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Bioresource Technology 292 (2019) 121922

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Strain-dependent variance in short-term adaptation effects of two xylose-fermenting strains of *Saccharomyces cerevisiae*

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ARTICLE INFO

Keywords:
Ethanol
Wheat straw hydrolysate
Inhibition
Short-term adaptation
Industrial *Saccharomyces cerevisiae* strains

ABSTRACT

The limited tolerance of *Saccharomyces cerevisiae* to the inhibitors present in lignocellulosic hydrolysates is a major challenge in second-generation bioethanol production. Short-term adaptation of the yeast to lignocellulosic hydrolysates during cell propagation has been shown to improve its tolerance, and thus its performance in lignocellulose fermentation. The aim of this study was to investigate the short-term adaptation effects in yeast strains with different genetic backgrounds. Fed-batch propagation cultures were supplemented with 40% wheat straw hydrolysate during the feed phase to adapt two different pentose-fermenting strains, CR01 and KE6-12. The harvested cells were used to inoculate fermentation media containing 80% or 90% wheat straw hydrolysate. The specific ethanol productivity during fermentation was up to 3.6 times higher for CR01 and 1.6 times higher for KE6-12 following adaptation. The influence of physiological parameters such as viability, storage carbohydrate content, and metabolite yields following short-term adaptation demonstrated that short-term adaptation was strain dependent.

1. Introduction

In order to facilitate the hydrolysis of lignocellulosic material in second-generation bioethanol production it must be pretreated under harsh conditions due to its recalcitrance. This leads to the production of inhibitors that decrease the microbial performance (Klinke et al., 2004). This is an even greater problem when higher substrate concentrations are used in the process (Koppram et al., 2014). The three main groups of inhibitors produced during the pretreatment of lignocellulosic material are organic acids, furans, and phenolics, as reviewed by Jönsson et al. (2013) (Jönsson, Alriksson, and Nilvebrant 2013). The mechanisms governing inhibition are complex and encompass many metabolic pathways and regulatory mechanisms, as reviewed by Palmqvist and Hahn-Hägerdal (2000b).

Xylose metabolism by recombinant *Saccharomyces cerevisiae* strains suffers from low xylose uptake rates compared to that of glucose (Van Maris et al., 2006). Moreover, xylose uptake rates have been shown to be reduced in the presence of glucose as the transporters involved have a higher affinity for glucose than for xylose (Kötter and Ciriacy, 1993; Meinander and Hahn-Hägerdal, 1997). This reduction in xylose uptake rate has been reported to lead to a lower capacity for ATP regeneration during pentose consumption (Bellissimi et al., 2009). This has also been confirmed in a metabolome study by Matsushika et al. (2013), where low ethanol productivity was explained by an imbalance between glycolytic and pentose phosphate pathway intermediates, which in turn leads to a decrease in ATP/AMP and GTP/GMP ratios. Since many responses of *S. cerevisiae* to inhibitors, such as proton efflux (Eraso and Gancedo, 1987; Verduyn et al., 1990; Viegas and Sá-Correia, 1991) and repair and maintenance (Allen et al., 2010; Hadi and Rehman, 1989; Modig et al., 2002) require ATP, xylose metabolism will be more affected by inhibitor stress than glucose metabolism.

Yeast cells are propagated in a cultivation step to obtain a suitable amount for inoculation of the fermentation medium. In the case of baker's yeast (*S. cerevisiae*), propagation is usually performed in aerated fed-batch cultivation using sucrose from molasses as the carbon source (Rose and Vijayalakshmi, 1978). *S. cerevisiae* is known to ferment sugar to ethanol even under aerobic conditions if the specific growth rate exceeds the so-called critical specific growth rate (Pronk et al., 1996), which has been shown to be strain dependent (Van Dijken et al., 2000). Fed-batch mode is used for yeast propagation so that the specific growth rate can be maintained below the critical specific growth rate, preventing the production of ethanol, while ensuring a high biomass yield (Stewart, 2017).

Oxygen is required for the synthesis of unsaturated fatty acids and sterols by *S. cerevisiae* (Kirsop, 1974) and, therefore, for cell growth. Industrial fermentation is performed without aeration, allowing the

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https://doi.org/10.1016/j.biortech.2019.121922
Received 3 May 2019; Received in revised form 26 July 2019; Accepted 27 July 2019
Available online 30 July 2019

growth of only a few generations before sterols and fatty acids become limiting. Volumetric productivity of fermentations inoculated with lower cell concentrations has been shown to be more affected by the inhibitors in lignocellulosic hydrolysates than when higher initial cell mass concentrations are used (Palmqvist and Hahn-Hägerdal, 2000a).

Other important aspects regarding inoculum cultures are cell viability and vitality. Viability is defined as the ability to reproduce, whereas vitality describes the metabolic activity of a culture (Kwolek-Mirek and Zadrag-Tecza, 2014; Lloyd and Hayes, 1995). Glycogen and trehalose are two major storage carbohydrates affecting the viability and vitality of *S. cerevisiae* during propagation (Parrou and François, 2001). Glycogen is the major energy-storing carbohydrate in yeast (Quain et al., 1981), and is used by the cell during the lag phase to synthesize sterols, fatty acids, and trehalose. Therefore, it is desirable for inoculum cultures to contain elevated intracellular concentrations of glycogen that can be used for the energy-demanding synthesis of sterols and fatty acids (Quain et al., 1981). Glycogen is accumulated in yeast cells in response to nitrogen or carbon limitation (Parrou et al., 1999). Trehalose is a non-reducing disaccharide that plays a protective role against stresses such as high osmolarity, nutrient depletion, starvation, high and low temperatures, and elevated ethanol concentration (D'Amore et al., 1991). It has been found to accumulate in response to stress and has been shown to prevent protein misfolding and aggregation (Singer and Lindquist, 1998). Elevated levels of intracellular trehalose have been shown to maintain cell viability during the initial stages of fermentation, and thereby increase carbohydrate utilization rates (Guldfeldt and Arnfborg, 1998). However, intracellular trehalose availability has been found not to affect cell growth or fermentative capacity (Jørgensen et al., 2002).

