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Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*

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Abstract

Wheat straw is an abundant agricultural residue which can be used as a raw material for bioethanol production. Due to the high xylan content in wheat straw, fermentation of both xylose and glucose is crucial to meet desired overall yields of ethanol. In the present work a recombinant xylose fermenting strain of *Saccharomyces cerevisiae*, TMB3400, cultivated aerobically on wheat straw hydrolysate, was used in simultaneous saccharification and fermentation (SSF) of steam pretreated wheat straw. The influence of fermentation strategy and temperature was studied in relation to xylose consumption, ethanol formation and by-product formation. In addition, model SSF experiments were made to further investigate the influence of temperature on xylose fermentation and by-product formation. In particular for SSF at the highest value of fibre content tested (9% water insoluble substance, WIS), it was found that a fed-batch strategy was clearly superior to the batch process in terms of ethanol yield, where the fed-batch gave 71% of the theoretical yield (based on all available sugars) in comparison to merely 59% for the batch. Higher ethanol yields, close to 80%, were obtained at a WIS-content of 7%. Xylose fermentation significantly contributed to the overall ethanol yields. The choice of temperature in the range 30–37 °C was found to be important, especially at higher contents of water insoluble solids (WIS). The optimum temperature was found to be 34 °C for the raw material and yeast strain studied. Model SSF experiments with defined medium showed strong temperature effects on the xylose uptake rate and xylitol yield.

Keywords: SSF; Ethanol; Xylose fermentation; Co-fermentation; Recombinant *Saccharomyces cerevisiae*; Wheat straw

1. Introduction

Wheat straw is an abundant agricultural residue in Europe and Asia, and can be used as a raw material for bioethanol production. Each year approximately 354 Mtonnes of wheat straw has been estimated to be available globally for ethanol production, of which Europe and Asia accounts for about 32 and 43%, respectively (Kim and Dale, 2004). Wheat straw is a lignocellulosic raw material that mainly consists of cellulose, lingocellulose (branched polymers of both hexoses and pentoses) and lignin. In wheat straw the pentoses are mainly xylose and arabinose. The overall ethanol yield is one of the most important economic factors for the ethanol production costs (von Sivers and Zacchi, 1996) and hence efficient conversion of all sugars in the raw material to ethanol is crucial. Consequently, due to the high xylan content in wheat straw, fermentation of xylose to ethanol is crucial to meet desired overall yields of ethanol. The most commonly used microorganism for industrial ethanol production; *Saccharomyces cerevisiae*, does not ferment pentoses. Therefore, xylose fermenting strains of *S. cerevisiae* have been constructed by introducing genes encoding for xylose isomerise (XI) from bacteria and fungi (Karhumaa et al., 2005; Kuyper et al., 2005), or genes encoding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from fungi (Kötter et al., 1990; Eliasson et al., 2000). Xylose and glucose share the same transport systems (Kilian and Uden, 1988; Meinander and Hahn-Hägerdal, 1997) and the affinity for xylose is approximately 200-fold lower than that of for glucose (Kötter and Ciriacy, 1993). Consequently, xylose transport into the cell is inhibited by glucose, which is a problem during co-fermentation of glucose and xylose with recombinant *S. cerevisiae*. In order to obtain co-fermentation it is thus necessary to keep the glucose concentration low.

By performing the enzymatic hydrolysis simultaneously with the fermentation, several advantages can be obtained. End-products of the hydrolysis, which inhibit cellulases or β-

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glucosidases, can be continuously removed (Eklund and Zacchi, 1995) and the capital investment cost is reduced (Wingren et al., 2003). Ethanol production by simultaneous saccharification and fermentation (SSF) (Takagi et al., 1977) has proved to give a significantly higher process productivity than separate hydrolysis and fermentation (SHF) (Alfani et al., 2000; Stenberg et al., 2000). Running SSF in a fed-batch mode, i.e. gradually adding fibre suspension to the bioreactor, may improve the process further. One important process advantage with fed-batch is that the viscosity can be maintained at a manageable level due to the gradual hydrolysis of the added fibres. Agitation is otherwise difficult at high WIS-contents (Ballesteros et al., 2002). Additionally, from a kinetic point of view, the glucose levels can be kept low allowing co-fermentation of xylose and glucose (Öhgren et al., 2006).

Studies on ethanol production from wheat straw, including, e.g. comparison of SHF with SSF (Alfani et al., 2000) and comparing wheat straw as a substrate with other substrates (Wyman et al., 1992) have been carried out in the past. However, there is no study on co-fermentation of glucose and xylose in SSF of wheat straw. Previously, the recombinant xylose fermenting strain *S. cerevisiae* TMB3400 (Wahlbom et al., 2003) have been used in SSF conversion of corn stover (Öhgren et al., 2006) and sugar cane bagasse (Rudolf et al., 2008) showing that co-fermentation of xylose and glucose can be achieved in SSF.

In the present work, the strain *S. cerevisiae* TMB3400 was used in SSF of pretreated wheat straw material, with the objective of determining suitable conditions for both glucose and xylose fermentation in an SSF process. Yields and by-product formation patterns for batch and fed-batch mode of operation were compared at different WIS-contents, and the optimal temperature for the SSF was determined. A comparison with USM21, the parental non-xylose fermenting strain of *S. cerevisiae* TMB3400, was also made. Yeast cells used in the SSF, were pre-cultivated in aerobic fed-batch fermentations on hydrolysate liquid in order to improve inhibitor tolerance.

