Furfural, 5-Hydroxymethyl Furfural, and Acetoin Act as External Electron Acceptors During Anaerobic Fermentation of Xylose in Recombinant *Saccharomyces cerevisiae*

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Abstract: The electron acceptors acetoin, acetaldehyde, furfural, and 5-hydroxymethylfurfural (HMF) were added to anaerobic batch fermentation of xylose by recombinant, xylose utilising *Saccharomyces cerevisiae* TMB 3001. The intracellular fluxes during xylose fermentation before and after acetoin addition were calculated with metabolic flux analysis. Acetoin halted xylitol excretion and decreased the flux through the oxidative pentose phosphate pathway. The yield of ethanol increased from 0.62 mol ethanol/mol xylose to 1.35 mol ethanol/mol xylose, and the cell more than doubled its specific ATP production after acetoin addition compared to fermentation of xylose only. This did, however, not result in biomass growth. The xylitol excretion was also decreased by furfural and acetaldehyde but was unchanged by HMF. Thus, furfural present in lignocellulosic hydrolysate can be beneficial for ethanolic fermentation of xylose. Enzymatic analyses showed that the reduction of acetoin and furfural required NADH, whereas the reduction of HMF required NADPH. The enzymatic activity responsible for furfural reduction was considerably higher than for HMF reduction and also in situ furfural conversion was higher than HMF conversion. *Biotechnol Bioeng* **78**: 172–178, 2002; DOI 10.1002/bit.10188
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INTRODUCTION

Xylitol is a major byproduct formed during anaerobic and oxygen-limited fermentation of xylose by recombinant *Saccharomyces cerevisiae* (Kötter and Ciriacy, 1993; Tantirungkij et al., 1993; Wahlbom et al., 2001; Walfridsson et al., 1995) and also by many natural xylose-fermenting eucaryotic organisms (du Preez, 1994; Jeffries, 1983; Skoog and Hahn–Hägerdal, 1988). This lowers the ethanol yield in ethanolic fermentation of lignocellulosic hydrolysates and reduces the economic feasibility of a future process (von Sivers and Zacchi, 1995).

Xylitol formation has been ascribed to the difference in cofactor preference for the two initial enzymes in the conversion of xylose (Bruinenberg et al., 1983a). The first enzyme, xylose reductase, which reduces xylose to xylitol (Chiang and Knight, 1960), can use both NADPH and NADH as a cofactor (Rizzi et al., 1988). The second enzyme, xylitol dehydrogenase (XDH), on the other hand, almost exclusively uses NAD^+ in the oxidation of xylitol into xylulose (Rizzi et al., 1989a). The less NADH consumed in XR reaction, the less NAD^+ is available for the XDH reaction and xylitol is excreted (Bruinenberg et al., 1983a).

Xylitol excretion in natural xylose-utilizing organisms has been circumvented by the addition of substances that act as electron acceptors (Alexander, 1986; Bruinenberg et al., 1983a; Ligthelm et al., 1989). Aldehydes and ketones such as acetaldehyde, acetoin, and acetone have been reduced to ethanol, butanediol, and 2-propanol, respectively, under simultaneous conversion of NADH to NAD^+ and concomitant decrease in xylitol excretion. Lignocellulosic hydrolysates contain aldehydes, and the most common non-aromatic ones are furfural and 5-hydroxymethylfurfural (HMF) (Heuser and Schott, 1923; Larsson et al., 1999). Furfural and HMF are reduced to furfuryl alcohol and 5-hydroxymethylfurfuryl alcohol (HMFAL), respectively, during yeast fermentation (Nemirovskii et al., 1989; Palmqvist et al., 1999a; Taherzadeh et al., 2000a; Villa et al., 1992). Furfural has been shown to act as an electron acceptor and decreased glycerol formation during glucose fermentation by *S. cerevisiae* (Palmqvist et al., 1999a). Furthermore, less xylitol was formed during fermentation of a lignocellulosic hydrolysate medium than during fermentation of a corresponding rich medium, which

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implies that substances present in the hydrolysate regenerate NAD^+ (Johansson et al., 2001).