Short-term adaptation effects on metabolism, as opposed to evolution caused by genetic mutations, have been observed in several studies where yeast was exposed to non-lethal concentrations of lignocellulosic hydrolysates during propagation (Alkasrawi et al., 2006; Nielsen et al., 2015; Zhang et al., 2019). Adapted cells showed higher viability, biomass production, and fermentation capacity during fermentation in a hydrolysate-containing medium, whereas the growth of unadapted cells was inhibited. Short-term adaptation has also been shown to enable growth in the presence of inhibitors at low pH (Narayanan et al., 2016). Nielsen et al. (2015) has also shown that short-term adaptation improves performance using simultaneous saccharification and co-fermentation of wheat straw hydrolysate (WSH). The cellular mechanisms underlying short-term adaptation are, however, still poorly understood.

The aim of this study was to investigate the effect of short-term adaptation of yeast to WSH during propagation, and to gain insight into the effects of strain background on adaptation. Two industrial xylose-metabolizing *S. cerevisiae* strains were compared. Cell propagation was performed in bioreactor fed-batch cultivations, and the viability and intracellular glycogen and trehalose contents of the propagated cells were determined. The fermentation efficiency was determined by quantifying the specific ethanol productivity during subsequent fermentation in media containing WSH.

2. Materials and methods

2.1. Raw material and pretreatment

The wheat straw used to produce the hydrolysate was grown near Køge, Denmark and was cut into lengths of ~80 mm using an RBS 260 straw shedder (Cormall, Denmark). The composition of the unpretreated wheat straw was determined after the material had been thoroughly dried and milled. The structural carbohydrates, lignin content, and extractives were determined using standardized procedures (Sluiter et al., 2010; Sluiter et al., 2008). The unpretreated wheat straw consisted of 39% (w/w) glucose, 23% (w/w) xylose, 2% (w/w) galactose, and 3% (w/w) arabinose equivalents and of 18% (w/w) lignin and 13% (w/w) extractives. The wheat straw was impregnated in a 0.2% (w/w) solution of sulfuric acid overnight. The resulting material was filter pressed to a dry matter content of 40% (w/w), and subsequently incubated in a steam pretreatment unit at 190 °C for 10 min, previously determined to be the optimal conditions (Linde et al., 2008). The steam-pretreated wheat straw slurry was thoroughly mixed and three representative samples were taken to determine the dry matter (15.1%) and water insoluble solids (WIS) content (10.5%). Soluble sugars, degradation products and total soluble sugars after acid hydrolysis were determined from the liquid fraction, using standardized National Renewable Energy Laboratory analytical procedures (Sluiter et al., 2006). The liquid fraction of the pretreated material contained 6.5 g L^{-1} glucose, 33.6 g L^{-1} xylose, 1.3 g L^{-1} formic acid, 4.2 g L^{-1} acetic acid, 0.1 g L^{-1} levulinic acid, 0.4 g L^{-1} 5-(hydroxymethyl)furfural (HMF), and 4.3 g L^{-1} furfural.

2.2. Enzymatic hydrolysis

In order to obtain the liquid WSH used in the experiments, the steam pretreated wheat straw slurry was first diluted to a WIS content of 10% (w/w). The enzyme cocktail Cellic Ctec 2 (Novozymes, Denmark) was added to the steam-pretreated wheat straw slurry at a concentration of 10 FPU g_{WIS}^{-1}. Enzymatic hydrolysis was performed in a stirred reactor at 45 °C, pH 4.8. Sugars and degradation products of the resulting WSH were determined using HPLC. The WSH contained 68.8 g L^{-1} glucose, 36.4 g L^{-1} xylose, 1.2 g L^{-1} formic acid, 4.7 g L^{-1} acetic acid, 0.6 g L^{-1} HMF, and 3.0 g L^{-1} furfural.

2.3. Microorganisms

The industrial *S. cerevisiae* strains used in this study, CR01 and KE6-12, were kindly provided by Taurus Energy AB, Sweden. These strains harbor the xylose-utilization genes Xyl1 (xylose reductase) and Xyl2 (xylitol dehydrogenase) from *P. stipitis* and overexpress the endogenous XKS1 (xylulokinase) gene. Both strains have also been subjected to evolutionary engineering to improve their xylose fermentation efficiency and lignocellulosic inhibitor tolerance. They were stored at −80 °C in a 30% (w/w) glycerol solution.

2.4. Cultivation

2.4.1. Seed cultivation

Before propagation, the frozen cell stock solutions were thawed and grown for 24 h in synthetic minimal medium containing 20 g L^{-1} glucose at pH 6.0. Other components in this medium were added at concentrations found to be optimal by Verduyn et al. (1992), with the exception of ammonium sulfate, which was replaced by 2.3 g L^{-1} urea. Incubation was performed at 30 °C on an orbital shaker (IKA, Germany) at 200 rpm (orbital diameter: 20 mm) in 250 mL shake flasks with a working volume of 50 mL.

2.4.2. Propagation

Aerobic, fed-batch propagation was performed in 3.6 L bioreactors (Infors, Switzerland). The batch medium consisted of 30 g L^{-1} sucrose (from molasses, sterilized using 0.2 µm nylon membrane filters), 5 g L^{-1} ammonium sulfate, 3 g L^{-1} potassium phosphate, 0.5 g L^{-1} magnesium sulfate, 0.033 mg L^{-1} D-biotin, and 0.1 g L^{-1} polypropylene glycol 2000. The batch cultivations, with a working volume of 0.5 L, were maintained at 30 °C, and at pH 5.0 by the addition of 2 M potassium hydroxide solution throughout propagation. A cascade control was triggered when the dissolved oxygen in the reactor decreased below 40%. Agitation was gradually increased from 800 rpm to 1000 rpm, and the air flow into the reactor from 1 vvm to 2 vvm. After the sucrose in the batch medium had been depleted, the feed was controlled so as to maintain a specific growth rate of 0.05 h^{-1}. All feed solutions contained a total concentration of 130 g L^{-1} sucrose and/or glucose, and 28 g L^{-1} xylose. In order to adapt the yeast to the WSH, a feed solution was

prepared using 80% (w/w) WSH. A reference solution was also prepared consisting of sucrose from beet molasses with 28 g L^{-1}xylose. Both feed solutions were supplemented with 28 g L^{-1} ammonium sulfate, 3 g L^{-1} potassium phosphate, 0.5 g L^{-1} magnesium sulfate, 0.033 mg L^{-1} D-biotin, and 0.1 g L^{-1} polypropylene glycol 2000. The feed solutions were pumped into the reactor at an exponentially increasing rate, until the batch working volume had doubled, thereby diluting the concentration in the feed solution to 40% (w/w) WSH. (The calculation of the feed rate is described below.) Samples were collected for analysis at the start and end of the feed phase, and cell dry weight (CDW) was determined at the end of the feed phase.