2. Material and methods

2.1. Raw material and pretreatment

The raw material used was wheat straw provided and pretreated by SAF-ISIS (F-40140, Soustons, France; http://www.safisis.com). The material was first impregnated with H_2SO_4 for 16 h at 20 °C, and pressed in a wine press at 100 bar to remove water and increase the solid content. This was followed by steam treatment at 19.5 bar for 150 s and subsequent rapid expansion to atmospheric pressure. The composition of the pretreated raw material is shown in Table 1. The water insoluble solid fraction was determined by a standard procedure developed by National Renewable Energy Laboratories (NREL) (Sluiter et al., 2004). The composition of the liquid fraction was determined by dilute acid hydrolysis followed by HPLC measurements (Ruiz and Ehrman, 1996).

2.2. Cell cultivation

The recombinant xylose fermenting strain of *S. cerevisiae* TMB3400 (Wahlbom et al., 2003) was used in the SSF experiments. Yeast cell mass to be used in SSF was produced by aerobic batch cultivation on glucose, followed by aerobic fed-batch cultivation on wheat straw hydrolysate liquid to improve inhibitor tolerance by adaptation as previously shown by Alkasrawi et al. (2006).

2.2.1. Preparation of inoculum

The strain was maintained on agar plates with the following composition: 10 g L^{-1} yeast extract, 20 g L^{-1} soy peptone, 20 g L^{-1} agar and 20 g L^{-1} glucose. Inoculum cultures were grown for 24 h in a 300 mL Erlenmeyer-flask containing 100 mL of sterile medium at pH 5.0 on a rotary shaker at a temperature of 30 °C and a shaker speed of 180 rpm. The composition of the medium was 16.6 g L^{-1} of glucose, 7.5 g L^{-1} of $(NH_4)_2SO_4$, 3.5 g L^{-1} of KH_2PO_4, 0.75 g L^{-1} $MgSO_4$, 10 mL L^{-1} of trace metal solution and 1 mL L^{-1} of vitamin solution. The trace metal and vitamin solutions were prepared according to Taherzadeh et al. (1996).

2.2.2. Aerobic batch cultivation

Aerobic batch cultivation was performed in a 2.5 L bioreactor (Biostat A, B. Braun Biotech International, Melsungen, Germany) at 30 °C. The medium contained the following components: 20.0 g L^{-1} of glucose, 20.0 g L^{-1} of $(NH_4)_2SO_4$, 10.0 g L^{-1} of KH_2PO_4, 2.0 g L^{-1} of $MgSO_4$, 27.0 mL L^{-1} of trace metal solution and 2.7 of mL L^{-1} vitamin solution. The trace metal and vitamin solutions were prepared according to Taherzadeh et al. (1996). The cultivation was initiated by adding 20.0 mL of inoculum to the batch. Aeration was maintained at 1.0 L min^{-1} and the stirrer speed was kept at 700 rpm. The pH was maintained at 5.0 by automatic addition of 3 M NaOH.

2.2.3. Aerobic fed-batch cultivation

Upon depletion of the ethanol produced in the batch phase, the feeding of pretreatment liquid was commenced. 1.0 L of pretreatment liquid was added during 16 h of cultivation. The feed rate was initially 0.04 L h^{-1} and increased linearly to 0.10 L h^{-1} over 16 h. The aeration was maintained at 1.4 L min^{-1} and the stirrer speed was kept at 800 rpm. DOT stayed above 30%

Table 1
The composition of the pretreated wheat straw

Content in solid fraction (%)			Concentration in the liquid fraction (g L^{-1})				
Glucan	Xylan	Lignin	Glucose[a]	Xylose[a]	Acetic acid	HMF	Furfural
54.5	3.5	34	10.9	51.2	5.0	0.6	1.7

[a] Both monomeric and oligomeric form is included.

throughout the cultivation. The pH was maintained at 5.0 by automatic addition of 3 M NaOH.

2.2.4. Cell harvest

The samples were centrifuged in 700 mL flasks using a HERMLE Z 513K centrifuge (HERMLE Labortechnik, Wehingen, Germany). The pellets were resuspended in 0.9% NaCl solution in order to obtain a cell suspension with a cell mass concentration of about 75 g L^{-1} dry weight. The time from the end of the fed-batch to the addition of the harvested cells to the SSF-fermentations, was no longer than 3 h.

2.3. Simultaneous saccharification and fermentation

All SSF-experiments were carried out in 2.5 L bioreactors (Biostat A, B. Braun Biotech International; Biostat A plus, Sartorius, Melsungen, Germany and BIOFLO III, New Brunswick Scientific, Edison, NJ, USA) with a final working weight of 1.4 kg. Prior to the experiments the empty bioreactors were autoclaved at 121 °C for 20 min.

