Xylose-Metabolizing *Saccharomyces cerevisiae* Strains Overexpressing the *TKL1* and *TAL1* Genes Encoding the Pentose Phosphate Pathway Enzymes Transketolase and Transaldolase

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Saccharomyces cerevisiae was metabolically engineered for xylose utilization. The *Pichia stipitis* CBS 6054 genes *XYL1* and *XYL2* encoding xylose reductase and xylitol dehydrogenase were cloned into *S. cerevisiae*. The gene products catalyze the two initial steps in xylose utilization which *S. cerevisiae* lacks. In order to increase the flux through the pentose phosphate pathway, the *S. cerevisiae* *TKL1* and *TAL1* genes encoding transketolase and transaldolase were overexpressed. A *XYL1*- and *XYL2*-containing *S. cerevisiae* strain overexpressing *TAL1* (S104-TAL) showed considerably enhanced growth on xylose compared with a strain containing only *XYL1* and *XYL2*. Overexpression of only *TKL1* did not influence growth. The results indicate that the transaldolase level in *S. cerevisiae* is insufficient for the efficient utilization of pentose phosphate pathway metabolites. Mixtures of xylose and glucose were simultaneously consumed with the recombinant strain S104-TAL. The rate of xylose consumption was higher in the presence of glucose. Xylose was used for growth and xylitol formation, but not for ethanol production. Decreased oxygenation resulted in impaired growth and increased xylitol formation. Fermentation with strain S103-TAL, having a xylose reductase/xylitol dehydrogenase ratio of 0.5:30 compared with 4.2:5.8 for S104-TAL, did not prevent xylitol formation.

D-Xylose is a pentose sugar found in the hemicellulose fraction of lignocellulosic materials (23). After pretreatment and hydrolysis of lignocellulosic materials, the hemicellulose fraction is liquefied to make the sugars accessible to fermenting microorganisms. Depending on the origin of lignocellulose, which can be softwood, hardwood, or agricultural waste, between 6 and 28% (percentage of total dry material) may consist of pentoses (23), of which xylose is the dominant one. Microorganisms able to ferment xylose are found among bacteria, yeasts, and filamentous fungi (14, 15, 38). In xylose-fermenting yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*, xylose is first reduced to xylitol, which then is oxidized to xylulose (14, 16, 38). The two enzymes catalyzing these reactions are xylose reductase (XR) and xylitol dehydrogenase (XDH). In xylose-utilizing bacteria, xylose is directly converted to xylulose by the enzyme xylose isomerase.

Saccharomyces cerevisiae, which is used industrially for the production of wine, beer, and potable and industrial ethanol, can utilize xylulose, which is phosphorylated to xylulose-5-phosphate and channelled via the pentose phosphate pathway (PPP) into glycolysis (Fig. 1) (12, 35, 42, 44). *S. cerevisiae* is suitable for transformation of genes encoding xylose-utilizing enzymes because of its (i) tolerance to inhibitors (25, 29), (ii) high ethanol tolerance (9), and (iii) growth under anaerobic conditions (3, 4). Other xylose-utilizing microorganisms as well as microorganisms genetically engineered for xylose utilization have qualities which render them less suitable for industrial fermentation of xylose-containing substrates: *P. stipitis* is very sensitive to inhibitors and also demands strict oxygenation (25, 39), recombinant *Escherichia coli* KO11 requires detoxified

substrates (28, 29, 43), and recombinant *Zymomonas mobilis* ferments only a few of the sugars present in lignocellulose hydrolysates (29, 45).

The genes encoding xylose isomerase from several bacteria have been cloned in *S. cerevisiae* (2, 20, 32). So far, all attempts at constructing an *S. cerevisiae* strain capable of fermenting xylose by cloning the xylose isomerase gene have failed. The *P. stipitis* genes *XYL1* and *XYL2* encoding an NADP(H)-dependent XR and an NAD⁺-dependent XDH, respectively, have been cloned in *S. cerevisiae* (17, 22, 41). The recombinant strains are able to utilize xylose, but the rate of xylose consumption is low and the formation of by-products reduces the yield of ethanol. The utilization of PPP metabolites has been suggested to be a bottleneck for efficient xylose/xylulose fermentation by *S. cerevisiae*, with the reactions of the nonoxidizing PPP catalyzed by the enzymes transaldolase (TAL) and transketolase (TKL) controlling the flux (13, 36).