In the present study, the quantitative response to the addition of electron acceptors was determined by calculating the intracellular fluxes (Wahlbom et al., 2001) during anaerobic xylose fermentation by recombinant *S. cerevisiae* TMB 3001 with and without acetoin present in the medium. Addition of acetaldehyde, furfural, and HMF was also investigated to study whether these compounds regenerated NAD^+ and lowered xylitol production. For interpretation of product formation, the cofactor preference was determined for four enzymes: acetaldehyde dehydrogenase, butanediol dehydrogenase, and the enzymatic activities responsible for the reduction of furfural and HMF, respectively.

MATERIALS AND METHODS

Organism, Medium, and Inoculum Preparation

The strain used in the experiments, *S. cerevisiae* TMB 3001, harbors genes for XR and XDH from *Pichia stipitis* and an extra copy of the endogenous gene for xylulokinase integrated into the chromosome, thereby ensuring stable expression of these enzymes (Eliasson et al., 2000).

In all fermentation experiments, a defined medium, including vitamins and trace elements, was used (Verduyn et al., 1990b). The medium was supplemented with ergosterol and unsaturated fatty acids, in the form of Tween 80 (Sigma, St. Louis, MO; Andreasen and Stier, 1953, 1954). Ergosterol and Tween 80 were dissolved in boiling 96% (v/v) ethanol to final concentrations of 0.01 g/L and 0.42 g/L, respectively. Xylose (50 g/L) and glucose (20 g/L) were used as carbon and energy sources. Antifoam (Dow Corning® Antifoam RD Emulsion, BDH Laboratory supplies, Poole, England) was added to a concentration of 0.5 mL per liter.

Precultures, 200 mL medium in 250 mL unbaffled shake-flasks, were incubated overnight at 30°C, 140 rpm in an orbital incubator (Gallenkamp INR-200, Leicester, UK). Bioreactors were inoculated with a cell mass concentration of 20 mg/L. Freshly distilled furfural was used throughout the study.

Cultivation Conditions

Batch fermentation was conducted anaerobically in computer-controlled glass bioreactors (Belach Bioteknik AB, Stockholm, Sweden) at 30°C with a stirring speed of 200 rpm. The working volume of the reactors was 800 mL, and the pH was automatically maintained at 5.5 with 3 *M* NaOH. Anaerobic conditions were ensured by continuous flushing with nitrogen containing less than 5 ppm O_2 (ADR class2 1A, AGA, Sweden) at a constant gas flow of 0.2 L/min controlled by mass flow meters (Bronkhorst HI-TECH, Ruurlo, The Netherlands). The off-gas condensers were cooled to 4°C.

Triplicate fermentation experiments were conducted for each of the three electron acceptors acetoin (Fluka AG, Buchs, Switzerland), furfural (ICN, Aurora, OH), and HMF (Sigma). The yields between triplicate fermentation experiments differed less than 15%. In each experiment, fermentation prior to the addition of electron acceptors served as control fermentation experiment.

Analyses

Glucose, xylose, xylitol, glycerol, acetate, and ethanol were analyzed by column liquid chromatography (CLC). A Gilson (Villiers-le-bel, France) CLC system equipped with an ion moderated partition chromatography column, Aminex HPX-87H (Bio-Rad, Hercules, CA), was used together with a Shimadzu (Kyoto, Japan) refractive index detector, RID-6A. The flow rate of the mobile phase consisting of 5 mM H_2SO_4 was set to 0.6 mL/min and the separation temperature to 45°C. The compounds were eluted during 26 min in the order given above.

Acetoin and 2,3-butanediol were analyzed on an Aminex HPX-87C (Bio-Rad) column using bidistilled water as mobile phase at a flow of 0.7 mL/min and 40°C. Analysis of furfural, furfuryl alcohol, and HMF were performed on a high-performance liquid chromatography system (Waters, Milford, MA) equipped with a BioSil-C18 column (Bio-Rad) using a UV detector (2487, Waters) operating at a wavelength of 220 nm for furfural and furfuryl alcohol and at 254 nm for HMF. The mobile phase consisted of 40% aqueous methanol supplied at a flow rate of 0.6 mL/min at room temperature. The relative standard deviation between two samples was below 1%.

The composition of the outgoing gas was continuously monitored by a carbon dioxide and oxygen monitor type 1308 (Brüel & Kjær, Copenhagen, Denmark) (Christensen et al., 1995) using photoacoustic and magnetoacoustic detection for CO_2 and O_2, respectively. The cell dry weight was determined by filtering a known volume of the culture broth through a 0.45-μm Supor membrane (Gelman Sciences, Ann Arbor, ML). After washing with three volumes of bidistilled water and drying in a microwave oven for 15 min at 900 W, the filter was weighed. The cell dry weight was determined in triplicate with a relative standard deviation of less than 2.5%.

