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ScienceDirect
Bioresource Technology 98 (2007) 1767–1773
BIORESOURCE TECHNOLOGY

Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors

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Received 22 March 2006; received in revised form 3 June 2006; accepted 3 July 2006
Available online 24 August 2006

Abstract

Adaptation of a xylose-utilizing genetically engineered strain of *Saccharomyces cerevisiae* to sugarcane bagasse hydrolysates by cultivation during 353 h using medium with increasing concentrations of inhibitors, including phenolic compounds, furaldehydes and aliphatic acids, led to improved performance with respect to ethanol production. The remaining xylose concentration in the medium at the end of the cultivation was 5.2 g l^{-1}, while it was 11 g l^{-1} in the feed, indicating that approximately half of the xylose was consumed. The performance of the adapted strain was compared with the parental strain with respect to its ability to ferment three bagasse hydrolysates with different inhibitor concentration. The ethanol yield after 24 h of fermentation of the bagasse hydrolysate with lowest inhibitor concentration increased from 0.18 g g^{-1} of total sugar with the non-adapted strain to 0.38 g g^{-1} with the adapted strain. The specific ethanol productivity increased from 1.15 g ethanol per g initial biomass per h with the non-adapted strain to 2.55 g g^{-1} h^{-1} with the adapted strain. The adapted strain performed better than the non-adapted also in the two bagasse hydrolysates containing higher concentrations of inhibitors. The adapted strain converted the inhibitory furaldehydes 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) at a faster rate than the non-adapted strain. The xylose-utilizing ability of the yeast strain did not seem to be affected by the adaptation and the results suggest that ethanol rather than xylitol was formed from the consumed xylose.

Keywords: Ethanol; Sugarcane bagasse; *Saccharomyces cerevisiae*; Adaptation; Fermentation inhibitors; Xylose utilization

1. Introduction

Ethanol is a possible alternative liquid fuel for vehicles. Traditional raw materials for ethanol production, such as sugarcane and maize, have the drawback that they serve as food and would represent a major part of the total production cost (Claassen et al., 1999). Lignocellulosic waste materials are abundant and represent a promising feedstock for industrial production of low-cost fuel ethanol (reviewed by Wheals et al. (1999)). Sugarcane bagasse represents one of the major lignocellulosic materials to be considered in most tropical countries since it has high carbohydrate and low lignin content, is readily available at the sugar mill site, and the cost for harvest, transport and storage has been borne by the sugar production (Triana et al., 1990; Pandey and Soccol, 2000). The use of bagasse for ethanol production would contribute to the diversification of the sugar industry, which is an urgent requirement for the sugarcane-based economies (Olguin et al., 1995; Gálvez, 2000).

Hydrolysis is required for the conversion of the polysaccharides in lignocellulose to fermentable sugars. However,

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0960-8524/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2006.07.021

during acid hydrolysis, compounds that inhibit fermenting microorganisms are formed as well (reviewed by Parajó et al. (1998); Palmqvist and Hahn-Hägerdal (2000); Hahn-Hägerdal et al. (2001)). Inhibitory compounds in lignocellulose hydrolysates include aromatic, primarily phenolic, compounds, furaldehydes, such as 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF), as well as aliphatic acids, such as formic, acetic and levulinic acid (Larsson et al., 1999). Removal or dilution of these compounds improves the fermentability of lignocellulose hydrolysates. Several different detoxification methods have been described and the effects on the chemical composition of lignocellulose hydrolysates have been investigated (Larsson et al., 1999). Although detoxification improves the fermentability of hydrolysates, it is for economical reasons desirable to limit the requirements for detoxification to a minimum (von Sivers et al., 1994). Adaptation of fermenting microorganisms to inhibitory hydrolysates is one of several possible strategies for dealing with inhibitor problems.

