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Prefermentation improves ethanol yield in separate hydrolysis and cofermentation of steam-pretreated wheat straw

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Abstract

Agricultural residues, such as wheat straw, are feasible substrates for ethanol fermentation provided that pentoses and hexoses can be converted efficiently. Separate hydrolysis and cofermentation (SHCF) constitute a framework for improvement of conversion efficiency, because it permits independent optimization of the enzymatic hydrolysis and cofermentation steps. A drawback is that the high glucose concentrations present in SHCF repress xylose utilization and constrain ethanol yields. To improve xylose utilization the xylose-rich hydrolyzate liquor was separated from glucose-rich solids and the phases were cofermented sequentially. Prefermentation of the xylose-rich hydrolyzate liquor followed by fed-batch cofermentation of glucose-rich prehydrolyzed solids enabled sequential targeting of xylose and glucose conversion. The aim was to improve the xylose conversion by lowering the glucose repression of the xylose uptake. Various prefermentation configurations and feed patterns for prehydrolyzed solids were examined. Prefermentation increased ethanol yields overall, and fed-batch prefermentation reduced xylitol production. The best results were obtained by balancing promotion of efficient xylose conversion with maintained yeast viability. Fed-batch prefermentation and a single addition of prehydrolyzed solids, elicited an ethanol yield of 0.423 $g \cdot g^{-1}$ and a xylitol yield of 0.036 $g \cdot g^{-1}$.

Keywords: *Saccharomyces Cerevisiae*, Xylose, Cofermentation, Prefermentation, Prehydrolysis, Lignocellulose, Ethanol

Background

Fermentative conversion of lignocellulosic biomass into ethanol provides a sustainable alternative that could partially replace traditional petroleum refining, but to successfully implement lignocellulosic technologies economic sustainability must be ensured. High final ethanol concentration and high ethanol yield has been identified as key factors for improved process economics [1]. To achieve these, efficient hydrolysis and fermentation as well as utilization of a variety of sugars present in the feedstock are necessary.

The main obstacles to efficient cofermentation of lignocellulose-derived sugars are the limitations of the microbial physiology that restricts efficient conversion of various substrates [2] and the ability to cope with a variety of inhibitors [3]. The wild-type strain of *Saccharomyces cerevisiae* is tolerant to many inhibitors that are generated by thermochemical pretreatment, but it is largely unable to convert pentoses into ethanol without genetic modification [4]. Exogenous genes that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) [5, 6], as well as xylose isomerase [7], have been introduced into the *S. cerevisiae* genome to enable assimilation of xylose. However, fermentation of xylose to ethanol by engineered *S. cerevisiae* is slower and generally results in lower ethanol yields than glucose fermentation [8]. This is likely because of limitations in capacity in the pentose phosphate shunt [9] and an imbalance in redox cofactors in engineered XR/XDH-pathways [10]. The cofactor imbalance between the NAD(P)H-consuming XR and NADH-producing XDH catalyzed reactions restricts flux through the engineered pathway, and causes xylitol production [11, 12]. Improvements have been

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made by altering the cofactor specificity of XR towards NADH [13, 14], and by overexpression of endogenous xylulokinase [9]. However, the distribution of products and the rate of conversion remain dependent on the balance and turnover of cofactors. The xylose conversion capacity, as well as tolerance of inhibitors, have been further improved in engineered *S. cerevisiae* strains by evolutionary engineering and adaptation strategies [15], but the slow cellular uptake of pentoses remains a constraint for efficient xylose conversion. Xylose is taken up by non-specific hexose transport mechanisms [16], and because their affinity for glucose is many-fold higher than for xylose [10] excessive amounts of glucose competitively inhibit the transporters and prevent efficient uptake of xylose. However, low concentrations of glucose have been shown to enhance the xylose uptake rate [17], which imply coconsumption [18]. The enhanced xylose uptake rate has been attributed to improved cofactor recycling [19], and the induction of genes expression for transporter systems [19] and glycolytic enzymes [20].

Fermentation design can provide a tool to improve xylose utilization and conversion efficiency by accommodating the substrate consumption patterns of the fermenting microorganism. Thus, various strategies have been proposed to optimize the conversion of biomass-derived glucose and xylose to ethanol, where enzymatic hydrolysis and fermentative conversion can be performed either sequentially (separate hydrolysis and cofermentation, or SHCF) or simultaneously in a single vessel (simultaneous saccharification and cofermentation, or SSCF). Opting for either strategy is generally a trade-off between optimal temperatures and inhibitory glucose concentrations during hydrolysis on the one hand (SHCF) and suboptimal temperatures and ethanol-inhibited cellulolysis on the other (SSCF). Whereas some studies have shown that SSCF-based designs generally result in higher yields [18, 21], the separate hydrolysis in SHCF-based designs enable optimization of the process conditions in the individual steps. Performing separate hydrolysis eliminates rate limiting effects of the hydrolysis on conversion rates, and problems associated with high viscosity during fermentation are alleviated by prior liquefaction of the solids. These properties become increasingly important as the solids load is increased in the process. Both strategies have advantages, and the choice is strain and feedstock dependent. Modifications to the fundamental strategies have been implemented to improve fermentation performance and substrate utilization [22–24]. Fed-batch design has been implemented to promote coconsumption in SHCF [22, 23]. Fed-batch designs, where a glucose-rich feed supported the xylose utilization, improved the overall ethanol yields, and lowered xylitol production in co-fermentation of steam-pretreated wheat straw with strains of xylose-fermenting *S. cerevisiae* [23]. Further, prefermentation has been implemented as a modification to SSCF to improve xylose utilization and ethanol yields [24, 25]. Depletion of glucose in the liquid fraction of whole spruce slurry, prior to enzyme addition in SSCF, reduced the competitive inhibition of the xylose uptake and increased ethanol yields [24]. The authors presupposed that the process significance would be even greater with xylose-rich feedstocks. When pretreated agricultural residues are used as substrate, which have higher xylose content than spruce, the use of prefermentation can be extended to encompass substantial xylose conversion. The high xylose concentration, in combination with low glucose concentration, in the hydrolyzate liquor provide glucose-to xylose ratios during prefermentation that kinetically favor xylose uptake [17]. By separating the hydrolyzate liquor from the lignocellulosic solids, the advantages of an SHCF strategy can be combined with the beneficial conditions for xylose conversion in prefermentation. The combined strategy features sequential targeting of xylose and glucose conversion with optimal temperatures and customization of the enzymatic hydrolysis and fermentation steps individually. It has previously been demonstrated with a 2-step batch-SSCF of AFEX-pretreated switchgrass that sequential targeting of xylose and glucose conversion improve xylose utilization and ethanol yields [25].