Cells were collected after fermentation, washed once with distilled water and treated with yeast protein extraction solution (Y-PER™, Pierce, Rockford, IL) for 30 min at room temperature. The mixture was centrifuged at 4°C, 4000 g for min and the supernatant was used for enzymatic assays.

Acetaldehyde dehydrogenase (Bruinenberg et al., 1983b) and butanediol dehydrogenase (Bruinenberg

et al., 1983a) activity were determined in cells collected from fermentation experiments in which acetoin had been added. The enzymatic activities responsible for the reduction of furfural to furfuryl alcohol and HMF to HMFAL, respectively, were assayed using the same reagent concentrations as for assaying butanediol dehydrogenase, using 10 m*M* furfural and HMF, respectively, as substrate. Assays were performed in cells collected from fermentation where furfural and HMF, respectively, had been added. In the following, these enzymatic activities are called furfuryl alcohol dehydrogenase and HMFAL dehydrogenase, respectively.

A modified version of a previously developed metabolic flux model (Wahlbom et al., 2001) was used to calculate the intracellular fluxes during xylose utilization with and without addition of acetoin. Tricarboxylic acid (TCA) cycle reactions and reactions for protein, RNA, and polysaccharide synthesis were omitted because no TCA cycle intermediates were excreted into the medium and because no growth occurred after glucose depletion. Glucose consumption was not studied; Therefore, the reaction for glucose phosphorylation was also excluded. One reaction for the conversion of acetoin to butanediol using NADH as cofactor was included. The resulting modified metabolic flux model included 19 compounds over which a mass balance was made, 17 intracellular reactions and 7 reactions for compounds that were exchangeable with the medium. Seven extracellular fluxes were measured: the uptake rates of xylose and the formation of carbon dioxide, ethanol, xylitol, glycerol, acetate, and butanediol. The measured specific productivities and specific consumption rates were calculated from the slope obtained from regression analysis of the change of concentration of at least five time points divided by the biomass concentration. The square of the residuals (R^2) of the xylose and xylitol were above 0.995, whereas the corresponding values were 0.96, 0.92, and 0.99 for acetate, glycerol, and butanediol, respectively. Of the consumed acetoin, 97% was recovered as butanediol. All fluxes except ethanol and carbon dioxide were used as measured fluxes. The measured values for carbon dioxide production were compared with the calculated ones and agreed within 91–97%.

RESULTS

S. cerevisiae TMB 3001 was grown anaerobically on a mixture of glucose and xylose. Glucose was consumed first and when it was depleted, no further growth of biomass was detected. The biomass increased from the inoculum concentration of 20 mg/L to on average 1.9 g/L. The cells continued to consume the remaining xylose, and at least four samples were withdrawn before addition of acetoin (6 g/L), furfural (3 g/L), and HMF (1.5 g/L), respectively. The period of xylose metabolism prior to addition of electron acceptor served as control experiment.

Acetoin was used as a model electron acceptor because it had been shown to decrease xylitol formation in *Candida utilis* (Bruinenberg et al., 1983a) and *Pachysolen tannophilus* (Ligthelm et al., 1989) and could replace the dihydroxyacetone phosphate dehydrogenase reaction as a redox sink in anaerobic cultures of *S. cerevisiae* (Björkqvist et al., 1997). Figure 1 shows in μmol/g biomass/h the measured (xylose, xylitol, glycerol, acetate, and butanediol) and calculated fluxes (all intracellular fluxes and the production of carbon dioxide and ethanol) during fermentation of xylose only and after acetoin addition (Fig. 1). Without acetoin addition, there was an almost equal utilization of NADH/NADPH in the xylose reductase step and around half of the produced xylitol was excreted. The flow between ribulose 5-phosphate and xylulose 5-phosphate was very low, and only small amounts of glycerol and acetate were produced. The ethanol yield was around 0.62 moles of ethanol/moles of xylose, which is 37% of the theoretical maximum yield (1.67 moles of ethanol/mole of xylose). When acetoin was consumed together with xylose, no extracellular xylitol was formed, and the fraction NADPH used by XR was lower. The flux in the oxidative pentose phosphate pathway was markedly reduced. The formation of glycerol decreased, but the formation of acetate increased 8-fold, and the ethanol yield more than doubled to 1.35 moles of ethanol/mole of xylose. The influence of acetaldehyde was also investigated, but because of difficulties to accurately measure the concentration in the bioreactor, it was not further analyzed. Acetaldehyde did, however, also decrease the xylitol excretion and lead to an increased ethanol production (data not shown).