Saccharomyces cerevisiae (baker's yeast), the microorganism that has traditionally been used for producing alcoholic beverages, has several positive features for ethanol production from lignocellulose. However, a disadvantage associated with *S. cerevisiae* is that it cannot metabolize xylose, an abundant sugar in hydrolysates of hardwoods and agricultural residues (Jeffries and Shi, 1999; Hahn-Hägerdal et al., 2001). Metabolic engineering can be used for the development of recombinant *S. cerevisiae* strains able to ferment xylose (Stephanopoulos et al., 1998; Hahn-Hägerdal et al., 2001). Previous investigations on fermentation of bagasse hydrolysates with a genetically engineered xylose-utilizing *S. cerevisiae* strain showed that removal of inhibitors was necessary for achieving good fermentation performance (Martín et al., 2002a,b).

The aims of the present work were to adapt a genetically engineered xylose-utilizing strain of *S. cerevisiae* to enzymatic hydrolysates of sugarcane bagasse and to compare the fermentative performance of the adapted strain with that of the non-adapted parental strain using bagasse hydrolysates with different inhibitor content.

2. Methods

2.1. Yeast strain

The strain used in this study, TMB 3001, is a CEN.PK derivative that expresses xylose reductase and xylitol dehydrogenase from the chromosomally integrated *Pichia stipitis* genes *XYL1* and *XYL2*, respectively, and over-expresses the homologous *XKS1* gene encoding xylulokinase (Eliasson et al., 2000). The strain was transferred from −80 °C and maintained on YPD-agar plates containing 10 g/l yeast extract (Merck, Darmstadt, Germany), 20 g/l peptone (Difco, Detroit, MI, USA), 20 g/l glucose (BDH, Poole, UK), and 20 g/l agar–agar (Merck).

2.2. Media

For the adaptation procedure, five different media with increasing inhibitor concentration (Table 1) were prepared by mixing three different bagasse hydrolysates. The hydrolysates were prepared by H_2SO_4-catalysed steam pretreatment of sugarcane bagasse followed by enzymatic hydrolysis as previously described (Martín et al., 2002a). The pH of all media was adjusted to 5.5 with 2 M NaOH and the media were filter sterilized and enriched with $(NH_4)_2SO_4$, $MgSO_4 \cdot 7\,H_2O$, KH_2PO_4, vitamins and minerals (Verduyn et al., 1992). All chemicals used were purchased from Merck (Darmstadt, Germany) if not otherwise stated. A sterile solution of glucose (BDH, Poole, England) and xylose (Acros Organics, New Jersey, USA) was added to the adaptation media to final concentrations of 30 and 11 g l^{-1}, respectively.

For evaluation of the adapted strain, three different media were used (Table 1). One of the media consisted of hydrolysate that was not diluted and is hereafter referred to as Hydrolysate SA100 (sulfuric acid, 100%). The two other media were prepared by dilution of Hydrolysate SA100 to a concentration of 50% and 75% (v/v) and are hereafter referred to as Hydrolysate SA50 and SA75, respectively. The concentrations of glucose and xylose in

Table 1
Sugar and inhibitor concentrations in the media used for adaptation of the yeast strain to bagasse hydrolysates (Inoculum, initial batch, feed 1–3) and for evaluation of the adapted strain (SA50, SA75, SA100)

Medium	Glucose (g l^{-1})	Xylose (g l^{-1})	Furaldehydes[a] (g l^{-1})	Aliphatic acids[b] (g l^{-1})	Phenols[c] (g l^{-1})
Inoculum	30	11	0.7	2.5	1.5
Initial batch	30	11	1.0	3.9	2.2
Feed 1	30	11	1.5	5.4	2.2
Feed 2	30	11	2.1	6.2	2.0
Feed 3	30	11	3.4	8.7	2.3
SA50	25.8	5.9	2.2	5.0	1.4
SA75	25.5	5.3	3.8	7.6	2.1
SA100	25.4	4.9	4.5	10.1	2.8

[a] Furfural and HMF.
[b] Formic, acetic and levulinic acid.
[c] Total phenolics determined using the Folin-Ciocalteu method.

Hydrolysate SA50 and SA75 were adjusted to similar levels as in SA100. The sugar and inhibitor concentrations are shown in Table 1. With the exception of glucose and xylose, the hydrolysates used for evaluation were supplemented with the same nutrients and prepared in the same way as the adaptation media.

