J Ind Microbiol Biotechnol (2010) 37:1211–1220
DOI 10.1007/s10295-010-0768-8

ORIGINAL PAPER

Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes

E. Tomás-Pejó · M. Ballesteros · J. M. Oliva · L. Olsson

Received: 5 April 2010 / Accepted: 14 June 2010 / Published online: 29 June 2010

Abstract An efficient fermenting microorganism for bioethanol production from lignocellulose is highly tolerant to the inhibitors released during pretreatment and is able to ferment efficiently both glucose and xylose. In this study, directed evolution was employed to improve the xylose fermenting *Saccharomyces cerevisiae* F12 strain for bioethanol production at high substrate loading. Adapted and parental strains were compared with respect to xylose consumption and ethanol production. Adaptation led to an evolved strain more tolerant to the toxic compounds present in the medium. When using concentrated prehydrolysate from steam-pretreated wheat straw with high inhibitor concentration, an improvement of 65 and 20% in xylose consumption and final ethanol concentration, respectively, were achieved using the adapted strain. To address the need of high substrate loadings, fed-batch SSF experiments were performed and an ethanol concentration as high as 27.4 g/l (61% of the theoretical) was obtained with 11.25% (w/w) of water insoluble solids (WIS).

Keywords Bioethanol · Xylose · Evolutionary engineering · Fed-batch · *S. cerevisiae*

E. Tomás-Pejó (✉) · M. Ballesteros · J. M. Oliva
CIEMAT, Biomass Unit, Av Complutense 22,
28040 Madrid, Spain
e-mail: mariaelia.tomas@ciemat.es

E. Tomás-Pejó · L. Olsson
Department of Chemical and Biological Engineering,
Industrial Biotechnology, Chalmers University of Technology,
41296 Göteborg, Sweden

Introduction

The depletion of the world petroleum supply as well as increased levels of greenhouse gases have recently stimulated extensive interest in optimizing fermentation processes for large-scale production of alternative biofuels such as bioethanol [1]. In this context, bioethanol produced from lignocellulose, the so-called second-generation bioethanol, is an interesting alternative. Lignocellulosic biomass such as agricultural residues, forest products or energy crops do not compete with food crops, and in addition, these side-streams from food production are also less expensive than conventional agricultural feedstocks [2].

Owing to the structural characteristics of the lignocellulosic materials, pretreatment is a crucial step for obtaining potentially fermentable sugars in the hydrolysis step. Steam explosion is one of the more widely exploited pretreatments. It offers several attractive features when compared to other pretreatment technologies, including the potential for significantly lower environmental impact, lower capital investment, larger potential for energy efficiency, less hazardous chemicals and conditions and complete sugar recovery [3]. During steam explosion pretreatment, fiber structure is altered and enzyme accessibility to cellulose is enhanced, which is reflected in an improvement in hydrolysis yields [4, 5]. Harsh conditions during pretreatment lead to a partial hemicellulose decomposition and the generation of toxic compounds derived from sugar degradation that could affect the proceeding hydrolysis and fermentation steps [6, 7]. Besides effecting cell growth, these inhibitors can also reduce sugar conversion rates during fermentation and consequently decrease the fermentation rate [8, 9].

Typically found inhibitors are furan derivates, weak acids, and phenolic compounds. Main furans are furfural and 5-hydroxymethyl furfural (HMF) derived from pentoses and hexoses degradation, respectively, which cause a prolonged lag phase during batch fermentation [7]. Acetic and formic acid are two of the most common weak acids found in lignocellulosic hydrolysates. Acetic acid is released due to the solubilization of acetyl groups present in hemicellulose and the formic acid derives from furfural and HMF degradation [10]. It is generally accepted that both acetic and formic acid influence yeast fermentation by reducing biomass formation [11], however, small amounts of acetic acid could increase glucose consumption rates and ethanol yields [12, 13]. Coumaric and ferulic acid are phenolic compounds characteristic of herbaceous biomass where they are esterified acting as a bond between lignin and hemicellulose [14, 15]. Its inhibition mechanism is not completely understood, but they may act on biological membranes, causing loss of integrity [16]. Vainillin and syringaldehyde are also aldehydes compounds derived from lignin degradation typically found in wheat straw hydrolysates.

The yeast *Saccharomyces cerevisiae* has been shown to be highly tolerant towards the discussed toxic compounds and the end product ethanol [17]. It is therefore one of the preferred microorganisms for ethanol production from lignocellulose. However, wild-type *S. cerevisiae* has the drawback of not being able to ferment pentoses, and one of the challenges of fermenting lignocellulosic hydrolysates is that an efficient ethanol producer that can utilize both hexoses and pentoses needs to be employed. One solution is to use recombinant *S. cerevisiae* modified with genes *XYL1* and *XYL2* from *Pichis stipitis* encoding for xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively [18–20]. It is known that XR and XDH have different co-factors preferences and xylitol is formed in order to maintain a redox balance during xylose fermentation [21]. The first enzyme involved in reduction of xylose to xylitol, XR, can use both NADPH and NADH as a co-factor. The second enzyme, XDH, uses NAD^+ almost exclusively in the oxidation of xylitol to D-xylulose. As mainly NADPH is utilized in the first reaction, NAD^+ is therefore not made available for the XDH reaction and consequently this leads to substantial xylitol production and therefore suboptimal ethanol yield on xylose [22].

