Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioresource Technology 96 (2005) 1670–1676
BIORESOURCE TECHNOLOGY

Ethanolic fermentation of acid pre-treated starch industry effluents by recombinant *Saccharomyces cerevisiae* strains

Jesus Zaldivar [a], Christophe Roca [a], Caroline Le Foll [a], Bärbel Hahn-Hägerdal [b], Lisbeth Olsson [a,*]

[a] *Center for Microbial Biotechnology, Technical University of Denmark, BioCentrum-DTU, Building 223, DK-2800 Kgs, Lyngby, Denmark*
[b] *Applied Microbiology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*

Received 8 March 2004; received in revised form 20 December 2004; accepted 22 December 2004
Available online 25 February 2005

Abstract

Two industrial effluents, a pre-fermentation effluent and a post-fermentation effluent from a wheat starch production plant, were used as substrates for fuel ethanol production in anaerobic batch cultures using minimal nutritional amendment. The performances of three metabolically engineered xylose-utilizing *Saccharomyces cerevisiae* strains: TMB 3001 expressing *XYL1*, *XYL2* and *XKS1*, redox metabolism modulated CPB.CR1 and glucose de-repressed CPB.CR2, as well as a reference strain CEN.PK 113-7D not fermenting xylose, were evaluated. For the recombinant strains a glucose consumption phase preceded the xylose consumption phase. In both effluents, biomass and ethanol production occurred predominantly during the glucose consumption phase, whereas xylitol and glycerol formation were predominant in the xylose consumption phase. Total specific ethanol productivities on glucose were 6-fold higher than on xylose in the pre-fermentation effluent and 15-fold higher than on xylose in the post-fermentation effluent. CPB.CR1 showed impaired growth compared to the two other xylose-utilizing strains, but displayed 18% increased ethanol yield in the post-fermentation effluent.

Keywords: Wheat starch effluent; Acid hydrolysis; Fuel ethanol; Metabolic engineering

1. Introduction

The reduction of atmospheric carbon dioxide has become an important issue worldwide and the utilization of fuel ethanol to reduce tailpipe emissions has the potential to contribute to a cleaner environment. Currently, fuel ethanol is made from crop sugars and its production cost is higher than that of oil-derived gasoline (Wyman, 1996). The utilization of cheap feedstocks such as lignocellulosic wastes (sugarcane bagasse, rice hulls, wheat straw) could potentially reduce the cost of fuel ethanol (Mielenz, 2001). Hydrolysates of lignocellulosic material will contain mixtures of sugars, including glucose, galactose, mannose, xylose and arabinose, and other constituents in variable proportions depending on the source (Wiselogel et al., 1996).

Several European countries (Swinnen et al., 1988), Canada (Boland, 1995) and Australia (Hunwick, 1980) use wheat starch as a feedstock for ethanol production. After grinding, the starch is hydrolysed and fermented to ethanol by industrial *Saccharomyces cerevisiae* strains. The remains, including wheat straw, constitute a waste material (Sosulski and Sosulski, 1994). Consequently, cellulose and hemicellulose in this material could be an additional source of sugar for ethanol production.

S. cerevisiae, the preferred microorganism for converting crop sugars into ethanol, is unable to utilize either xylose or arabinose. Hence, genes from the xylose-utilizing yeast *Pichia stipitis*, encoding xylose

* Corresponding author. Tel.: +45 45 25 26 77; fax: +45 45 88 41 48.
E-mail address: lo@biocentrum.dtu.dk (L. Olsson).

0960-8524/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2004.12.034

reductase and xylitol dehydrogenase, have been inserted (Kötter and Ciriacy, 1993; Tantirungkij et al., 1993; Walfridsson et al., 1997). This strategy, together with an over-expression of xylulokinase (Ho and Chang, 1989), has resulted in—among others—*S. cerevisiae* TMB 3001 (Eliasson et al., 2000). *S. cerevisiae* TMB 3001 has been evaluated in enzymatic hydrolysates of sugarcane bagasse, and it was shown that the ethanol yield in these hydrolysates was lower than in comparable reference substrates (Martín et al., 2002a). It was also found that TMB 3001 could use the xylose in the medium at a low consumption rate. Furthermore, it has been shown that pre-treatment by steam-explosion, and possible detoxification, influenced the sugar consumption rates by *S. cerevisiae* TMB 3001 significantly, whereas the ethanol yield was only marginally influenced (Martín et al., 2002a,b). Consequently, inhibiting compounds present in lignocellulosic hydrolysates influence the rate of sugar consumption and yield of ethanol (as reviewed by Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000a,b). Furthermore, the sensitivity towards fermentation inhibitors has recently been shown to be highly strain dependent (Martín and Jönsson, 2003).

