on growth was reached by detoxification with a GAC application doses of 4 g GAC per 100 g of C5 stream and higher (Fig. 3). The sugar consumption by the yeast showed a similar trend; in cultures with C5 sugar stream treated with more than 2 g GAC per 100 g stream, the sugar consumption was completed within 45 h (Fig. 3, Table S4).

Ethanol production was observed in the cultures on detoxified C5 streams supplemented with YP. The highest titre of ethanol (18.7 g L^{-1}) with a yield of 0.32 g per g of consumed sugars was observed on C5 stream medium detoxified using 4 g GAC per100 g stream (Table S4). This yield is circa 63 % of the theoretical value of 0.51 g per g consumed sugars [14].

Fermentations at 0.2 L scale were conducted on detoxified C5 stream using 4 g GAC per 100 g stream, containing approximately 50 g L^{-1} total sugars. This stream was supplemented with yeast extract and peptone (C5 + YP), and the culture was run under micro-aerophilic conditions (Fig. 4, Table 2). In the culture on reference medium, xylose was completely consumed within 46 h of cultivation, while glucose was consumed very rapidly. The results on the detoxified C5 stream were very similar to that of the reference culture. On both fermentations, the major product was ethanol at yields of 0.43 and 0.46 g per g of consumed sugars from the reference medium and the detoxified C5 stream, respectively (Table 2).

3.4. Upscaling of fermentations of sugar streams to bioethanol

The sugar streams produced at pilot scale were used as substrates for fermentations at 10 L working volume. The sugar content of the concentrated C6 and detoxified C5 streams is shown in Table 1. The fermentability of these streams was firstly determined in shake flasks, using dilutions in a similar manner as in sections 3.2 and 3.3. The results showed that the C6 stream diluted at 50 % (w/v) in medium with YP and salts was fermentable by *S. cerevisiae* and that all glucose was consumed and converted to ethanol at a high yield. Therefore, this condition was chosen for the upscaled fermentation (Supplementary materials, Fig. S7, Table S5).

During the 10-L fermentation of the C6 stream (50 % w/v), growth started after a lag phase of approx. 2.5 h, and all glucose (210 g L^{-1}) was consumed after 26.5 h (Fig. 5). The main fermentation products were ethanol and glycerol (Fig. 5, Table 2). An ethanol yield of 0.48 g per g of consumed sugars was obtained (Table 2), while in shake flasks this was 0.44 g per g (Table S5). The glucose consumption rate was 7.9 g L^{-1} h^{-1} (Table 2), with an ethanol production rate of 3.9 g L^{-1} h^{-1}. A low concentration of xylose was present in the medium (16 g L^{-1}) and remained mostly unused (10.6 g L^{-1} at the end of the fermentation). The cell dry weight at the end of the fermentation corresponded to 6.4 g L^{-1}. A carbon balance was performed using the collected data of the substrate composition and product formation and resulted in 97 % of carbon recovery.

The C5 stream resulting from the upscaled detoxification treatment was fermentable by *S. passalidarum* when diluted at 15 % (w/v) in

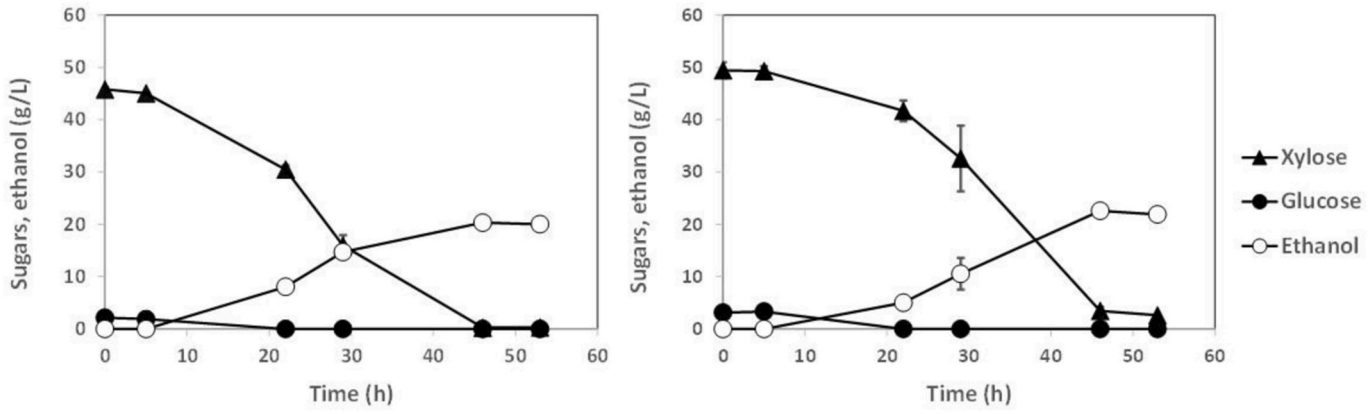

Fig. 4. Ethanol production by *S. passalidarum* cultures at 0.2 L scale in bioreactors on medium with (left) a mix of xylose and glucose (reference culture) and (right) on detoxified beech C5 stream (4 g GAC per 100 g stream).

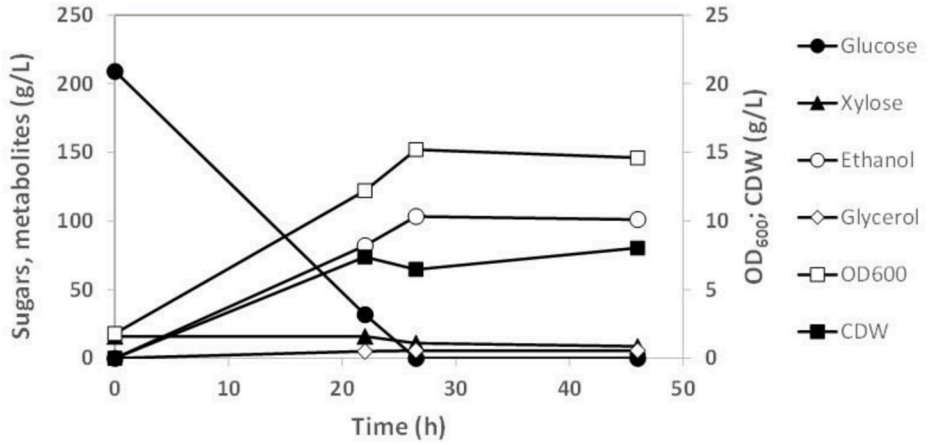

Fig. 5. Fermentation of C6 stream from upscaled fractionation of beech wood chips by *S. cerevisiae* in a 20-L bioreactor with 10 L working volume. The C6 stream was diluted at 50 % (w/v) and supplemented with yeast extract, peptone and salts.