2.4.3. Cell harvest

Cells were harvested for inoculum by centrifugation (3800xg, 5 min), followed by washing with 9 g L^{-1} sterile sodium chloride (NaCl) solution. The cell pellets were then resuspended in the fermentation medium.

2.4.4. Fermentation

Batch fermentation was performed in 500 mL screw-top shake flasks (Duran, Germany), with a one-way valve connected to the cap (Eppendorf, Germany) to allow for carbon dioxide release. Another connection allowed for sterile sampling through a swabable valve. The working volume for fermentation was 200 mL. The fermentation medium contained 80% (w/w) or 90% (w/w) WSH supplemented with 2.3 g L^{-1} urea, 3 g L^{-1} potassium phosphate, 0.5 g L^{-1} magnesium sulfate, and 0.033 mg L^{-1} D–biotin. The fermentation broth was inoculated with 1 g_{CDW} L^{-1} of yeast, and incubated on an orbital shaker at 150 rpm, at 30 °C, for 48 h. The weight loss of the flask and contents (due to carbon dioxide release) was monitored and was used to determine the progress of fermentation over time. Samples were taken for the analysis of cell viability and CDW at 24 and 48 h.

2.5. Analytical methods

2.5.1. Cell density measurements

The optical density was determined by measuring the absorbance of the cell culture at a wavelength of 600 nm (OD_{600nm}) using a Genesys 20 spectrophotometer (Thermo Scientific, USA). The OD obtained from filtered samples was subtracted to compensate for the background color of the medium. CDW was determined by filtering appropriate volumes (containing a minimum of 10 mg_{CDW} and a maximum of 40 mg_{CDW} on the filter) of cell culture through a pre-dried and weighed 0.45 µm polyethersulfone membrane (Sartorius, Germany). The filters containing samples were washed with deionized water and dried again using a microwave oven at a power output of 385 W for 15 min, before final weighing.

2.5.2. Metabolite and inhibitor analysis

The concentrations of extracellular metabolites, sugars, and inhibitors were determined by HPLC, using a refractive index detector (Jasco, Italy). Measurements were performed on filtered samples (0.2 µm nylon membrane filters, VWR, USA). Glucose, xylose, arabinose, formic acid, acetic acid, HMF, and furfural were separated using a Rezex ROA-Organic Acid H^+ column at a flow rate of 0.8 mL min^{-1}, at 80 °C, using 5 mM sulfuric acid solution as eluent. Sucrose, fructose, mannose, and galactose were separated using a Rezex RPM Monosaccharide Pb^+ column at a flow rate of 0.6 mL min^{-1}, at 85 °C, using Milli-Q water as eluent. Both columns were purchased from Phenomenex (USA).

2.5.3. Intracellular glycogen and trehalose measurements

The intracellular glycogen and trehalose contents were measured using the Glycogen Assay Kit (Abnova, Taiwan) and the Trehalose Assay Kit (Megazyme, Ireland). Samples were taken at the start (15 h) and end of the feed phase (52 h). The samples were prepared for assaying by washing the cells in 9 g L^{-1} NaCl solution and subsequently resuspending the cell pellet in 20 mM sodium acetate buffer (pH 4.8). The washing and buffer solutions were cooled, and the cells were continually kept on ice and centrifuged at 0 °C. After resuspension in the buffer, the cells were frozen using liquid nitrogen, and stored at −80 °C until analysis. Prior to assaying, the samples were inactivated by boiling for 10 min. The cells were then lysed using 1 g g_{CDW}^{-1} borosilicate glass beads (diameter: 3 mm, Sigma Aldrich, USA) in a TissueLyser (Qiagen, Germany) for 10 min.

2.5.4. Viability measurements

Cell samples were centrifuged (3800xg, 5 min) and resuspended in 9 g L^{-1} NaCl solution to avoid interference from autofluorescence of the WSH during imaging. The cells were then stained with propidium iodide (6 mg L^{-1}) and incubated for 5 min at room temperature. The propidium ion is excluded by the intact membrane of viable cells and thus propidium iodide exclusively stains cells that have irreparable membrane damage (i.e. non-viable cells). The stained cells were imaged on an inverted fluorescence microscope using both bright field and red fluorescent filters and were counted using Image-Pro Plus 6 software (Media Cybernetics, USA). At least 100 cells were imaged for each sample.

2.6. Calculations

2.6.1. Feed rate

The feed rate [L h^{-1}] for propagation was calculated using Equation (1):

$$F(t) = \frac{\mu_s S_i V(t_0)}{S_F} \exp(\mu_s t) \quad (1)$$

where μ_s is the desired, constant specific growth rate during the feed phase [h^{-1}], S_i is the concentration of sucrose at the start of the batch phase [g L^{-1}], $V(t_0)$ is the working volume of the culture when starting the feed [L], S_F is the concentration of sucrose in the feed solution [g L^{-1}], and t is the time that has expired since starting the feed.

2.6.2. Biomass yield

Specific biomass yields [g g^{-1}] were calculated using Eq. (2):

$$Y_{x/s} = \frac{c_x}{c_{s,0} - c_s} \quad (2)$$

where c_s is the residual concentration of sugars at time t [g L^{-1}], $c_{s,0}$ is the concentration of sugars at the time of inoculation [g L^{-1}], and c_x is the concentration of biomass at a given time [g L^{-1}].

2.6.3. Ethanol yield

Ethanol yields [g g^{-1}] were calculated using Eq. (3):

$$Y_{e/s} = \frac{c_p}{c_{s,0} - c_{s,t}} \quad (3)$$

where $c_{s,t}$ is the concentration of sugars the yeast can convert at a given time [g L^{-1}], $c_{s,0}$ is the initital concentration of sugars the yeast can convert [g L^{-1}]; and c_e is the concentration of ethanol at a given time [g L^{-1}].

2.6.4. Specific ethanol productivity

The specific ethanol productivity [g_e $g_x^{-1} h^{-1}$] was calculated using Equation (4):

$$q_e = \frac{\Delta c_e}{(\frac{\Delta c_x}{\Delta t})} \quad (4)$$

where Δc_e is the difference in ethanol concentration over a given time period Δt [g L^{-1}], Δc_x is the difference in cellular biomass concentration over the same time period Δt [g L^{-1}]; and Δt is the duration of the time period [h].