2.3. Adaptation of the yeast strain to bagasse hydrolysate

The yeast strain was transferred to an agar plate containing 50% (v/v) of a hydrolysate with low levels of inhibitors (Martín et al., 2002a), in addition to the normal components of the YPD medium. After a 72-h incubation at 30 °C, a loopful of cells was inoculated in a 250-ml baffled Erlenmeyer flask containing 100 ml of the "Inoculum medium" (Table 1) and incubated during 20 h at 30 °C with agitation (150 rpm) in an orbital incubator (Gallenkamp, Leicester, UK). Cell growth was monitored by turbidity measurements using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). The cells were harvested in the exponential phase by centrifugation (J-25I, Beckman, Fullerton, CA, USA) at 1200*g* for 10 min, washed once with 0.9% (w/v) NaCl and used for inoculation of the fermentor.

The cultivation was performed in a 150-ml homemade fermentor with magnetic stirring and automatic control of the temperature and pH (Radiometer A/S, Copenhagen, Denmark). The "Initial batch medium" (Table 1) was added to the fermentor, which was then inoculated to a final biomass concentration of 0.2 g l^{-1} (DW (dry weight)).

The fermentation started as a batch culture (20 h). When the glucose was depleted, the feed was started using "Feed 1" medium (Table 1). Fresh medium was continuously fed using a peristaltic pump (Easy-load II, Cole-Parmer Instrument Co., Barrington, IL, USA) with a dilution rate of 0.1 h^{-1}. The working volume was kept at 100 ml by continuous removal of fermentation broth with a peristaltic pump (P-1, Pharmacia Biotech, Uppsala, Sweden). When 50% of the medium had been consumed "Feed 2" medium was added to the remaining "Feed 1" without stopping the feed. When 50% of the new medium had been consumed the rest of the "Feed 2" was added. The "Feed 3" medium was added in the same way. Consequently, the inhibitor concentration in the feed increased steadily throughout the cultivation from 0.7 to 3.4 g/l and 2.5 to 8.7 g/l for furaldehydes and aliphatic acids, respectively. The cultivation continued for 353 h (including the batch culture). Samples for HPLC-analysis were taken twice a day.

2.4. Fermentation

The performance of the adapted strain, i.e. the strain which was isolated from the adaptation culture after 353 h, was evaluated in fermentations of bagasse hydrolysates. The fermentations were performed in 50-ml fermentation vessels sealed with rubber stoppers and with a working volume of 45 ml. The fermentation vessels were equipped with cannulas for carbon dioxide removal and sampling. Inoculation was made to a biomass concentration of approximately 0.2 g l^{-1} (DW). Incubation was performed at 30 °C with magnetic stirring and the fermentation continued for 48 h under aseptic conditions. Parallel fermentations using both the adapted and the non-adapted strains were carried out. Samples for HPLC-analysis were withdrawn after 0, 6, 12, 24, 36, and 48 h.

2.5. Analyses

All samples were filtered through a 0.20 μm filter and diluted prior to HPLC analysis. The analysis was performed using a Waters HPLC (Waters, Milford, MA, USA) using the EZChrom software (Scientific Software, Inc., San Ramón, CA, USA). Glucose, xylose, ethanol, xylitol, acetic acid, formic acid and levulinic acid were separated on an Aminex HPX-87 H column (Bio-Rad, Hercules, CA, USA) operating at 45 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1} and detection was performed with an RI-detector (Shimadzu RID-6 A, Kyoto, Japan).

Furfural, HMF and furfuryl alcohol were separated with a BioSil C18 column (Bio-Rad) using a UV-detector (Waters 2487 Dual Absorbance Detector) set at 230 nm and operating at room temperature. The mobile phase consisted of 40% (v/v) aqueous methanol (BDH), adjusted to pH 3 with concentrated hydrochloric acid and supplied at a flow rate of 0.6 ml min^{-1}.