In this study, various SHCF-based cofermentation strategies for the conversion of glucose and xylose to ethanol were examined. Two wheat straw slurries with various inhibitor concentrations, prepared by dilute acid catalyzed steam-explosion, were used. The pentose-rich hydrolyzate liquor in the slurries was separated from the hexose-rich solids to enable sequential targeting of xylose and glucose conversion. Cofermentation was performed by a xylose-fermenting and inhibitor tolerant strain of *S. cerevisiae* in a 2-step process, where prefermentation of the hydrolyzate liquor was followed by feeding of enzymatic hydrolysate, which consisted of prehydrolyzed unwashed solids. The hypothesis was that xylose could be converted with greater efficiency and render higher ethanol yields under the more favorable conditions for xylose conversion in the sequential fermentation steps than by conventional SHCF. Various feed patterns during prefermentation and for the addition of enzymatic hydrolyzate were examined to improve the xylose conversion and maximize the ethanol yield.

Methods

Microorganisms

Fermentation was performed with the non-commercial recombinant *Saccharomyces cerevisiae* KE6-12 strain (Taurus Energy AB), which harbors genes from

Scheffersomyces stipitis (formerly *Pichia stipitis*) that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) and overexpressing endogenous xylulokinase (XK), thus enabling xylose conversion. The strain was developed by evolutionary engineering [26] on the industrial strain *S. cerevisiae* TMB3400 [27] to improve inhibitor tolerance and xylose conversion capacity. Stock culture aliquots contained a mass fraction of 20 % glycerol in water and were stored at −80 °C.

Raw material and preprocessing

Two batches of wheat straw that were pretreated with dilute acid-catalyzed steam explosion under various conditions were procured from SEKAB E-Technology AB (Örnsköldsvik, Sweden). The slurries were denoted *severe* or *mild* slurry, respectively, based on the relative inhibitor concentrations in the hydrolyzate liquor. The *severe* slurry was prepared by impregnation with dilute H_2SO_4, pH 2.4, and steam-pretreatment at 190 °C for 15 min, and had a water-insoluble solids (WIS) content of 13.9 wt % and a total dry-matter (DM) content of 20.1 wt %. The *mild* slurry was prepared by impregnation of wheat straw with dilute H_2SO_4, pH 1.7, and steam-pretreatment at 187 °C for 8 min, and had a WIS content in the range of 11.1–12.7 wt % and a total DM content in the range of 17.5–18.2 wt %. The pH of the slurries was adjusted to 5 with 12.5 M NaOH, and the hydrolyzate liquor was separated from the solids by filtration using a hydraulic press (HP5 M, Fischer Maschinenfabrik). The unwashed solid fraction of the *severe* slurry had a WIS content of 34 wt % after filtration, versus between 38 and 44 wt % WIS in the unwashed solid fraction of the *mild* slurry.

Cultivation of yeast

The precultures were cultivated in 250 ml shake flasks with 150 ml of sterile minimal medium, containing 20 $g{\cdot}L^{-1}$ glucose, 20 $g{\cdot}L^{-1}$ xylose, 7.5 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$, 3.75 $g{\cdot}L^{-1}$ KH_2PO_4, and 0.75 $g{\cdot}L^{-1}$ $MgSO_4$ 7 H_2O. The media was supplemented with 1 $mL{\cdot}L^{-1}$ vitamin solution and 10 $mL{\cdot}L^{-1}$ trace element solution, per Taherzadeh et al. [28]. The pH of the medium was adjusted to 5.5 with 5 M NaOH in all precultures, all of which were inoculated with 300 µl of the stock cell aliquots. The preculture was incubated at 30 °C on an orbital shaker (LabTherm, Kühner) at 180 rpm for 24 h.

The cultivations were performed in a sterilized 2 L Labfors bioreactor (Infors AG) equipped with two six-blade Rushton turbines. The reactor diameter to impeller diameter ratio was 3, and the reactor height to diameter ratio was 1.7. The yeast was propagated with aerobic batch cultivation on molasses followed by aerobic fed-batch cultivation on wheat straw hydrolyzate liquor and molasses. The batch cultivation was performed with 50 $g{\cdot}L^{-1}$ molasses solution that was supplemented with 23.5 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$, 3 $g{\cdot}L^{-1}$ KH_2PO_4, 2.25 $g{\cdot}L^{-1}$ $MgSO_4$ ·7 H_2O, 33 $\mu g{\cdot}L^{-1}$ biotin, and 120 ppm Vitahop (BetaTec). The molasses (Nordic Sugar A/S) contained 40 wt % fermentable sugars (sucrose, fructose, and glucose), lactic acid (0.034 $g{\cdot}g^{-1}$), and acetic acid (0.008 $g{\cdot}g^{-1}$). The cultivation was initiated by inoculation with the preculture. The batch cultivation was performed with 0.5 L working volume, with a constant aeration rate of 1 vvm, pH maintained at 5.2 and an agitation rate of 700 rpm. The batch phase was concluded when all sugars were consumed, as indicated by the evolution of carbon dioxide and oxygen in the bioreactor gas effluent.

The fed-batch phase was initiated after fermentable sugars were depleted in the batch phase. The feed solution comprised diluted wheat straw hydrolyzate liquor that was supplemented with 150 $g{\cdot}L^{-1}$ of molasses. The hydrolyzate liquor in the feed solution brought about inhibitor concentrations in the final working volume that corresponded to the concentrations in a broth with a 7.5 wt % WIS load. The purpose of the hydrolyzate liquor in the fed-batch phase was to improve yeast tolerance by short-term adaptation of the cultivated yeast to the environmental conditions in the fermentation experiments, per Nielsen et al. [29]. The feed solution was pulse-fed to the bioreactor at a constant rate for 20 h to a final working volume of 1.5 L. The reactor was aerated by sparging at a constant rate of 1 vvm, based on the final volume, and the pH was maintained at 5.2 automatically with sterile 2.5 M NaOH solution.

The propagated yeast was harvested by centrifugation (3800×*g*, 10 min) and washed with 9 $g{\cdot}L^{-1}$ sterile NaCl solution. The cell pellets were resuspended in sterile 9 $g{\cdot}L^{-1}$ NaCl solution to yield inocula with a cell dry matter concentration of 120 $g{\cdot}L^{-1}$.