To investigate whether lignocellulosic hydrolysate components could also decrease the xylitol excretion, addition of furfural and HMF was also investigated. Before addition of furfural, xylitol was the main product and only small amounts of ethanol, acetate, and glycerol were formed (Fig. 2). The succinate concentration remained constant. The xylitol excretion ceased after the addition of furfural, which was stoichiometrically converted into furfuryl alcohol. There was an increase in acetate production from 0.6 g/L to 0.9 g/L. Also glycerol and succinate production increased, from 3.5 g/L to 4.2 g/L and from 0.18 g/L to 0.98 g/L, respectively. The concentration of ethanol, on the other hand, decreased slightly. Contrary to the conversion of acetoin to butanediol, the conversion of furfural to furfuryl alcohol was not linear (Fig. 2). Therefore, the intracellular metabolic fluxes could not be determined.

As opposed to the case with furfural, no decrease in xylitol excretion was observed after HMF addition (Fig. 3). HMF was consumed at a slower rate than furfural. Neither the oxidized nor the reduced product of HMF is commercially available, so these products could not be analyzed. There was an increase in glycerol and acetate production, but contrary to furfural, HMF had no effect on the ethanol or succinate production. Similar to fur-

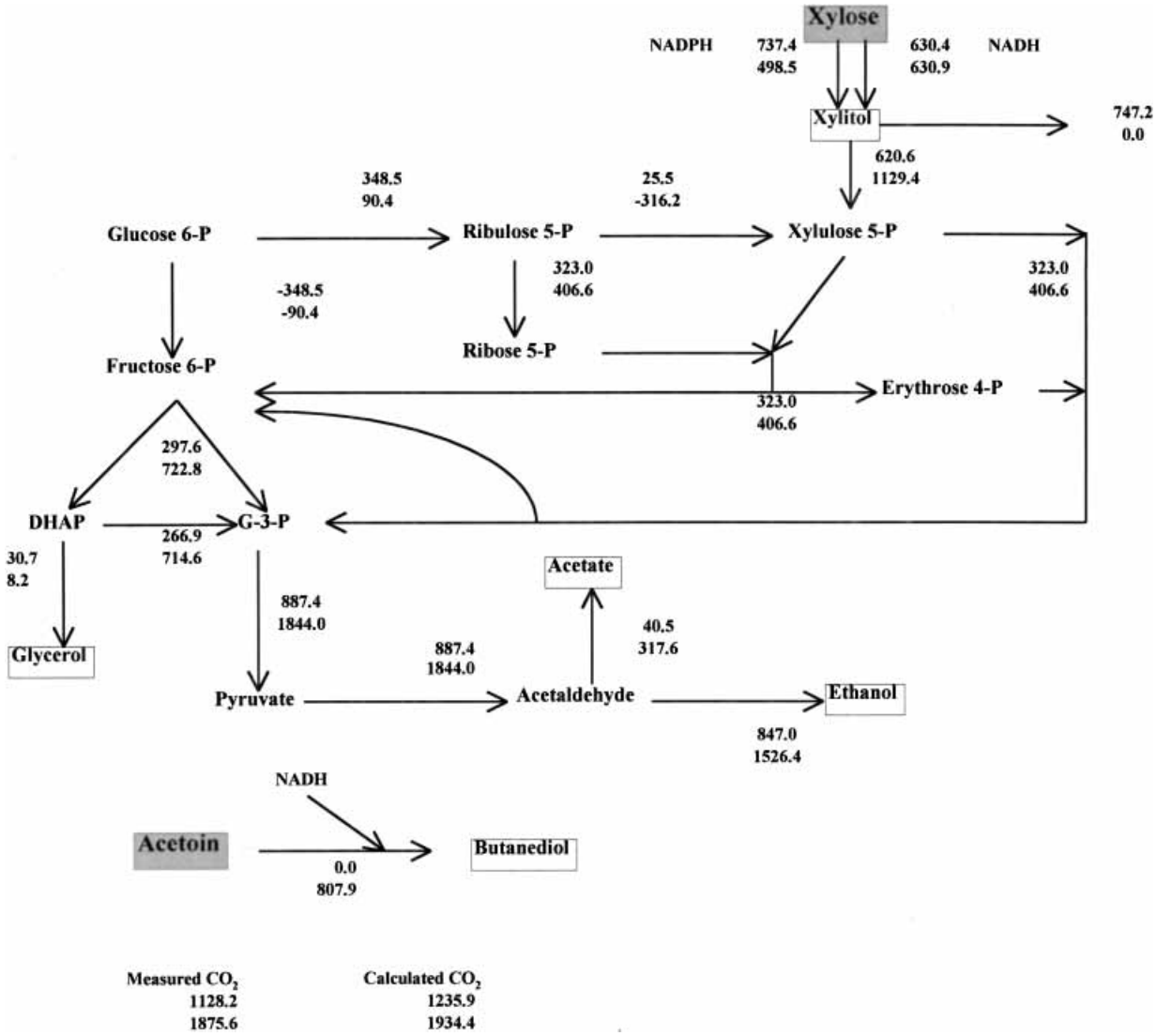

Figure 1. The fluxes in *S. cerevisiae* TMB 3001 during anaerobic xylose fermentation (upper values) and after acetoin addition (lower values). DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate. All fluxes are given in μmol/g biomass/h. Grey boxes indicate substrate and substances enclosed by a box are excreted into the medium.

fural conversion the HMF conversion was non-linear so the intracellular fluxes were not determined.