The total content of phenolic compounds was quantified colorimetrically by using the Folin–Ciocalteu method (Singleton et al., 1999) and a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Vanillin (4-hydroxy-3-methoxybenzaldehyde) was used as the calibration standard. The dry weight of the cell mass was determined by filtering 5 ml of the samples through 0.45 μm pre-weighed filters (Gelman Sciences, Ann Arbour, MI, USA). The filtered cells were then washed with 15 ml ultra-pure water (Millipore) and dried in a microwave oven (Whirlpool, MI, USA) during 8 min at the power of 3.5 and weighed. OD (optical density) measurements of the inoculum were performed at 620 nm using the U-1100 spectrophotometer (Hitachi).

3. Results

The performance of the yeast strain during the adaptation process is displayed in Fig. 1. The glucose in the "Initial batch medium" was depleted readily while one third of the xylose was consumed during the 20 h that this stage lasted. After switching to a continuous feed of medium, the glucose concentration typically remained at a low level although retardation in the glucose consumption was observed occasionally. At the same time, a decrease in xylose consumption, ethanol formation and turbidity of the medium was observed. This indicates difficulties in the metabolic activity, which could be due to problems in the adaptation of the yeast strain to the increased toxicity

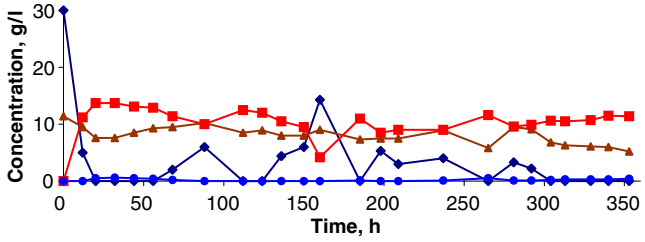

Fig. 1. Consumption of glucose (◆) and xylose (▲) and formation of ethanol (■) and xylitol (●) during the adaptation to bagasse hydrolysates.

of the medium. After 160 and 240 h, the feeding was therefore stopped temporarily and a batch regime was established for a few hours. When the turbidity measurements indicated that the cell growth was restored, the continuous feeding was restarted. Bacterial contamination was not detected during the cultivation.

The xylose consumption improved after switch to the batch mode and also during the last 60 h of the continuous feeding (Fig. 1). The xylose concentration at the end of the cultivation was only 5.2 g l^{-1}. Since the xylose concentration in the feed was 11 g l^{-1}, around 50% of the xylose was consumed.

The ethanol formation remained relatively constant during the cultivation, except during the already mentioned periods of retardation in glucose and xylose utilization, when the ethanol concentration also decreased proportionally to the decrease in sugar consumption. A steady increase in ethanol concentration (statistically confirmed using the software STATGRAPHICS Plus 2.1 for Windows) was observed at the end of the fermentation. The increase in ethanol concentration coincided with an increase in xylose consumption. Hardly any xylitol formation was seen (Fig. 1). The low xylitol formation together with that the increase in ethanol concentration coincided with the increase in xylose consumption suggest ethanol formation from xylose. There were no indications that the xylose-utilizing ability of the yeast strain was affected by the adaptation process.

The fermentation of Hydrolysate SA50 proceeded much faster when the adapted strain was used (Fig. 2A). The maximum ethanol yield in the fermentation with the adapted strain was reached 12 h earlier than in the fermentation with the non-adapted strain (Fig. 2A). After 24 h, the glucose was depleted in the fermentation with the adapted strain, whereas approximately half of the initial glucose was still not consumed by the non-adapted strain. The xylose consumption, albeit slow, was faster for the adapted than for the non-adapted strain (not shown). The ethanol yield on total sugar after 24 h and the specific and volumetric productivities were more than twice as high for the non-adapted strain. The biomass yield was higher for all fermentations with the adapted strain (Table 2).