medium with YP and salts (Table S6). At this dilution level, all sugars were consumed, and ethanol was produced at a high yield and therefore, this concentration was selected for the upscaled 10-L fermentation. During cultivation of the C5 stream, a lag phase of approx. 3.5 h was observed, followed by the growth phase where sugar consumption started. The batch phase ended after approx. 30 h. The products of the fermentation were ethanol, xylitol and yeast cell biomass (Fig. 6, Table 3). The ethanol yield of 0.38 g per g of consumed sugar was higher than the yield of 0.34 g per g obtained in the shake flask experiment (Table S6), indicating more favourable ethanol production conditions at larger scale.

The fed-batch phase started at 42 h by feeding additional C5 stream. At this point, ethanol production was observed (Fig. 6). After 56 h, feeding of the C5 stream stopped and the cultivation continued for 14 h. The average rate over the feeding phase was circa 133 g C5 stream per h. Part of the sugar accumulated already in the first hours of the fed-batch phase and continued thereafter (Fig. 6), indicating that the feeding rate was higher than the sugar uptake rate. In the fed-batch phase, 10 g L^{-1} of ethanol were additionally produced, indicating a consumption of sugars from the feed. A final ethanol concentration of 30.6 g L^{-1} was reached. The ethanol yield in this period (0.33 g per g of consumed sugar) was slightly lower than the yield observed during the batch phase. The sugar consumption rate and ethanol volumetric productivity were also higher in the batch phase compared to the fed-batch phase, despite a lower amount of yeast biomass being formed in the latter phase (Table 3).

Table 3
Fermentation parameters of a fed-batch fermentation in a bioreactor by *S. passalidarum* on YP + salts medium with beech C5 sugar stream (15 %, w/v) from the upscaled fractionation and detoxification process. The starting volume of the culture medium was 10 L, and contained mainly xylose, with residual glucose at 2.6 g L^{-1}. Abbreviation: nd, not determined.

	batch phase		fed-batch phase	overall
	t = 0 h	t = 39 h	t = 65 h	t = 0–65 h
sugars, g L^{-1}	54.3	0	38.2	
sugars from feed, g L^{-1}			68.0	
xylitol, g L^{-1}	0	1.9	0	
cell dry weight, g L^{-1}	nd	9.8	8.8	
Sugar consumption, g L^{-1}	54.3		29.8	84.1
Sugar consumption rate, g L^{-1} h^{-1}	2.0		1.3	–
Ethanol production, g L^{-1}	20.8		9.8	30.6
Ethanol yield, g g^{-1}	0.38		0.33	0.36
Ethanol productivity, g L^{-1} h^{-1}	0.78		0.44	–

3.5. Co-utilization of sugars in a single culture

By fermenting the sugar streams separately, the fermentation process would consist of at least two separate fermentation reactors, with the possibility of uncomplete utilization of sugars, in particular of the xylose in the C6 streams. Conditions where all sugars in the streams could be

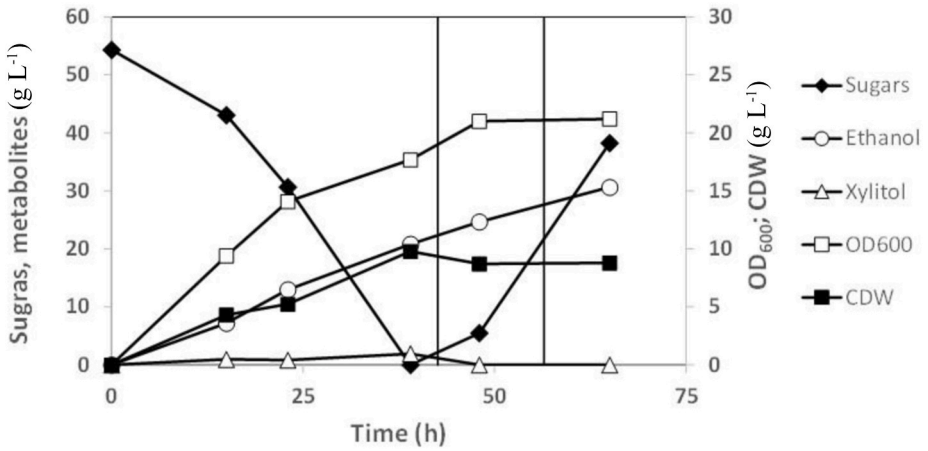

Fig. 6. Growth and ethanol production by *S. passalidarum* in medium with beech wood pellet C5 stream from upscaled detoxification. The batch fermentation (10 L working volume) was started with 15 % (w/v) of C5 stream. In the fed-batch phase an additional amount of C5 substrate of 18 % w/v was fed. Feeding started after 42 h and lasted till 56 h, indicated by the vertical lines.

utilized in a single bioreactor would make the process more efficient. With this aim, the streams from the upscaled fractionation process were used as feedstocks in three fermentation experiments carried out using shake flasks.

1) Cultivation of *S. passalidarum* on a mixture of C6 and C5 streams supplemented with YP + salts. The fermentation profile of a culture grown on a mixture of C5 and C6 streams, with a total starting concentration of sugars of 118 g L^{-1} (69.5 g L^{-1} of glucose and 48.3 g L^{-1} of xylose) is shown in Fig. 7A. The strain fermented both glucose and xylose, the consumption rate of glucose being higher. After 161 h of cultivation, glucose and xylose were completely consumed and the ethanol titre was 28 g L^{-1} with a yield of 0.24 g ethanol per g of consumed sugar and a production rate of 0.17 g L^{-1} h^{-1} (Fig. 7A, Table 4).
2) Co-cultivation of *S. passalidarum* and *S. cerevisiae* on a mixture of C5 and C6 streams supplemented with YP + salts. In this experiment, the same mixture of C6 and C5 streams as in condition 1) was used as substrate, and both strains were inoculated simultaneously. The consumption of glucose was complete after 17 h, most likely by the *S. cerevisiae* strain (Fig. 7B). Also, xylose was consumed partially, and ethanol was produced during the first 17 h of cultivation (Fig. 7B, Table 4). Thereafter, xylose consumption continued by *S. passalidarum* and xylitol production started, reaching a final concentration of 13.7 g L^{-1} while the ethanol content decreased. Apparently, ethanol production by *S. passalidarum* was hampered due to the conditions of the co-culture. However, the ethanol yield and production rate were relatively high during the first 17 h of cultivation (Table 4).