Activities of four key enzymes in acetoin, furfural, and HMF conversion were assayed in cells collected from respective fermentation (Table I). Acetaldehyde dehydrogenase activity was low and NADPH specific. Butanediol dehydrogenase, which converts acetoin to 2,3-butanediol, was NADH specific, which is in agreement with previous studies (Hcidlas and Tressl, 1990). Furfuryl alcohol dehydrogenase had the highest activity of all measured enzymes and also used NADH as a cofactor. The activity of HMFAL dehydrogenase was considerably lower and favored NADPH as cofactor.

DISCUSSION

Earlier studies of xylose fermentation by recombinant *S. cerevisiae* have focused on the performance and product formation in media containing xylose alone or supplied with glucose (Kötter and Ciriacy, 1993; Tantirungkij et al., 1993; Wahlbom et al., 2001; Walfridsson et al., 1995). Often xylitol has been produced in considerable yields due to intracellular depletion of NAD^+. Addition of the aldehydes and ketones like acetaldehyde (Ligthelm et al., 1989), acetone (Alexander, 1986; Ligthelm et al., 1989), and acetoin (Bruinenberg et al., 1983a; Ligthelm et al., 1989), to xylose fermentation by the naturally xylose fermenting yeasts *P. tannophilus* and *C. utilis* regenerated NAD^+ and prevented xylitol excretion. Also, *S. cerevisiae* TMB 3001 decreased the anaerobic xylitol excretion upon acetoin addition and as with *C. utilis* there was a large increase in the acetate production (Bruinenberg et al., 1983a). Hence, the response of recombinant *S. cerevisiae* on acetoin addition was similar to that of natural xylose utilizing yeasts.

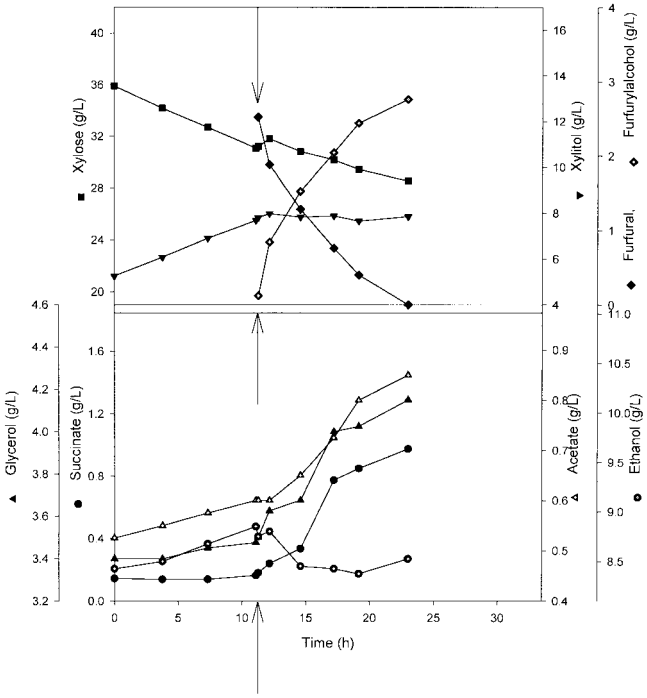

Figure 2. The effect of furfural addition (indicated by arrows) to *S. cerevisiae* TMB 3001 during xylose fennentation. The graph is a representative example of fermentation experiments carried out in triplicate.