Hydrolysate SA75 contained much higher concentrations of inhibitors than SA50, with regard to furaldehydes, aliphatic acids as well as phenolics (Table 1). Although this

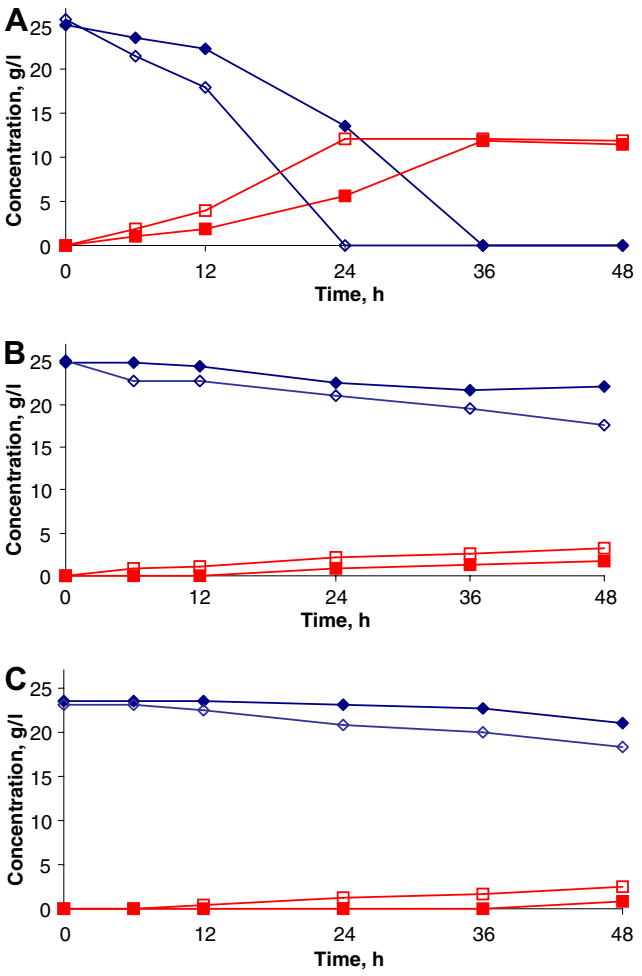

Fig. 2. Glucose consumption (◆)and ethanol formation (■) during batch fermentation of Hydrolysate SA50 (A), SA75 (B) and SA100 (C) with the adapted (open symbols) and the non-adapted (closed symbols) strain.

resulted in poor fermentability (Fig. 2B), the adapted strain performed better than the non-adapted. The specific productivity of the adapted strain was three times higher than that of the non-adapted strain, while the ethanol yield was two times higher. Ethanol formation was detected already after 6 h with the adapted strain but only after 24 h with the non-adapted strain.

Even if the ethanol yield and productivity were very modest, Hydrolysate SA100 was to some extent fermentable with the adapted strain, whereas the non-adapted strain gave almost no ethanol (Table 2). Ethanol fermentation was detected after 12 h for the adapted strain and after 48 h for the non-adapted strain (Fig. 2C).

The fermentation inhibitors furfural and HMF were converted faster by the adapted strain than by the non-adapted strain. For Hydrolysate SA50, both strains completely converted furfural to furfuryl alcohol within 24 h (Fig. 3), but the maximal conversion rate (0.150 g l^{-1} h^{-1}) achieved by the adapted strain was twice as high as that for the non-adapted strain (0.075 g l^{-1} h^{-1}). HMF was converted at a slower rate than furfural. For Hydrolysate SA75, 74% of the initial furfural and 40% of the initial

Table 2
Yields and productivities obtained in the evaluation of the adapted and the non-adapted strains

Strain	Hydrolysate concentration (%) (v/v)	$Y^{24}_{E/TS}$[a] (g g^{-1})	$Y^{max}_{E/TS}$[b] (g g^{-1})	Q_{max}[c] (g l^{-1} h^{-1})	q_{max}[d] (g g^{-1} h^{-1})	$Y_{X/TS}$[e] (g g^{-1})
Adapted	50	0.38	0.38	0.51	2.55	0.029
	75	0.07	0.11	0.09	0.45	0.012
	100	0.05	0.09	0.05	0.25	0.012
Non-adapted	50	0.18	0.38	0.23	1.15	0.020
	75	0.03	0.06	0.03	0.15	0.006
	100	0	0.03	0	0	0.009

[a] Ethanol yield on total sugar after 24 h.
[b] Maximum ethanol yield on total sugar.
[c] Maximum volumetric productivity.
[d] Maximum specific productivity.
[e] Biomass yield on total sugar.