In addition, the co-culture described above was grown on reference medium with similar sugar concentrations as in the C5 and C6 streams mixture but using pure glucose and xylose as carbon sources. Under these conditions, all sugars were consumed within 41 h and ethanol was the main product (Fig. 8A). In this case, xylose consumption by *S. passalidarum* resulted in ethanol production, and the levels of xylitol remained below 3.5 g L^{-1}.

3) Sequential fermentation of C5 and C6 streams in the same culture. To improve ethanol production from the C5 sugars stream, sequential fermentations were performed with an initial phase of *S. passalidarum* growing on C5 stream followed by a second phase where *S. cerevisiae* and C6 sugar stream are added to the culture. Ethanol was produced from xylose in the first phase of 41 h (Fig. 7C). In the second phase, glucose was rapidly consumed within 23 h and mainly ethanol was produced. The total amount of sugars in the sequential fermentation culture (168 g L^{-1} of glucose and xylose) was completely consumed after 65 h of cultivation except for 6 g L^{-1} of xylose in the second phase (Fig. 7C). The ethanol yield was 0.41 g per g of consumed sugar and the production rate was 1.06 g L^{-1} h^{-1} (Table 4). The results of a sequential fermentation on reference medium with pure glucose and xylose as carbon sources were similar (Fig. 8B, Table 4).

4. Discussion

In this section, the results previously presented are further discussed in the context of experimental data available in scientific literature. Furthermore, general considerations are presented in relation to the combination of technologies and the implications of their selection for process upscaling and further needed optimisation.

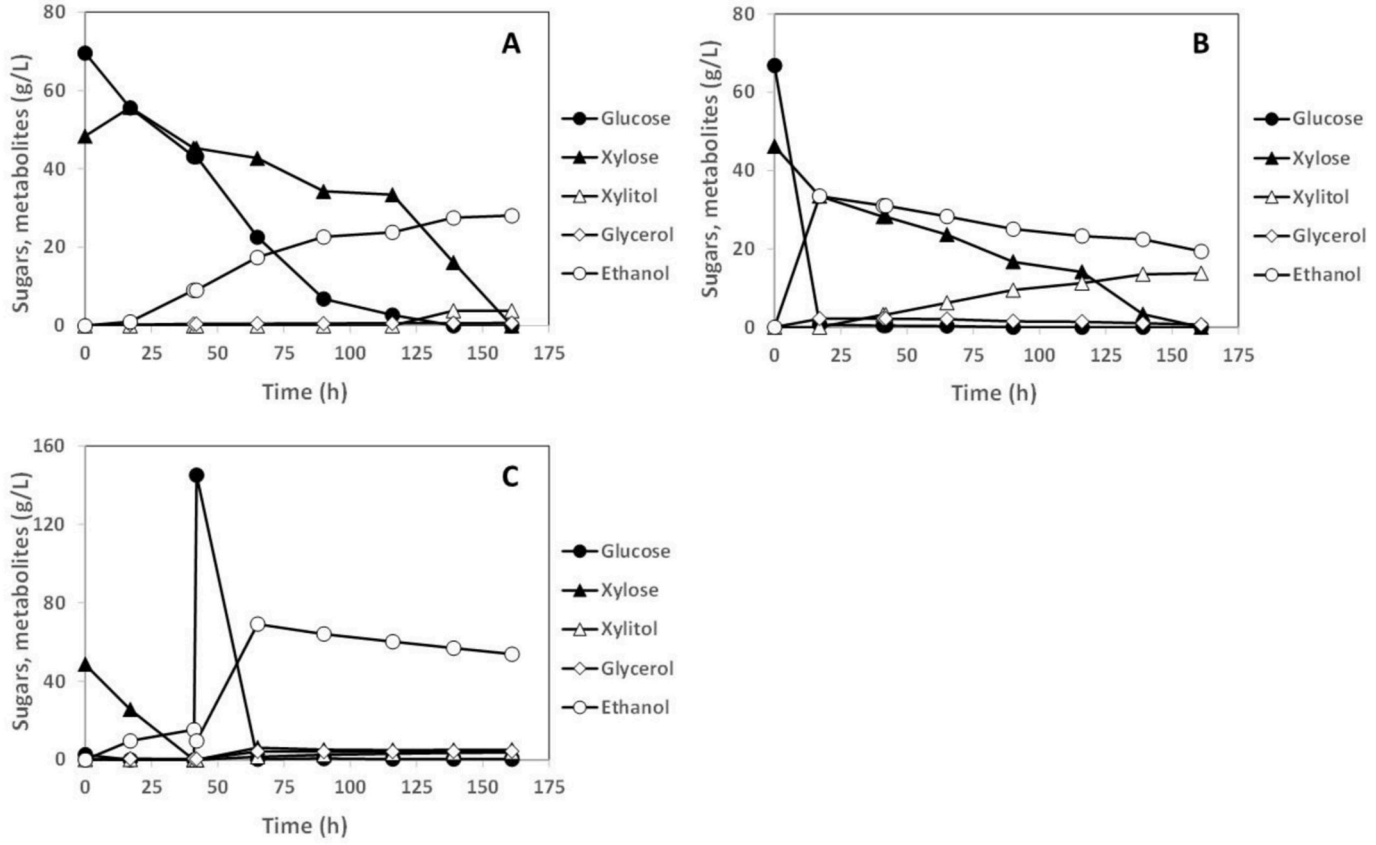

Fig. 7. Substrate consumption and product formation in (A) a monoculture of *S. passalidarum* on a mixed substrate of beech C5 and C6 sugar streams; (B) a co-culture of *S. passalidarum* and *S. cerevisiae* on the mixed substrate; (C) a sequential fermentation of *S. passalidarum* on C5 stream followed by *S. cerevisiae* on C6 sugar stream added at 41 h. Shake flasks of 100 mL volume with 30 mL culture medium were used. The sequential fermentation was started with 20 mL medium and after 41 h supplemented with 10 mL medium. All fermentations were carried out at 30 °C with 150 rpm shaking speed.