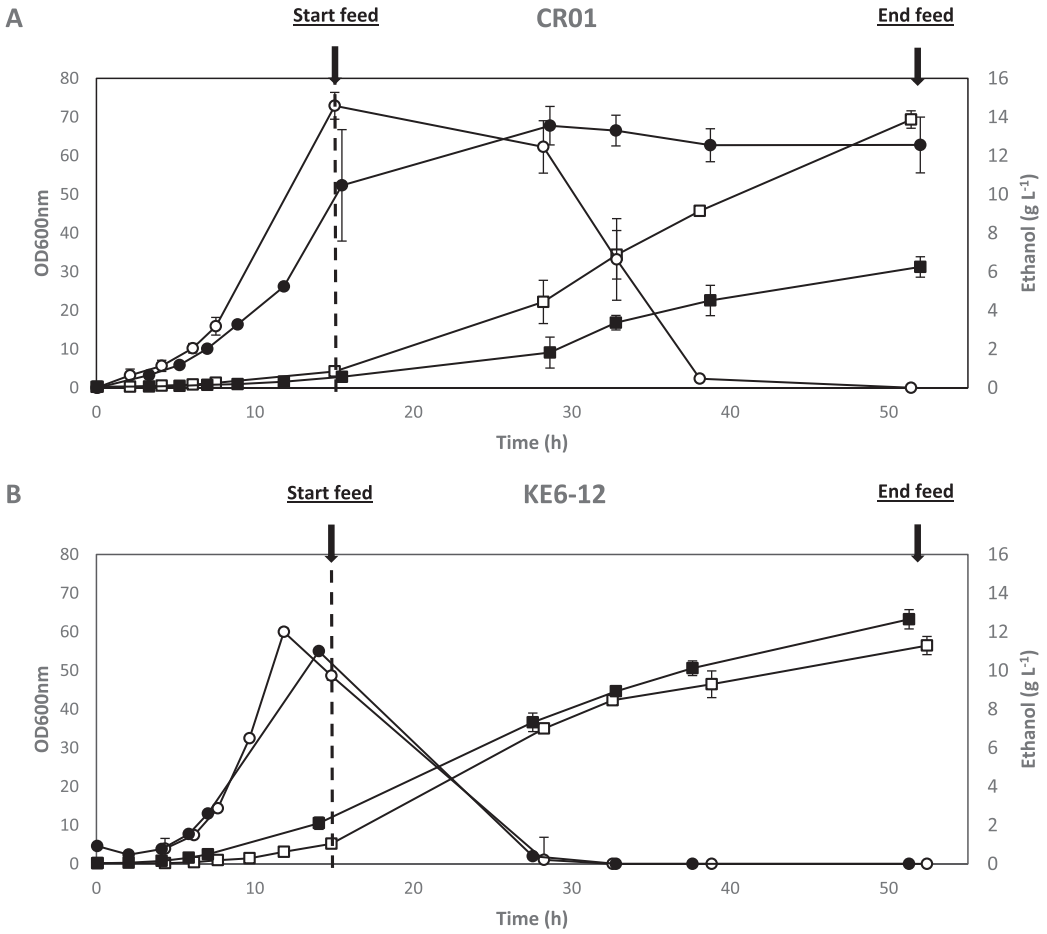

Fig. 1. Overview of fed-batch propagation of *S. cerevisiae* strains CR01 (A) and KE6-12 (B). Variation in OD_{600nm} (□, ■) and ethanol concentration (○, ●) as a function of time during propagation in the control medium (no wheat straw hydrolysate) (□, ○) and in the adaptation medium (containing 40% (w/w) wheat straw hydrolysate) (■, ●) during the feed phase. The dotted line indicates the start of the feed phase, which is preceded by a batch phase. Arrows indicate when samples were taken to determine CDW, intracellular glycogen and trehalose contents, and viability. Values given are the average of two biological replicates, and the error bars depict the standard error.

3. Results & discussion

3.1. Propagation of yeast in the presence of inhibitors

The propagation of the pentose-fermenting yeast strains, CR01 and KE6-12, in fed-batch was monitored throughout. The maximum specific growth rates (μ_{max}) in the batch phase were 0.23 ± 0.00 h^{-1} (CR01) and 0.29 ± 0.01 h^{-1} (KE6-12), respectively. The lower maximum specific growth rate of CR01 resulted in a 2 h longer batch phase than for KE6-12 (Fig. 1), indicating that the basic metabolic capacity of these strains is different.

After an initial batch phase (15 h), feeding was started using a solution containing sugar from beet molasses (control medium), or 80% (w/w) WSH supplemented with beet molasses (adaptation medium) and continued until the batch working volume had doubled, thereby diluting the WSH in the propagation medium to a final concentration of 40% (w/w) WSH. During cultivation of CR01 in the control medium, the cells completely consumed the ethanol during the feed phase (38 h; Fig. 1A) whereas adaptation in the WSH medium led to the accumulation of ethanol throughout the feed phase, to a final concentration of 12.6 g L^{-1} (Fig. 1A). In cultures with KE6-12, the ethanol produced during the batch phase was completely consumed at the end of the feed phase under both conditions, as expected (Fig. 1B). KE6-12 showed faster ethanol consumption than CR01 during the feed phase; the ethanol being completely consumed after 28 h (Fig. 1B). The addition of WSH to the feed phase had no effect on the ethanol consumption of KE6-12.

Ethanol accumulation during the cultivation of *S. cerevisiae* under aerobic conditions indicates overflow metabolism. It was expected that both strains would have a critical specific growth rate above 0.05 h^{-1}, which was the specific growth rate maintained during the feed phase. The observed ethanol accumulation when using CR01 shows that the presence of WSH reduced the critical specific growth rate to below 0.05 h^{-1}. Thus, CR01 was in a different metabolic state during propagation than KE6-12. This could indicate differences in adaptation mechanisms between the strains. It could also be that the inhibitors had a greater effect on the metabolism of CR01 than on KE6-12.

At the end of the feed phase in the adaptation medium, the OD_{600nm} of CR01 was 31.3, compared to 69.4 in the control medium (Fig. 1A). The lower cell concentration following adaptation indicates a significant decrease in cell mass yield. No such effect was observed on cell growth for KE6-12 (Fig. 1B).