Yeasts strains to be used for second-generation bioethanol production have to cope with challenging conditions that are inherent to the industrial process such as high concentration of inhibitory products, osmotic problems due to high sugar concentrations, high process temperatures, ethanol inhibition, and growth conditions that are not well controlled [23]. Tolerance of these multiple stresses is likely to be a complex phenotype involving several cellular mechanisms and it will be difficult to find the targets to perform efficient metabolic engineering. Several approaches such as, evolutionary engineering or adaptative strategies are appearing as a promising alternatives to develop more tolerant yeasts [24]. Directed adaptation of yeast to inhibitory prehydrolysate is one interesting strategy to enhance the process efficiency and it results in higher xylose consumption rates and fermentation yields improving fermentation performance [25].

Simultaneous saccharification and fermentation (SSF) process has been used since the early 1990s, being one of the most successful methods for ethanol production [26]. By performing SSF experiments, the ethanol yield is increased by minimizing end-product inhibition from glucose on the enzymatic action. Furthermore, the employment of two separate reactors for saccharification and fermentation is avoided, thereby minimizing capital costs. However, there are several drawbacks when performing conventional SSF processes from lignocellulose, one of them being that high substrate loading is limited by the high viscosity [27]. In this context, fed-batch SSF, in which substrate is fed continuously by scheduled additions has been shown as a suitable configuration as the substrate is gradually degraded and the viscosity is kept low [28]. This strategy results in higher substrate loading, which is necessary for reaching higher ethanol concentration.

Furthermore, when employing xylose fermenting yeast, xylose transport into the cell is slow due to the competition between glucose and xylose for the same transporter [29]. Fed-batch strategies can reduce glucose accumulation in the media allowing more efficient co-fermentation of xylose and glucose [8].

The aim of the present work was to adapt a genetically engineered xylose fermenting yeast to improve its ability to ferment toxic wheat straw hydrolysates. Adapted and parental strains were compared in terms of ethanol production, by-products formation and sugar consumption. Furthermore, different fed-batch SSF processes were performed in order to reach high substrate loading for improving the ethanol concentration produced from steam pretreated wheat straw.

Materials and methods

Raw material and pretreatment

Wheat straw had the following composition (% dry weight): cellulose, 40.7; hemicellulose, 27.6 (xylan, 23.7; galactan, 2.6; and arabinan, 1.3); lignin, 17.0; and ash 4.7 [30]. It was milled using a laboratory hammer mill to a particle size smaller than 5 mm and stored at room temperature until it was used.

Two wheat straw batches were pretreated at 210 and 200°C for 5 min without acid impregnation in a steam explosion unit equipped with a reactor vessel of 2-l working volume.

After pretreatment, the material was recovered in a cyclone and the wet material was cooled down to 40°C. One portion of both recovered slurries was vacuum filtered, resulting in two fractions: a liquid fraction, referred to as *prehydrolysate*, and a solid fraction, referred to as the *water insoluble solids (WIS) fraction*. The prehydrolysate obtained at 210°C and 5 min was used for adaptative purposes and in fermentation tests. The prehydrolysate obtained at 200°C and 5 min was only employed in fermentation experiments.

Microorganism and media

S. cerevisiae F12 [*S. cerevisiae* F *his3*::YIploxZEOXR/XDH/XK] was used in all experiments as fermenting microorganism. It is a recombinant strain engineered to harbor xylose-consuming traits. The strain has been modified by over-expressing the endogenous gene encoding xylulokinase (XK) and by introduction of the genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis* [21].

The preinoculum for all experiments was grown in 2-l Erlenmeyer flasks containing 500 ml of Delft medium as follows: 20 g/l glucose, 20 g/l xylose, 7.5 g/l $(NH_4)_2SO_4$, 14 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 2 ml/l trace metal solution and 1 ml/l vitamin solution [31]. The preculture was grown for 24 h at 150 rpm and 30°C. The preculture was harvested by centrifugation at 6,500 rpm for 5 min and 4°C. The supernatant was discarded and the pellet was washed with sterile 0.9% NaCl and then centrifuged again. The supernatant was removed and the cells were weighed and diluted with sterile water to obtain the desired inoculum size of 1 g/l cells (dry weight).

Analytical methods

The raw material and WIS fraction were analyzed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, LAP-004, LAP-017, and LAP-019) [32]. Dry weight of WIS and slurry was determined by drying samples at 105°C for 24 h (LAP-001).

Most of the sugars present in the prehydrolysates were in oligomeric form, due to that a mild acid post-hydrolysis (4% (v/v) H_2SO_4, 120°C and 30 min) was performed for obtaining monomeric sugars for analysis.

Ethanol, sugars, xylitol, and glycerol were analyzed by high-performance liquid chromatography (HPLC) in Waters Ultimate 3000 chromatograph with a refractive index detector. An Aminex HPX-87H Bio-Rad-Ion exclusion column operated at 60°C with 5 mM H_2SO_4 as mobile phase (0.6 ml/min) was used for separation.

HMF, furfural, vanillin, syringaldehyde, coumaric and ferulic acid were analyzed by HPLC (Agilent, Waldbronn, Germany) employing an Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) at 65°C. The mobile phase contained 89% 5 mM H_2SO_4 and 11% acetonitrile at flow rate of 0.7 ml/min. A 1050A Photodiode-Array detector (Agilent, Walsbronn, Germany) was employed for detection.

Formic and acetic acid were quantified with a modular HPLC (Waters, Milfors, MA) using a 410 Water refractive index detector. An Aminex HPX-87H (Bio-Rad Labs, Hercules, CA) column maintained at 65°C and a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min were employed.