2.3.1. Batch SSF

The SSF batch experiments with a working weight of 1.4 kg were carried out at temperatures of 30, 34 or 37 °C with a content of 7 or 9% water insoluble solids (WIS), for 96 h. To obtain the desired WIS-content, the pretreatment slurry was diluted with sterile deionised water. The SSF medium was supplemented by: 0.5 g L^{-1} $NH_4H_2PO_4$, 0.025 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 1.0 g L^{-1} yeast extract. The experiments were initiated by adding cell suspension resulting in a concentration of 4 g dry weight L^{-1} in the batch. The enzyme preparation, Xylanase XL, was obtained from SAF-ISIS (Souston, France) and had a cellulase activity of 46.5 FPU g^{-1} and a β-glucosidase activity of 65.6 IU g^{-1}. This was used together with Novozyme 188 (Novozymes A/S, Bagsvaerd, Denmark) with a β-glucosidase activity of 339 IU g^{-1}. The amount of enzyme added corresponded to a cellulase activity of 21.0 FPU g^{-1} glucan, and the amount Novozyme 188 added corresponded to a β-glucosidase activity of 25.5 IU g^{-1} glucan, resulting in a total β-glucosidase activity (SAF-ISIS and Novozyme 188 combined) of 56.5 IU g^{-1} glucan. The pH was maintained at 5.0 throughout the fermentation by automatic addition of 3 M NaOH. Samples for HPLC-analyses were taken at the fermentation times 0, 2, 4, 6, 8, 24, 48, 72 and 96 h.

2.3.2. Fed-batch SSF

The SSF fed-batch experiments were performed with a final working weight of 1.4 kg for 96 h at 34 °C. The initial WIS-content was 4 and 6%, respectively. The slurry was added pulse-wise according to Table 2. After repeated slurry additions the total WIS-content, i.e. added and initial amount, corresponded to a WIS-content of 7 and 9% WIS, respectively. The pH, cell mass concentration, nutrient concentration, and enzyme loadings were the same as in the batch SSF experiments.

2.3.3. Model SSF experiments

Model SSF experiments without fibres were carried out for 96 h with an initial volume of 1.4 L at temperatures of 30, 34 and 37 °C. Either a defined medium (i.e. only sugars and nutrients) or a hydrolysate liquid from wheat straw was used. Both media contained the following nutrients: 8.0 g L^{-1} of $(NH_4)_2SO_4$, 3.8 g L^{-1} of KH_2PO_4, 0.8 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 10.7 mL L^{-1} of trace metal solution and 1.4 mL L^{-1} of vitamin solution. Before commencing the experiment, glucose and xylose were added to obtain the initial concentrations in an SSF with 7% WIS. In the experiments with defined medium, pure xylose and glucose were used to obtain the right initial concentrations, whereas hydrolysate liquid was used in the other case. In order to simulate the release of glucose during hydrolysis in SSF (in a simple fashion), a glucose solution (460 g L^{-1}) was fed at a constant rate during the experiment. The total amount of glucose added corresponded to the amount of glucose in form of glucan in SSF with 7% WIS. No xylose was used in the feed, since most of the xylose is already present in the liquid in monomeric form after the pretreatment (Table 1). The experiments were initiated by adding cell suspension resulting in a concentration of 4 g dry weight L^{-1} in the batch.

2.4. Analysis

2.4.1. Cell mass concentration

The cell mass concentration during the aerobic cell cultivation was measured from duplicate 10 mL samples. The samples were centrifuged ($1000 \times g$) for 5 min at 3000 rpm (Z200 A, HERMLE Labortechnik). The supernatants were discarded, and the pellets were washed with 0.9% NaCl solution and centrifuged a second time. The pellets were dried at 105 °C overnight and subsequently weighed.

2.4.2. HPLC-analysis

Samples of the fermentation liquid were centrifuged ($16{,}000 \times g$) in 2 mL eppendorf tubes at 14,000 rpm for 5 min (Z 160M, HERMLE Labortechnik). The supernatant was filtered using 0.2 μm sterile filters and the filtered samples were stored at −20 °C. Analyses of the most common metabolites and substrates were made using HPLC. The sugar concentrations were determined using a polymer column (Aminex HPX-87P, Bio-Rad Laboratories, München, Germany) at 85 °C. MilliQ-water was used as eluent, with a flow rate of 0.6 mL min^{-1}. Ethanol, glycerol, acetate, HMF and furfural were analysed using an Aminex HPX-87H column (Bio-Rad Laboratories) at 60 °C. The eluent used was 5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1}. The compounds of interest were detected with a refractive index detector (Waters 2410, Waters, Milford, MA, USA).

2.4.3. Yield calculations

The ethanol yields were calculated based on the total amount of fermentable sugars added to the fermentations, i.e. the sum of glucose and xylose (monomers and oligomers) present in the liquid fraction of the added pretreatment slurry, along with the glucose and xylose in the form of glucan and xylan in the fibres. The theoretical weight of glucose and xylose released during the

hydrolysis are (due to the addition of water when the glycosidic bonds are hydrolysed) 1.11 times the weight of glucan and 1.13 times the weight of xylan, respectively.

3. Results

Yeast strains used (both the USM21 and the TMB3400 strain) were cultivated on wheat straw hydrolysate liquid in an aerobic fed-batch to achieve adaptation to the hydrolysate medium, as has previously been shown important (Alkasrawi et al., 2006). The most suitable process temperature was determined, and the improved ethanol yield was quantified from the difference between the xylose utilising TMB3400 and its non-xylose utilising parental strain USM21 in batch SSF. Subsequently, different modes of operation, aiming at a higher xylose conversion, were studied by varying the feed strategy and the WIS-content. The temperature influence on product distribution was finally further investigated by model SSF experiments with both defined medium and hydrolysate liquid from wheat straw.