The biomass yields at the end of the feed phase were lower in the adaptation medium than in the control medium for both strains (Fig. 2A). The decrease in biomass yield was 60% for CR01 and 22% for KE6-12. Nielsen et al. (2015) also reported a 20% decrease in cell mass yield after short-term adaptation of KE6-12 during fed-batch propagation. Inhibitors affect ATP net generation and thereby cell mass yield decreases as stated by Palmqvist and Hahn-Hägerdal (2000b). Nevertheless, the drastic decrease in biomass seen for CR01 (60%) was not

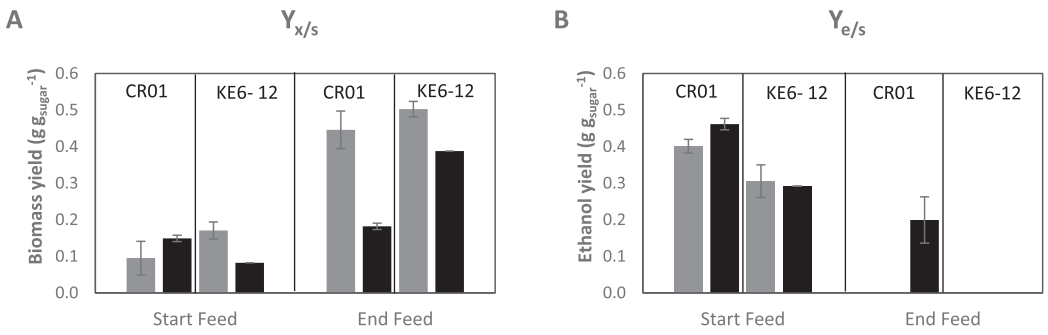

Fig. 2. Yields from total sugars during propagation of CR01 and KE6-12. Yields are shown for the production of Biomass (A) and Ethanol (B) on total sugars for propagation in the control medium containing no hydrolysate (■) and for propagation in the adaptation medium containing 40% (w/w) wheat straw hydrolysate (■) during the feed phase. Values given are the average of two biological replicates and the error bars depict the standard error.

expected. This reinforces the observation that the inhibitors had a greater negative affect on CR01 than on KE6-12.

During the batch phase, the ethanol yield of CR01 was higher than that of KE6-12 (0.43 g g^{-1} vs. 0.30 g g^{-1}, Fig. 2B). This difference can be explained by the high xylitol yields observed for KE6-12 (0.18 ± 0.03 $g_{xylitol}$ g_{xylose}^{-1}, data not shown), which were 7.6 times higher than for CR01 (0.02 ± 0.01 $g_{xylitol}$ g_{xylose}^{-1}, data not shown). The differences in the ethanol and xylitol yields further support the hypothesis that there are significant differences in the metabolism of these two strains.

The ethanol yield of CR01 in the adaptation medium at the end of the feed phase was 0.20 g g^{-1} (Fig. 2B), whereas it was below the detection limit in the control medium, and in both media with KE6-12 (Fig. 2B). This indicates that the decrease in biomass yield is mainly due to ethanol accumulation, and not the accumulation of other by-products. Indeed, no glycerol or acetate accumulation was observed when propagating CR01 in the adaptation medium (data not shown).

3.2. Intracellular glycogen and trehalose contents during propagation

The intracellular glycogen and trehalose contents at the start of the feed phase were all below 1 mg g_{CDW}^{-1} (Fig. 3), which was expected, as the cells grew at high specific growth rates during the batch phase. Yeast metabolism will channel uridine diphosphate (UDP) glucose to cell wall synthesis at high specific growth rates. However, when growth slows down, UDP glucose is instead channeled to glycogen production (Küenzi and Fiechter, 1972; Wilson et al., 2010). Thus, the glycogen content was expected to increase during the feed phase. The glycogen content was found to increase during the feed phase in all cases except for CR01 in the adaptation medium (Fig. 3A and B), which was below the detection limit of the assay (0.08 mg g_{CDW}^{-1}). One explanation of the lack of glycogen accumulation in CR01 under this condition is that at growth above the critical specific growth rate the cells experience glucose repression, and UDP glucose is therefore channeled away from glycogen synthesis. There were significant differences in the glycogen accumulation between propagation cultures in the control medium of CR01 (19.6 ± 6.4 $g_{glycogen}$ g_{CDW}^{-1}; Fig. 3A) and KE6-12 (3.9 ± 1.4 $g_{glycogen}$ g_{CDW}^{-1}, Fig. 3B).

At the end of the feed phase, a higher trehalose content was observed in CR01 cultures in the control medium, than in the adaptation medium (38.8 $g_{trehalose}$ g_{CDW}^{-1}, vs. 18.3 $g_{trehalose}$ g_{CDW}^{-1}, respectively; Fig. 3C). Trehalose accumulation by *S. cerevisiae* has been shown to be induced by stress but also depends on growth conditions (D'Amore et al., 1991; De Virgilio et al., 1993). Therefore, it was expected that the exposure of *S. cerevisiae* to inhibitors would cause the accumulation of trehalose, which is not what is observed for CR01. However, a 1.3-fold increase in trehalose content was observed during the adaptation of KE6-12 compared to the control medium (28.9 $g_{trehalose}$ g_{CDW}^{-1}vs. 50.7 $g_{trehalose}$ g_{CDW}^{-1}, respectively; Fig. 3D). Nielsen et al. (2015) reported a decrease in trehalose content in KE6-12 using a similar setup. The relatively low cell mass yields reported by Nielsen et al. (2015) (0.24–0.21 $g_{biomass}$ g_{sugar}^{-1}), indicate that the cells may have been growing above the critical specific growth rate during propagation. A possible explanation of the decrease in trehalose content upon adaptation of CR01 is that there is a shortage of UDP glucose required to synthesize trehalose in the cells.

3.3. Fermentation with short-term adapted yeast

The fermentation performance of CR01 and KE6-12 and CR01, propagated in media with and without WSH, was evaluated in anaerobic, batch cultivations using media containing 80% (w/w) or 90% (w/w) WSH. The media compositions were chosen after screening different concentrations and finding that a 10% (w/w) difference in WSH allowed a suitable experimental range. Ethanol production started earlier with adapted CR01 than with the unadapted CR01 yeast (Fig. 4A) in the medium containing 80% (w/w) WSH. Similar behavior was seen in the medium containing 90% (w/w) WSH, where adapted CR01 started producing ethanol after 22 h, while unadapted CR01 did not produce any ethanol during the course of the experiment (48 h, Fig. 4B). Glucose consumption followed a similar pattern. In 80% (w/w) WSH medium the adapted CR01 yeast depleted the glucose after 26 h, compared to 43 h in the case of the unadapted CR01 (data not shown). In 90% (w/w) WSH medium, adapted CR01 depleted all the glucose in 43 h, whereas the unadapted CR01 did not deplete the glucose within 48 h (data not shown). During fermentation of 80% WSH after 48 h, 22% of xylose was consumed by unadapted cultures and 46% by adapted cultures of CR01 (Fig. 5). Using 90% WSH, adapted cultures of CR01 had consumed up to 57% of xylose after 48 h. These cultures produced similar biomass concentrations (Table 1).