Due to a number of limitations in the cellular xylose metabolism (recently reviewed by Hahn-Hägerdal et al., 2001; and Zaldivar et al., 2001), further improvements of the recombinant xylose fermenting *S. cerevisiae* strains are necessary. Xylose reductase and xylitol dehydrogenase have different co-factor preferences, which results in xylitol formation at the expense of ethanol formation and concomitantly reduced ethanol yield (Bruinenberg et al., 1984). Metabolic engineering of the NADPH producing glucose 6-phosphate dehydrogenase reaction has lead to increased ethanol yield in xylose fermentation (Jeppsson et al., 2002, 2003a). An alternative way to modulate the redox metabolism is to alter the ammonium assimilation, which has been demonstrated to improve the ethanol yield on xylose in defined mineral medium with a glucose–xylose mixture (Roca et al., 2003).

It has earlier been shown that manipulations of the cascade responsible for glucose repression can be engineered to result in more simultaneous sugar utilization (as reviewed by Olsson and Nielsen, 2000). *S. cerevisiae* CPB.CR2 is a glucose de-repressed xylose-utilizing strain, and it exhibits improved xylose consumption rate during chemostat cultivation on a mixture of glucose and xylose (Roca et al., 2004).

In the present study, two acid pre-treated and enzymatically hydrolysed effluents from a wheat starch industry were used as substrates for anaerobic batch fermentation with three metabolically engineered xylose-utilizing *S. cerevisiae* strains: TMB 3001 expressing *XYL1*, *XYL2* and *XKS1*, redox metabolism modulated CPB.CR1 and glucose de-repressed CPB.CR2, as well as a reference strain CEN.PK 113-7D not fermenting xylose. The aim was to evaluate how the metabolically engineered strains performed in industrial substrates.

2. Methods

2.1. Fermentation substrates

The wheat starch industry effluent substrates were derived from the pre-fermentation step and the distillation step in a wheat-based ethanol plant. Effluents were heated to 98 °C, and after addition of 2% (w/v) sulfuric acid they were kept at 98 °C for 2 h (a slight modification of the procedure described by Magee and Kosaric, 1985) to release the sugars from the hemicellulose fraction. After cooling, pH was adjusted to 5.0 with $Ca(OH)_2$. To allow the release of monomeric glucose from the starch fraction, effluents were incubated with amyloglucosidase (Novozymes, Bagsværd, Denmark), 130 μl/l effluent at 37 °C for 24 h. The concentrations of sugars in the pre- and post-fermentation effluents are summarized in Table 1. The xylose concentration in these effluents was low and 50 g/l of xylose was added to the pre-fermentation effluent and 25 g/l to the post-fermentation effluent, bringing the initial concentrations of xylose to 54.1 g/l and 29.5 g/l, respectively. The post-fermentation effluent was also supplemented with 10 g/l of glucose, so that the initial concentration of glucose was 120 g/l in the pre-fermentation effluent and 13.5 g/l in the post-fermentation effluent, respectively (Table 1). After supplementation with sugars, three filtration steps followed: firstly, Schleicher & Schüell (Dassel, Germany) Nr 589[3] paper, secondly, Pall-Gelman (Ann Arbor, MI) 0.45-μm membrane filter paper, and thirdly, Pall-Gelman 0.22-μm. Except for Ergosterol (15 mg/l) and Tween 80 (660 mg/l) no other nutrients were added.

2.2. Saccharomyces cerevisiae strains

CEN.PK113-7D (*MAT*a *MAL2-8*^{c} *SUC2*) was used as a reference strain (Entian and Kötter, 1998). *S. cerevisiae* TMB 3001 (*MAT*a *MAL2-8*^{c} *SUC2 XYL1 XYL2*

Table 1
Compositions of the pre- and post-fermentation effluents

Component	Pre-fermentation effluent	Post-fermentation effluent
Arabinose (g/l)	5.2	6.5
Galactose (g/l)	0.3	0.7
Glucose (g/l)	120	3.5 (13.5)[a]
Mannose (g/l)	0	0
Xylose (g/l)	4.1 (54.1)[a]	4.5 (29.5)[a]

[a] Number within brackets refer to the concentration in the fermentation substrate after supplementation with glucose and xylose, respectively.