3.1. Influence of temperature on SSF

Batch SSF experiments were made with TMB3400 at 30, 34 and 37 °C at 7 and 9% WIS, respectively, in order to investigate the temperature influence on process performance. The impact of the temperature was much greater when using 9% WIS than at 7% WIS (Table 2). At 7% WIS there was only a small difference in ethanol yield on added sugars between the examined temperatures, but 37 °C was slightly less favourable than 30 and 34°. However, with a WIS-content of 9% the differences were much larger and 37 °C was clearly unfavourable, giving an ethanol yield of only 0.18 g g^{-1} on total available sugars, i.e. glucan, xylan, glucose and xylose (Row 6, Table 2). Furthermore, more xylose was consumed at 30 °C at both 7 and 9% WIS compared to the other temperatures, but not necessarily resulting in a higher ethanol yield. An ethanol yield of 0.26 g g^{-1} at 30 °C and 0.29 g g^{-1} at 34 °C with a WIS-content of 9% implies that 34 °C is the most suitable temperature for SSF (Rows 2 and 4, Table 2). This temperature was therefore chosen in the following fed-batch experiments.

3.2. Comparison of TMB3400 and USM21

A comparison between the recombinant xylose fermenting strain TMB3400 and its non-xylose fermenting parental strain USM21 was made by performing SSF at a WIS-content of 7%. As expected, in fermentations with USM21 the concentration of xylose decreased only slightly. The converted xylose was found to be quantitatively reduced to xylitol. In contrast, a large fraction of xylose could be fermented to ethanol by TMB3400. An ethanol yield of 0.30 g g^{-1} on total available sugars, i.e. glucan, xylan, glucose and xylose (corresponding to 59% of the theoretical), and 0.38 g g^{-1} (corresponding to 75% of the theoretical) was obtained for USM21 and TMB3400, respectively (Rows 1 and 11, Table 2). Xylose consumed by USM21 was quantitatively converted into xylitol, whereas no more than 16% of the xylose resulted in xylitol formation by TMB3400.

3.3. Influence of WIS-content and feed strategy – batch vs. fed-batch

To investigate the effect of substrate feed during SSF, batch and fed-batch SSF experiments were compared at two different WIS-contents: 7 and 9%. A final ethanol concentration around 35 g L^{-1}, corresponding to an ethanol yield of 0.40 g g^{-1} on total available sugars (glucose, glucan, xylose and xylan) was obtained in fed-batch experiment in which the initial WIS-content of 4% was increased to a total WIS-content of 7% during the first 12 h (Row 7, Table 2). This corresponds to nearly 80% of the theoretical yield, and as much as 74% of all xylose avail-

Table 2
Summary of SSF experiments

Strain	Temperature (°C)	WIS (%)	Glucose (g L^{-1})	Xylose (g L^{-1})	Glycerol (g L^{-1})	Xylitol (g L^{-1})	Ethanol (g L^{-1})	Ethanol yield[a] (g g^{-1})	Ethanol yield[b] (%)
TMB3400	30	7	0.9	8.6	6.7	2.6	32.9	0.38	75
TMB3400	30	9	2.7	23.7	5.9	1.3	32.7	0.26	51
TMB3400	34	7	0.7	16.9	5.0	2.5	32.9	0.38	75
TMB3400	34	9	8.1	33.3	4.2	1.7	33.2	0.29	59
TMB3400	37	7	1.1	15.7	4.6	4.1	30.5	0.36	71
TMB3400	37	9	28.7	34.6	1.0	1.3	20.8	0.18	35
TMB3400	34	4–7[c]	1.5	8.5	5.5	4.3	34.7	0.40	78
TMB3400	34	4–7[d]	0.2	5.7	4.8	4.1	33.1	0.40	78
TMB3400	34	6–9[e]	3.7	25.9	4.5	1.6	38.1	0.36	71
TMB3400	34	6–9[f]	2.2	22.7	4.5	1.9	37.3	0.35	69
USM21	34	7	0.5	22.41	1.3	4.7	26.0	0.30	59

All concentrations are for 96h of SSF.

[a] Based on the total amount of glucose and xylose in the (pretreated) raw material.

[b] Yield of theoretical, based on the total amount of glucose and xylose in the (pretreated) raw material.

[c] Fed-batch SSF with 4% WIS initially, 4 additions during 12 h to a final WIS-content of 7%.

[d] Fed-batch SSF with 4% WIS initially, 8 additions during 24 h to a final WIS-content of 7%.

[e] Fed-batch SSF with 6% WIS initially, 4 additions during 12 h to a final WIS-content of 9%.

[f] Fed-batch SSF with 6% WIS initially, 8 additions during 24 h to a final WIS-content of 9%.