Enzymatic hydrolysis of the solid fractions

The unwashed solid fractions from the filtered slurries of pretreated wheat straw were hydrolyzed enzymatically prior to the cofermentation (Fig. 1) in a Terrafors rotating drum reactor (Infors AG) that was agitated by free-fall mixing, yielding a glucose-rich enzymatic hydrolyzate. The unwashed solid fractions of the *severe* and *mild* slurries, containing both solids and hydrolyzate liquor, were diluted with distilled water. The dilution of the solid fraction of the *severe* slurry yielded a WIS load of 20 wt % in the enzymatic hydrolysis, and the dilution of the solid fractions of the *mild* slurry yielded WIS loads of 26 and 32 wt % in the enzymatic hydrolyzes, dependent on the WIS content in the original slurry. The dilution was constrained by the target 10 wt % WIS in the SHCF experiments. Hydrolysis was performed at 45 °C for 96 h with an enzyme load of 9 $FPU{\cdot}g^{-1}$ WIS and constant

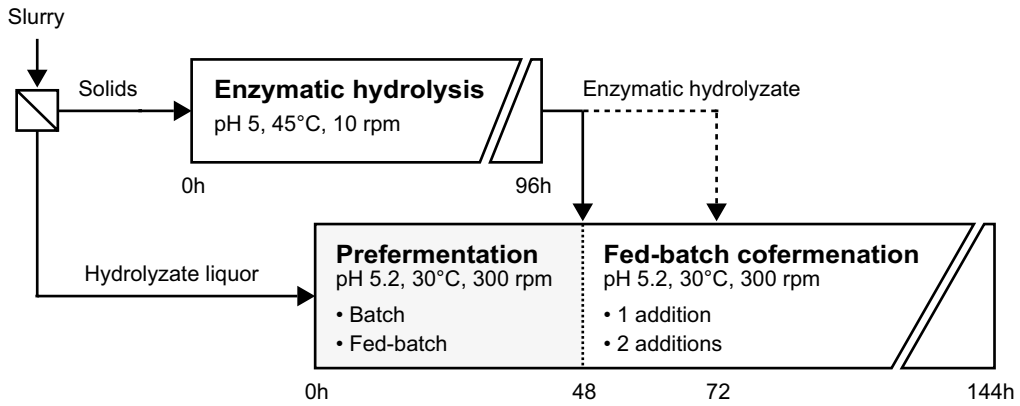

Fig. 1 Schematic of SHCF strategies

reactor revolution at 10 rpm. Cellic CTec2 enzyme preparation (Novozymes A/S) with a filter paper activity of 98 FPU·g^{-1}, as determined per Adney and Baker [30], was dispersed in the dilution-water to promote even distribution of enzymes. The pH was maintained at 5 by manual addition of 5 M sterile NaOH solution.

Separate hydrolysis and cofermentation

All cofermentations were performed in sterilized 2 L Labfors bioreactors (Infors AG) equipped with an anchor impeller and a pitched six-blade turbine. The reactor diameter to impeller diameter ratio was 1.5 for the anchor impeller and 1.7 for the pitched six-blade turbine, and the reactor height to diameter ratio was 1.7. All cofermentations were supplemented with 0.5 g·L^{-1} $(NH_4)_2HPO_4$, 0.125 mL·L^{-1} Vitahop (BetaTec), and 0.4 mL·L^{-1} Antifoam RD Emulsion (Dow Corning), all based on the final volume. The pH in the fermentation broths were maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution.

SHCF with prefermentation

The prefermentation of hydrolyzate liquor followed by fed-batch cofermentation of enzymatic hydrolyzate was performed with a final working weight of 1.5 kg; a total WIS load of 10 wt %, based on the final weight; a yeast load of 5 g·L^{-1} of dry matter (DM), based on the final volume; and an overall enzyme load of 10 FPU·g^{-1} WIS.

The fermentations were performed sequentially by prefermentation of the hydrolyzate liquor, followed by cofermentation of the enzymatic hydrolyzate (Fig. 1). The prefermentation of the hydrolyzate liquor was performed using either of two feed strategies: batch or fed-batch. In batch prefermentation, the entire amount of separated hydrolyzate liquor was supplied at outset and cofermented for 48 h. For fed-batch prefermentation, a two-step process was adopted. Approximately 25 wt % of the separated hydrolyzate liquor was cofermented in batch for 12 h, and the remaining 75 wt % was fed linearly to the fermentor for the subsequent 36 h. The amount of hydrolyzate liquor at outset was set to surpass minimum required liquid level in the fermentor (≥200 mL) to ensure reliable pH and temperature control. Regardless of mode, the full yeast load, 5 g·L^{-1}, was pitched at outset and 1 FPU·g^{-1} WIS of Cellic CTec2, based on total ingoing WIS, was added after 4 h to hydrolyze solubilized oligosaccharides. The hydrolyzate liquor was cofermented under anaerobic conditions at 30 °C for 48 h, and the bioreactor was agitated at 300 rpm.

Two feed strategies were used for the following fed-batch cofermentation of the enzymatic hydrolyzate: (i) all enzymatic hydrolysate was added after 48 h, and (ii) half of the enzymatic hydrolyzate was added after 48 h, and the remaining half added after 72 h. Enzymes, equivalent to a total enzymatic activity of 9 FPU·g^{-1} WIS, were carried over with the addition of enzymatic hydrolyzate, resulting in total cellulolytic activity of 10 FPU·g^{-1} WIS, based on the total ingoing WIS content. The temperature was maintained at 30 °C, and agitation was maintained at 300 rpm. The experiments were terminated after a total fermentation time of 144 h.

Model fermentations

Model cofermentations were performed with a final working weight of ~1.4 kg using *mild* and *severe* hydrolyzate liquor from the pretreated wheat straw slurries. The hydrolyzate liquor was diluted with distilled water to inhibitor and sugar concentrations that corresponded to a WIS load of 10 wt %. Further, the hydrolyzate liquor was supplemented with glucose, corresponding to 81 % yield in enzymatic hydrolysis of the solid fraction. The supplemented hydrolyzate liquor mimicked the composition

of enzymatically hydrolyzed slurry. An enzyme load of 2 FPU·g^{-1} WIS was applied to hydrolyze solubilized oligosaccharides, and the fermentor was inoculated with a yeast load of 5 g·L^{-1} DM. All components were added at the outset. The model cofermentation mimicked a batch SHCF, but avoided the bias from influence of solid material and hydrolysis limitations in the cofermentation. The hydrolyzate liquor was cofermented under anaerobic conditions at 30 °C for 144 h, and the bioreactor was agitated at 300 rpm.