Adaptation performance

Adaptation was performed by sequential transfer of cultures to diluted prehydrolysate medium with increasing concentration of inhibitors. Prehydrolysate for adaptation was obtained after filtration of the slurry pretreated at 210°C and 5 min. It was first subjected to a mild acid post-hydrolysis (4% (v/v) H_2SO_4, 120°C and 30 min) in order to obtain monomeric sugars. The prehydrolysate corresponded to a slurry with 15.2% (w/w) of WIS and it was diluted to prehydrolysates corresponding to 5, 6, 7, 8, and 9% (w/w) of WIS and supplemented with 5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 1 ml/l trace metal solution and 1 ml/l vitamin solution. Sugar concentration was fixed in all cases at 15.6 g/l xylose and 3.6 g/l glucose according to the sugar composition in the prehydrolysate obtained after pretreatment at 210°C and 5 min.

The adaptation process was performed in a 250-ml shake flask with 100 ml of medium. The initial shake flask cultivation was performed with the most diluted prehydrolysate (equivalent to 5% (w/w) of WIS) and it was inoculated with an inoculum corresponding to 1 g/l (dw) cells. Each culture was grown at pH 5.0 for 48 h at 32°C in a rotary shaker. Culture aliquot of 0.5 ml from one shake flask was transferred to the following shake flask. Cells were grown twice at the same prehydrolysate dilution before transferring the cells to more concentrated prehydrolysate resulting in about 50 generations of growth at each prehydrolysate dilution and a total of 250 generations during the total adaptation procedure.

When reaching the most concentrated prehydrolysate, isolation of the cells was performed by withdrawing 100 µl of culture and transferring the volume to agar plates. The agar plates contained prehydrolysate equivalent to 9%

(w/w) of WIS, 10 g/l yeast extract, 20 g/l mycopeptone, 20 g/l agar and sugar concentrations were fixed at 15.6 g/l of xylose and 3.6 g/l of glucose corresponding to the same sugar concentration as in the non-diluted prehydrolysate.

Prehydrolysates fermentability

Different prehydrolysate fermentation tests were performed in 100-ml shake flasks containing 50 ml of fermentation medium. Fermentation tests were performed in duplicate, which showed very good reproducibility (±5%). Two different prehydrolysates from steam exploded wheat straw pretreated at 200 and 210°C for 5 min, with different concentration of inhibitors resulting after pretreatment, were used for comparison of the evolved and parental strains. Both prehydrolysates were diluted with deionized water to give final concentrations, based on % (w/w) WIS content present in the pretreated slurry, of 4, 6, and 8%. Sugar and salts composition was as follows (independently of prehydrolysate dilution): 40 g/l xylose, 10 g/l glucose, 5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 1 ml/l trace metal solution and 1 ml/l vitamin solution. Prehydrolysates containing glucose and xylose were sterilized separately from the salt solution. All experiments were performed for 140 h in a rotatory shaker at 150 rpm and 32°C. pH was adjusted at 5.0 with 5 M KOH. Initial cell mass concentration level was 1 g/l (dw) cells.

Fed-batch simultaneous saccharification and fermentation process

Whole slurries produced at 210 and 200°C for 5 min were used as initial substrate loading in fed-batch SSF experiments. Slurry obtained at 200°C for 5 min was used in fed-batch SSF experiments with the parental strain, since the parental strain was completely inhibited by the slurry obtained at 210°C. Evolved strain was used in fed-batch SSF experiments with slurry pretreated at 210°C for 5 min. Slurries were supplemented with 5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 1 ml/l trace metal solution and 1 ml/l vitamin solution. The experiments were performed in a 2-l fermentor with a final working weight of 1.2 kg. All fed-batch SSF experiments were carried out at 32°C and 150 rpm for 140 h. The initial slurry loading was 6% (w/w) of WIS. The washed WIS was added at 12, 24, 36, or 48 h according to the different feeding profile with the aim of reaching a substrate loading as high as 11.25% (w/w) of WIS content. Owing to the addition of wet material, the medium volume changed along the process. The pH was maintained at 5.0 by automatic addition of 5 M KOH. The initial cell mass concentration was 1 g/l (dw) cells in the fermenter.

The enzyme mixture was obtained from Novozymes A/S (Bagsværd, Denmark) and consisted of NS50013 (60 FPU/ml) supplemented with β-glucosidase NS50010 (900 IU/ml). The enzyme loading was 15 FPU/g cellulose and 15 IU/g cellulose, respectively. In order to maintain a constant enzyme to substrate ratio, extra enzyme was added together with extra substrate.

All fed-batch SSF experiments were preceded by an enzymatic prehydrolysis step for 3 h at 50°C in the same 2-l fermentor.

Results and discussion

A comparison of the evolved and parental strain was performed in terms of sugar consumption rates and final ethanol concentration using two different prehydrolysates from steam exploded wheat straw during batch fermentations. Slurries obtained at different pretreatment conditions were also used in fed-batch SSF experiments for reaching substrate loading as high as 11.25% (w/w) of WIS.

Steam explosion pretreatment

Two slurries resulting after pretreatment at 200 and 210°C, respectively, were filtered in order to obtain prehydrolysates and WIS fractions. WIS was washed thoroughly with tap water in order to wash out embedded inhibitory compounds. The WIS content in the slurry after pretreatment was 16.6% (w/w) at 200°C and 15.2% (w/w) at 210°C.

The effect of temperature and time on hydrolysis and formation of degradation products has been described as the severity factor R_o being higher when increasing the temperature [33, 34]. A rise in temperature from 200 to 210°C means an increase in R_o from 3.63 to 3.94. Previous studies have concluded that optimum R_o for pretreating wheat straw was between 3 and 4.5 [35]. As depicted in Table 1, higher R_o factor was reflected in higher inhibitor concentration in prehydrolysate obtained at 210°C and 5 min compared to the one obtained at 200°C. Although the higher temperatures resulted in increased removal of hemicelluloses from the WIS fraction and in improved saccharification of cellulose, it also promotes higher sugar degradation [36, 37], thus, lower sugar concentration was obtained in the prehydrolysate pretreated at harsher conditions (Table 1).