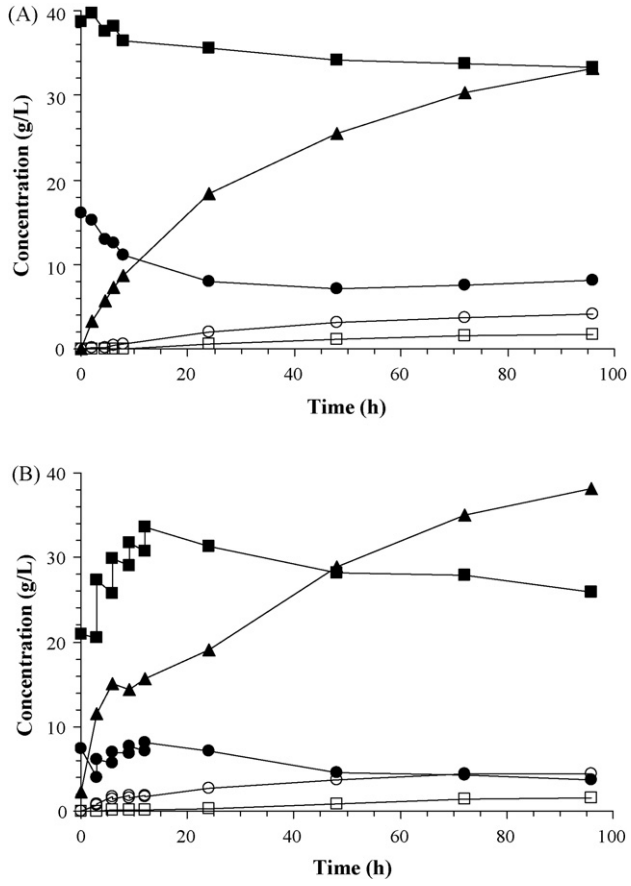

Fig. 1. Measured concentrations during SSF of wheat straw at 34 °C, showing glucose (●), xylose (■), ethanol (▲), glycerol (◯) and xylitol (□) in batch SSF with 9% WIS (A) and fed-batch SSF with 6–9% WIS (B).

able was consumed. The reference experiment, i.e. the batch SSF with 7% WIS, gave a final ethanol concentration of 33 g L^{-1} and an ethanol yield of 0.38 g g^{-1} (Row 1, Table 2). However, when increasing the WIS-content, the difference in fermentation performance between batch and fed-batch increased sharply, most likely due to higher amounts of inhibitors present in the slurry. SSF with 9% WIS in batch mode (Fig. 1A) only resulted in an ethanol yield of 0.29 g g^{-1} on total available sugars, implying inhibition of the yeast. When changing to fed-batch mode (Fig. 1B) a significant improvement was seen, and a final ethanol concentration of 38 g L^{-1} and an ethanol yield of 0.36 g g^{-1} was obtained (Fig. 2). Furthermore, the relative improvement in xylose uptake was larger at 9% WIS compared with 7% WIS. Twice as much xylose was consumed in fed-batch compared with batch at 9% WIS (Fig. 3). One can therefore conclude that the main reason behind the higher overall ethanol yields in fed-batch is that a much larger fraction of xylose is converted. The final xylitol concentration increased when changing from batch to fed-batch at 7% WIS. However, at 9% WIS, the xylitol concentration did not increase as much when changing from batch to fed-batch. This finding correlates well with the larger increase in ethanol production observed for fed-batch at 9% WIS, compared to the slight increase at 7% WIS. The glycerol production, in contrast, did not seem to be much affected when switching from batch to fed-batch mode (Table 2).

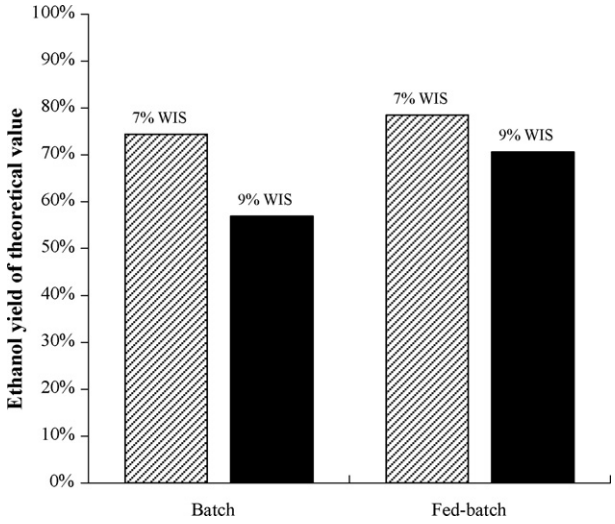

Fig. 2. Comparison of ethanol yield in batch and fed-batch SSF of wheat straw at different WIS-contents using TMB3400. At 9% WIS a 20% difference in yield can be seen between batch and fed-batch, in comparison with only a 4% difference at 7% WIS.

Two different feed strategies, i.e. addition of the fibre slurry during either 12 or 24 h, were also tested to investigate if the addition rate of slurry had any influence on the SSF. No significant differences in final yields were found (Rows 7–10, Table 2).

3.4. Model SSF experiments

Experiments without added fibres aiming at qualitatively modelling SSF conditions were carried out at three different temperatures: 30, 34 and 37 °C, respectively, to further investigate the influence of the temperature on by-product distribution. This was done using either defined medium (i.e. only sugars and nutrients), or hydrolysate liquid from the pretreatment of wheat straw. One principal advantage in the model SSF experiments is that the change in cell mass can be followed, which is not possible during SSF of the straw material due to the difficulty

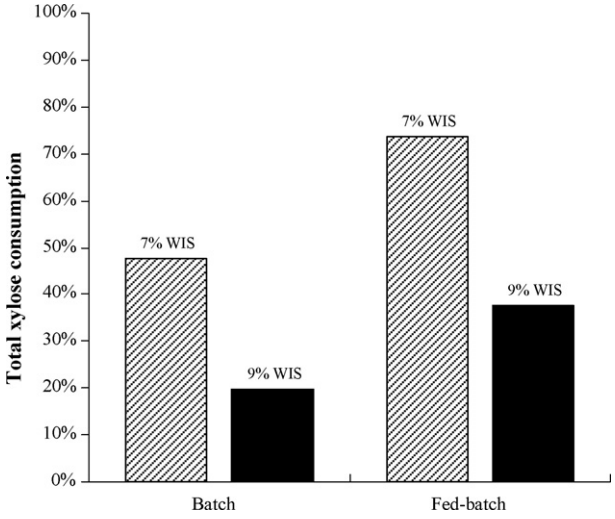

Fig. 3. Comparison of total xylose consumption in batch and fed-batch SSF of wheat straw at two different WIS-contents using TMB3400.