We constructed recombinant *S. cerevisiae* strains in which the expression levels of *XYL1* and *XYL2* varied. In addition, the genes *TKL1* and *TAL1* were overexpressed individually and together in *S. cerevisiae* containing the *XYL1* and *XYL2* genes. The fermentation of xylose and xylose-glucose mixtures with the different recombinant strains was investigated, and the influence of different oxygenation levels was studied.

MATERIALS AND METHODS

Strains. *E. coli* DH5 α [F⁻ ϕ 80d Δ lacZ Δ M15 Δ (lacZYA-argF) U169 deoR recA1 endA1 hsdR17(r_K⁻ m_K⁺) supE44 λ -thi-1 gyrA96 relA1] (GIBCO BRL, Gaithersburg, Md.) was used for subcloning. *S. cerevisiae* GPY55-15B α (*leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir⁺*) was obtained from Gregg Payne, University of California, Berkeley. This strain is referred to as H158 and was used as a host for transformation. The recombinant yeast strains constructed are listed in Table 1.

Media. Yeast strains were grown in a synthetic complete (SC) medium (37) (0.67% Difco yeast nitrogen base without amino acids supplemented with adenine [13.5 mg liter⁻¹], arginine [348 mg liter⁻¹], aspartic acid [266 mg liter⁻¹], histidine [58 mg liter⁻¹], inositol [36 mg liter⁻¹], isoleucine [525 mg liter⁻¹],

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FIG. 1. Proposed metabolic pathways for xylose and glucose in yeasts. Abbreviations: XK, xylulokinase; Ribulose 5P, ribulose-5-phosphate; Ribose 5P, ribose-5-phosphate; Xylulose 5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; G3P, glyceraldehyde-3-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; FDP, fructose diphosphate.

leucine [262 mg liter⁻¹], lysine [91 mg liter⁻¹], methionine [149 mg liter⁻¹], phenylalanine [83 mg liter⁻¹], serine [105 mg liter⁻¹], threonine [119 mg liter⁻¹], tryptophan [82 mg liter⁻¹], tyrosine [18 mg liter⁻¹], uracil [22 mg liter⁻¹], and valine [117 mg liter⁻¹]. For selection of the transformants either leucine alone or leucine and uracil were omitted from the medium. Sugars (xylose and glucose) were used at different concentrations.

Nucleic acid manipulations. Standard techniques for nucleic acid manipulation were used as described by Sambrook et al. (31). Restriction enzymes and other modifying enzymes were purchased from Boehringer Mannheim Scandinavia AB, Bromma, Sweden.

Yeast strain transformation. Yeast strain transformation was performed by the LiAc method (34).

Construction of *XYL1* *XYL2* plasmids. The *XYL1* gene cloned between the phosphoglycerate kinase (PGK) promoter and terminator (26) in plasmid pUA103 (17) was cleaved out with *Xba*I, separated by gel electrophoresis, and eluted with GeneClean (United States Biochemical). It was then filled in for blunt end generation with Klenow enzyme and ligated into the *Hind*III site of pKB102 (5), which had previously been made blunt ended. The *Hind*III site of pKB102 is located between the alcohol dehydrogenase (ADH1) promoter and terminator (1). The *XYL1*-ADH1 promoter fragment was next cleaved out of pKB102 with *Bam*HI and ligated into the *Bam*HI site of pMA91 (26) containing a PCR-amplified *XYL2* gene, resulting in pMW103 (Fig. 2A). The *XYL2* gene in pMA91-*XYL2* was cleaved out with *Bgl*II, separated by gel electrophoresis, and eluted from the gel. It was then filled in with Klenow enzyme and ligated into the *Hind*III site of pKB102, which had previously been made blunt ended. The *XYL2*-ADH1 promoter fragment was then cleaved out of pKB102 and ligated into the *Bam*HI site of pUA103, resulting in pMW104 (Fig. 2B).

***TKL1* and *TAL1* plasmids.** Multicopy vectors containing the genes for *TKL* (*TKL1*) and/or *TAL* (*TAL1*) in plasmid GS42 were previously constructed (33). The plasmids containing the *TKL1* and/or *TAL1* genes, GS42-*TKL*, GS42-*TAL*, and GS42-*TKL*-*TAL*, were generously supplied by Ine Schaaff-Gerstenschläger (Darmstadt, Germany).