Acetic acid was the predominant inhibitory compound in both prehydrolysates. It was released owing to the solubilization of the acetyl groups present in the hemicellulose. Formic acid was formed due to the degradation of furfural and HMF [10], which resulted from pentose and hexose degradation, respectively [7]. Besides these inhibitors, some phenolic compounds characteristic of

Table 1 Composition of the pretreated wheat straw at two different conditions

	200°C–5 min	210°C–5 min
WIS		
Component (% dry weight, w/v)		
Glucose	62.0	67.0
Xylose	12.0	4.0
Lignin	29.9	29.2
Ashes	1.2	3.2
Prehydrolysate		
Monosaccharides (g/l)		
Glucose	5.0	3.6
Xylose	33.0	15.6
Galactose	1.1	1.1
Arabinose	0.6	0.6
Inhibitors (g/l)		
Furfural	1.4	1.6
HMF	0.3	0.4
Acetic acid	5.2	6.8
Formic acid	2.1	2.8
Coumaric acid	0.04	0.05
Ferulic acid	0.05	0.07
Vanillin	0.1	0.1
Syringaldehyde	0.03	0.03

herbaceous biomass were also detected. Ferulic and coumaric acid were derived from cinnamic acid and are acting as linkages between lignin and hemicellulose [38]. Vanillin is derived from guaiacyl propane units and syringaldehyde is produced from syringylpropane units present in lignin [39].

Considering the WIS compositions (Table 1), the glucan proportion after pretreatment was higher in the pretreated material compared to the raw material (44.8%) due to the solubilization of hemicellulosic sugars during pretreatment leaving lignin and cellulose mainly in the solid fraction.

Prehydrolysates fermentation

Two prehydrolysates from steam-exploded wheat straw with different concentration of inhibitory compounds (Table 1) were used for comparing the parental and evolved strain in terms of sugar consumption rates, final ethanol concentration, and production rates. No differences were observed regarding glucose consumption rate when using more diluted prehydrolysates (corresponding to slurries with 4 and 6% (w/w) of WIS) and the glucose was completely consumed during the first 12 h of fermentation (Fig. 1). With less toxic prehydrolysate (200°C and 5 min) diluted to 8% (w/w) of WIS, the parental strain consumed glucose completely after 40 h, whereas the adapted strain exhausted glucose after 12 h (Fig. 1a). Nevertheless, glucose consumption was slower when using more concentrated prehydrolysate (8% (w/w)) obtained at 210°C. In this case, glucose was completely consumed after 48 h when using the adapted strain, whereas glucose was present in the media almost to the end of the process when the parental strain was used (Fig. 1b). Previous studies have also shown that phenolic compounds, furaldehydes and aliphatic acids present in sugarcane bagasse hydrolysates slow down glucose fermentation [25]. Furthermore, in the same study adaptation of *S. cerevisiae* to growth in bagasse hydrolysate resulted in higher glucose consumption rates.

During the fermentations the cellular capacity to consume xylose was assessed. In all cases, the adapted strain performed better showing higher amount of consumed xylose, expressed as percentage of the total xylose (40 g/l) (present in the media at the beginning of the fermentation) consumed after 140 h (Fig. 2). In the best case, 85% consumption of the total xylose was reached at 140 h when using the adapted strain with less toxic prehydrolysate at higher dilution (lowest amount of inhibitory compounds in the broth). It was remarkable that the highest differences in xylose consumption between the adapted and parental strain were found when using more concentrated prehydrolysates corresponding to 8% (w/w) of WIS and improvements of 53 and 67% with prehydrolysates

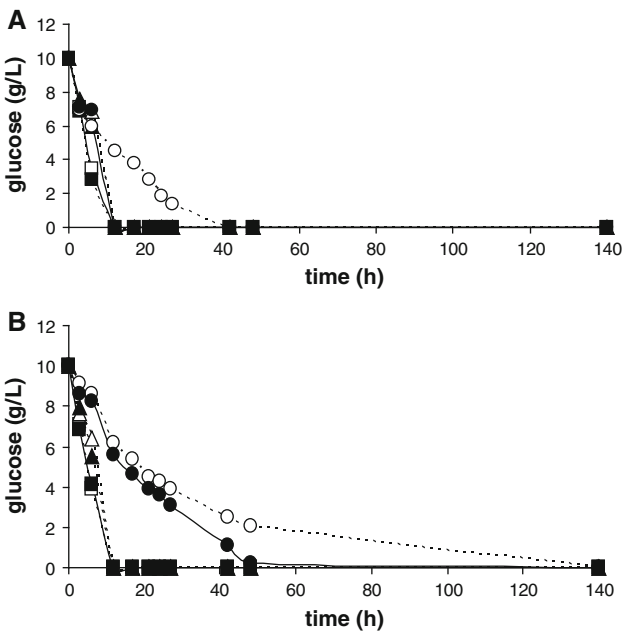

Fig. 1 Glucose consumption in 140-h-long fermentation tests with diluted prehydrolysates obtained at **a** 200°C and **b** 210°C for 5 min. Dilution corresponding to (*square*) 4%, (*triangle*) 6% and (*circle*) 8% (w/w) of WIS in the slurry. *Dotted lines* with *opened symbols* mean parental strain. *Black lines* with *closed symbols* mean adapted strain

obtained at 200 and 210°C, respectively, were observed (Fig. 2). This proved the success of the directed evolution with regard to improve sugar consumption rates. Faster sugar utilization by the adapted strain showed that the ability to tolerate inhibitors was enhanced. An evolved *S. cerevisiae* TMB3001C5 strain has demonstrated 19% higher final ethanol concentration as well as faster xylose consumption rate in a synthetic medium containing glucose and xylose when comparing with the parental strain [40]. It is not clear if an improvement also could be found in lignocellulosic hydrolysates.