XKS1) contained the *XYL1* and *XYL2* genes from *P. stipitis* coding for xylose reductase and xylitol dehydrogenase, respectively, and the endogenous *XKS1* coding for xylulose kinase chromosomally integrated (Eliasson et al., 2000). *S. cerevisiae* CPB.CR1 (*MAT*a *MAL2-8^c SUC2 gdh1Δ XYL1 XYL2 XKS1*) was additionally disrupted in *gdh1* (Roca et al., 2003), and *S. cerevisiae* CPB.CR2 (*MAT*a *SUC2 MAL2-8^c mig1ΔXYL1 XYL2 XKS1*) was disrupted in *mig1* (Roca et al., 2004). The four strains will in the following be referred to as **REF** for CEN.PK113-7D, **TMB** for *S. cerevisiae* TMB 3001, **CR1** for *S. cerevisiae* CPB.CR1 and **CR2** for *S. cerevisiae* CPB.CR2.

2.3. Fermentation conditions

Batch fermentation was performed in in-house manufactured fermenters with a volume of 1.4 l, equipped with one Rushton turbine, containing 1 l effluent. Technical quality nitrogen (AGA, Copenhagen, Denmark) containing less than 5 ppm O_2 was flushed through the vessels (0.5 lpm) to maintain an anaerobic environment, and the exhaust gas passed through a reflux cooler maintained at 2 °C to minimize ethanol evaporation. pH was maintained at 5.0 with 2 M NaOH. Fermentation was performed at 30 °C with stirring at 500 rpm. Carbon dioxide was monitored with an acoustic gas-analyser (Brüel & Kjaer type 1308, Nærum, Denmark). Reactors were inoculated to an initial biomass concentration of 20 mg DW/l with pre-cultures grown in unbaffled standing flasks at 30 °C for 15 h on the same effluent medium (pre- or post-fermentation) as used in the following fermentation.

2.4. Analytical methods

Cell growth was monitored by absorbance at 600 nm (Hitachi U-1100, Tokyo, Japan) and by gravimetric determination of dry weight (Olsson and Nielsen, 1997).

Samples for analyses of sugars, sugar alcohols and extracellular metabolites were filtered through 0.45-μm-pore-size acetate filters (Osmonics, Minnetonka, MN) and stored at −20 °C until analyzed by HLPC (as described by Zaldivar et al., 2002).

2.5. Calculations

Yields of biomass and ethanol on sugars for the reference strain were determined at the time of glucose depletion (30 and 10 h in pre- and post-fermentation effluents, respectively) since this strain is unable to utilize xylose. For the recombinant strains the end of fermentation (192 and 181 h in pre- and post-fermentation effluents, respectively) was considered.

Specific ethanol productivity was based on the volumetric productivity divided by the biomass at the time of glucose exhaustion for the reference strain and at the end of the fermentation for the recombinant strains. Specific ethanol productivity was expressed in g/g DW per h.

Xylose uptake rate was calculated using the equation: $r_{xyl} = dC_{xylose}/dt^*1/DW$, where DW is the biomass concentration. Xylose uptake rate was expressed in g/g DW per h.

3. Results

3.1. Biomass growth, sugar consumption and ethanol formation

The filtration-sterilization step following the enzymatic hydrolysis removed particles contributing to the turbidity of the effluents, which allowed determination of biomass concentrations (Fig. 1A). Cellular growth (and ethanol production) occurred predominantly as a result of glucose consumption. Growth resulted in biomass concentrations of 10.7, 9.7, 8.7 and 10.6 g/l, respectively, in strains REF, TMB, CR1 and CR2 grown in pre-fermentation effluent, and 3.1, 3.1, 2.9 and 3.5 g/l, respectively, in post-fermentation effluent. Specific growth rates for the *gdh1Δ* strain (CR1) were the lowest, 0.24 and 0.27 h^{-1}, respectively, in pre- and post-fermentation effluents, compared to REF, in which they were highest, 0.31 and 0.34 h^{-1} (Table 2).

In fermentation lasting 8 days, a phase of rapid glucose consumption preceded the slow xylose consumption phase (Fig. 1B). In the pre-fermentation effluent, glucose exhaustion occurred in 30–36 h in all cases and in the post-fermentation effluent in 10 h, strain CR1 being the exception in both effluents (Fig. 1B). As expected, the reference strain was unable to consume xylose. The consumption of xylose by CR1 was lowest in both effluents, i.e., at the end of fermentation, 55% of the xylose remained unconsumed in the pre-fermentation effluent and 35% in the post-fermentation effluent. For strains CR2 and TMB, respectively, less than 12% and 7% remained in pre- and post-fermentation effluents, respectively, at the end of fermentation. In pre-fermentation effluent CR2 exhibited the highest specific xylose consumption rate (0.038 g/g h), whereas in post-fermentation effluent the highest specific xylose consumption rate was shown by TMB (0.065 g/g h) (Table 2).