Preparation of crude cell extract. Transformants were grown at 30°C in an SC

FIG. 2. Physical maps of the expression vectors pMW103 (A) and pMW104 (B) containing the *P. stipitis* genes *XYL1* and *XYL2* encoding XR and XDH. PGK p, PGK promoter; PGK t, PGK terminator; ADH p, ADH promoter; ADH t, ADH terminator.

medium with selection until mid-logarithmic phase. The cells were harvested by centrifugation at 3,000 × g for 5 min and washed in 0.1 M Na-phosphate buffer, pH 7.0. The pellet was resuspended in disintegration buffer (0.1 M Na-phosphate, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) and vortexed together with an equal volume of glass beads (0.5-mm diameter) twice for 5 min at 4°C. The disintegrated cell mixture was centrifuged at 13,000 rpm (Heraeus microcentrifuge) for 5 min at 4°C, and the supernatant was stored at -70°C until it was analyzed for protein concentration and enzyme activities.

Protein concentration determination. Protein concentration was measured according to the Bradford method (8), with bovine serum albumin as the standard.

Enzyme assays. XR was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm (40) with the following final concentrations: Na-phosphate buffer, 0.1 M (pH 7); xylose, 0.2 M; and NADPH, 0.15 mM. XDH was measured by monitoring the reduction of NAD at 340 nm with the following final concentrations: Tris-HCl, 0.1 M (pH 7); MgCl₂, 1 mM; xylitol, 50 mM; and NAD, 5 mM. *TKL* and *TAL* activities were measured according to the method of Bruinenberg (10). The specific activities of the enzymes were expressed as micromoles of converted substrate per milligram of protein per minute (which is equivalent to units per milligram).

Cultivation conditions. Xylose and xylose-glucose utilization was studied with shake flasks at 30°C in an orbital shaker (150 rpm) unless otherwise indicated. Aerobic cultivations were performed in 1-liter baffled Erlenmeyer flasks with 200 ml of media. For oxygen-limited conditions, 500-ml flasks were filled with 300 ml of media. For anaerobic conditions, 500-ml flasks were filled with media and plugged with rubber stoppers. Outlet of gas was secured by inserting a cannula through the rubber stopping. Agitation was achieved with a magnetic stirrer. The xylose concentration was 20 g liter⁻¹, and the xylose and glucose concentrations varied from 12 and 128 g liter⁻¹ to 113 and 10 g liter⁻¹, respectively.

Analytical methods. Glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by high-performance liquid chromatography (Varian, Sunnyvale, Calif.) using an Aminex ion-exclusion HPX-87H cation-

TABLE 1. Recombinant *XYL1* *XYL2* yeast strains (and reference strain) with their genes overexpressed^a

Strain	Overexpressed gene(s)
H158 (reference strain)	
S103	
S103-TKL	<i>TKL1</i>
S103-TAL	<i>TAL1</i>
S103-TKL-TAL	<i>TKL1</i> , <i>TAL1</i>
S104	
S104-TKL	<i>TKL1</i>
S104-TAL	<i>TAL1</i>
S104-TKL-TAL	<i>TKL1</i> , <i>TAL1</i>

^a The genes *XYL1*, *XYL2*, *TKL1*, and *TAL1* encode XR, XDH, *TKL*, and *TAL*, respectively.

TABLE 2. In vitro enzyme activities of XR, XDH, TKL, and TAL for the recombinant yeast strains

Strain	Sp act (U mg of protein ⁻¹) of:			
	XR	XDH	TKL	TAL
H158 (reference strain)	0.021	0.001	ND ^a	ND
S103	0.5	30.1	0.068	0.069
S103-TAL	0.5	30.2	ND	1.1
S103-TKL-TAL	0.5	29.8	0.92	0.93
S104	4.2	5.8	0.068	0.068
S104-TAL	4.0	5.7	ND	1.0
S104-TKL-TAL	4.2	5.7	0.97	0.92

^a ND, not determined.

exchange column (Bio-Rad, Richmond, Calif.) at 65°C with 5 mM H₂SO₄ as the mobile phase. The compounds were detected with a refractive-index detector (410 differential refractometer; Waters, Milford, Mass.). Cell growth was estimated from the optical density at 600 nm.