Samples were not taken early enough to determine whether there was any difference in lag phase between the different yeast strains, in the two media tested. However, differences in the times at which ethanol production started and in the times at which glucose was depleted indicate that the adapted CR01 yeast had a shorter lag phase than the unadapted CR01. It is known that glycogen is utilized as a source of energy during the lag phase (Quain et al., 1981) and a high glycogen content thus may indicate a shorter lag phase. However, cultures of adapted CR01 had lower glycogen contents (Fig. 3A) than the unadapted yeast, while the fermentation performance was clearly improved.

In the case of KE6-12, no clear difference in ethanol production was observed between adapted cells and unadapted cells (Fig. 4C and D), or in glucose (data not shown) and xylose consumption (Fig. 5) in either fermentation medium, seemingly due to the fact that unadapted cultures already performed well under these conditions. Nielsen et al.

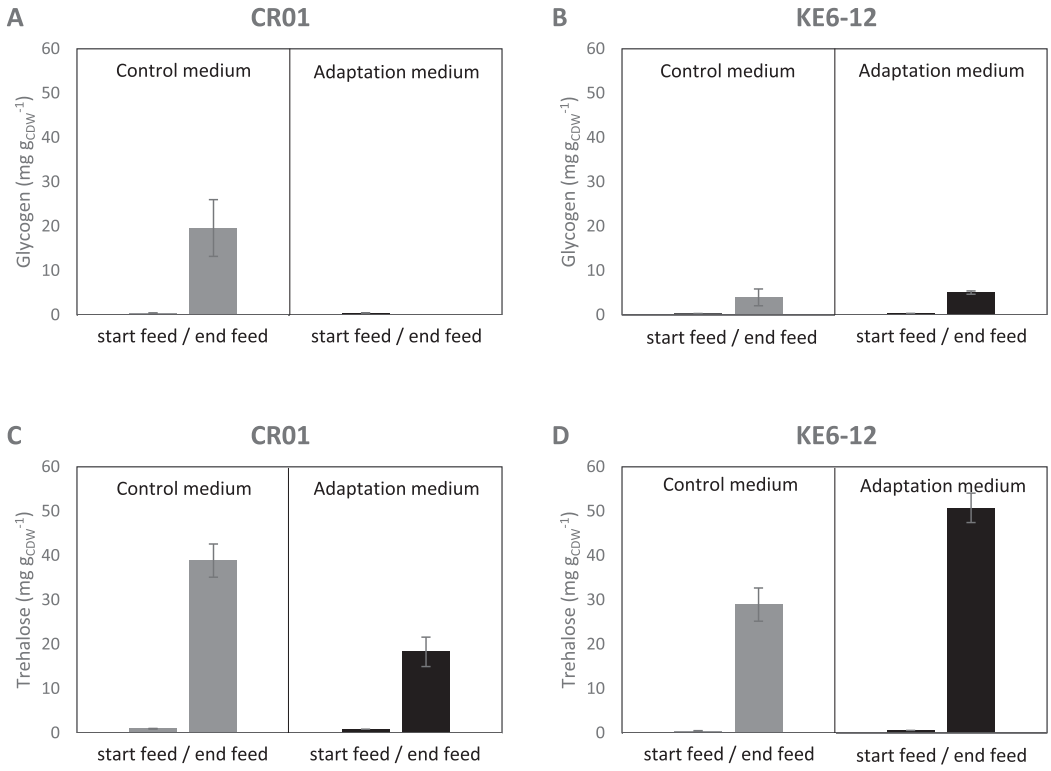

Fig. 3. Intracellular content of glycogen (A, B) and trehalose (C, D) during fed-batch propagation of CR01 (A, C) and KE6-12 (B, D). Samples were analyzed at the start of feeding and end of feeding as indicated in Fig. 1. Given values are for cultures propagated in the control medium (no adaptation, grey) and in the adaptation medium containing 40% (w/w) wheat straw hydrolysate (adaptation, black) during the feed phase. Values given are the average of two biological replicates, and the error bars depict the standard error.