Analytical procedures

Extracellular metabolites, inhibitors, and sugars were measured by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system that was equipped with an RID-10A refractive index detector (Shimadzu). Samples for carbohydrate analysis with low pH (from hydrolyzate liquors) were adjusted to pH 5 with $CaCO_3$ and centrifuged in 10 mL tubes (960×*g*, 5 min). All samples were centrifuged (16,000×*g*, 3 min), and the supernatants filtered through 0.20 μm syringe filters (GVS Filter Technology). Filtered samples were stored at −20 °C until analysis.

Extracellular metabolites, organic acids, and degradation products in the hydrolyzate liquors and fermentation broth were analyzed by isocratic ion-exchange chromatography using an Aminex HPX-87H column (Bio-Rad Laboratories) at 50 °C. The eluent was 5 mM H_2SO_4, applied at a flow rate of 0.5 mL·min^{-1}. Sugars and xylitol in the hydrolyzate liquors and fermentation broth were quantified by isocratic ion-exchange chromatography on an Aminex HPX-87P column (Bio-Rad Laboratories) at 85 °C. Deionized water was used as the eluent at a flow rate of 0.5 mL·min^{-1}.

Dry matter content (DM) of solids and water-insoluble solids (WIS) were measured per Sluiter et al. [31], and Sluiter et al. [32], respectively. Soluble carbohydrates, monomeric sugars that were released into solution during pretreatment, and pretreatment degradation products were quantified by acid hydrolysis and HPLC per Sluiter et al. [33]. Further, structural carbohydrates, lignin, and ash contents of the water-insoluble fraction of the wheat straw slurries were measured per Sluiter et al. [34].

Calculation of yields

Ethanol yields were calculated at two levels: an overall yield and a metabolic yield. The overall ethanol yield was based on total supplied glucose and xylose; i.e. the sum of glucose and xylose present in the slurries, including monomers, oligomers, and polymers. The metabolic ethanol yield was based on consumed glucose and xylose. The mass of glucose and xylose available in the form of polymers in the WIS were corrected with factors 1.111 and 1.136, respectively, to account for the addition of water to the monomeric units during hydrolysis. The percentage of maximum theoretical ethanol yield was based on a theoretical stoichiometric yield of 0.51 g·g^{-1} on glucose and xylose.

Results and discussion

Material preprocessing and composition

Wheat straw was pretreated using dilute acid-catalyzed steam pretreatment at two conditions to yield two slurries denoted *mild* and *severe* slurry, based on the relative inhibitor concentrations in the hydrolyzate liquor. The lower inhibitor concentrations in the *mild* slurry was attributed to lower degradation, due to the shorter hold-up time, and also to some extent to higher dilution in the pretreatment. The *severe* slurry had a water-insoluble solids (WIS) content of 13.9 wt %, versus 11.1 to 12.7 wt % for the *mild* slurry. The variation between barrels of the *mild* slurry was likely due to sedimentation in the storage vessel at the demonstration-scale pretreatment site. The *mild* slurry with WIS content of 12.7 wt % was used in the evaluated fermentations of *mild* slurry. The *mild* slurry with lower WIS content (11.1 wt %) was used to illustrate the limitations with the proposed cofermentation strategy. The applied pretreatments solubilized mainly hemicellulosic sugars. Consequently, hydrolyzate liquors that were rich in xylose and hemicellulosic oligomers, and cellulose-rich solids were obtained. The composition of the WIS fractions and the sugar and inhibitor concentrations of the hydrolyzate liquors are listed in Table 1.

The separation of hydrolyzate liquors from the lignocellulosic solids by filtration, in combination with the compositional differences between the phases (Table 1), enabled the sequential targeting of xylose and glucose conversion. The hydrolyzate liquor contained most of the xylose and inhibitors and the unwashed lignocellulosic solids harbored mainly cellulose and lignin. This allowed the use of feeding schemes that mitigate the impact of inhibitors and effect more favorable glucose-to-xylose ratios for xylose utilization could be implemented.

Enzymatic hydrolysis of the solid fractions

The enzymatic hydrolysis of the retained solids after filtration was performed at high WIS loads, because of the separation of hydrolyzate liquor from the lignocellulosic solids. The dilution of the solid fractions was further constrained by the intended WIS load of 10 wt % in the SHCF experiments and the aim to maximize separation between the bulk fractions of glucose and xylose.

The unwashed solid fraction of the *severe* slurry was diluted from a WIS content of 34 wt % in the retained solids to the 20 wt % used in the enzymatic hydrolysis.

Table 1 Composition of structural carbohydrates and lignin in the water-insoluble fractions of the pretreated materials and sugar compositions and prevalence of inhibitory compounds in hydrolyzate liquors

	Severe wheat straw slurry	*Mild* wheat straw slurry
Steam-pretreated material (% of dry weight)		
Glucan	51.4	50.7
Xylan	1.7	1.0
Galactan	0.0	*BDL*
Arabinan	0.1	0.05
Mannan	0.1	0.07
Acid-soluble lignin	0.7	0.6
Acid-insoluble lignin	33.8	29.8
Lignin ash	6.9	10.2
Total determined	94.6	92.4
Hydrolyzate liquor ($g \cdot L^{-1}$)		
Glucose	14.5	8.7
Xylose	32.6	35.4
Galactose	*BDL*	0.9
Arabinose	3.5	1.8
Mannose	1.8	0.7
Formic acid	1.6	0.8
Acetic acid	8.5	5.6
Levulinic acid	*BDL*	0.5
HMF	1.3	0.3
Furfural	7.7	3.7

BDL below detection limit

The unwashed retained solids from the *mild* slurry was diluted from a WIS content of 44 to the 26 wt % WIS applied in the enzymatic hydrolysis. Sufficient water for dilution was available to provide even distribution of enzyme preparation and sufficient mixing. Liquefaction became apparent after a few hours, and an extensive degradation of solids occurred during the enzymatic hydrolysis. The enzymatic hydrolysis was performed to produce a glucose-rich enzymatic hydrolysate, and not evaluated further.

Performing the enzymatic hydrolyses at high WIS loads imply the risk of lower sugar yields in hydrolysis. It has been shown for several lignocellulosic substrates that greater substrate loads decrease the corresponding hydrolysis yield [35]. The effect has been attributed to product inhibition [36, 37], inhibition by sugar-derived inhibitors and lignin [38, 39], and mass transfer limitations and other effects that are related to the increased WIS loads [40]. However, inhibition primarily affects the hydrolysis rate— not the maximum conversion or yield— given sufficient time. Minor inefficiencies in the hydrolysis could be rectified with the employed cofermentation strategy, because the enzymes were carried over from the enzymatic hydrolysis to the subsequent cofermentation step, and provided an additional period of hydrolysis. Despite the possibility to partially rectify hydrolysis inefficiencies obtaining high sugar yields in the hydrolysis was important to successfully carry out the devised SHCF-design.