RESULTS

Expression of the heterologous genes for XR and XDH. The *XYL1* and *XYL2* genes were both cloned into the yeast expression vector pMA91 under the control of the PGK or ADH1 promoter, resulting in the vectors pMW103 and pMW104 (Fig. 2). H158 transformed with pMW103 (S103) and pMW104 (S104) expressed *XYL1* and *XYL2* constitutively. In S103, in which *XYL1* was expressed under the control of the ADH1 promoter and *XYL2* was expressed under the control of the PGK promoter, the activities of XR and XDH were 0.5 and 30.1 U mg⁻¹, respectively (Tables 1 and 2). In S104, in which *XYL1* was expressed under the control of the PGK promoter and *XYL2* was expressed under the control of the ADH1 promoter, the XR and XDH activities were 4.2 and 5.8 U mg⁻¹, respectively.

Construction of *S. cerevisiae* strains containing *XYL1* and *XYL2* and overproducing TKL and/or TAL. The strains S103 and S104 were transformed with the multicopy plasmids GS42-TKL, GS42-TAL, and GS42-TKL-TAL and selected on SC medium-Leu-uracil plates. The new transformants (S103-TKL, S103-TAL, S103-TKL-TAL, S104-TKL, S104-TAL, and S104-TKL-TAL) overexpressed *TKL1* and *TAL1* at rates approximately 14 times those of the reference strains, S103 and S104 (Tables 1 and 2). In vitro measurements of TAL and TKL activities for strain S104-TKL-TAL resulted in specific enzyme activities of 0.97 U mg⁻¹ (TKL) and 0.92 U mg⁻¹ (TAL), compared with 0.068 U mg⁻¹ (TKL and TAL) for the reference strain.

Growth of recombinant *S. cerevisiae* strains on xylose plates. The recombinant strains S103 and S104 containing the *P. stipitis* genes *XYL1* and *XYL2* and in addition overproducing transketolase and transaldolase were streaked on minimal xylose plates and incubated for 3 days (Fig. 3). S103 and S104, producing low levels of XR and high levels of XDH and medium levels of XR and XDH, respectively, showed a growth pattern similar to that of the reference strain *S. cerevisiae* H158. The same was observed for transformants S103-TKL and S104-TKL, in which TKL was overproduced. However, when TAL was overproduced in *XYL1* *XYL2* transformants (S103-TAL and S104-TAL), growth was considerably improved. The same was observed when both TKL and TAL were overproduced (S103-TKL-TAL and S104-TKL-TAL). Thus, overproducing TAL enhanced the growth of recombinant *S. cerevisiae*, irrespective of the activities of the enzymes XR, XDH, and TKL. Strains S103 and S104 showed the same growth pattern on

FIG. 3. Growth of yeast strains on SC medium plates containing 20 g of xylose liter⁻¹ (after 3 days of incubation at 30°C). For relevant genotypes of the strains see Table 1. GS42 was the empty host vector for *TKL1* and *TAL1*. (A) Section 1, H158 (reference strain); section 2, S103; section 3, S103-GS42; section 4, S103-TKL; section 5, S104-TAL; section 6, S104-TKL-TAL. (B) Section 7, H158 (reference strain); section 8, S104; section 9, S104-GS42; section 10, S104-TKL; section 11, S104-TAL; section 12, S104-TKL-TAL.

xylose, despite the fact that in S103 the ratio of XR to XDH is 0.5:30 and in strain S104 it is 4.2:5.8.

Cultivation of strains S103-TAL and S104-TAL in xylose. Growth and product formation of S103-TAL and S104-TAL in shake flask cultivations under aerobic conditions were studied. When strains S103-TAL and S104-TAL were cultivated in a minimal medium with 20 g of xylose liter⁻¹, the main product was biomass with xylitol as a by-product (Fig. 4). No ethanol was produced from xylose. For S103-TAL, 10.4 g of xylose liter⁻¹ was consumed within 138 h and 0.2 g of xylitol liter⁻¹ was produced. For S104-TAL, 19.8 g of xylose liter⁻¹ was

FIG. 4. Aerobic shake flask cultivations of S103-TAL (A) and S104-TAL (B) in SC medium-Leu-uracil containing 20 g of xylose liter⁻¹. Symbols: ◇, xylose; ○, xylitol; △, glycerol; ⊞, acetic acid; ⊕, optical density at 600 nm (OD 600).

consumed within 138 h and 3.6 g of xylitol liter⁻¹ was produced. Thus, S104-TAL, having an XR-to-XDH ratio of 4.2:5.8, consumed more xylose than S103-TAL, which could not be deduced from growth on plates (Fig. 3).