Higher amount of consumed xylose was reflected in higher volumetric ethanol productivities and higher final ethanol concentrations (Table 2). The adapted strain performed better than the parental strain in all tests with regard to final ethanol concentration (after 140 h) and volumetric productivity. The highest ethanol concentration (13.1 g/l) was reached with the adapted strain during fermentation of the most diluted prehydrolysate (low level of toxic compounds). The adapted strain showed an increase in the ethanol concentration of 45 and 20%, respectively, with both prehydrolysates obtained at 200 and 210°C at the lowest dilution (8% w/w). Fermentation with the adapted strain also revealed an increase in the volumetric ethanol productivities, which should be beneficial from an economic point of view leading to reduced fermentation time.

Glycerol is produced during anaerobic growth in *S. cerevisiae* as a redox sink to maintain the cytosolic redox balance [41]. Glycerol yield on glucose is usually found to be around 0.1 g/g during anaerobic growth conditions [21, 42] and it is known that during fermentation processes, major amounts of glycerol are produced during glucose consumption as a consequence of biomass production [43]. In the present study, higher glycerol levels in fermentation tests were observed with the adapted strain indicating better cell growth with adapted strain.

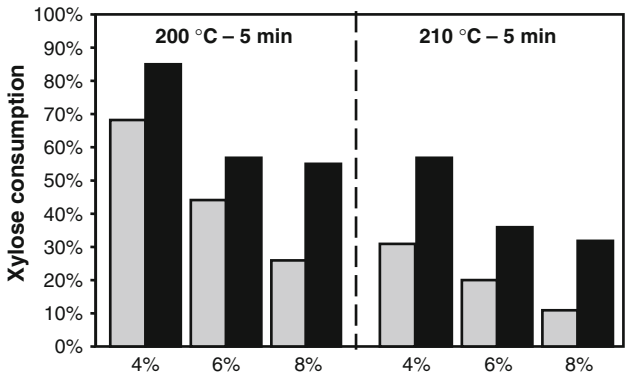

Fig. 2 Xylose consumption (expressed as % of the total xylose present in the medium) in 140-h-long fermentations tests with prehydrolysates obtained at 200 and 210°C for 5 min diluted to 4, 6, and 8% (w/w) of WIS. Parental strain is represented in *grey color* and adapted strain is represented in *black color*

In spite of considerable xylose consumption, very low amounts of xylitol were formed during the prehydrolysate fermentation tests (Table 2), which points out that the conditions prevailing in the hydrolysate lead to redox reactions that balance the redox imbalance that results during xylose fermentation in yeast using the XR/XDH pathway. The presence of substances such as aldehydes and ketones that act as electron acceptors can lead to reduced xylitol formation supplying NAD^+ for the XDH reaction and redirecting product formation toward ethanol production at expense of xylitol formation [22]. In the present study, sugar degradation products such as furfural present in lignocellulosic prehydrolysates, which is reduced to furfuryl alcohol, acts as electron acceptor reducing xylitol excretion [44].

The low xylitol formation together with low amount of residual xylose coincided with the improvement in final ethanol concentration, suggesting an improved ethanol yield on xylose.

The power of continuous evolution is based on its effectiveness in producing the desired effects and the possibility to select cells with advantageous properties under conditions that reflect the characteristics of an industrial process. In liquid media, adequate strains to function in a particular environment evolve over time and eventually replace the parental population as a consequence of adaptation by selection [45]. Since inhibitors released during pretreatment of lignocellulosic biomass could reduce glucose and xylose conversion during fermentation as well as decrease ethanol productivities [9, 46], adaptation has been shown as an effective strategy for increasing tolerance to the toxic compounds [12, 25], which should be reflected in higher ethanol consumption rates, final ethanol titers, and productivities.

S. cerevisiae F12 has previously been shown to be a very inhibitor tolerant strain in several studies [9, 21]. In the present work, adaptation of a recombinant xylose-fermenting *S. cerevisiae* F12 were performed to further increase its inhibitor tolerance towards toxic compounds in wheat straw hydrolysates. Adaptation resulted in an evolved population with even higher inhibitor tolerance, higher sugar consumption rates and higher volumetric productivities and ethanol yields compared to the parental strain.

Fed-batch simultaneous saccharification and fermentation process

Since the energy consumption in the distillation step depends on the level of ethanol, higher substrate loading is needed for an efficient ethanol production process. The use

Table 2 Ethanol production, ethanol yield and productivity. and by-products formations during fermentation of diluted prehydrolysates with adapted and parental *S. cerevisiae* F12 strains