Table 4
Fermentation parameters of single strain, co- and sequential fermentations on beech C5 and C6 sugars, and on pure glucose and xylose, in YP + salts medium.

culture		*S. passalidarum*	co-culture	sequential	co-culture	sequential
Carbon source		beech	beech	beech	pure	pure
Fermentation time	h	161	17[a]	65	41	65
Glucose consumption	g L^{-1}	69.5	66.1	140.0	52.6	118.3
Xylose consumption	g L^{-1}	48.3	12.7	28.7	32.6	26.1
Ethanol production	g L^{-1}	28.1	33.5	69.2	30.7	52.5
Ethanol yield[b]	g g^{-1}	0.24	0.42	0.41	0.36	0.36
Ethanol productivity	g L^{-1} h^{-1}	0.17	1.97	1.06	0.75	0.81

[a] No ethanol was produced after 17 h of fermentation; the fermentation continued until complete consumption of the xylose with xylitol as the product (13.7 g L^{-1}).
[b] The ethanol yield is calculated based on the weight of the consumed sugars.

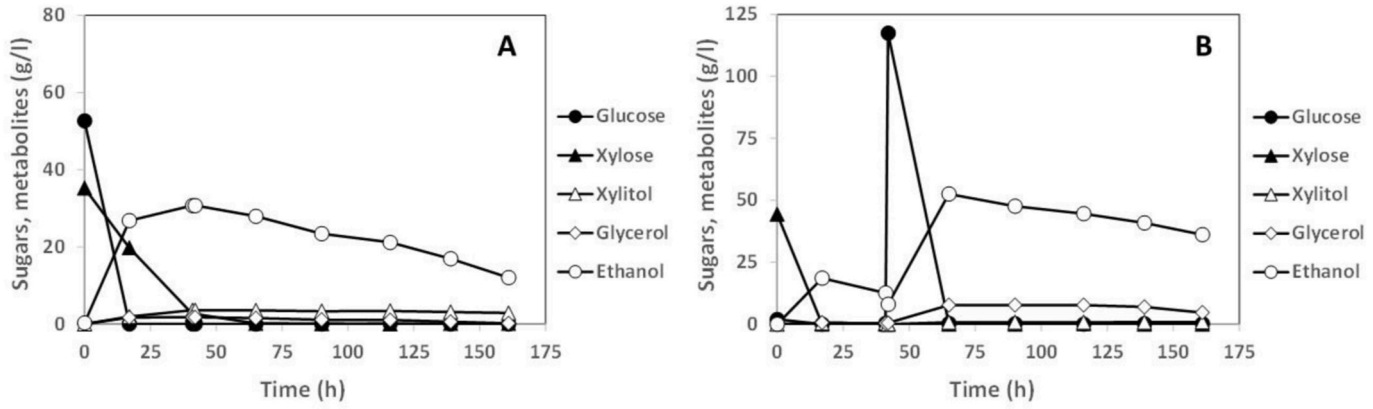

Fig. 8. Substrate consumption and product formation in (A) a co-culture of *S. passalidarum* and *S. cerevisiae* on a mix of pure glucose and xylose; (B) a sequential fermentation of *S. passalidarum* on xylose followed by inoculation with *S. cerevisiae* and addition of glucose at 41 h.

Cellulosic pulp obtained from acetone organosolv fractionation of beech wood chips had a glucan content of 68 % of the dry matter and lignin content (15 % of the dry matter) lower than in the starting biomass (Table S1). Glucan content was relatively lower than in previously reported work (see P-BEC-3 in Ref. [6]), due to a shorter fractionation time. However, the pulp was readily digestible during enzymatic hydrolysis, with a solubilisation of approx. 97 wt% of the glucan into glucose. The resulting C6 sugar stream showed limited amount of fermentation inhibitors, making the biorefinery approach via acetone organosolv and enzymatic hydrolysis highly suitable for fermentation. Nonetheless, the total sugar concentration in this stream after enzymatic hydrolysis was relatively low (67 g kg^{-1}) compared to the desirable substrate concentrations for glucose fermentation (>100 g kg^{-1}). This was due to the moderate pulp consistency used for upscaled hydrolysis (10 % w/w). Other strategies for increasing the sugar throughput in this biorefining step at large scale include high solid loading reactor designs as well as operation in conventional hydrolysers using approaches such as enzyme/biomass fed in batches or multiple stages [29–31].

The C5 sugar stream resulting from lignin precipitation and solvent rectification contained significant amounts of inhibitors. Detoxification by carbon adsorption has shown potential for improving the fermentability of this stream. Removal of inhibitors at the dosages applied were consistent with reported data for other activated carbon materials [15,18,19,32]. For hydrolysates obtained from sorghum leaves and hardwood chips, furfural removal between 93 and 98 % have been reported at dosages between 2.5 and 5 g AC per 100 g (acidic) hydrolysate [19,33]. For acetic acid, the removal is typically lower (<37 %) due to the lesser affinity of organic acids especially at acidic conditions. Typically, prohibitive carbon dosages are required for substantial organic acid removal [33]; whereas combination of activated carbon treatment with pH adjustment and/or evaporation can be more effective for removal of organic acids. Carbohydrates in the form of oligomeric compounds are also of relevance for process development. Relatively low amounts of oligomers are found in the C5 and C6 streams (<8 wt% of total sugars). However, most ethanol-fermenting yeasts do not metabolise these molecules and thus they end up in the residual fermentation broth.