Yields of biomass on sugars (Y_{sx}) for the reference strain were 0.09 and 0.22 g/g, respectively, in pre- and post-fermentation effluents. For the recombinant strains, yields were 0.05–0.06 g/g in pre-fermentation effluent and 0.07–0.08 g/g in post-fermentation effluent (Table 2). As the post-fermentation effluent is a waste

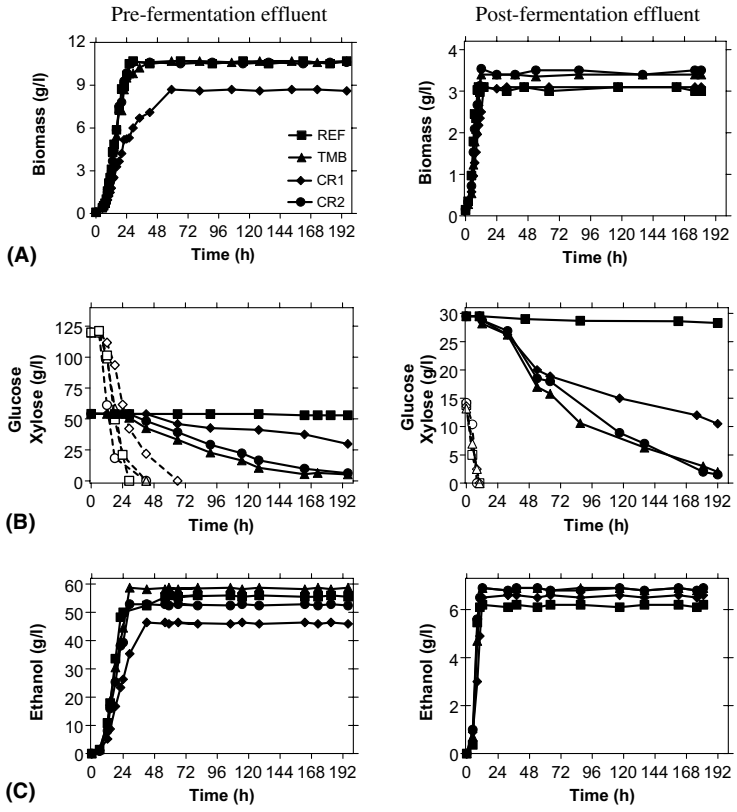

Fig. 1. Time course of biomass concentration (A), glucose and xylose consumption (B), and ethanol production (C) in acid hydrolysed pre-fermentation effluent (left) and post-fermentation effluent (right). (■) REF: reference strain, (▲) TMB: *S. cerevisiae* TMB 3001; (◆) CR1: *S. cerevisiae* CPB.CR1; (●) CR2: *S. cerevisiae* CPB.CR2. B: Filled symbols represent xylose, hollow symbols glucose.

Table 2
Summary of results in anaerobic batch fermentation of acid hydrolysed effluents

Substrate	Pre-fermentation effluent				Post-fermentation effluent			
Strain	REF[a]	TMB	CR1	CR2	REF	TMB	CR1	CR2
μ_{max}[b]	0.31	0.31	0.24	0.28	0.34	0.32	0.27	0.32
Y_{sx}[c]	0.09	0.06	0.05	0.06	0.22	0.07	0.07	0.08
Y_{se}[d]	0.47	0.34	0.27	0.30	0.46	0.16	0.15	0.16
r_{EtOH}[e]	0.180	0.031	0.027	0.026	0.200	0.012	0.013	0.011
r_{xyl}[f]	0	0.035	0.012	0.038	0	0.065	0.035	0.053
Y_{xx}[g]	0	0.11	0.06	0.08	0	0.22	0.11	0.17