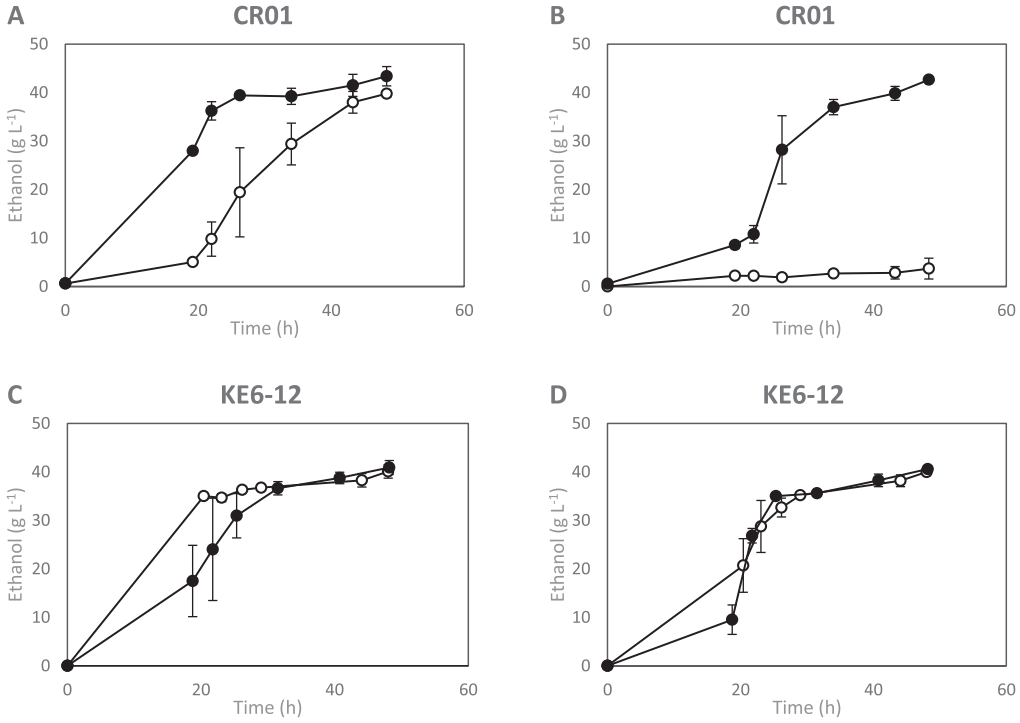

Fig. 4. Ethanol produced (g L^{-1}) during fermentation by CR01 (A, B) and KE6-12 (C, D) in medium containing 80% (w/w) (A, C) or 90% (w/w) (B, D) wheat straw, using unadapted cells (○) and cells adapted in 40% (w/w) wheat straw hydrolysate (●). Values given are the average of two biological replicates, and the error bars depict the standard error.

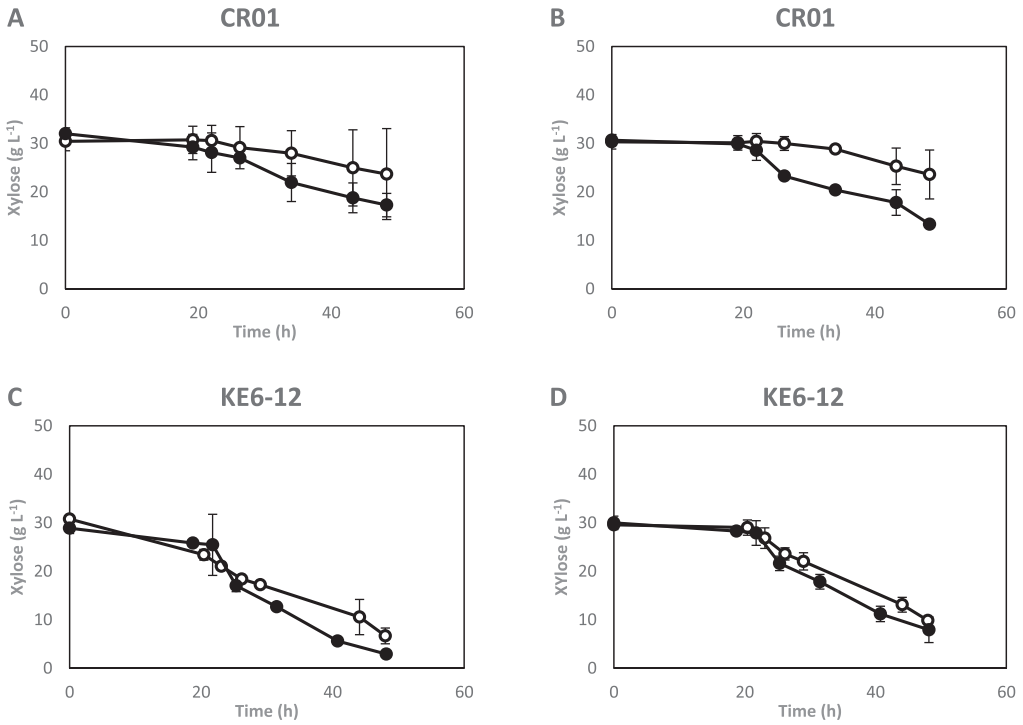

Fig. 5. Xylose (g L^{-1}) during fermentation by CR01 (A, B) and KE6-12 (C, D) in medium containing 80% (w/w) (A, C) or 90% (w/w) (B, D) wheat straw, using unadapted cells (○) and cells adapted in 40% (w/w) wheat straw hydrolysate (●). Values given are the average of two biological replicates, and the error bars depict the standard error.

(2015) reported a significant improvement in fermentation performance for KE6-12 following adaptation; however, the WSH used for adaptation in their study contained higher concentrations of acetic acid (2.2-fold), furfural (2.6-fold), and HMF (1.7-fold) than that used in the present work and thereby was more inhibitory to the unadapted cultures than the WSH used in the current study. This indicates that only in a highly inhibitory fermentation medium is it required to adapt the yeast to achieve improved fermentation performance using short–term adaptation.

3.4. Ethanol productivity during fermentation

The specific ethanol productivity (i.e. ethanol production rate normalized to cellular dry weight, $g_{ethanol}\ g_{CDW}^{-1}h^{-1}$) exhibited by CR01 during fermentation of 90% (w/w) WSH was higher for the adapted yeast (13.2 ± 0.1 $g_{ethanol}\ g_{CDW}^{-1}h^{-1}$) than for the unadapted yeast (3.7 ± 1.9 $g_{ethanol}\ g_{CDW}^{-1}h^{-1}$ (Fig. 6A). The KE6-12 strain showed a similar trend (17.1 ± 2.0 $g_{ethanol}\ g_{CDW}^{-1}h^{-1}$ vs. 9.7 ± 0.8 $g_{ethanol}\ g_{CDW}^{-1}h^{-1}$ (Fig. 6B). No clear differences were observed in fermentation with either strain in the fermentation medium containing 80% (w/w) WSH (Fig. 6A and B). The difference in performance of the yeasts in the two different fermentation media indicates that the effects of short-term adaptation are more pronounced in media with the higher inhibitor concentrations. Increased variability in ethanol productivity data for KE6-12 following adaptation is also reported by (Nielsen et al., 2015). The increase in ethanol productivity following short-term adaptation of strain CR01 was 3.6-fold in the 90% (w/w) WSH medium (Fig. 6A), while the KE6-12 strain showed only a 1.6-fold increase (Fig. 6B). The results of this study show that CR01has an inherently lower tolerance to WSH than KE6-12, which could explain why the effects adaptation were more pronounced in this strain. Mapping the genetic backgrounds of these strains may reveal why the molecular mechanisms governing short-term adaptation improve the fermentation performance of CR01 more than KE6-12.