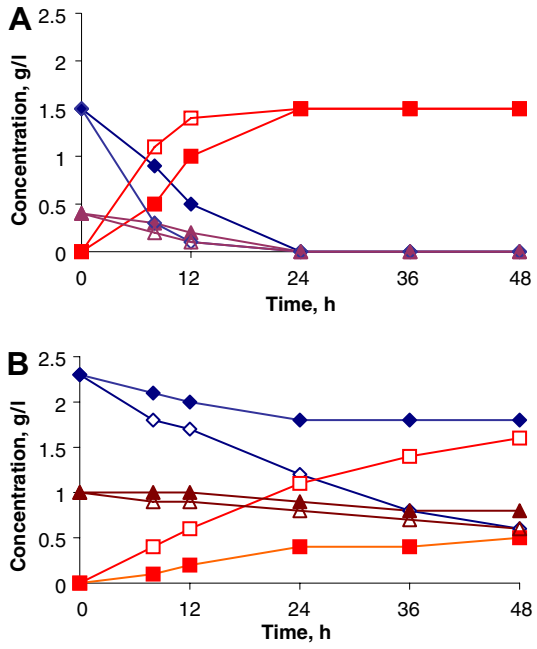

Fig. 3. Conversion of furfural (◆) and HMF (▲) and formation of furfuryl alcohol (■) during batch fermentation of Hydrolysate SA50 (A) and SA75 (B) with the adapted (open symbols) and the non-adapted (closed symbols) strain.

HMF were converted by the adapted strain after 48 h, whereas only 22% of the furfural and 20% of the HMF were converted with the non-adapted strain. Although only a minor part of the furaldehydes in Hydrolysate SA100 was converted, a clear difference in favor of the adapted strain was observed.

4. Discussion

During the adaptation to media with increasing concentration of bagasse hydrolysate, the yeast strain was tuned for performing the cell functions in presence of environmental stress in the form of high concentrations of toxic substances. Faster utilization of sugar and conversion of the furaldehydes by the adapted strain indicated that the ability of the yeast strain to tolerate and to transform inhibitors was improved.

The potential of the metabolically engineered xylose-utilizing *S. cerevisiae* strain TMB 3001 for the fermentation of sugarcane bagasse hydrolysates has previously been demonstrated (Martín et al., 2002a,b). However, the performance of TMB 3001 in undetoxified hydrolysates was considerably poorer than in detoxified hydrolysates (Martín et al., 2002b). Successful adaptation of yeasts to softwood prehydrolysates and rice straw hemicellulose hydrolysates suggests that this is a feasible approach to increase the resistance to fermentation inhibitors (Keller et al., 1998; Silva and Roberto, 2001). However, adaptation of xylose-utilizing recombinant strains of *S. cerevisiae* has to our knowledge not been reported previously.

Although a considerable part of the xylose was consumed at the end of the fermentation, the amount of xylitol produced was still very low, which is a prerequisite for achieving high ethanol yields. It is possible that the low xylitol level is related to the furaldehyde content of bagasse hydrolysates, which contain higher levels of furfural than HMF (Fig. 3) (Martín et al., 2002a,b). Xylitol production by genetically engineered *S. cerevisiae* strains carrying the genes encoding xylose reductase and xylitol dehydrogenase from *Pichia stipitis* has been attributed to a redox cofactor imbalance due to problems with the regeneration of NAD^+ (reviewed by Hahn-Hägerdal et al., 2001). Wahlbom and Hahn-Hägerdal (2002) found that the reduction of furfural to furfuryl alcohol is associated with oxidation of NADH, while the reduction of HMF to 5-hydroxymethylfurfuryl alcohol instead is associated with oxidation of NADPH. As a consequence, reduction of furfural would be expected to be beneficial by counteracting the redox cofactor imbalance causing xylitol production by contributing to the regeneration of NAD^+.