Strategies for separate hydrolysis and cofermentation

In the first cofermentation step, the prefermentation, xylose conversion was targeted. The prefermentation of the hydrolyzate liquors separated from *mild* and *severe* slurry were conducted in batch and fed-batch. In the second cofermentation step, the fed-batch cofermentation, glucose conversion was targeted, and remaining xylose coconsumed. The enzymatic hydrolyzate obtained from the enzymatic hydrolysis of the solid fractions, which contained high amounts of glucose, was fed to the prefermented hydrolyzate liquor. One or two additions of enzymatic hydrolyzate were investigated. The aim was to elucidate the impact of number of additions of enzymatic hydrolyzate, independently and in combination with different prefermentation configurations, on xylose utilization and ethanol yield.

Xylose utilization in batch prefermentation

Batch prefermentation of the separated *mild* hydrolyzate liquor resulted in depletion of glucose and consumption of 88 % of the xylose. The deviation from the mean in the experiments was less than 1 %. The consumed xylose equaled 69 % of the total available xylose in the used slurry. Batch prefermentation of separated *mild* hydrolyzate liquor elicited higher xylose utilization than with *severe* hydrolyzate liquor. The glucose in the *severe* hydrolyzate liquor was depleted and 77 % of the available xylose in the hydrolyzate was consumed, which equaled 48 % of the total available xylose in the used slurry. The substantial consumption of xylose during batch prefermentation reduced the extent of xylose coconsumption needed after the addition of enzymatic hydrolyzate.

The addition of enzymes after the depletion of measured glucose in the fermentation broth was intended to supply low amounts of monomeric glucose from solubilized oligomers to promote coconsumption and facilitate xylose uptake. However, declining xylose uptake rates were seen in the batch prefermentations of *mild* and *severe* hydrolyzate liquors after depletion of measured glucose (Figs. 2a, c, 3a), indicating that not enough glucose was available in the solubilized oligomers to sustain coconsumption and facilitate xylose utilization.

Batch prefermentation of *mild* and *severe* hydrolyzate liquor elicited metabolic ethanol yields of 0.34 and 0.33 $g \cdot g^{-1}$, respectively. The higher ethanol

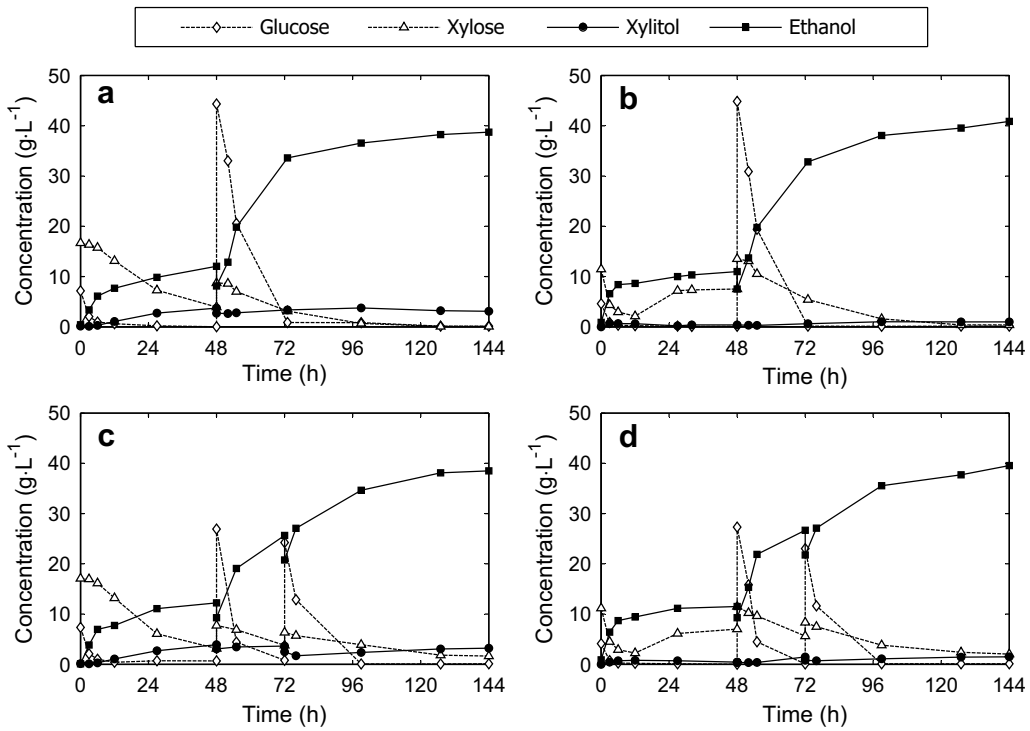

Fig. 2 Time courses for SHCF of *mild* wheat straw slurry with 48 h of (**a**) batch or (**b**) fed-batch prefermentation followed by one addition of enzymatic hydrolyzate and 48 h of (**c**) batch or (**d**) fed-batch prefermentation followed by two additions of enzymatic hydrolyzate

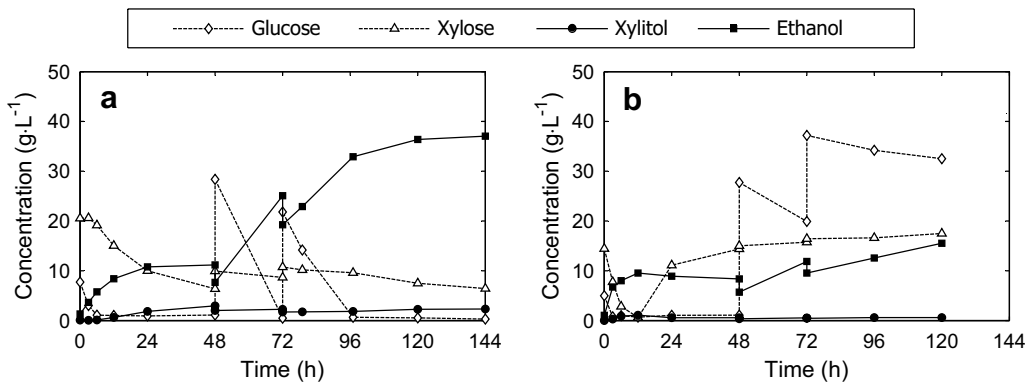

Fig. 3 Time courses for SHCF of *severe* wheat straw slurry with 48 h of (**a**) batch or (**b**) fed-batch prefermentation followed by two additions of enzymatic hydrolysate

concentrations and metabolic ethanol yields obtained with *mild* hydrolyzate liquor were attributed to the higher xylose utilization (Table 2) and the lower inhibitor concentrations (Table 1), respectively.