It is important to note that extensive evaporation (to dry matter contents >50 wt%) was performed in this work for practical purposes (i.e. hydrolysate preservation, storage, transport). This evaporation step could have a significant impact on biorefinery operational costs. For technology deployment, increases in pulp consistency used during enzymatic hydrolysis would produce C6 streams that do not require further concentration. In the case of C5 streams, further process optimisation could be envisioned in the integration of detoxification via carbon adsorption and other operations such as (falling-film) evaporation or membrane filtration to the required concentrations for fermentation [21,13,23]. Further process optimisation regarding sugar concentrations in the C5 stream may come from improved pulp washing efficiency and use of continuously-operated fractionation reactors, where process intensification through liquid flow recycles can be implemented. This type of developments is highly dependent on process scale and configuration, therefore efforts on this area at a relevant scale and under continuous operation are needed. Additionally, identifying tolerance levels of fermentative strains becomes indispensable in process design and optimisation, since it allows identifying minimal detoxification requirements or best combination of unit operations. For instance, evaporation/membrane filtration operations can be used to increase sugar concentration to levels suitable for fermentation (>60 g L^{-1}) while partially removing organic acids and furfural; carbon adsorption treatment can be applied subsequently to remove remaining (heavier) inhibitors.

The *S. cerevisiae* strain Ethanol Red® showed high performance on media containing beech wood C6 stream. During cultivation at 0.2 L scale under controlled conditions, 192 g L^{-1} of glucose was converted into 91 g L^{-1} of ethanol, with an ethanol yield of 0.49 g per g of consumed glucose which is more than 95 % of the theoretical yield (0.51 g per g of consumed sugar). The substrate consumption rate was

slightly lower in medium with C6 stream than in medium with pure glucose, however, more research is needed to assess a possible inhibitory effect of beech C6 substrate. Extra nutrients in the form of yeast extract and peptone were required for high and fast substrate consumption.

The C6 sugar stream used as substrate in the 10 L volume fermentation contained 209 g L^{-1} of glucose and, due to the xylan found in the pulp, 16 g L^{-1} of xylose. The glucose was completely consumed within 28 h, while the xylose remained mostly unused (Table 2). The glucose consumption rate of 7.9 g L^{-1} h^{-1} at 10 L scale was higher than at 0.2 L scale (6.4 g L^{-1} h^{-1}). In the upscaled fermentation, a slightly lower glycerol titre was observed, and the ethanol yields on consumed sugar were similar, indicating that larger scale bioethanol production is feasible using this substrate. Fermentation of the concentrated C5 sugar stream was inhibited at concentrations of 20 % or higher (w/v) in nutrient-rich medium. Organic acids, furanic and phenolic compounds were present in low amounts in the C5 sugar stream (Table 1), which required detoxification. The detoxified stream was fermentable at sugar concentrations of 50–60 g L^{-1} by *S. passalidarum*. In 0.2 L cultures under controlled conditions, more than 22 g L^{-1} of ethanol was produced from circa 52 g L^{-1} of sugars, mainly from xylose, with an ethanol yield of 0.46 g per g of consumed sugar (Table S4). This corresponds to 90 % of the theoretical yield of 0.51 g ethanol per g of sugar. In general, the maximum yield is not reached in xylose-to-ethanol fermentations by native yeast strains. For these fermentations, oxygen is required to neutralize the redox imbalance which originates in a different co-factor preference of the first two enzymes involved in xylose conversion, xylose reductase (XR) and xylitol dehydrogenase (XDH). For *Candida shehatae* cultures on xylose, the maximal theoretical yield was calculated at 0.62 C (carbon)-$mol_{ethanol}$ per C (carbon)-mol_{xylose} (corresponding to 0.475 g of ethanol per g of xylose) for an oxygen requirement of 0.33 mol_{oxygen} per mol_{xylose} linked to the cofactors of the xylose reductase [34]. *S. passalidarum* can utilize xylose under anaerobic or microaerobic conditions producing high ethanol yields [14,16,35–37]. Further research with *S. passalidarum* fermentations of beech wood sugars under controlled conditions of oxygen supply are necessary to optimize ethanol production.

The C5 sugar stream from the upscaled detoxification process was fermentable when diluted at 15 % (w/v), resulting in a medium with approx. 55–59 g L^{-1} of sugars. This was a similar starting sugar concentration as observed in the tests with detoxified C5 stream at laboratory scale under the same conditions (4 g GAC per 100 g sugar stream) (Tables S4 and S6). During fed-batch cultivation (Fig. 6), the strain grew in the presence of approx. 30 % (w/v) C5 stream, while in the screening test, no growth was observed in the shake flasks at a 20 % (w/v) dilution (80 g L^{-1} of sugars) (Table S6). During the fed-batch cultivation, the sugar concentration in the culture did not exceed 54 g L^{-1}. The lower concentration of sugars could play a role on the higher tolerance to the substrate. Growth of the same *S. passalidarum* strain was shown on higher concentrations of 90 and 140 g L^{-1} of pure xylose [36,38]. Another possible reason for this higher tolerance during the 10-L fed-batch cultivation might be the adaptation of *S. passalidarum* to low concentrations of inhibitors or conversion of these during the batch phase. Also, the higher content of yeast biomass in this period may contribute to increased tolerance to inhibitors. Table 5 shows the conversion of lignocellulosic sugars by *S. passalidarum* reported in literature. Most research described in literature was carried out at laboratory scale, and this manuscript describes the largest scale fermentation to date with this strain.

It is interesting to notice that in the upscaled fermentations, the media were supplemented with a reduced amount of peptone compared to the laboratory scale tests (1 versus 20 g L^{-1}). This modification created conditions more comparable to those used at industrial scale. Under these conditions, the performance of the strains was at least comparable to the laboratory scale tests, indicating that peptone was not required for efficient ethanol production.

The fermentation of both C5 and C6 streams in a single bioreactor would be an interesting option to simplify the process and possibly reduce equipment and operational costs compared to the use of two separate bioreactors at industrial scale. The results obtained growing *S. passalidarum* on a mixed C6 and C5 stream containing 118 g L^{-1} of total sugars showed that the strain can utilize both glucose and xylose simultaneously, but at a relatively low rate. Furthermore, both sugars were utilized only after a long fermentation time (161 h). These results are in agreement with earlier reports, where co-fermentation of glucose and xylose by this strain was studied in control media under aerobic or anaerobic conditions [35]. In a mixture of glucose and xylose, *S. passalidarum* showed a preference towards glucose utilization, which can be explained by the repression of genes related to xylose metabolism by glucose [39].