[a] REF: *S. cerevisiae* CEN.PK113-7D; TMB: *S. cerevisiae* TMB 3001; CR1: *S. cerevisiae* CPB.CR1; CR2: *S. cerevisiae* CPB.CR2.
[b] Maximum specific growth rate (h^{-1}).
[c] Biomasss yield (g biomass/g available sugar). REF cannot utilize xylose, therefore available sugars were restricted to 120 g/l glucose and 13.5 g/l glucose, respectively, in pre- and post-fermentation effluents. For the recombinant strain, available sugars (arabinose excluded), were 174.1 and 43 g/l, respectively, in pre- and post-fermentation effluents.
[d] Ethanol yield (g ethanol/g available sugar). Y_{se} for REF was calculated on glucose and for the recombinant strains on the mixture glucose and xylose.
[e] Specific ethanol productivity (g ethanol/g DW h), calculated as the volumetric productivity (g/l h) divided by the biomass at the time of glucose exhaustion, 30 and 10 h, respectively, in pre- and post-fermentation effluents, for the REF and at the end of the culture for the recombinant strains.
[f] Specific xylose consumption rate (g xylose/g biomass h), calculated by the equation: specific uptake rate = $dC_{xylose}/dt^*1/DW$.
[g] Xylitol yield (g xylitol/g xylose), calculated by dividing the final xylitol concentration by the initial xylose concentration in the reactor.

effluent after distillation (vinasse), it is possible that residues derived from dead cells contributed to the higher cell mass production in this effluent. A biomass yield of around 0.1 g/g glucose is normally achieved in anaerobic batch cultivations, whereas a lower biomass yield can result under stressful growth conditions. Peak values of ethanol concentration were 56, 59, 46 and 53 g/l, respectively, for strains REF, TMB, CR1 and CR2 grown in the pre-fermentation effluent and 6.2, 6.9, 6.6 and 6.9 g/l, respectively, in the post-fermentation effluent (Fig. 1C). The yields of ethanol on available sugars (Y_{se}) for the reference strain were 0.47 and 0.46 g/g,

respectively, in pre- and post-fermentation effluents (Table 2). For the recombinant strains ethanol yields were in the range 0.27–0.34 g/g in pre-fermentation effluent and 0.15–0.16 g/g in post-fermentation effluent (Table 2). Since the REF strain does not consume xylose, calculations were based on 120 g/l glucose, whereas in the case of the recombinant strains, the available sugars (glucose + xylose, arabinose excluded) were 174.1 g/l. In the post-fermentation effluent, sugars available for the reference strain were 13.5 g/l, whereas it was 43 g/l for the recombinant strains. Specific ethanol productivity was the highest in the REF strain, 0.18 and 0.20 g/g h (calculated on glucose) in pre- and post-fermentation effluents, respectively. For the metabolically engineered strains specific productivities (calculated on glucose and xylose) were very low, which reflects the fact that the xylose consumption rate was much lower than the glucose consumption rate. The highest value among the recombinant strains in the pre-fermentation effluent was 0.031 g/g h for TMB, whereas in the post-fermentation effluent values were in the range 0.011–0.013 g/g h.

3.2. By-products formation

During the fermentations, major amounts of glycerol were produced during glucose consumption (Fig. 1 and Table 3), where glycerol was produced as a consequence of biomass production. During this phase the majority of the ethanol was produced. In both effluents the CR1 strain (*Δgdh1*) produced most glycerol at the time of glucose exhaustion, i.e., 13.1 g/l and 1.2 g/l, respectively, in pre- and post-fermentation effluents. During the second part of the fermentations xylose was consumed (Fig. 1), glycerol and xylitol being major products and ethanol a minor product. At the end of cultivation, the glycerol concentrations reached 16.6, 19.0 and 19.0 g/l, respectively, in the pre-fermentation effluent for TMB, CR1 and CR2, and 3.0, 4.3 and 3.7 g/l, respectively, in post-fermentation effluent (Table 3). As a consequence of the poor fermentation on xylose, unusually high glycerol to ethanol ratios were found in these fermentations.

The formation of xylitol occurred only during the xylose consumption phase, and in both effluents, the final concentrations were similar, although the xylose concentration in the pre-fermentation effluent was almost double that in the post-fermentation effluent (Table 3). The highest concentrations were achieved with TMB, i.e., 6.0 and 6.6 g/l in pre- and post-fermentation effluents, respectively (Table 3). The yields of xylitol on consumed xylose were respectively, 0.12, 0.14, and 0.09 g/g for strains TMB, CR1 and CR2, respectively, in the pre-fermentation effluent and 0.24, 0.17 and 0.18 g/g, respectively, in post-fermentation effluent.