Cultivation of strain S104-TKL-TAL in xylose with various oxygenation levels. The influence of oxygen in xylose cultivations was studied for S104-TKL-TAL, since this strain showed superior growth compared with S103. The strain was cultivated in minimal xylose (20 g liter⁻¹) media in shake flasks with three different levels of oxygenation, i.e., under aerobic, oxygen-limited, and anaerobic conditions (Fig. 5). The maximum growth rates were estimated to be 0.02 h⁻¹ (aerobic), 0.01 h⁻¹ (oxygen limited), and 0.004 h⁻¹ (anaerobic). With decreasing oxygenation the biomass formation was reduced and the xylitol production was increased. S104-TKL-TAL produced no ethanol from xylose.

The effect of cultivating S104-TAL in mixtures of xylose and glucose. The utilization of xylose in the presence of glucose was studied for S104-TAL under oxygen-limited conditions. Five xylose/glucose ratios were investigated (ratios are in grams of xylose liter⁻¹ to grams of glucose liter⁻¹): 12:128, 28:88, 53:53, 81:23, and 113:10 (Fig. 6). Xylose and glucose were consumed simultaneously. The consumption of xylose was faster in the presence of glucose, with a range of 0.14 to 0.43 g liter⁻¹ h⁻¹ from the lowest xylose/glucose ratio to the highest xylose/glucose ratio. After glucose was depleted, the rate of xylose con-

sumption became much lower. Xylitol production started only when glucose was depleted and no additional ethanol was produced from xylose. The total levels of xylose consumption during the fermentation of the five different sugar mixtures were as follows: 12 g liter⁻¹ (after 50 h for the 12:128 ratio), 20 g liter⁻¹ (after 118 h for the 28:88 ratio), 20 g liter⁻¹ (after 118 h for the 53:53 ratio), 23 g liter⁻¹ (after 118 h for the 81:23 ratio), and 34 g liter⁻¹ (after 118 h for the 113:10 ratio). The maximum growth rate of S104-TAL in the five substrates was between 0.1 h⁻¹ (low xylose/glucose ratio) and 0.07 h⁻¹ (high xylose/glucose ratio).

DISCUSSION

In this study we have constructed *S. cerevisiae* strains containing the *P. stipitis* genes *XYL1* and *XYL2* encoding the xylose-degrading enzymes XR and XDH. The genes were placed in the yeast expression vector pMA91 under the control of the PGK promoter and the ADH1 promoter, respectively. When transformed into *S. cerevisiae*, the two vectors (pMW103 and pMW104) resulted in different levels of XR and XDH (Table 2) depending on promoter choice and the direction of the *XYL1* gene in relation to the *XYL2* gene. The *XYL1* *XYL2* transformants S103 and S104 behaved similarly and showed very limited growth on xylose (Fig. 3) even though they constitutively express the genes for XR and XDH and are able to

FIG. 5. S104-TKL-TAL cultivated under three different levels of oxygenation in SC medium-Leu-uracil containing 20 g of xylose liter⁻¹. (A) Aerobic cultivation; (B) oxygen-limited cultivation; (C) anaerobic cultivation. Symbols: ◇, xylose; ○, xylitol; ⊕, optical density at 600 nm (OD 600).

FIG. 6. Oxygen-limited cultivations of S104-TAL in SC medium-Leu-uracil with different mixtures of xylose and glucose. (A) Twelve grams of xylose liter⁻¹ and 128 g of glucose liter⁻¹; (B) 28 g of xylose liter⁻¹ and 88 g of glucose liter⁻¹; (C) 53 g of xylose liter⁻¹ and 53 g of glucose liter⁻¹; (D) 81 g of xylose liter⁻¹ and 23 g of glucose liter⁻¹; (E) 113 g of xylose liter⁻¹ and 10 g of glucose liter⁻¹. Symbols: $\diamond$, xylose; $\square$, glucose; $\circ$, xylitol; $\triangle$, glycerol; $\boxplus$, acetic acid; $\oplus$, ethanol; $\odot$, optical density at 600 nm (OD 600).

further metabolize xylulose, the product of the two enzyme reactions.