Fermentation of mixed C6 and C5 sugar streams by a co-culture of *S. passalidarum* and *S. cerevisiae* both inoculated simultaneously was tested herein to determine the effect of the fermentation products on each strain. In this culture, fast consumption of glucose, most probably by *S. cerevisiae*, was observed. During the first 17 h of the cultivation, 12.7 g L^{-1} of xylose were consumed by *S. passalidarum*, which contributed to reach an ethanol titre of 33 g L^{-1}. After this point, xylose consumption continued at a low rate, with concomitant production of xylitol as main product. Xylitol is the first intermediate in the route for

Table 5
Ethanol production from cellulosic feedstocks by fermentation with *S. passalidarum* strains. Abbreviations: Glc, glucose; Xyl, xylose; Gal, galactose; Man, mannose; Rha, rhamnose; Ara: arabinose.

S. passalidarum strain	Fermentation system and volume	Carbon source	Sugar type and content, g L^{-1}	Ethanol titre g L^{-1}	Ethanol yield g g^{-1}	Ref.
MYA-4345, mutant obtained by mutagenesis and protoplast fusion	Flask, batch 50 mL	Liquid fraction of pre-treated wheat straw	Glc, Xyl 33	4.5	0.40	[27]
NN245, adapted on wood hydrolysate	Bioreactor, batch 2 L	Hydrolysate of maple hardwood AFEX hydrolysate of corn stover	Glc, Xyl 100 Glc, Xyl, cellobiose 69	38 23	0.37 0.39	[16]
NRRL Y-27907, UV mutant adapted to acetic acid	Flask, batch 200 mL	Auto-hydrolysate of *Eucalyptus globulus* wood chips	Glc, Xyl, cellobiose 87	30	0.39	[28]
NRRL Y-27907	Bioreactor, fed-batch 0.7 L	Hydrolysate of alkali-treated sugar cane bagasse	Glc, Xyl 58	23.3	0.46	[29]
NN245, adapted on cellulosic hydrolysates	Flask, batch 50 mL	AFEX pre-treated corn stover	Glc, Xyl, cellobiose 110	39	0.45	[30]
CBS 10155	Bioreactor, batch 10 L	C5 sugar stream from acetone organosolv fractionated beech wood	Xyl, Glc, Gal, Man, Rha, Ara 54	20.8	0.38	This study
CBS 10155	Bioreactor, fed-batch 10 L	C5 sugar stream from acetone organosolv fractionated beech wood	Xyl, Glc, Gal, Man, Rha, Ara 92	30.6	0.36	This study

xylose assimilation by *S. passalidarum*, and its formation from xylose is catalysed by xylose reductase enzymes. Accumulation of xylitol indicates an imbalance in the NADH-NAD^+ pool in the microorganism, as co-factor availability and regeneration is a key parameter for the metabolic activities for xylitol metabolism [14,35,37]. Besides non-optimal aeriation, other factors could have influenced xylitol accumulation. Farias et al. [40] described co-cultures of *Saccharomyces* sp. with *S. passalidarum* NRRL Y-27907 grown on a mixed hydrolysate from sugarcane bagasse and molasses, where the main sugars were sucrose (approx. 40 g L^{-1}) and xylose (approx. 50 g L^{-1}). In these co-cultures, the xylose was consumed faster, and both sugars were totally consumed before 80 h of cultivation, where ethanol was the main product (30.2 g L^{-1}).

A sequential culture in which firstly *S. passalidarum* was cultivated on C5 stream followed by addition of C6 stream and inoculation with *S. cerevisiae* resulted in rapid sugar consumption and ethanol production at high yield. This way, the fermentation by *S. passalidarum* was unaffected by the presence of *S. cerevisiae* as well as high substrate and ethanol contents. The C5 to C6 sugar ratio in the sequential cultivation experiment was approx. 1:3.5 (40 g L^{-1} xylose, 140 g L^{-1} glucose), and it could be further optimized. The actual ratio of C5 and C6 sugars in the used beech wood was approximately 1:2. Results of the sequential fermentation can be compared with the results of the separate fermentation of the same batches of the C5 and C6 streams (Table 4, Table S5). The ethanol yield of the sequential fermentation was the same as the ethanol yield of the combined separate fermentations, i.e., 0.41 g per g of consumed sugars. However, the ethanol productivity in the sequential fermentation was lower because the fermentation time was prolonged by nearly one day as compared to the fermentation time of the C5 stream only under similar conditions.

Insight in the relation between pre-treatment/fractionation and fermentability of 2G sugars are a key element in the technical and economic feasibility of 2G bioethanol biorefineries at industrial scale. Ferreira & Taherzadeh [41] have highlighted that some of the knowledge gaps in simulation and techno-economic analyses of organosolv pre-treatment for ethanol production include the lack of knowledge in terms of by-product formation, product recovery from complex mixtures (namely lignin and non-toxic hemicellulose sugars), and solvent recovery. Organosolv processes have the advantage of producing three separate product streams that each bring potential cost reduction benefits. The high purity lignin can be used for various purposes in chemicals and materials and has potentially an added value [41]. The relatively high purity of the C6 pulp stream helps minimise enzyme dose requirement, decreasing the associated costs of enzyme production which is known to be a significant cost element in bioethanol production [42–44]. Low levels of inhibitors achieved by the combination of the fractionation with detoxification of the C5 streams leads to effective fermentation [18–20,32,33]. This is important in order to limit fermenter volume and costs, but most importantly to allow for C5 sugar valorisation. Similarly, having two separate streams of C5 and C6 sugars allows for optimisation of the fermentation process, either in separate reactors or combined in a vessel, reducing vessel size and thereby investments. Finally, effective fermentations allow for a high ethanol product concentration, reducing product recovery costs. However, as previously mentioned, further research is needed to demonstrate the integration of most cost-efficient operations at appropriate scale to inform technical and economic feasibility studies of 2G bioethanol biorefinery concepts based on organosolv fractionation and novel fermentation approaches.

5. Conclusions

Organosolv fractionation of beech wood chips performed at a pilot scale (70 kg d.w.) resulted in a fermentable glucose-rich sugar stream, a xylose-rich sugar stream, and lignin. The xylose-rich C5 stream contained fermentation inhibitors, and detoxification using granulated activated carbon was adapted to remove these components in a scalable process.