4. Discussion

The aim of this study was to evaluate the performance of differently engineered strains in two industrial effluents. The two effluents led to different fermentation profiles with a higher ethanol production rate for the pre-fermentation effluent, but a faster xylose consumption rate in the post-fermentation effluent. The recombinant strains performed differently as well. The *gdh1* deleted strain (CR1) had the lowest specific growth rate in both effluents; 30% lower than the TMB strain. This phenotype, i.e. specific growth rates 45–55% lower than the reference strain, has previously been reported for other *gdh1* disrupted strains (Miller and Magasanik, 1990; Nissen et al., 2000). Concomitant with the reduced growth rate, the CR1 strain had the lowest xylose consumption rate (Fig. 1), which has previously been observed in defined medium with a glucose and xylose mixture (Roca et al., 2003). This metabolic engineering strategy is based on manipulation of ammonia metabolism, but unlike the previous study, where ammonium sulfate was used as nitrogen source in defined mineral medium, the effluents were not supplemented with ammonium in the present investigation. Despite this fact, an 18% improved ethanol yield, based on sugars consumed, was found for the CR1 strain in the post-fermentation substrate, whereas the CR1 strain presented the lowest ethanol yields based both on total sugars and sugars consumed in the pre-fermentation effluent. It is not clear which components in the medium the yeast cells used as nitrogen source in these experiments.

Table 3
Xylitol and glycerol formation at the time of glucose exhaustion and at end of the cultivations

Substrate	Pre-fermentation effluents				Post-fermentation effluents			
Strain	REF	TMB	CR1	CR2	REF	TMB	CR1	CR2
Xylitol concentration at glucose exhaustion (g/l)[a]	nd[b]	nd	nd	nd	nd	nd	nd	nd
Xylitol concentration at end of cultivation (g/l)	0.6	6.0	3.3	4.2	0.4	6.6	3.3	5.1
Glycerol concentration at glucose exhaustion (g/l)[a]	6.0	8.0	13.1	7.0	0.7	0.8	1.2	0.9
Glycerol concentration at end of cultivation (g/l)	8.8	16.6	19.0	19.0	0.6	3.0	4.3	3.7

[a] The time for glucose exhaustion was for strains REF, TMB, CR1 and CR2 30, 36, 60 and 35 h in pre-fermentation effluent and 10, 10, 12 and 10 h in the post-fermentation effluent, respectively.
[b] nd—Not detected.

The formation of glycerol and xylitol seemed to be related in the recombinant strains (Fig. 2). As a consequence of its modified redox balance, CR1 formed relatively more glycerol than xylitol in the pre-fermentation effluent than was found in all the other fermentation experiments. The same has been observed in defined medium containing a mixture of glucose and xylose (Roca et al., 2003). Another approach to metabolically engineering the redox metabolism involves reduction of the flux through the NADPH producing part of the pentose phosphate shunt, by reduction of the glucose-6-phosphate dehydrogenase activity (Jeppsson et al., 2002). Also, in this case, a reduced xylitol production was concomitant with increased ethanol yield, but decreased xylose consumption rate and increased glycerol yields resulted. However, by increasing the xylose reductase levels in the glucose-6-phosphate dehydrogenase deleted strain, the xylose consumption rate could be restored, albeit at the expense of a decreased ethanol yield and increased glycerol yield (Jeppsson et al., 2003b). A simultaneous over-expression of glutamate dehydrogenase 2 with the deletion of glutamate dehydrogenase 1 also led to restored xylose consumption rate, in this case with an improved ethanol yield but increased xylitol yield (Roca et al., 2003). Since redox equivalents tie the cellular metabolism together, the results indicate that considerable fine-tuning of the redox metabolism would be necessary to achieve both decreased xylitol yield and restored or even improved xylose consumption rates.

All recombinant strains, including the *mig1* deleted CR2 strain, utilized sugars sequentially so that xylose was mainly utilized when glucose had been exhausted (Fig. 1A). Xylose consumption rate (up to 0.065 g/g biomass h) was low for both effluents compared to what can be achieved during growth on a defined medium (e.g. 0.089 g xylose/g biomass h using TMB 3001, Roca et al., 2004). Thus, deletion of *MIG1* did not improve the xylose consumption rate as such, nor did it result in simultaneous consumption of glucose and xylose. The same has been observed in defined medium (Roca et al., 2004). Increased xylose consumption rate has only been achieved in *S. cerevisiae* CPB.CR2 in carbon-limited chemostat cultivation at low glucose concentration and it has been attributed to an increased transport capacity (Roca et al., 2004). The high glucose concentrations in the industrial effluents used in the present study most probably saturated the hexose transport system. In the absence of a specific xylose transporter in *S. cerevisiae* (Busturia and Lagunas, 1986), the transport of xylose is carried out by the hexose transport system (Özcan and Johnston, 1999; Hamacher et al., 2002). The affinity of the transport system for glucose is in the range of 2–25 mM (Walsh et al., 1994) whereas the affinity for xylose is 50- to 80-fold lower (Kotyk, 1967). K_m-values of 0.16–0.19 M (Busturia and Lagunas, 1986) and 1.5 M (Kötter and Ciriacy, 1993), for the high and low affinity transport of xylose, respectively, have been found. The vast difference in the affinity of the transport system for the two sugars suggests that sequential utilization of glucose and xylose in presently available recombinant xylose-utilizing strains of *S. cerevisiae* originates from competition for the same transport system rather than from glucose repression.