Extra copies of the *S. cerevisiae* *TAL1* gene encoding TAL greatly improved growth on xylose, supporting earlier suggestions that the level of TAL in *S. cerevisiae* limits growth on xylose (13, 22, 33, 36). TAL converts sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to erythrose-4-phosphate and fructose-6-phosphate. The intermediates glyceraldehyde-3-phosphate and fructose-6-phosphate are shared with glycolysis. As has been suggested, the bottleneck in the PPP may be due to competition for glyceraldehyde-3-phosphate (13). The addition of extra copies of only the *TKL1* gene encoding TKL did not contribute to improved growth on xylose. TKL catalyzes the conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and

glyceraldehyde-3-phosphate (Fig. 1). Similar results were recently obtained when the *P. stipitis* gene encoding TKL (*TKT*) was cloned under the control of the pyruvate decarboxylase promoter into *XYL1 XYL2* integrants of *S. cerevisiae* (27). When the integrants were grown aerobically on minimal xylose media, a doubling of the generation time (from 10 to 20 h) compared with that of the reference strain was observed. The authors suggested that a simultaneous overexpression of the *P. stipitis* genes for TKL and TAL could increase the growth rate on xylose. However, the present study shows that overexpression of both *TKL1* and *TAL1* has the same effect as overexpression of only *TAL1*. Recently, overexpression of the gene encoding xylulokinase has also been shown to improve the xylose utilization rate (19a). Thus, both xylulokinase and TAL

appear to limit the flux through the PPP in natural *S. cerevisiae* strains.

The fermentation of xylose-glucose mixtures under oxygen-limited conditions improved the rate of xylose utilization (Fig. 6). The metabolized xylose is used for growth and/or ethanol production. Both sugars were consumed simultaneously, indicating that transport of xylose is not limiting. After glucose was depleted, the utilization of xylose was much slower. This was probably due to a combination of two independent phenomena. On the one hand, the transport of xylose may have been limited because of the low xylose concentration. Xylose is believed to be taken up by the same transport systems as glucose but with much higher K_m values (11). On the other hand, efficient ethanol production requires that the enzymes pyruvate decarboxylase and alcohol dehydrogenase channel pyruvate to acetaldehyde and ethanol (6, 7). These enzymes are activated through a series of events involving key intermediary metabolites of both the upper and lower parts of glycolysis (6). The fact that xylitol production started immediately when glucose was consumed indicates that fermentation of xylose alone results in concentrations of intermediary metabolites too low for induction of key ethanologenic enzymes. The *P. stipitis* XR can utilize both NADH and NADPH as cosubstrates, whereas XDH exclusively uses NAD⁺. In vitro measurements have shown that XR in *S. cerevisiae* transformants and in *P. stipitis* has a preference for NADPH (17, 39). This leads to an accumulation of NADH resulting from the oxidation of xylitol to xylulose by the NAD⁺-dependent XDH. In the absence of oxygen NADH cannot be reoxidized. As a result xylitol accumulates and is excreted. This has also been observed in *P. stipitis* grown on xylose under anaerobic conditions (24, 30). In strain S103, high-level XDH activity and low-level XR activity did not prevent xylitol formation. The formation of xylitol was more pronounced during fermentations with low levels of oxygenation, indicating a redox imbalance in the recombinant *S. cerevisiae* strains. Recently two redox sinks which may prevent xylitol formation have been detected in *P. stipitis*: (i) an alternative cyanide-insensitive oxidase (21) believed to regenerate the NAD⁺ required for polyol oxidation and (ii) a D-arabinitol dehydrogenase that may act as a redox balancer by reducing D-ribulose to D-arabinitol (18). These activities are not present in native *S. cerevisiae* strains and could explain why recombinant *S. cerevisiae* produces xylitol under conditions in which *P. stipitis* does not.