Fermentation of the C6 and C5 sugar streams has been developed using a different yeast strain for each stream, based on sugar preference and strain performance. The fermentation process has been validated at 10-L scale for each stream, reaching high yields and titres of ethanol. The best results were obtained on the C6 stream, where titres above 10 % w/v of ethanol were reached in the fermentation. Sequential fermentation of the sugar streams was also tested as a means to simplify the fermentation process (single reactor).

This work presents insights in the relation between pre-treatment/fractionation and fermentability of 2G sugars and shows the importance of simultaneous development of technologies at relevant scales.

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Author contributions

TV, RMV and RHAMvdV designed and performed fermentation experiments and data analysis. KD, PAB and ATS designed and performed fractionation experiments and data analysis. TV, AMLC and KD supervised the research and drafted the manuscript. ATS and AMLC participated in acquisition of funding. All authors revised and approved the final version of the manuscript.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2023.107003.

References

[1] J. Han, R. Willems, J. Van Hal, X. Seykens, C. Bekdemir, CO2 Neutral Heavy-Duty Engine Concept with RCCI Combustion Using Seaweed-Based Fuels, SAE International, 2020.
[2] ePURE Association Website, 2023. https://www.epure.org/resources-statistics/statistics-infographics/. (Accessed 27 October 2023).
[3] S.S. Hassan, G.A. Williams, A.K. Jaiswal, Moving towards the second generation of lignocellulosic biorefineries in the EU: drivers, challenges, and opportunities, Renew. Sustain. Energy Rev. 101 (2019) 590–599.
[4] IEA Bioenergy Webpage, 2023. https://www.ieabioenergy.com/installations/. (Accessed 27 October 2023).
[5] A. Smit, W. Huijgen, Effective fractionation of lignocellulose in herbaceous biomass and hardwood using a mild acetone organosolv process, Green Chem. 19 (22) (2017) 5505–5514.
[6] A.T. Smit, M. Verges, P. Schulze, A. van Zomeren, H. Lorenz, Laboratory- to pilot-scale fractionation of lignocellulosic biomass using an acetone organosolv process, ACS Sustain. Chem. Eng. 10 (32) (2022) 10503–10513.
[7] T. Su, D. Zhao, M. Khodadadi, C. Len, Lignocellulosic biomass for bioethanol: recent advances, technology trends, and barriers to industrial development, Curr. Opin. Green Sustainable Chem. 24 (2020) 56–60.