The ethanol yields of the adapted yeast strains calculated after 48 h of fermentation showed a clear increase in the case of CR01 (0.37 $g_{ethanol}\ g_{sugar}^{-1}$), but not for KE6-12 (0.00 $g_{ethanol}\ g_{sugar}^{-1}$), compared to the unadapted strains (data not shown). Zhang et al. (2019) also reported no significant differences in ethanol yields following adaptation, however they did observe improved xylose conversion. Similarly, in the

Table 1

Strain		CR01				KE6-12			
Adaptation (% WSH)	Fermentation (% WSH)	$C_{x,0h}$[a] (g L^{-1})	$C_{x,48h}$[b] (g L^{-1})	V_{0h}[c] (%)	V_{48h}[d] (%)	$C_{x,0h}$[a] (g L^{-1})	$C_{x,48h}$[b] (g L^{-1})	V_{0h}[c] (%)	V_{48h}[d] (%)
0	80	1	3.2 ± 0.9	96 ± 3	95 ± 3	1	3.8 ± 0.1	98 ± 1	90 ± 5
40	80	1	3.5 ± 0.2	97 ± 1	95 ± 1	1	3.0 ± 0.3	97*	97*
0	90	1	0.9 ± 0.3	96 ± 3	86 ± 1	1	4.1 ± 0.5	97 ± 3	93 ± 0
40	90	1	3.1 ± 0.2	97 ± 2	94 ± 3	1	2.4 ± 0.3	97*	97*

[a] Cellular biomass concentration at inoculation of fermentation medium.
[b] Cellular biomass concentration at end of fermentation. Values shown given are averages of two biological replicates, and the error is the standard error.
[c] Viability at inoculation of fermentation medium. Values shown given are averages of two replicates.
[d] Viability at end of fermentation. Values shown given are averages of two replicates.
* No replicate.

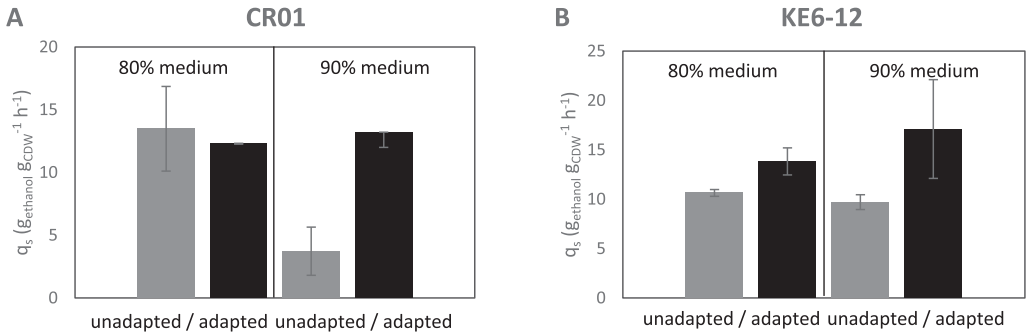

Fig. 6. Specific ethanol productivity (q_s) during fermentation of wheat straw hydrolysate by strains CR01 (A) and KE6-12 (B). Specific ethanol productivities were calculated over 48 h of fermentation and expressed as ($g_{ethanol}$ g_{CDW}^{-1} h^{-1}). Fermentation was performed in 80% and 90% (w/w) wheat straw hydrolysate medium. The performance of unadapted yeast (grey) was compared to that of adapted yeast (black). Values given are the average of four replicate fermentations (two fermentations per propagation), and error bars depict the standard deviation.

current work improved xylose consumption was observed following adaptation for CR01 (Fig. 5). The differences in ethanol yield and productivity between the two yeast strains are due to differences in biomass production during fermentation. Unadapted KE6-12 produced significantly more biomass during the fermentation of 90% (w/w) WSH than adapted KE6-12 (4.1 g L^{-1} vs. 2.4 g L^{-1}, Table 1).

3.5. Viability during hydrolysate fermentation

In the fermentation of 90% (w/w) WSH medium by the unadapted CR01 yeast, a decrease in overall viability, from 96% (0 h) to 86% (48 h), and a constant cell mass concentration were observed (Table 1). However, no clear decrease in viability was seen in the adapted CR01 yeast, while the cell mass concentration increased from 1.0 g L^{-1} (0 h) to 3.1 g L^{-1} (48 h). Considering the low ethanol productivity observed for the unadapted CR01 in 90% (w/w) WSH (Fig. 6), the viability of these cultures after 48 h was remarkably high. No other significant differences in viability were observed between the start and the end of fermentation (Table 1). One reason for the apparently high viability could be the use of the propidium iodide stain, which does not determine metabolic activity but membrane integrity. Thus, the actual percentage of metabolically active cells in fermentation may have been lower. Different methods of determining cell vitality have been shown to provide different information on the cellular metabolic state (Kwolek-Mirek and Zadrag-Tecza, 2014). The methods available to evaluate viability, especially in hydrolysates, are limited. Thus, expanding fermentation analysis to include the determination of vitality could be a valuable next step towards understanding short-term adaptation.

Higher cell mass concentrations were found following the fermentation of both media (80% and 90% WSH) by unadapted KE6-12 than by adapted KE6-12. In the 80% (w/w) WSH medium, the cell mass concentrations were 3.8 g L^{-1} for unadapted and 3.0 g L^{-1} for adapted KE6-12, while in the 90% (w/w) WSH medium, the corresponding cell mass concentrations were 4.1 g L^{-1} and 2.4 g L^{-1} KE6-12 (Table 1). As a similar amount of total sugar was consumed during fermentation by adapted and unadapted KE6-12 (data not shown), the specific ethanol production rate for unadapted KE6-12 was lower than for adapted KE6-12 (Fig. 6B).

4. Conclusions

The specific ethanol productivity during fermentation increased for both CR01 (3.6-fold) and KE6-12 (1.6-fold) strains after short-term adaptation during propagation. The critical specific growth rate of CR01 during propagation was shown to decrease below 0.05 h^{-1} in the presence of 40% (w/w) WSH, whereas this was not observed for KE6-12. The results of this study showed that both the strain background and the hydrolysate concentration in the fermentation medium influence the extent of the short-term adaptation effect. Gaining a better understanding of the effects on yeast of short-term adaptation to hydrolysates will help improve the performance and consistency of second-generation ethanol production. We believe that this study has provided the basis for further investigations into this topic.

Acknowledgements

This work was supported by The Swedish Energy Agency, project number 41252-1. LO and YN acknowledge Chalmers Area of Advance Energy for their support of lignocellulose fermentation at Industrial Biotechnology Division. Taurus Energy AB is thanked for the yeast strains supplied. Rakesh Koppram, Taurus Energy AB is thanked for fruitful discussions.

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