	4% (w/w) of WIS		6% (w/w) of WIS		8% (w/w) of WIS	
	Non-adapted	Adapted	Non-adapted	Adapted	Non-adapted	Adapted
200°C–5 min[a]						
$Xylitol_{140\ h}$ (g/l)	2.4	4.34	0.90	2.12	–	–
$Glycerol_{140\ h}$ (g/l)	0.8	1.05	0.87	1.06	0.18	0.78
$Ethanol_{140\ h}$ (g/l)	10.88	13.11	7.54	8.60	4.08	7.40
$Q_{etoh24\ h}$ (g/lh)	0.21	0.24	0.17	0.21	0.1	0.20
$Q_{etoh48\ h}$ (g/lh)	0.15	0.18	0.11	0.13	<0.1	0.12
$Y_{etoh/cs}$[c]	0.30	0.30	0.29	0.29	0.23	0.25
210°C–5 min[b]						
$Xylitol_{140\ h}$ (g/l)	0.49	2.12	0.49	1.29	–	–
$Glycerol_{140\ h}$ (g/l)	0.70	1.05	0.88	1.09	0.16	1.10
$Ethanol_{140\ h}$ (g/l)	7.87	11.84	5.98	9.23	4.00	5.09
$Q_{etoh24\ h}$ (g/lh)	0.20	0.26	0.19	0.24	0.10	0.12
$Q_{etoh48\ h}$ (g/lh)	0.12	0.17	0.11	0.16	<0.1	<0.1
$Y_{etoh/cs}$[c]	0.37	0.37	0.36	0.39	0.36	0.25

[a] Prehydrolysate corresponding to a slurry with 16.6% (w/w) of WIS and diluted with deionized water to 4, 6, and 8% (w/w) of WIS

[b] Prehydrolysate corresponding to a slurry with 15.2% (w/w) of WIS and diluted with deionized water to 4, 6, and 8% (w/w) of WIS

[c] Ethanol yield was based on the consumed glucose and xylose at 140 h

Table 3 Residual sugars, ethanol production, and yields during different 140-h-long fed-batch SSF experiments

Strain	Pretreatment conditions	Initial substrate	WIS content % (w/w)					Residual xylose (g/l)	Residual glucose (g/l)	$EtOH_{140h}$ (g/l)	$Y^{a}_{ET/TS}$	% theoret.[b]
			6 h	12 h	24 h	36 h	48 h					
S. cerevisiae F12 parental strain	200°C–5 min	Slurry 6% (w/w) of WIS			9.23		11.25	2.8	–	24.3	0.25	49
				9.23		11.25		0.2	–	21.8	0.23	44
			9.23		11.25			0.8	–	24.8	0.26	50
				8.33	10	11.25		1.3	–	23.1	0.24	47
S. cerevisiae F12 adapted strain	210°C–5 min[c]	Slurry 6% (w/w) of WIS			9.23		11.25	<0.1	–	27.4	0.31	61

[a] Ethanol yield was based on the total glucose and xylose content present in the pretreated wheat straw

[b] Assuming ethanol yield on glucose and xylose present in the pretreated wheat straw of 0.51 g/g

[c] No-growth neither ethanol production were shown with the parental strain and material pretreated at these conditions

of high substrate loading implies higher viscosity, which leads to mixing problems, resulting in uneven distribution of yeast, enzyme, substrate, pH and temperature. By using a fed-batch strategy, the problems associated with high viscosity can be overcome. With the aim of reaching substrate loading as high as 11.25% (w/w) of WIS, different fed-batch SSF experiments were performed (Table 3). The adapted strain was used in fed-batch SSF experiments from material pretreated at 210°C, which was not fermentable by the parental strain.

In order not to loose any sugar present in the liquid fraction, whole slurry should preferably be used instead of the WIS fraction. However, the liquid fraction also contains the sugar degradation products potentially inhibitory for enzymes and yeast. If the whole slurry is used, high substrate loading means higher concentration of inhibitors and at too high slurry content complete inhibition of yeast may take place. To avoid a complete inhibition, whole slurry was used for the initial substrate loading (6% w/w WIS) and washed WIS was employed for different substrate additions.

The fed-batch configurations performed in the present study as well as residual sugars, ethanol production, and yields are shown in Table 3. *S. cerevisiae* parental strain (F12) was totally inhibited, no growth nor ethanol production were found, when using the whole slurry obtained at 210°C for 5 min as initial substrate loading (6% WIS) (data not shown). In contrast, the parental strain performed

well in fed-batch SSF experiments with material pretreated at 200°C for 5 min (Fig. 3a).

High ethanol concentration was obtained in previous studies using wheat straw slurry exploded at 210°C and 5 min with the parental strain [47]. Despite pretreating the wheat straw at the same conditions, it was found that the inhibitor concentrations in the prehydrolysate were higher in the present study. Compared to earlier pretreated wheat straw, we assume that the difference was due to the effect that chip size had on sugar and lignin degradation during the pretreatment. In the preceding work, most particles had a size around 10 mm, by contrast, in the present work, raw material was milled to a particle size smaller than 5 mm before pretreatment. Some earlier studies have concluded that using smaller particle sizes is reflected in the higher release of degradation products during pretreatment using steam explosion [48].

The highest ethanol concentration obtained with the parental strain was 24.8 g/l, which corresponds to an ethanol yield of 50% of the theoretical yield based on the glucose and xylose present in the material pretreated at 200°C and 5 min. It is not known how much residual sugar was bound to the solid part at the end of the cultivation and therefore it can not be concluded whether suboptimal enzymatic hydrolysis or fermentation is the major reason that higher yields are not obtained. In the best feeding profile, all the substrate was added during the first 24 h, which is in agreement with previous studies, concluding that fed-batch strategies with earlier substrate addition resulted in higher ethanol production [8, 30]. An explanation may be the decreased cell viability along the progress of the SSF leading to less efficient cellular metabolism at the end of the process. Maximum residual xylose was found when the last feed pulse took place at 48 h (Table 3). Xylose transport into the cell is carried out by the glucose transport system and the affinity for xylose is approximately 200-fold lower than for glucose [49]. Consequently, xylose uptake competes with glucose uptake and it is necessary to keep the glucose concentration low to promote xylose uptake. Thus, later substrate addition meant the addition of more glucose, which competes with xylose uptake during longer time.