The concentrations of aliphatic acids and furaldehydes in Hydrolysate SA50 were within the range of the concentrations found in the adaptation medium when the cultivation had proceeded for around 200 h. Therefore, the yeast strain was adapted for around 150 h to a medium with an inhibitor concentration that was approximately equal to or higher than in Hydrolysate SA50. The inhibitor concentration of

Hydrolysate SA75 was equivalent to that of the adaptation medium used during the final hours of the cultivation. Hence, it is probable that the adaptation of the yeast strain to the highest concentrations was only in an initial phase when the continuous feed was stopped. The inhibitor concentration in Hydrolysate SA100 was higher than in any of the adaptation media. Therefore, the yeast strain was not properly adapted for dealing with the high inhibitor concentrations in Hydrolysate SA100, which was also apparent from the relatively poor performance in this hydrolysate.

Hydrolysate SA100 was produced under quite severe conditions and contained relatively high inhibitor concentrations, for instance 4.5 g l^{-1} furaldehydes and more than 10 g l^{-1} aliphatic acids. This can be compared with 1.5–1.6 g l^{-1} furaldehydes and 5.2–5.5 g l^{-1} aliphatic acids in two enzymatic hydrolysates of sugarcane bagasse (Martín et al., 2002a). A third hydrolysate containing 4.5 g l^{-1} furaldehydes and 7.4 g l^{-1} aliphatic acid was not fermentable under the conditions used (Martín et al., 2002a). Two other sugarcane bagasse hydrolysates (Martín et al., 2002b) contained 1.3–2.1 g l^{-1} furaldehydes and 4.8–5.9 g l^{-1} aliphatic acids. Reported concentrations of furaldehydes in different acid hydrolysates made from sugarcane bagasse varied between 0.2 and 3.6 g l^{-1}, while the concentration of acetic acid varied between 1.3 and 16.7 g l^{-1} (reviewed by Parajó et al., 1998). Thus, the concentrations of furaldehydes (2.2 g l^{-1}) and aliphatic acids (5.0 g l^{-1}) in SA50, the least inhibitory hydrolysate in this study, were still considerable. The concentration of the undissociated form of acetic acid should not exceed 5 g l^{-1} in the medium for growth to occur in an anaerobic batch fermentation of *S. cerevisiae* (Taherzadeh et al., 1997).

Less is known regarding the effect of the phenolic compounds. Treatment of sugarcane bagasse hydrolysates with laccase, an enzyme that specifically removes the phenolic inhibitors (Jönsson et al., 1998; Larsson et al., 1999) resulted in a major improvement of the fermentability (Martín et al., 2002b) showing that phenolic compounds greatly contributed to the inhibition. However, the hydrolysates in that study (Martín et al., 2002b) contained higher total concentration of phenolics, 4.1–4.5 g l^{-1}, than SA100, which contained a concentration (2.8 g l^{-1}) comparable with the concentration in the three hydrolysates studied by Martín et al. (2002a) (2.8–3.2 g l^{-1}). Martín et al. (2002a) also found that although the total concentration of phenolics in the three different bagasse hydrolysates examined was rather similar, the concentrations of separate phenolic compounds were widely different, which makes assessments of the toxicity based on the total concentration of phenolics difficult considering that different phenolic compounds have very different effect on *S. cerevisiae* (Ando et al., 1986; Larsson et al., 2000).

Fermentation with the adapted strain revealed a considerable increase in the ethanol productivity. This would decrease the length required for the fermentation, which should be beneficial from an economic point of view. The analyses suggested that the adapted strain was able to reduce toxic furaldehydes to the corresponding alcohols at a faster rate than the parental strain. It is possible that the use of even higher inhibitor concentrations in the adaptation medium would result in further improvements of the strain. The use of adapted strains or strains that have been genetically engineered to withstand high inhibitor concentrations (Larsson et al., 2001a,b) decreases the need for more extensive measures against problems with fermentation inhibitors, such as detoxification. In conclusion, the results clearly indicate the great potential in using adaptation as a tool for improving the tolerance of yeast strains to inhibitors in lignocellulose hydrolysates.

Acknowledgements

The Swedish National Energy Administration and the University of Matanzas are gratefully acknowledged for financial support.

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