Xylose utilization in fed-batch prefermentation

To address the inability to sustain coconsumption of xylose and glucose by liberating glucose from the solubilized oligomers in the hydrolyzate liquors, the fed-batch prefermentation strategy was implemented. The fed-batch layout was intended to continuously supply low amounts of glucose and promote coconsumption. The glucose-to-xylose ratio of the hydrolyzate liquors was such (Table 1) that a low measured glucose concentration in the bioreactor could be maintained with a feed of hydrolyzate liquor (Figs. 2b, d, 3b), which kinetically favored xylose consumption [17]. However, lower xylose utilization was observed with fed-batch prefermentation,

Table 2 Summary of prefermentation results

	Severe wheat straw slurry		Mild wheat straw slurry	
	Batch	Fed-batch	Batch	Fed-batch
Residual sugars and end-products [g·L^{-1}]				
Glucose	0.1	0.1	0.0	0.1
Xylose	6.4	14.4	3.7	7.3
Ethanol	11.2	8.4	12.1	11.2
Xylitol	2.9	0.6	3.8	0.4
Metabolic ethanol yield [g·g^{-1}][a]	0.329	0.322	0.342	0.353
% of theoretical	64.4	63.1	67.1	69.2
Xylose utilization [%]	77.3	48.6	88.2	76.9
Xylitol yield [g·g^{-1}][b]	0.136	0.041	0.137	0.017

[a] Based on consumed glucose and xylose and related to a maximum theoretical yield of 0.51 g·g^{-1}

[b] Based on consumed xylose

as compared with batch, with both hydrolyzate liquors (Table 2). On average 77 and 49 % of xylose available in the *mild* and *severe* hydrolyzate liquor, respectively, was consumed; as compared to 88 and 77 %, respectively, in batch. The deviation from the mean in fed-batch prefermentation experiments was less than 3 %. The underlying reason for the lower xylose consumption was that the feed rate of xylose-rich hydrolyzate liquor exceeded the xylose uptake rate. In combination with preferential consumption of glucose, this resulted in the accumulation of xylose during the fed-batch phase of the prefermentation (Figs. 2b, d, 3b). Although lower xylose utilizations were achieved with fed-batch prefermentation, substantial fractions of total available xylose in the slurries were consumed. The consumed xylose in *mild* and *severe* hydrolyzate liquor after fed-batch prefermentation corresponded to 60 and 31 %, respectively, of total available xylose.

The lower xylose consumptions in fed-batch prefermentation were mirrored in the obtained ethanol concentrations (Table 2), but did not have a significant effect on the metabolic ethanol yields (Table 2). Metabolic ethanol yields of 0.353 and 0.322 g·g^{-1} were obtained with fed-batch prefermentation of *mild* and *severe* hydrolyzate liquor, respectively, as compared to 0.342 and 0.329 g·g^{-1} with batch prefermentation. The deviations from the mean metabolic ethanol yields were less than 2 % in all instances. Fed-batch prefermentations were also accompanied with lower xylitol production than batch prefermentation. Batch prefermentation of *mild* and *severe* hydrolyzate liquor resulted in xylitol yields of 0.136 and 0.134 g·g^{-1}, respectively, whereas only 0.017 g·g^{-1} respectively 0.041 g·g^{-1} were produced in fed-batch prefermentation. The deviations from the mean in batch prefermentation experiments were less than 2 %, and less than 7 % in fed-batch prefermentation experiments. The difference in xylitol production between the prefermentation configurations was attributed to the higher xylose consumption rate in batch prefermentation. High xylose consumption rates create a metabolic bottleneck because of an imbalance of cofactors in engineered XR/XDH-pathways [11, 12], whereas it has been shown that lower consumption rates suppress xylitol production [41]. The lower xylose consumption in fed-batch prefermentation in combination with continuous availability of low concentrations of furaldehydes, which act as external electron sinks for the regeneration of cofactors [42], likely alleviated the cofactor imbalance.

Effect of prefermentation on overall xylose utilization

The selected feed strategy during prefermentation had several intertwined consequences on fermentation performance during the subsequent fed-batch cofermentation of enzymatic hydrolyzate. The different prefermentation configurations had different impacts on fermentative capacity of the yeast during the fed-batch cofermentation of enzymatic hydrolyzate, which was primarily attributed to changes in the viability.

Comparison of overall cofermentation outcome between batch and fed-batch prefermentation of *severe* hydrolyzate liquor followed by two additions of enzymatic hydrolyzate (Fig. 3) exemplify the different consequences in a high inhibitor concentration context. Batch prefermentation followed by fed-batch cofermentation of enzymatic hydrolyzate elicited a xylose utilization of 71 % and an overall ethanol yield of 0.381 g·g^{-1}. In contrast, the corresponding strategy with fed-batch prefermentation resulted in low glucose conversion and, seemingly, no xylose consumption after fed-batch prefermentation of *severe* hydrolyzate liquor (Fig. 3b). Fed-batch prefermentation supplied lower initial concentrations of inhibitors than batch prefermentation, but the continuous feed of hydrolyzate liquor seemingly exhausted the yeast. The continuous feed of inhibitors during fed-batch prefermentation exceeded the detoxification rate, which resulted in the accumulation of non-metabolized furaldehydes (data not shown). The furaldehydes were not fully converted during the sequent fed-batch cofermentation of enzymatic hydrolyzate. In contrast, the yeast was resilient to the higher initial inhibitor concentrations in batch prefermentation. The yeast was capable of promptly detoxifying the hydrolyzate liquor during batch prefermentation and after each addition of enzymatic hydrolyzate (data not shown). The decreased viability and fermentative capacity of the yeast was likely due to ceased growth and inhibitory effects [3]. The yeast cells

were assumed to be particularly sensitive to exposure to inhibitors during the prefermentation of xylose-rich hydrolyzate liquor, because the conversion of xylose does not support growth efficiently [43].