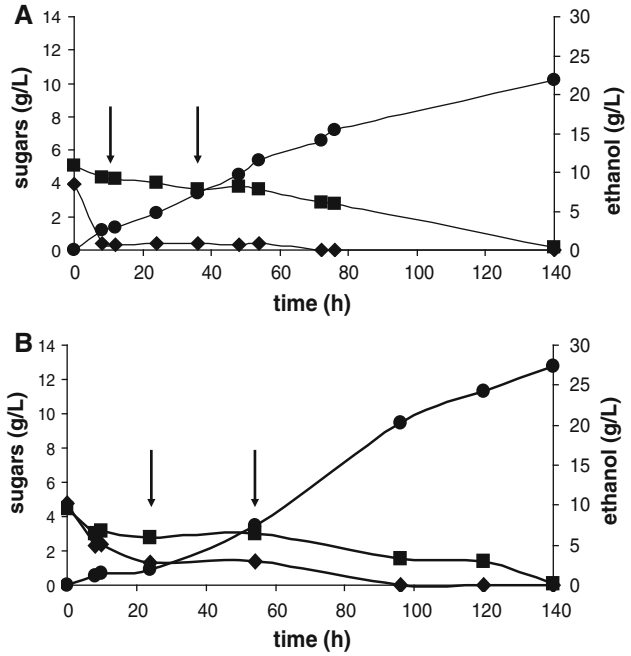

Fig. 3 Fed-batch SSF with slurry at 6% of WIS as initial substrate loading. **a** Substrate pretreated at 200°C for 5 min, parental strain and addition pulses at 12 and 36 h. **b** Substrate pretreated at 210°C for 5 min, evolved strain and addition pulses at 24 h and 48 h. (*filled circle*) Ethanol, (*filled diamond*) glucose and (*filled square*) xylose concentration

Steam-exploded wheat straw pretreated at 210°C was not a feasible substrate for fed-batch SSF with the parental strain. However, after an adaptation process, evolved *S. cerevisiae* F12 was able to ferment slurry obtained at 210°C because the strain showed improved inhibitor tolerance. In order to investigate the efficiency of the adapted strain, a fed-batch strategy for the adapted strain (addition at 24 and 48 h) was chosen according to the best previous results with the parental strain in order to check if residual xylose was lowered and the ethanol production was increased when using the adapted strain compared to what could be achieved using the parental strain.

In Fig. 3, fed-batch experiments with the lowest (Fig. 3a) and the highest (Fig. 3b) ethanol concentration are represented. As can be observed in Fig. 3b, the adapted strain produced an ethanol concentration as high as 27.4 g/l, which meant 61% of the theoretical ethanol yield. Furthermore, the residual xylose was lower when comparing with the fed-batch SSF experiments with the parental strain. The theoretical ethanol yield was assessed assuming ethanol yields on glucose and xylose of 0.51 g/g. These yields were overestimated since previous studies concluded that ethanol yield on glucose and xylose for *S. cerevisiae* F12 were 0.46 and 0.26 g/g, respectively [21].

Ethanol yields on total sugars of 0.27 g/g were achieved in previous studies with the parental strain, which are in agreement with yields obtained in the present study [17, 50]. However, the ethanol yield on total sugar was also increased to 0.31 g/g when using the adapted strain.

Conclusions

Adaptation of a recombinant xylose-fermenting *S. cerevisiae* F12 to wheat straw hydrolysates with increasing concentration of inhibitors resulted in an evolved population showing higher sugar consumption rates and higher

ethanol concentrations and productivities than the parental strain. The evolved strain performed better than the parental in fermentation tests using the prehydrolysate with higher inhibitor concentration and a dilution corresponding to 8% of WIS, showing 20% more final ethanol concentration and 65% more xylose consumption compared to the parental strain.

Using fed-batch SSF approaches, substrate loading as high as 11.25% (w/w) of WIS was used without mixing problems or inhibition problems. The parental strain was not able to ferment slurries obtained at 210°C for 5 min owing to the high concentration of toxic compounds, but those slurries were fermentable with the adapted strain showing an ethanol concentration as high as 27.4 g/l. Less residual xylose remain, and higher xylose consumption rates were also observed with the adapted strain.