[8] A.P. Jacobus, J. Gross, John H. Evans, Sandra R. Ceccato-Antonini, Andreas K. Gombert, *Saccharomyces cerevisiae* strains used industrially for bioethanol production, Essays Biochem. 65 (2) (2021) 147–161.
[9] K. Robak, M. Balcerek, Current state-of-the-art in ethanol production from lignocellulosic feedstocks, Microbiol. Res. 240 (2020), 126534.
[10] C.-G. Liu, Y. Xiao, X.-X. Xia, X.-Q. Zhao, L. Peng, P. Srinophakun, F.-W. Bai, Cellulosic ethanol production: progress, challenges and strategies for solutions, Biotechnol. Adv. 37 (3) (2019) 491–504.
[11] M.G. Wiebe, Y. Nygård, M. Oja, M. Andberg, L. Ruohonen, A. Koivula, M. Penttilä, M. Toivari, A novel aldose-aldose oxidoreductase for co-production of D-xylonate and xylitol from D-xylose with *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 99 (22) (2015) 9439–9447.
[12] A.K. Pandey, M. Kumar, S. Kumari, P. Kumari, F. Yusuf, S. Jakeer, S. Naz, P. Chandna, I. Bhatnagar, N.A. Gaur, Evaluation of divergent yeast genera for fermentation-associated stresses and identification of a robust sugarcane distillery waste isolate *Saccharomyces cerevisiae* NGY10 for lignocellulosic ethanol production in SHF and SSF, Biotechnol. Biofuels 12 (1) (2019) 40.
[13] C. Chen, K. Ma, Q. Zhu, F. Tan, Y. Wang, M. He, G. Hu, A method for concentration of monosaccharide and removal of inhibitors during hydrolysate pretreatment for improved bioethanol production, J. Clean. Prod. 260 (2020), 120999.
[14] K.A. Selim, S.M. Easa, A.I. El-Diwany, The xylose metabolizing yeast *Spathaspora passalidarum* is a promising genetic treasure for improving bioethanol production, Fermentation 6 (1) (2020) 33.
[15] F. Carvalheiro, L.C. Duarte, S. Lopes, J.C. Parajó, H. Pereira, F.M. Gírio, Evaluation of the detoxification of brewery's spent grain hydrolysate for xylitol production by *Debaryomyces hansenii* CCMI 941, Process Biochem. 40 (3) (2005) 1215–1223.
[16] H.C.T. Veras, N.S. Parachin, J.R.M. Almeida, Comparative assessment of fermentative capacity of different xylose-consuming yeasts, Microb. Cell Factories 16 (1) (2017) 1–8.
[17] P.T. Pienkos, M. Zhang, Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates, Cellulose 16 (4) (2009) 743–762.
[18] Y. Chen, B. Dong, W. Qin, D. Xiao, Xylose and cellulose fractionation from corncob with three different strategies and separate fermentation of them to bioethanol, Bioresour. Technol. 101 (18) (2010) 6994–6999.
[19] C. Sarawan, T. Suinyuy, Y. Sewsynker-Sukai, E.G. Kana, Optimized activated charcoal detoxification of acid-pretreated lignocellulosic substrate and assessment for bioethanol production, Bioresour. Technol. 286 (2019), 121403.
[20] X. Guo, A. Cavka, L.J. Jönsson, F. Hong, Comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production, Microb. Cell Factories 12 (1) (2013) 93.
[21] L. Pan, M. He, B. Wu, Y. Wang, G. Hu, K. Ma, Simultaneous concentration and detoxification of lignocellulosic hydrolysates by novel membrane filtration system for bioethanol production, J. Clean. Prod. 227 (2019) 1185–1194.
[22] P. Makoś-Chełstowska, E. Słupek, J. Gebicki, Extractive detoxification of feedstocks for the production of biofuels using new hydrophobic deep eutectic solvents – experimental and theoretical studies, J. Mol. Liq. 308 (2020), 113101.
[23] P. Schulze, M. Leschinsky, A. Seidel-Morgenstern, H. Lorenz, Continuous separation of lignin from organosolv pulping liquors: combined lignin particle formation and solvent recovery, Ind. Eng. Chem. Res. 58 (9) (2019) 3797–3810.
[24] A.T. Smit, A. van Zomeren, K. Dussan, L.A. Riddell, W.J.J. Huijgen, J.W. Dijkstra, P.C.A. Bruijnincx, Biomass pre-extraction as a versatile strategy to improve biorefinery feedstock flexibility, sugar yields, and lignin purity, ACS Sustain. Chem. Eng. 10 (18) (2022) 6012–6022.
[25] M. Diallo, A.D. Simons, H. van der Wal, F. Collas, B. Houweling-Tan, S.W. Kengen, A.M. López-Contreras, L-Rhamnose metabolism in *Clostridium beijerinckii* strain DSM 6423, Appl. Environ. Microbiol. 85 (5) (2019), e02656-18.
[26] B. Gilbert-López, J.A. Mendiola, L.A. van den Broek, B. Houweling-Tan, L. Sijtsma, A. Cifuentes, M. Herrero, E. Ibáñez, Green compressed fluid technologies for downstream processing of *Scenedesmus obliquus* in a biorefinery approach, Algal Res. 24 (2017) 111–121.
[27] C. Tanase, A. Mocan, S. Coșarcă, A. Gavan, A. Nicolescu, A.-M. Gheldiu, D. C. Vodnar, D.-L. Muntean, O. Crișan, Biological and chemical insights of beech (*Fagus sylvatica* L.) bark: a source of bioactive compounds with functional properties, Antioxidants 8 (9) (2019) 417.
[28] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification, Bioresour. Technol. 74 (1) (2000) 17–24.
[29] J. Zhang, W. Hou, J. Bao, Reactors for High Solid Loading Pretreatment of Lignocellulosic Biomass, Bioreactor Engineering Research and Industrial Applications II, 2016, pp. 75–90.
[30] K. Liu, J. Zhang, J. Bao, Two stage hydrolysis of corn stover at high solids content for mixing power saving and scale-up applications, Bioresour. Technol. 196 (2015) 716–720.
[31] Y.H. Jung, H.M. Park, D.H. Kim, J. Yang, K.H. Kim, Fed-batch enzymatic saccharification of high solids pretreated lignocellulose for obtaining high titers and high yields of glucose, Appl. Biochem. Biotechnol. 182 (2017) 1108–1120.
[32] R. Gupta, Hemansi, S. Gautam, R. Shukla, R.C. Kuhad, Study of charcoal detoxification of acid hydrolysate from corncob and its fermentation to xylitol, J. Environ. Chem. Eng. 5 (5) (2017) 4573–4582.
[33] J.M. Lee, R.A. Venditti, H. Jameel, W.R. Kenealy, Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*, Biomass Bioenergy 35 (1) (2011) 626–636.
[34] C. Bideaux, J. Montheard, X. Cameleyre, C. Molina-Jouve, S. Alfenore, Metabolic flux analysis model for optimizing xylose conversion into ethanol by the natural C5-fermenting yeast *Candida shehatae*, Appl. Microbiol. Biotechnol. 100 (3) (2016) 1489–1499.
[35] X. Hou, Anaerobic xylose fermentation by *Spathaspora passalidarum*, Appl. Microbiol. Biotechnol. 94 (1) (2012) 205–214.
[36] Y.K. Su, L.B. Willis, T.W. Jeffries, Effects of aeration on growth, ethanol and polyol accumulation by *Spathaspora passalidarum* NRRL Y-27907 and *Scheffersomyces stipitis* NRRL Y-7124, Biotechnol. Bioeng. 112 (3) (2015) 457–469.
[37] R.M. Cadete, A.M. de las Heras, A.G. Sandström, C. Ferreira, F. Gírio, M.-F. Gorwa-Grauslund, C.A. Rosa, C. Fonseca, Exploring xylose metabolism in *Spathaspora* species: XYL1.2 from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*, Biotechnol. Biofuels 9 (1) (2016) 167.
[38] T.M. Long, Y.-K. Su, J. Headman, A. Higbee, L.B. Willis, T.W. Jeffries, Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*, Appl. Environ. Microbiol. 78 (16) (2012) 5492–5500.
[39] L.E. Ribeiro, F.M. Albuini, A.G. Castro, V.J. Campos, G.B. de Souza, J.G. Mendonça, C.A. Rosa, T.A. Mendes, M.F. Santana, W.B. da Silveira, Influence of glucose on xylose metabolization by *Spathaspora passalidarum*, Fungal Genet. Biol. 157 (2021), 103624.
[40] D. Farias, F. Maugeri Filho, Co-culture strategy for improved 2G bioethanol production using a mixture of sugarcane molasses and bagasse hydrolysate as substrate, Biochem. Eng. J. 147 (2019) 29–38.
[41] J.A. Ferreira, M.J. Taherzadeh, Improving the economy of lignocellulose-based biorefineries with organosolv pretreatment, Bioresour. Technol. 299 (2020), 122695.
[42] D. Klein-Marcuschamer, P. Oleskowicz-Popiel, B.A. Simmons, H.W. Blanch, The challenge of enzyme cost in the production of lignocellulosic biofuels, Biotechnol. Bioeng. 109 (4) (2012) 1083–1087.
[43] S. Heiss-Blanquet, D. Zheng, N.L. Ferreira, C. Lapierre, S. Baumberger, Effect of pretreatment and enzymatic hydrolysis of wheat straw on cell wall composition, hydrophobicity and cellulase adsorption, Bioresour. Technol. 102 (10) (2011) 5938–5946.
[44] D. Ouyang, H. Chen, N. Liu, J. Zhang, X. Zhao, Insight into the negative effects of lignin on enzymatic hydrolysis of cellulose for biofuel production via selective oxidative delignification and inhibitive actions of phenolic model compounds, Renew. Energy 185 (2022) 196–207.
[45] T. de Vrije, K. Dussan, R. van de Vondervoort, P. Bonouvrie, A. Smit, A. López-Contreras, Production of Bio-Ethanol from Beech Wood Pellets via Mild Acetone Organosolv Fractionation, 30th European Biomass Conference and Exhibition, EUBCE 2022, ETA-Florence Renewable Energies, 2022, pp. 849–850.