When hydrolyzate liquor and enzymatic hydrolyzate from *mild* slurry was utilized, the xylose fermenting capacity was sustained throughout 144 h of cofermentation. However, lower xylose utilization was obtained after fed-batch cofermentation of enzymatic hydrolyzate when fed-batch prefermentation was applied, as compared to the corresponding strategy with batch prefermentation (Table 3). Fed-batch prefermentation with one or two additions of enzymatic hydrolyzate elicited xylose utilizations of 98 and 91 %, respectively; whereas the corresponding cofermentations with batch prefermentation elicited xylose utilizations of 97 and 93 %, respectively. The deviations from the means for experiments employing fed-batch prefermentation was less than 3 %. The primary reason was higher residual xylose concentrations at the end of fed-batch cofermentations of enzymatic hydrolysate when fed-batch prefermentation was applied (Table 3). This suggests that fed-batch prefermentation negatively influences the xylose fermenting capacity.

Effect of prefermentation on overall xylitol production

Strategies employing fed-batch prefermentations elicited lower xylitol yields than those with batch prefermentation (Table 3). The xylitol production was decreased with fed-batch prefermentation, regardless of whether *mild* or *severe* slurry was used, and effected lower overall xylitol yields at the end of the fed-batch cofermentation of enzymatic hydrolyzate (Table 3). Because of the sequential targeting of xylose and glucose conversion in the fermentation steps, the xylitol productions during prefermentation were major determinants of total xylitol production. After prefermentation little xylitol was produced, because glucose was the predominant substrate and remaining xylose was mainly coconsumed.

For all practical purposes xylitol production represents a loss of carbon that could be converted to ethanol. The lower xylitol production in strategies employing fed-batch prefermentation contributed to the xylose conversion efficiency and, thus, the higher overall ethanol yields obtained. Despite lower xylose utilization the overall ethanol yields were higher than for strategies employing batch prefermentation (Table 3). The higher overall ethanol yields were correlated with the decreased xylitol production obtained with fed-batch prefermentation.

Effect of number of additions of enzymatic hydrolyzate

The multiple additions of enzymatic hydrolyzate during the fed-batch cofermentation provided the means to lower glucose concentrations in the fermentation broth, as compared to a single addition. Fed-batch SHCF of steam-pretreated wheat straw has previously been shown to enhance xylose utilization [22, 23]. However, regardless of chosen prefermentation configuration a single addition of enzymatic hydrolyzate elicited higher xylose utilization and ethanol yields than corresponding fed-batch cofermentations with two additions (Table 3). The yeast exhibited decreased xylose fermenting capacity with repetitive addition of *mild* enzymatic hydrolyzate

Table 3 Summary of results after prefermentation of hydrolyzate liquor followed by fed-batch cofermentation of enzymatic hydrolyzate

Fig.	Description	Residual sugars and end-products				Xylose utilization	Yields		
		Glucose	Xylose	Xylitol	Ethanol		Ethanol[a]		Xylitol[b]
		$g \cdot L^{-1}$	$g \cdot L^{-1}$	$g \cdot L^{-1}$	$g \cdot L^{-1}$	%	$g \cdot g^{-1}$	%	$g \cdot g^{-1}$
	S, Model	0.1	5.3	2.0	28.9	75.9	0.346	67.9	0.120
	M, Model	0.1	1.2	5.7	32.2	96.1	0.360	70.6	0.191
2a	*S, B, 2*	0.3	6.4	2.3	37.0	70.6	0.381	74.8	0.135
2b	*S, FB, 2*	32.5	17.5	0.6	16.0	*ND*	0.164	32.2	*ND*
3a	*M, B, 1*	0.1	0.1	3.1	38.7	98.0	0.401	78.5	0.112
3b	*M, FB, 1*	0.1	0.4	1.0	40.9	96.9	0.423	82.9	0.036
3c, 4b	*M, B, 2*	0.1	1.6	3.2	38.5	92.4	0.398	78.1	0.123
4a	*M, B, 2*[c]	0.5	4.7	1.4	27.3	*ND*	0.287	56.2	*ND*
3d	*M, FB, 2*	0.1	2.0	1.5	39.6	90.9	0.409	80.2	0.058

S severe slurry; *M* mild slurry; *B* batch prefermentation; *FB* fed-batch prefermentation; *1* 1 addition of enzymatic hydrolyzate; *2* 2 additions of enzymatic hydrolyzate, *ND* not determined

[a] Ethanol yield based on total supplied glucose and xylose and related to the maximum theoretical yield (0.51 $g \cdot g^{-1}$)

[b] Xylitol yield based on total consumed xylose

[c] 12, M, B, 2 with insufficient prehydrolysis

that contained inhibitors. The trend was evidenced by higher final residual xylose concentrations (Table 3).

Because xylose is converted at lower rates than glucose [8], the fermentation time becomes of essence. The distributed multiple additions of enzymatic hydrolyzate effectively reduce the average time available for conversion of xylose that resides in the enzymatic hydrolyzate. Another aspect relates to the addition of inhibitors. The measured inhibitors were predominantly present in the separated hydrolyzate liquors, and were to a large extent converted to less toxic entities during the prefermentation. However, contributions of inhibitors were made with every addition of enzymatic hydrolyzate, because 20–30 wt % of the total amount of hydrolyzate liquor in the slurry after pretreatment remain with the solids after the filtration. In the experiment series with *mild* slurry the yeast was able to convert furfural irrespective of prefermentation mode and number of additions of enzymatic hydrolyzate. HMF, on the other hand, was only fully converted with batch prefermentation and one addition of enzymatic hydrolyzate (data not shown). Fed-batch prefermentation decreased the conversion of HMF, and in combination with two additions of enzymatic hydrolyzate no HMF was converted after prefermentation (data not shown). The trend in furaldehyde conversion was correlated to that of the xylose fermenting capacity. The aggregated effect of fed-batch prefermentation and multiple additions of enzymatic hydrolysate elicited increasingly higher residual xylose concentrations (Table 3), and thus lower xylose utilization. This finding was indicative of that decreases in viability of the yeast occurred with both continuous and repetitive addition of substrate that contained inhibitors.

Maximization of ethanol yield

The highest overall ethanol yield was obtained with low concentrations of inhibitor in the slurry, because it permitted feeding schemes that maximized xylose conversion efficacy while the viability of the fermenting microorganism was sustained. The best results were obtained with fed-batch prefermentation and a single addition of enzymatic hydrolyzate, with an overall ethanol yield of 0.423 $g \cdot g^{-1}$, xylose utilization of 98 %, and the lowest xylitol production at 0.036 $g \cdot g^{-1}$. The deviations from the means were below 4 % in all instances. The strategy combined lower xylitol productions and higher ethanol yields elicited by fed-batch prefermentation with the lower effect of inhibitors associated with one addition of enzymatic hydrolyzate. A trade-off existed between promoting efficient xylose conversion with substrate feeding and maintaining yeast viability. Ethanol yield was maximized with a balance between them.