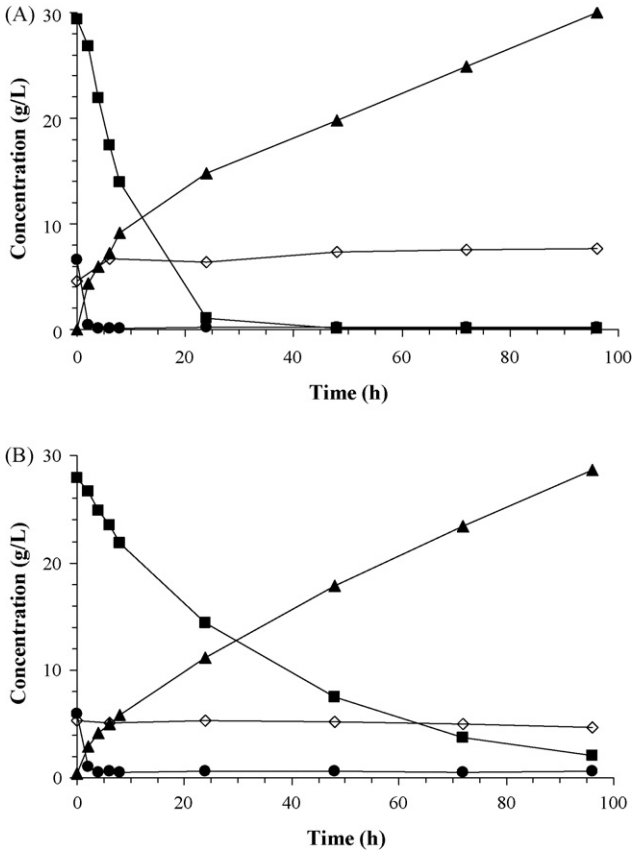

Fig. 4. Measured concentrations during model SSF at 34 °C with defined medium (A) and hydrolysate liquid (B), showing glucose (●), xylose (■), ethanol (▲) and cell mass (◊).

in separating cell mass and other solids. When defined medium was used, amounts of glucose and xylose equivalent to the initial concentrations in a SSF with 7% WIS were added before commencing the SSF. In both cases a constant feed of glucose was used.

In the model SSF experiment using defined medium, all xylose was consumed in less than 24 h (Fig. 4A) and all three temperatures gave similar ethanol yields, but with a slightly higher yield at 34 °C (Row 2, Table 3). In experiments with hydrolysate liquid, the ethanol yields were somewhat lower, but again very similar for the three different temperatures. The consumption rate of xylose was clearly reduced in hydrolysate liquid compared to defined medium. However, almost all xylose was consumed in all three cases after 96 h (Fig. 4B). In all cases, independent of medium and temperature, the glucose levels decreased to almost zero very rapidly, and remained very low throughout the experiment. The final cell mass was strongly affected by temperature when a defined medium was used. At 30 °C the cell mass more than doubled, increasing with 4.4 g L^{-1} dry weight over 96 h, while the cell mass only increased by 1 g L^{-1} at 37 °C. In contrast, the cell mass slightly decreased during the model SSF with hydrolysate liquid for all temperatures.

A clear increase of the xylose consumption rate with temperature was found for the case of defined medium, but not for hydrolysate liquid (Table 4). During the first 8 h, a rapid consumption of xylose was observed, reaching as high as 0.39 g xylose g $cells^{-1}$ h^{-1} at 37 °C for defined medium. The consumption rate differed significantly between the three temperatures, with a difference in the xylose consumption rate of 25% between 30 and 37 °C. Furthermore, the xylose consumption was around twice as fast with defined medium in comparison to hydrolysate liquid. The xylitol yield on xylose in defined medium was around three times the yields obtained in hydrolysate liquid (Table 4) and also the glycerol production rate was higher in defined medium in comparison to hydrolysate liquid.

4. Discussion

The current work has shown that ethanol yields up to 80% of the theoretical, based on total available sugars from wheat straw, can be obtained by co-fermentation of glucose and xylose in SSF with TMB3400 at a WIS-content of 7%. At a higher and thus more desirable WIS-content of 9%, up to 71% of the theoretical ethanol yield could be obtained. This can be seen in relation to yields of 64% with 5% WIS and 59% with 11% WIS obtained with SSF of corn stover (Öhgren et al., 2006), as well as yields of 69% with 5% WIS and 59% with 7.5% WIS obtained with SSF of sugar cane bagasse (Rudolf et al., 2008). Evidently, significantly higher yields were achieved with SSF of wheat straw in comparison to previous studies using TMB3400.

Table 3
Summary of model SSF experiments with TMB3400

Medium	Temperature (°C)	Glucose (g L^{-1})	Xylose (g L^{-1})	Glycerol (g L^{-1})	Xylitol (g L^{-1})	Ethanol (g L^{-1})	Change in cell mass[a] (g dry weight L^{-1})	Ethanol yield[b] (g g^{-1})	Ethanol yield[c] (%)
Defined	30	0.2	0.1	6.2	6.7	28.7	4.4	0.42	82
Defined	34	0.2	0.1	7.6	6.7	30.0	3.0	0.43	84
Defined	37	0.2	0.1	8.7	5.2	28.5	1.0	0.39	76
Hydrolysate liquid	30	0.4	1.6	7.4	2.6	27.2	−0.5	0.37	73
Hydrolysate liquid	34	0.6	2.1	7.9	3.5	28.6	−0.6	0.36	71
Hydrolysate liquid	37	0.2	3.0	6.6	5.8	28.1	−0.3	0.36	71

The total amounts of sugars in the experiments corresponded to a WIS-content of 7%. A constant feed rate of glucose was used to simulate the sugar release in SSF. All concentrations are for 96h of SSF.