References

1. Aristidou A, Penttila M (2000) Metabolic engineering application to renewable resource utilization. Curr Opin Biotechnol 11:187–198
2. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G (2006) Bio-ethanol—the fuel of tomorrow from the residues of today. Trends Biotechnol 24:549–556
3. Mosier N, Wyman CE, Dale BD, Elander RT, Lee YY, Holtzapple M, Ladisch CM (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686
4. Jorgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod Biorefin 1:119–134
5. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY (2005) Coordinated development of leading biomass pretreatment technologies. Bioresour Technol 96:1959–1966
6. Olsson L, Hahn-Hägerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18:312–331
7. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanism of inhibition. Bioresour Technol 74:25–33
8. Olofsson K, Rudolf A, Liden G (2008) Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*. J Biotechnol 134:112–120
9. Panagiotou G, Olsson L (2007) Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rate. Biotechnol Bioeng 96:250–258
10. Oliva JM, Saez F, Ballesteros I, González A, Negro MJ, Manzanares P, Ballesteros M (2003) Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Appl Biochem Biotechnol 105(1–3):141–154
11. Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Biochem Biotechnol 66:10–26
12. Keating JD, Panganiban C, Mansfield SD (2006) Tolerance and adaptation of ethanologenic yeast to lignocellulosic inhibitory compounds. Biotechnol Bioeng 93:1196–1206
13. Graves T, Neelakan V, Narendranath KD, Power R (2006) Effect of pH and lactic or acetic acid on ethanol productivity by *Saccharomyces cerevisiae* in corn mash. J Ind Microbiol Biotechnol 33:469–474
14. Buranov AU, Mazza G (2008) Lignin in straw of herbaceous crops. Ind Crop Prod 28:237–259
15. Sun RC, Sun XF, Wang SQ, Zhu W, Wang XY (2002) Ester and ether linkages between hydroxycinnamic acids and lignins from wheat, rice, rye, and barley straws, maize stems, and fast-growing poplar wood. Ind Crops Prod 15:179–188
16. Almeida JRM, Modig T, Petersson A, Hahn-Hägerdal B, Liden G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349
17. Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007) Towards industrial pentose-fermenting yeast strains. Appl Biochem Biotechnol 74:937–953
18. Eliasson A, Christensson C, Wahlbom F, Hahn-Hägerdal B (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL1, XYL2* and *XKS1* in mineral medium chemostat cultures. Appl Environ Microbiol 66:3381–3386
19. Ho NWY, Zhengdao C, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859
20. Matsushika A, Inoue H, Murakami K, Takimura O, Sawayama S (2009) Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Bioresour Technol 100:2392–2398
21. Sonderegger M, Jeppsson H, Larsson C, Gorwa-Grauslund MF, Boles E, Olsson L, Spencer-Martins I, Hahn-Hägerdal B, Sauer U (2004) Fermentation performance of engineered and evolved xylose-Fermenting *Saccharomyces cerevisiae* strains. Biotechnol Bioeng 87:90–98
22. Wahlbom CF, Hahn-Hägerdal B (2002) Furfural, 5-hydroxymethyl furfural, and acetoin act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 78:172–178
23. Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Biochem Biotechnol 56:17–34
24. Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and hydrolxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460
25. Martin C, Marcet M, Almazan O, Jonsson LJ (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767–1773
26. Wyman CE, Spindler DD, Grohmann K (1992) Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol. Biomass Bioenerg. 3:301–307
27. Rosgaard L, Andric P, Dam-Johansen K, Pedersen S, Meyer AS (2007) Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. Appl Biochem Biotechnol 143:27–40
28. Rudolf A, Alkasrawi M, Zacchi G, Liden G (2005) A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. Enzyme Microb Technol 37:195–204
29. Meinander NQ, Boels I, Hahn-Hägerdal B (1999) Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1 and XYL2 from *Pichia stipitis* with and without overexpression of TAL1. Bioresour Technol 68:79–87
30. Tomás-Pejó E, Oliva JM, González A, Ballesteros I, Ballesteros M (2009) Bioethanol production from wheat straw by

thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. Fuel 88:2142–2147
31. Verduyn C, Postma E, Scheffers A, Van Dijken JP (1990) Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136:95–403
32. NREL. National Renewable Energy Laboratory (2007) Chemical Analysis and Testing Laboratory Analytical Procedures. Golden, CO
33. Hendriks ATW, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100:10–18
34. Wu M, Chang K, Gregg DJ, Boussaid A, Beatson RP, Saddler JN (1999) Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwood. Appl Biochem Biotechnol 77–79:47–54
35. Alfani A, Gallifuoco F, Saporosi A, Spera A, Cantarella M (2000) Comparison of SHF and SSF process for the bioconversion of steam-exploded wheat straw. J Ind Microbiol Biotechnol 25:184–192
36. Ballesteros I, Oliva JM, Negro MJ, Manzanares P, Ballesteros M (2002) Enzymic hydrolysis of steam exploded herbaceous agricultural waste (*Brassica carinata*) at different particule sizes. Process Biochem 38:187–192
37. Kabel MA, Bos G, Zeevalking J, Voragen AGJ, Schols HA (2007) Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour Technol 98:2034–2042
38. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11
39. García-Aparicio M, Ballesteros I, González A, Oliva JM, Ballesteros M, Negro MJ (2006) Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl Biochem Biotechnol 129:278–288
40. Sonderegger M, Sauer U (2003) Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol 69:1990–1998
41. Bakker BM, Overkamp KM, Van Maris AJA, Kottler P, Luttik MAH, Van Dijken JP, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37
42. Nissen TL, Schulze U, Nielsen J, Villadsen J (1997) Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology 143:203–218
43. Zaldivar J, Roca C, Le Foll C, Hahn-Hägerdal B, Olsson L (2005) Ethanolic fermentation of acid-treated starch industry effluents by recombinant *Saccharomyces cerevisiae* strains. Bioresour Technol 96:1670–1676
44. Karhumaa K, Sanchez RG, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 6:5
45. Sauer U (2001) Evolutionary engineering of industrially important microbial phenotypes. Adv Biochem Eng Biotechnol 73:129–169
46. Helle S, Cameron D, Lam J, White B, Duff S (2003) Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineering strain of *S* cerevisiae. Enzyme Microb Technol 33:786–792
47. Tomás-Pejó E, Oliva JM, Ballesteros M, Olsson L (2008) Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting *Saccharomyces cerevisiae* strains. Biotechnol Bioeng 100:1122–1131
48. Ballesteros I, Oliva JM, Navarro AA, González A, Carrasco J, Ballesteros M (2000) Effect of chip size on steam explosion pretreatment of softwood. Appl Biochem Biotechnol 84–86:97–110
49. Hamacher T, Becker J, Gardonyi M, Hahn-Hägerdal B, Boles E (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. Microbiology 148:2783–2788
50. Olsson L, Soerensen HR, Dam BP, Christensen H, Krogh KM, Meyer AS (2006) Separate and simultaneous enzymatic hydrolysis and fermentation of wheat hemicellulose with recombinant xylose utilizing *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 129–132:117–129