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REFERENCES

- Ammerer, G. 1983. Expression of genes in yeast using the *ADCI*-promoter. *Methods Enzymol.* **101**:192-210.
- Amore, R., M. Wilhelm, and C. P. Hollenberg. 1989. The fermentation of xylose and analysis of the expression of *Bacillus* α -acetolactate decarboxylase genes in yeast. *Appl. Microbiol. Biotechnol.* **30**:351-357.
- Andreasen, A. A., and T. J. B. Stier. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* **41**:23-26.
- Andreasen, A. A., and T. J. B. Stier. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* **43**:271-281.
- Blomqvist, K., M.-L. Suihko, J. Knowles, and M. Penttilä. 1991. Chromosomal integration and expression of two bacterial α -acetolactate decarboxylase genes in brewer's yeast. *Appl. Environ. Microbiol.* **57**:2796-2803.
- Boles, E., J. Heinisch, and F. K. Zimmermann. 1993. Different signals control the activation of glycolysis in the yeast *Saccharomyces cerevisiae*. *Yeast* **9**:761-770.
- Boles, E., and F. K. Zimmermann. 1993. Induction of pyruvate decarboxylase in glycolysis mutants of *Saccharomyces cerevisiae* correlates with the concentration of three-carbon glycolytic metabolites. *Arch. Microbiol.* **160**:324-328.
- Bradford, M. M. 1976. Photometric methods for protein determination. *Anal. Biochem.* **72**:248-254.
- Brown, S. W., S. G. Oliver, D. E. F. Harrison, and R. C. Righelato. 1981. Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. *Eur. J. Appl. Microbiol. Biotechnol.* **11**:151-155.
- Bruinenberg, P. M., J. P. van Dijken, and W. A. Scheffers. 1983. An enzymic analysis of NADPH production and consumption in *Candida utilis*. *J. Gen. Microbiol.* **129**:965-971.
- Busturia, A., and R. Lagunas. 1986. Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **132**:379-385.
- Chiang, L.-C., C.-S. Gong, L.-F. Chen, and G. T. Tsao. 1981. D-Xylulose fermentation to ethanol by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **42**:284-289.
- Ciriacy, M., and H. Porep. 1986. Conversion of pentoses to ethanol by bakers' yeast, p. 677-679. In E. Magnien (ed.), *Biomolecular engineering in European Community*. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
- Hahn-Hägerdal, B., J. Hallborn, H. Jeppsson, L. Olsson, K. Skoog, and M. Walfridsson. 1993. Pentose fermentation to alcohol, p. 231-290. In J. N. Saddler (ed.), *Bioconversion of forest and agricultural plant residues*. CAB International, Wallingford, United Kingdom.
- Hahn-Hägerdal, B., H. Jeppsson, L. Olsson, and A. Mohagheghi. 1994. An interlaboratory comparison of the performance of ethanol-producing microorganisms in a xylose-rich acid hydrolysate. *Appl. Microbiol. Biotechnol.* **41**:62-72.
- Hahn-Hägerdal, B., H. Jeppsson, K. Skoog, and B. A. Prior. 1994. Biochemistry and physiology of xylose fermentation by yeasts. *Enzyme Microb. Technol.* **16**:933-943.
- Hallborn, J., M. Walfridsson, U. Airaksinen, H. Ojamo, B. Hahn-Hägerdal, M. Penttilä, and S. Keränen. 1991. Xylitol production by recombinant *Saccharomyces cerevisiae*. *Bio/Technology* **9**:1090-1095.
- Hallborn, J., M. Walfridsson, M. Penttilä, S. Keränen, and B. Hahn-Hägerdal. 1995. A short-chain dehydrogenase gene from *Pichia stipitis* having D-arabinitol dehydrogenase activity. *Yeast* **11**:839-847.
- Heinisch, J., K. Vogelsang, and C. P. Hollenberg. 1991. Transcriptional control of yeast phosphofructokinase gene expression. *FEBS Lett.* **289**:77-82.
- Ho, N. W. Y. Personal communication.
- Ho, N. W. Y., P. Stevis, S. Rosenfeld, J. J. Huang, and G. T. Tsao. 1983. Expression of the *E. coli* isomerase gene by a yeast promoter. *Biotechnol. Bioeng. Symp.* **13**:245-250.
- Jeppsson, H., N. J. Alexander, and B. Hahn-Hägerdal. 1995. Existence of cyanide-insensitive respiration in the yeast *Pichia stipitis* and its possible influence on product formation during xylose utilization. *Appl. Environ. Microbiol.* **61**:2596-2600.
- Kötter, P., and M. Ciriacy. 1993. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **38**:776-783.
- Ladisch, M. R., K. W. Lin, M. Voloch, and G. T. Tsao. 1983. Process considerations in the enzymatic hydrolysis of biomass. *Enzyme Microb. Technol.* **5**:82-102.
- Ligthelm, M. E., B. A. Prior, and J. C. du Preez. 1988. The oxygen requirements of yeasts for the fermentation of D-xylose and D-glucose to ethanol. *Appl. Microbiol. Biotechnol.* **28**:63-68.
- Lindén, T., and B. Hahn-Hägerdal. 1989. Fermentation of lignocellulose hydrolysates with yeasts and xylose isomerase. *Enzyme Microb. Technol.* **11**:583-589.
- Mellor, J., M. J. Dobson, N. A. Roberts, M. F. Tuite, J. S. Emtage, S. White, P. A. Lowe, T. Patel, A. J. Kingsman, and S. M. Kingsman. 1983. Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene* **24**:1-14.
- Metzger, M. H., and C. P. Hollenberg. 1994. Isolation and characterization of the *Pichia stipitis* transketolase gene and expression in a xylose-utilising *Saccharomyces cerevisiae* transformant. *Appl. Microbiol. Biotechnol.* **42**:319-325.
- Ohta, K., D. S. Beall, M. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. *Appl. Environ. Microbiol.* **57**:893-900.
- Olsson, L., and B. Hahn-Hägerdal. 1993. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Process Biochem.* **28**:249-257.
- Rizzi, M., C. Klein, C. Schulze, N. A. Bui-Thanh, and H. Dellweg. 1989. Xylose fermentation by yeasts. 5. Use of ATP balances for modeling oxygen-limited growth and fermentation of the yeast *Pichia stipitis* with xylose as carbon source. *Biotechnol. Bioeng.* **34**:509-514.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a

- laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. Sarthy, A. V., B. L. McConaughy, Z. Lobo, J. A. Sundstrom, C. E. Furlong, and B. D. Hall. 1987. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **53**:1996–2000.
 33. Schaaff-Gerstenschläger, I., T. Miosga, and F. K. Zimmermann. 1994. Genetics of pentose phosphate pathway enzymes in *Saccharomyces cerevisiae*. Bioresour. Technol. **50**:59–64.
 34. Schiestl, R. H., and D. Gietz. 1989. High efficiency transformation of intact yeast cells by single stranded nucleic acids as carrier. Curr. Genet. **16**:339–346.
 35. Senac, T., and B. Hahn-Hägerdal. 1990. Intermediary metabolite concentrations in xylulose- and glucose-fermenting *Saccharomyces cerevisiae* cells. Appl. Environ. Microbiol. **56**:120–126.
 36. Senac, T., and B. Hahn-Hägerdal. 1991. Effects of increased transaldolase activity on D-xylulose and D-glucose metabolism in *Saccharomyces cerevisiae* cell extracts. Appl. Environ. Microbiol. **57**:1701–1706.
 37. Sherman, F., G. Fink, and J. B. Hicks. 1983. Methods in yeast genetics. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 38. Skoog, K., and B. Hahn-Hägerdal. 1988. Xylose fermentation. Enzyme Microb. Technol. **10**:66–80.
 39. Skoog, K., and B. Hahn-Hägerdal. 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Appl. Environ. Microbiol. **56**:3389–3394.
 40. Smiley, K. L., and P. L. Bolen. 1982. Demonstration of D-xylose reductase and D-xylitol dehydrogenase in *Pachysolen tannophilus*. Biotechnol. Lett. **4**:607–610.
 41. Tantirungkij, M., N. Nakashima, T. Seki, and T. Yoshida. 1993. Construction of xylose-assimilating *Saccharomyces cerevisiae*. J. Ferment. Bioeng. **75**:83–88.
 42. Ueng, P. P., C. A. Hunter, C.-S. Gong, and G. T. Tsao. 1981. D-Xylulose fermentation in yeasts. Biotechnol. Lett. **3**:315–320.
 43. von Sivers, M., G. Zacchi, L. Olsson, and B. Hahn-Hägerdal. 1994. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. Biotechnol. Prog. **10**:555–560.
 44. Wang, P. Y., C. Shopsis, and H. Schneider. 1980. Fermentation of a pentose by yeasts. Biochem. Biophys. Res. Commun. **94**:248–254.
 45. Zhang, M., C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science **267**:240–243.