[a] Change = end concentration − start concentration.

[b] Based on the total amount of glucose and xylose in the (pretreated) raw material.

[c] Yield of theoretical, based on the total amount of glucose and xylose in the (pretreated) raw material.

Table 4
Model SSF experiments with TMB3400 showing parameters for the first 8 h

Medium	Temperature (°C)	Xylose consumption rate (g xylose g cells^{-1} h^{-1})	$Y_{(xylitol/xylose)}$ (g g^{-1})	Glycerol production rate (g glycerol g cells^{-1} h^{-1})
Defined	30	0.30	0.32	0.05
Defined	34	0.33	0.34	0.06
Defined	37	0.39	0.29	0.07
Hydrolysate liquid	30	0.14	0.11	0.03
Hydrolysate liquid	34	0.15	0.09	0.03
Hydrolysate liquid	37	0.15	0.13	0.03

The main factor behind the different yields was found to be a different extent of xylose conversion. In all SSF experiments at 34 °C, even those performed in batch mode, xylose was taken up already from the start. This was most likely possible due to the favourable ratios between xylose and glucose concentrations in the SSF. The initial xylose to glucose ratio at a WIS-content of 9% was for example >2 (Fig. 1A). Improved xylose conversion, and higher ethanol yields, was obtained when using a gradual addition of the slurry. This can be understood by an even more favourable xylose to glucose ratio. In the example shown in Fig. 1B, the obtained ratio stayed above 4 during the entire fed-batch. This favours xylose uptake, since high glucose concentrations may prevent xylose uptake due to competition for transporters as discussed earlier (Kilian and Uden, 1988; Kötter and Ciriacy, 1993; Meinander and Hahn-Hägerdal, 1997).

S. cerevisiae strains harbouring the eukaryotic xylose utilisation pathway (i.e. xylose reductase, XR, and xylitol dehydrogenase, XDH) generally form xylitol as a by-product during anaerobic conditions, hampering the ethanol yields. While XR from, e.g. *Candida utilis* can utilise only NADPH (Bruinenberg et al., 1983), the XR from *Pichia stipitis*, which is expressed in TMB3400, can use either NADH or NADPH as a cofactor (Verduyn et al., 1985) (Fig. 5). The dual cofactor dependence of XR on NADH and NADPH may prevent a complete regeneration of NAD^+ which is needed for the XDH reaction (Kötter and Ciriacy, 1993; Jeffries and Jin, 2004), and hence xylitol is secreted into the medium. Xylitol may also be formed due to the action of unspecific reductases, like *GRE3* (Träff et al., 2001). Interestingly, more glycerol but less xylitol was produced with TMB3400 than with USM21 (Table 2). This was previously also observed when performing SSF on corn stover (Öhgren et al., 2006). XR has been shown to reduce dihydroxyacetone phosphate to glycerol (Jeppsson et al., 2003) and in this reaction NAD^+ is generated. This might explain the higher glycerol and lower xylitol concentrations obtained by TMB3400.

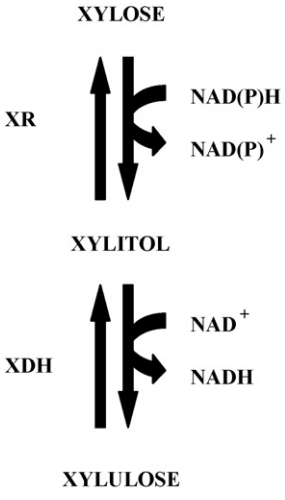

Fig. 5. Enzymes (xylose reductase, XR and xylitol dehydrogenase, XDH) and co-factors used by TMB3400 for xylulose formation from xylose.

SSF is often run at a temperature of 37 °C, as a compromise between the optimal temperatures for the yeast and the cellulolytic enzymes: about 30 and 55 °C, respectively. However, it has lately been suspected that this is not necessarily a good choice of temperature when it comes to co-fermentation of glucose and xylose. Rudolf et al. (2008) concluded that more xylose was consumed by TMB3400 at 32 °C in comparison with 37 °C during SSF of sugar cane bagasse. A temperature of 35 °C was also used by Öhgren et al. (2006) in SSF of corn stover. One reason for this may be that the rate of hydrolysis – obviously affected by temperature – is lowered and a lower rate of hydrolysis gives a slower release of glucose. As discussed above, this may favour xylose in the competition for transporters. However, also inhibition effects may play a role, and the yeast tolerance to inhibitors at temperatures closer to the optimal for growth, may be higher.