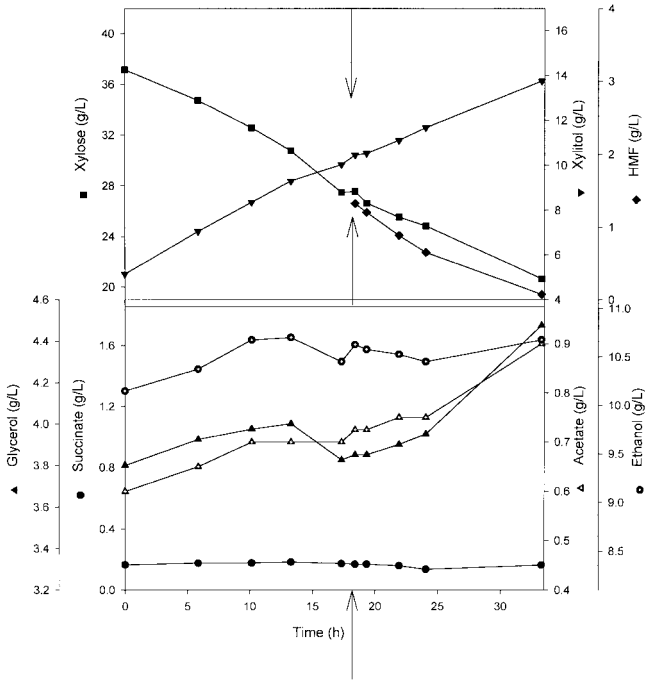

Figure 3. The effect of HMF addition (indicated by arrows) *S. cerevisiae* TMB 3001 during xylose fermentation. The graph is a representative example of fermentation experiments carried out in triplicate.

The utilization of electron acceptors in industrial xylose fermentation, as has previously been suggested (Alexander, 1986), would hardly be an economical alternative. However, non-detoxified lignocellulosic hydrolysate contains substances that act as electron acceptors that regenerate NAD^+. Furfural and HMF, which are formed by thermal degradation of pentoses (Antal et al., 1991; Dunlop, 1948) and hexoses (Antal et al., 1990; Heuser and Schott, 1923), respectively, are the two most common aldehydes (Larsson et al., 1999). These substances inhibit ethanolic glucose fermentation by causing longer lag phase (Taherzadeh et al., 2000a; Sanchez and Bautista, 1988) and lower growth rate (Boyer et al., 1992; Palmqvist et al., 1999b; Sanchez and Bautista, 1988). However, during xylose fermentation, xylitol excretion decreased after addition of furfural because NADH was oxidized to NAD^+ during its reduction to furfuryl alcohol. HMF, which requires NADPH for reduction, did not affect xylitol secretion. Furfural present in lignocellulosic hydrolysate can thus be beneficial for xylose fermentation and furfural reduction most likely contributed to low xylitol yields and increased ethanol yields during fermentation of lignocellulosic hydrolysate by recombinant, xylose-utilizing *S. cerevisiae* (Johansson et al., 2001). Previously, it has been demonstrated that furfural (Palmqvist et al., 1999b) and acetoin (Björkqvist et al., 1997) can replace glycerol production as a way of regeneration of NAD^+ during anaerobic glucose fermentation by *S. cerevisiae*. In the present study, the ethanol yield did not increase in response to furfural addition. Instead, a small consumption of ethanol was detected. This was interpreted as a cellular requirement for NADH for furfural reduction. However, ethanol is a difficult substance to follow in fermentation. Because of its low boiling point, a substantial fraction evaporates when the bioreactor is continuously flushed with nitrogen (Wahlbom et al., 2001). NADH is formed not only from oxidation of xylitol to xylulose but also from ethanol oxidation to

Table I. Activities of acetaldehyde dehydrogenase, butanediol dehydrogenase, furfuryl, and HMFAL dehydrogenase using different cofactors.