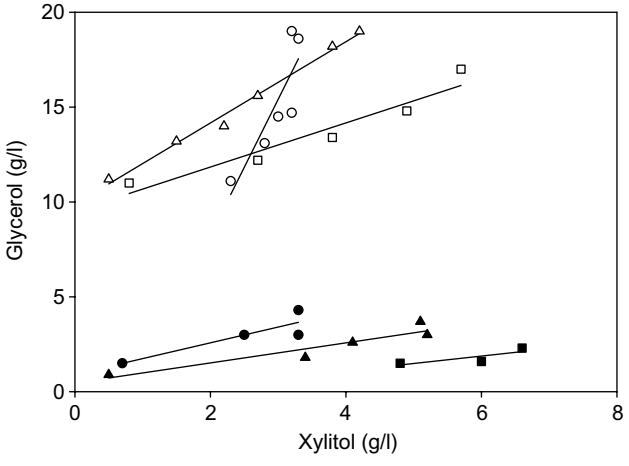

Fig. 2. Glycerol formation as a function of xylitol production in strains TMB (□), CR1 (○) and CR2 (△). Hollow symbols represent fermentations in the pre-fermentation effluent and filled symbols fermentations in the post-fermentation effluent.

Evaluation of recombinant strains in lignocellulosic hydrolysates is crucial since fuel ethanol production will be based on these substrates rather than laboratory media. The present study shows that a lignocellulosic hydrolysate influences the performance of yeast negatively, both due to its response towards a variety of degradation products in the hydrolysate and due to the fact that the sugar content might be high (and stress inducing). The difference in xylitol yield found in the two hydrolysates investigated showed that the cellular redox balances were influenced by the constituents of the lignocellulosic hydrolysates. Even though one of the metabolic engineering concepts tested involved modified ammonium assimilation, it was demonstrated that this strategy could also be successful in a medium without ammonia (as in the post-fermentation effluent).

Acknowledgements

We thank Dr. Bruno Jarry, Orsan-Amylum for kindly providing the substrates; pre- and post-fermentation effluents from a wheat to starch plant. The work on fermentation of lignocellulosic hydrolysates at the Center for Microbial Biotechnology at the Technical University of Denmark was supported under the European Commission Framework V, contract no. QLK3-CT-1999-00080.

References

Boland, T., 1995. Canadian ethanol industry seeks balanced expansion. The Energy Independent 1, 4–5.

Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P., Scheffers, W.A., 1984. NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. Appl. Microbiol. Biotechnol. 19, 256–260.

Busturia, A., Lagunas, R., 1986. Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 132, 379–385.

Eliasson, A., Christensson, C., Wahlbom, C.F., Hahn-Hägerdal, B., 2000. Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL1 XYL2* and *XKS1* in mineral media chemostat cultivations. Appl. Environ. Microbiol. 66, 3381–3386.

Entian, K.-D., Kötter, P., 1998. Yeast mutant and plasmid collections. In: Brown, A.J.P., Tuite, M.F. (Eds.), Methods in Microbiology. Academic Press, London, pp. 431–449.

Hahn-Hägerdal, B., Wahlbom, C.F., Gardonyi, M., van Zyl, W.H., Cordero Otero, R.R., Jönsson, L.J., 2001. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv. Biochem. Eng./Biotechnol. 73, 53–84.

Hamacher, T., Becker, J., Gárdonyi, 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.

Ho, N.W.Y., Chang, S.-F., 1989. Cloning of yeast xylukinase gene by complementation of *E. coli* and yeast mutations. Enzyme Microb. Technol. 11, 417–421.

Hunwick, R.J., 1980. The separation of carbohydrate and protein from wheat for the production of energy and food: conventional and proposed processes. Food Technol. Australia 32, 458–466.