The complex issue of by-product formation during SSF becomes even more complex when it comes to co-utilisation of different substrates. The temperature appears to have a clear impact on the product formation pathways. The results from the model SSF experiments with defined medium showed that glycerol production is dependent on temperature, as reported previously (Carvalheiro et al., 1999). Yeast cells need glycerol for heat-shock proteins to, e.g. protect and repair cellular structures, and it has been shown that the expression levels of both *GPD1* and *GPD2* are affected by temperature (Siderius et al., 2000) and that heat shock prolongs the intensive period of *GPD1* transcription (Kajiwara et al., 2000). In the experiments with defined medium more glycerol was formed at higher temperature, along with a lower final xylitol concentration (Table 3). The xylitol concentration reached the highest value after 24 h, after which the concentration decreased again. The peak value coincided with the time of xylose depletion (Fig. 6A). This suggests that when xylose is depleted, the glucose feed is insufficient to provide ATP for maintenance and glycerol production purposes. It is possible that NAD^+ generated in the reduction of DHAP to

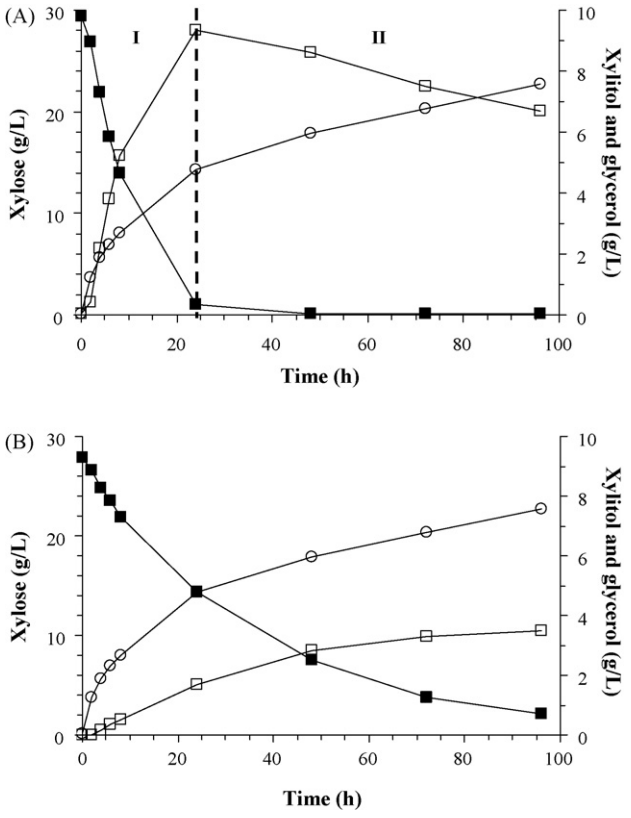

Fig. 6. Measured concentrations during model SSF at 34 °C with defined medium (A) and hydrolysate liquid (B), showing xylose (■), xylitol (□) and glycerol (◯). With defined medium (A), the xylitol concentration is increasing (phase I) until all xylose is consumed, then it starts decreasing (phase II). In model SSF with hydrolysate liquid (B) no xylitol consumption is observed.

G-3-P is used by the XDH catalysed oxidation of xylitol to xylulose. Then xylulose may, via fructose-6-P or glyceraldehyd-3-P, form glycerol with the concomitant net formation of 2/3 ATP. In contrast, when using hydrolysate liquid the xylose was not fully depleted (Fig. 6B). Hence, there is no need for uptake of xylitol since new xylitol is formed continuously from xylose (Fig. 5). Furthermore, when using hydrolysate as the medium, the difference in glycerol production between the temperatures was smaller. Here the highest temperature no longer gave the highest amount of glycerol, but the lowest (Table 3).

The reason for large differences in xylose consumption rate between the two different media, being twice as fast for defined medium in comparison to hydrolysate liquid, may be due to a more inhibiting environment in the hydrolysate liquid. In defined medium there was a clear difference in xylose consumption rate between the three temperatures and also a slight difference in xylitol yield during the first 8 h (Table 4). A possible explanation may be that XR and XDH have different optimal temperatures: the optimal activity has been found at 60 °C for XR (Rizzi et al., 1988) and at 35 °C for XDH (Rizzi et al., 1989), which means that XDH is operating in a more suitable temperature than XR. Hence, the equilibrium is pushed to xylulose (Fig. 5) by increased temperature and consequently the xylose uptake rate is increased. The reason why the xylitol yield is slightly lower at 37 °C, in comparison to 30 and 34 °C, may be an increased glycerol production, which in turn could be affected by an increased XR activity (since XR reduces dihydroxyacetone phosphate to glycerol) when the temperature is increased. Interestingly, the temperature effects observed with defined medium is less pronounced with hydrolysate liquid. Possibly other factors such as inhibition and the presence of additional electron acceptors cancel out some of the temperature effects. Furthermore, the xylose consumption rate in hydrolysate liquid was only around half of the xylose consumption rate achieved in defined medium. Nevertheless, the xylitol yield on xylose in defined medium was around three times higher than that obtained in hydrolysate liquid. Also the glycerol production rate was higher in defined medium in comparison to hydrolysate liquid for all temperatures (Table 4). Further work is needed to establish whether this is due to additional electron acceptors in the medium, or due to other effects.

In conclusion, we here demonstrate that it is possible to produce ethanol from wheat straw by SSF with relatively high WIS-content and at the same time achieve high final ethanol concentration (38 g L^{-1}) and a reasonable yield (80% of the theoretical) due to co-fermentation of glucose and xylose. However, the SSF process needs to be carefully designed in terms of temperature and feed profile to reach this yield. There is no doubt that fed-batch is the most feasible method when using high WIS-content (9%) in the SSF. Thereby the inhibitors can be kept at a lower level, along with a lower glucose concentration, to facilitate xylose consumption. In addition, it has been shown that the choice of temperature is important when designing SSF, not least for obtaining a good co-fermentation of glucose and xylose.

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