Enzyme	Specific activity (NADH) (mU/mg protein)	Specific activity (NADPH) (mU/mg protein)
Acetaldehyde dehydrogenase	0	24 ± 2.4
Butanediol dehydrogenase	120 ± 17	7 ± 0.8
Furfuryl alcohol dehydrogenase	490 ± 22	22 ± 0.7
HMFAL dehydrogenase	2.2 ± 0.7	22 ± 1.4

The values are the averages and standard deviations of at least four measurements.

acetaldehyde. In the presence of furfural, the cell seems to face a shortage instead of a surplus of NADH. If furfural had been added at a rate corresponding to its reduction rate, the ethanol yield would probably have increased. This fermentation strategy has been successfully explored for the fermentation of a non-detoxified lignocellulosic hydrolysate with wild-type *S. cerevisiae* (Taherzadeh et al., 1999; Taherzadeh et al., 2000b). The presented results have implications for fermentation of complex substrates. These may include substances like furfural that influence cellular redox balance and thereby affect product formation. It is therefore important to account also for such substances and not only investigate the main carbon source.

Different cofactors were preferred for the reduction of furfural and for the reduction of HMF, respectively. The NADH/NADPH ratio of utilization for furfural reduction was about 20:1, whereas it was 1:10 for HMF. The difference in cofactor preference not only explains the difference in the product formation but may also suggest a difference in inhibition mechanism. NADPH is required in the synthesis of amino acids and nucleotides, so the conversion of HMF competes with biomass synthesis and cell growth. The enzymatic activities responsible for furfural reduction were considerably higher than for HMF reduction. Also, the in situ furfural reduction was higher than the HMF reduction, which is in agreement with previous studies (Larsson et al., 1999; Taherzadeh et al., 2000a). The specific in vitro activity of the two enzyme(s) that reduce furfural and HMF using NADPH was the same, 0.022 U/mg protein, which indicates it may be the same enzyme.

For the calculation of the intracellular fluxes, a modified version of a previously developed metabolic flux model (Wahlbom et al., 2001) was used. The correlation between calculated and measured values for the carbon dioxide production was good, and the model provided results in addition to those experimentally determined. The oxidative pentose phosphate pathway produced NADPH for the first step of the xylose utilization during fermentation of xylose only. However, after acetoin addition, NADPH was instead produced in the oxidation of acetaldehyde to acetate by the NADPH-dependent enzyme acetaldehyde dehydrogenase. Thus, less carbon was lost as carbon dioxide in the oxidative pentose phosphate pathway. The shift in flux at the metabolic junction at acetaldehyde may be governed by the reduced $NADH/NAD^+$ ratio caused by acetoin (Björkqvist et al., 1997), which inhibited alcohol dehydrogenase 1 (Fan and Plapp, 1999) and favored acetate formation. Together with the decrease in xylitol excretion, this lead to a higher flux in the lower part of glycolysis where ATP is produced. The specific ATP production was calculated to 856.6 μmol/g biomass/h during fermentation of xylose only and 1835.8 μmol/g biomass/h after acetoin addition. Thus, the cell used the extra NADH oxidation power to produce more ATP. Few yeast other than *S. cerevisiae* are capable of strict anaerobic growth on glucose (Visser et al., 1990). Recently, *P. stipitis* transformed with an *S. cerevisiae URA1* gene was shown to grow anaerobically on glucose (Shi and Jeffries, 1998). Anaerobic growth of yeasts on xylose has not yet been reported, and it has been suggested to be due to insufficient ATP formation during anaerobic xylose metabolism (Rizzi et al., 1989b). The maintanance energy for growth on xylose by *P. stipitis* has been estimated to around 1 mmol ATP/g biomass/h (Rizzi et al., 1989b). For anaerobic growth on glucose by *S. cerevisiae*, it has been estimated to be below 1 mmol ATP/g biomass/h (Verduyn et al., 1990a). During cometabolism of acetoin, the recombinant, xylose-fermenting strain *S. cerevisiae* TMB 3001 produced approximately twice as much ATP as the estimated maintenance energy, so that slow growth would have been possible. The findings indicate that other factors than shortage of ATP may limit anaerobic growth of recombinant *S. cerevisiae* or that higher maintenance energy is required during growth on xylose.

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