The reduction in residual xylose concentrations and decreased xylitol production (Table 3), compared to the model fermentations, indicate that the sequential targeting of xylose and glucose conversion is a feasible way to improve xylose conversion with SHCF-based conversion strategies. The proposed strategy leaves degrees of freedom in the design to implement feeding schemes that accommodate the traits of various fermenting microorganisms, as to mitigate the inhibitory effects, sustain yeast viability and maximize xylose conversion.

Method limitations

The necessitated high WIS enzymatic hydrolysis constituted a weakness of the proposed method, because the hydrolysis outcome had profound effect on the cofermentation. The shortcoming was evidenced by the difference between cofermentations of *mild* slurry with an original WIS content of 11.1 and 12.7 wt % (Table 3; Fig. 4). Batch prefermentation followed by two additions of enzymatic hydrolyzate was applied as cofermentation strategy.

The dilution of the retained solids after filtration of the *mild* slurry with the lower WIS content (11.1 wt %) was severely constrained by the required WIS load of 10 wt % in the SHCF. Hence, the retained solids were only diluted to a WIS load of 32 wt % in the enzymatic hydrolysis step. The enzymatic hydrolysis resulted in inferior hydrolysis because of various reasons associated with high WIS applications [35–37]. With inferior hydrolysis the lignocellulosic solids that remained in the enzymatic hydrolyzate were partially hydrolyzed and fermented simultaneously during the fed-batch cofermentation and the benefits of the preparatory hydrolysis of the unwashed solids waned. The simultaneous hydrolysis and cofermentation was undesirable in the SHCF, because the solids were partially hydrolyzed under suboptimal conditions at 30 °C during the fed-batch cofermentation. The drawback was not necessarily a lower yield given sufficient time, but that hydrolysis rate became a limiting factor for the fermentation rate. This constrained the final ethanol yield for the limited cofermentation period. An increase in temperature during the fed-batch cofermentation of the enzymatic hydrolyzate would have been needed to accommodate hydrolysis of remaining solids and avoid severe limitations to the fermentation rate. The illustrated limitation puts emphasis on the necessity of high sugar yields in preparatory enzymatic hydrolysis to implement the sequential targeting of xylose and glucose conversion with this strategy.

Conclusions

Prefermentation of hydrolyzate liquor followed by fed-batch separate hydrolysis and cofermentation improved ethanol yields, yet batch and fed-batch prefermentation

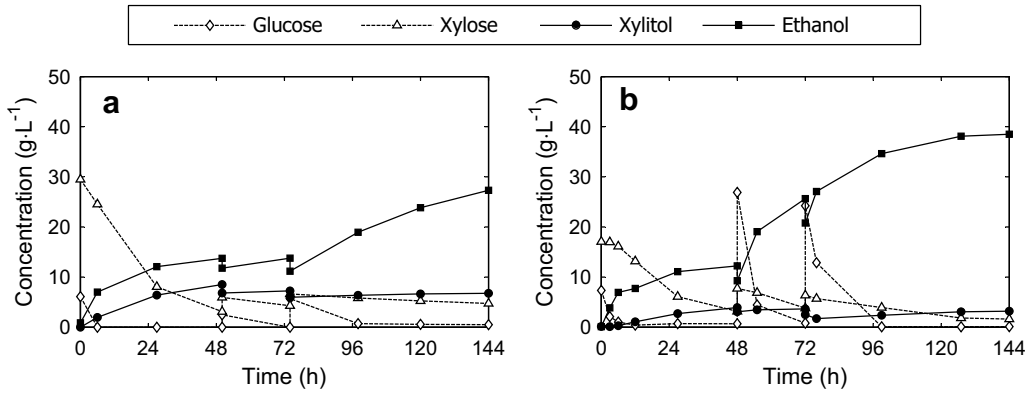

Fig. 4 Effects of insufficient prehydrolysis on fermentation behaviour. Comparison of SHCF of *mild* wheat straw slurry with batch prefermentation and two additions of enzymatic hydrolyzate, where (**a**) is with insufficiently hydrolyzed solids and (**b**) is with adequate hydrolysis

had different impacts on the cofermentation. Under influence of lower inhibitor concentrations fed-batch prefermentation resulted in lower xylitol production during all steps of the fermentation and prompted higher final ethanol yields compared to corresponding cofermentations with batch prefermentation. Under influence of high inhibitor concentrations, sustained fermentation capacity was paramount to obtain improved ethanol yield. *S. cerevisiae* KE6-12 was resilient to high inhibitor concentrations, but succumbed to continuous exposure to inhibitors. Regardless of type of slurry, continuous feed during prefermentation of the hydrolyzate liquor and multiple additions of enzymatic hydrolyzate—and their combination—appeared to hamper the fermentative capacity and exhaust the cells. The viability of the yeast, not glucose repression of xylose metabolism, appeared to be the limiting factor for higher ethanol yields in the cofermentations. The best results were obtained with *mild* slurry, applying fed-batch prefermentation and a single addition of enzymatic hydrolyzate. An ethanol yield of 0.423 g·g^{-1}, based on supplied glucose and xylose, and the lowest xylitol production, 0.036 g·g^{-1}, was obtained. A trade-off existed between promoting xylose conversion with substrate feeding and maintaining yeast viability, and ethanol yield was maximized with a balance between them.

Abbreviations

DM: dry matter; FPU: filter paper unit; HMF: 5-hydroxymethylfurfural; HPLC: high-performance liquid chromatography; SHCF: separate hydrolysis and cofermentation; SSCF: simultaneous saccharification and cofermentation; vvm: gas volume flow per unit of liquid volume per minute; WIS: water-insoluble solids; XDH: xylitol dehydrogenase; XK: xylulokinase; XR: xylose reductase.

Authors' contributions

FN participated in the conception and design of the study, performed the experiments and wrote the manuscript. GZ participated in the conception and design of the study and critically reviewed the manuscript. OW and MG assisted in interpreting the data, participated in the editing and preparation of the final manuscript, and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

The study was part of the project "Process development for combined pentose and hexose fermentation", which was co-financed by Taurus Energy AB. GZ is a shareholder and member of the board at Taurus Energy AB. Remaining authors declares no financial competing interests and all authors declare that they have no non-financial competing interests.

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