Jeppsson, M., Johansson, B., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2002. Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl. Environ. Microbiol. 68, 1604–1609.

Jeppsson, M., Johansson, B., Ruhdal-Jensen, P., Hahn-Hägerdal, B., Gorwa-Grauslund, M., 2003a. The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. Yeast 20, 1263–1272.

Jeppsson, M., Träff, K., Johansson, B., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2003b. Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. FEMS Yeast Res. 3, 167–175.

Kotyk, A., 1967. Properties of the sugar carrier in baker's yeast. 2. Specificity of transport. Folia Microbiol. 12, 121–131.

Kötter, P., Ciriacy, M., 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 38, 776–783.

Magee, R.J., Kosaric, N., 1985. Bioconversion of hemicellulosics. Adv. Biochem. Eng. 32, 61–93.

Martín, C., Galbe, M., Wahlbom, C.F., Hahn-Hägerdal, B., Jönsson, L.J., 2002a. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using xylose-utilising *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 31, 274–282.

Martín, C., Galbe, M., Nilvebrant, N.-O., Jönsson, L.J., 2002b. Comparison of the fermentability of enzymatic hydrolysates of sugarcane bagasse pretreated by steam explosion using different impregnating agents. Appl. Biochem. Biotechnol. 98–100, 699–716.

Martín, C., Jönsson, L.J., 2003. Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. Enzyme Microb. Technol. 32, 386–395.

Mielenz, J.R., 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4, 324–329.

Miller, S.M., Magasanik, B., 1990. Role of the NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. 172, 4927–4935.

Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J., 2000. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. Yeast 16, 463–474.

Olsson, L., Hahn-Hägerdal, B., 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol. 18, 312–331.

Olsson, L., Nielsen, J., 1997. On-line and in situ monitoring of biomass in submerged cultivations. TIBTECH 15, 517–522.

Olsson, L., Nielsen, J., 2000. The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: utilization of industrial media. Enzyme Microb. Technol. 26, 785–792.

Özcan, S., Johnston, M., 1999. Function and regulation of the yeast hexose transporters. Microbiol. Molec. Biol. Rev. 63, 554–569.

Palmqvist, E., Hahn-Hägerdal, B., 2000a. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour. Technol. 74, 17–24.

Palmqvist, E., Hahn-Hägerdal, B., 2000b. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33.

Roca, C., Nielsen, J., Olsson, L., 2003. Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl. Environ. Microbiol. 69, 4732–4736.

Roca, C., Haack, M.B., Olsson, L., 2004. Engineering of carbon catabolite repression in recombinant xylose fermenting *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 63, 578–583.

Sosulski, K., Sosulski, F., 1994. Wheat as a feedstock for fuel ethanol. Appl. Biochem. Biotechnol. 45-6, 169–180.

Swinnen, J.F., Jacobs, P.A., Uytterhoeven, J.B., Tollens, E.F., 1988. An economic and simulation approach for renewable natural resources: ethanol production in the EEC: a case study. Biomass 15, 143–154.

Tantirungkij, M., Nakashima, N., Seki, T., Yoshida, T., 1993. Construction of xylose-assimilating *Saccharomyces cerevisiae*. J. Ferment. Bioeng. 75, 83–88.

Walfridsson, M., Anderlund, M., Bao, X., Hahn-Hägerdal, B., 1997. Expression of different levels of enzymes from the *Pichia stipitis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilization. Appl. Microbiol. Biotechnol. 48, 218–224.

Walsh, M.C., Smits, H.P., Scholte, M., van Dam, K., 1994. Affinity of glucose transport in *Saccharomyces cerevisiae* is modulated during growth on glucose. J. Bacteriol. 176, 953–958.

Wiselogel, A., Tyson, S., Johnsson, D., 1996. Biomass feedstock resources and composition. In: Wyman, C.E. (Ed.), Handbook on Bioethanol: Production and Utilization. Taylor and Francis, USA, pp. 105–118.

Wyman, C.E., 1996. Ethanol production from lignocellulosic biomass: overview. In: Wyman, C.E. (Ed.), Handbook on Bioethanol: Production and Utilization. Taylor and Francis, USA, pp. 1–18.

Zaldivar, J., Nielsen, J., Olsson, L., 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol. 56, 17–34.

Zaldivar, J., Borges, A., Johansson, B., Smits, H.P., Villas-Bôas, S.G., Nielsen, J., Olsson, L., 2002. Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 59, 436–442.