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Optimal Growth and Ethanol Production from Xylose by Recombinant *Saccharomyces cerevisiae* Require Moderate D-Xylulokinase Activity

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D-Xylulokinase (XK) is essential for the metabolism of D-xylose in yeasts. However, overexpression of genes for XK, such as the *Pichia stipitis XYL3* gene and the *Saccharomyces cerevisiae XKS* gene, can inhibit growth of *S. cerevisiae* on xylose. We varied the copy number and promoter strength of *XYL3* or *XKS1* to see how XK activity can affect xylose metabolism in *S. cerevisiae.* The *S. cerevisiae* genetic background included single integrated copies of *P. stipitis XYL1* and *XYL2* driven by the *S. cerevisiae TDH1* promoter. Multicopy and single-copy constructs with either *XYL3* or *XKS1,* likewise under control of the *TDH1* promoter, or with the native *P. stipitis* promoter were introduced into the recombinant *S. cerevisiae.* In vitro enzymatic activity of XK increased with copy number and promoter strength. Overexpression of *XYL3* and *XKS1* inhibited growth on xylose but did not affect growth on glucose even though XK activities were three times higher in glucose-grown cells. Growth inhibition increased and ethanol yields from xylose decreased with increasing XK activity. Uncontrolled XK expression in recombinant *S. cerevisiae* is inhibitory in a manner analogous to the substrate-accelerated cell death observed with an *S. cerevisiae tps1* mutant during glucose metabolism. To bypass this effect, we transformed cells with a tunable expression vector containing *XYL3* under the control of its native promoter into the FPL-YS1020 strain and screened the transformants for growth on, and ethanol production from, xylose. The selected transformant had approximately four copies of *XYL3* per haploid genome and had moderate XK activity. It converted xylose into ethanol efficiently.

Xylose utilization is critical for the successful fermentation of biomass to fuels and chemicals (7). Although a few xylose-fermenting yeasts are found in nature (12, 19), *Saccharomyces cerevisiae* is used ubiquitously for industrial ethanol production. However, S. *cerevisiae* cannot assimilate xylose, so engineering S. *cerevisiae* for xylose utilization has focused on adapting the xylose metabolic pathway from the xylose-utilizing yeast *Pichia stipitis* (15, 18, 30, 35). In this organism, xylose is converted into xylulose by two oxidoreductases. First, xylose is reduced to xylitol by an NAD[P]H^+-linked xylose reductase (XR) (34), and then xylitol is oxidized to xylulose by an NAD^+-linked xylitol dehydrogenase (XDH) (23). Finally, D-xylulokinase (XK) phosphorylates D-xylulose into D-xylulose-5-phosphate (X5P), which is metabolized further via the pentose phosphate pathway (PPP) and glycolysis (14). Early attempts at engineering xylose metabolism expressed only *XYL1* and *XYL2,* which code for XR and XDH, from *P. stipitis* in *S. cerevisiae* (15, 18, 30, 35) because *S. cerevisiae* can ferment xylulose (3, 26, 36). Recombinant S. *cerevisiae* expressing *XYL1* and *XYL2* could grow on xylose, but ethanol production from xylose was not significant because a substantial portion of the consumed xylose was converted into xylitol (15, 18, 29).

Recombinant *S. cerevisiae* transformed with a single copy of *XYL1* and multiple copies of *XYL2* accumulate xylulose (13). This suggests that the native level of XK activity in *S. cerevisiae* limits xylose assimilation when genes for the two preceding enzymes are overexpressed, which was consistent with earlier findings. Ho et al. (8) reported that overexpression of an endogenous S. *cerevisiae* XK gene (*ScXKS1*) with *XYL1* and *XYL2* increased ethanol production and decreased xylitol production from xylose. However, this observation has remained controversial. Rodriguez-Pena et al. (24) showed that overexpression of *XKS1* in *S. cerevisiae* inhibits growth on pure D-xylulose. Other studies by Toivari et al. (33) and Richard et al. (22) did not show an inhibitory effect from XK overexpression, but Johansson et al. (16) found that overexpression of *ScXKS1* reduced xylose consumption by 50 to 80% in S. *cerevisiae* transformants, even though it increased the yield of ethanol from xylose. Johansson et al. (16) cautioned against the unmodulated overexpression of *ScXKS1.*

In the present study, we investigated the effect of overexpressing *XYL3* and *XKS1* on xylose metabolism by recombinant *S. cerevisiae* that was also expressing *XYL1* and *XYL2* driven by a strong constitutive *S. cerevisiae* promoter. Overexpression of either *XYL3* or *XKS1* was detrimental to cell growth on xylose but not on glucose. To bypass this inhibition, we integrated multiple copies of *XYL3* with its native *P. stipitis* promoter into the *S. cerevisiae* genome by using a tunable expression vector that allows various expression levels by achieving different integrated copy numbers (21).

MATERIALS AND METHODS

Strains and plasmids. The microbial strains and plasmids used in this study are listed in Table 1. Jin-Ho Seo at Seoul National University provided *S. cerevisiae* L2612 (*MAT*a *leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal*$^+$) and the **plasmid** pY2XK. K. D. Wirttrup at the Massachusetts Institute of Technology provided the Neo^r-based Ty-d tunable expression vector pITy4 (21). *Escherichia*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. cerevisiae L2612	*MAT* a *trp1-112 leu2-1* ura3-52	4
S. cerevisiae FPL-YS10	*MAT* a *trp1-112 leu2::LEU2-TDH1*$_P$*-XYL1-TDH1*$_T$	13
S. cerevisiae FPL-YS1020	*MAT* a *trp1-112 leu2:LEU2-TDH1*$_P$*-XYL1-TDH1*$_T$ *ura3::URA3-TDHI*$_P$*-XYL2-TDH1*$_T$	13
S. cerevisiae FPL-YSX3	*MAT* a *trpl-112 leu2:LEU2-PsXYL1 ura3::URA3-PsXYL2 Ty3::NEO-PsXYL3*	This study
S. cerevisiae FPL-YS314	*S. cerevisiae* FPL-YS1020(pRS314)	This study
S. cerevisiae FPL-YS424	*S. cerevisiae* FPL-YS1020(pRS424)	This study
S. cerevisiae FPL-YS2831	*S. cerevisiae* FPLYS1020(pYPR2831)	This study
S. cerevisiae FPL-YS31N	*S. cerevisiae* FPL-YS1020(pYS31N)	This study
S. cerevisiae FPL-YS32N	*S. cerevisiae* FPL-YS1020(pYS32N)	This study
S. cerevisiae FPL-YS31	*S. cerevisiae* FPL-YS1020(pYS31)	This study
S. cerevisiae FPL-YS32	*S. cerevisiae* FPL-YS1020(pYS32)	This study
S. cerevisiae FPL-YS41	*S. cerevisiae* FPL-YS1020(pYS41)	This study
S. cerevisiae FPL-YS42	*S. cerevisiae* FPL-YS1020(pYS42)	This study
Plasmids		
pRS314	*TRP1* CEN/ARS	28
pRS424	*TRP1* 2μm origin	5
pYPR2831	*TRP1* 2μm origin *TDH1*$_P$ and *TDH1*$_T$	10
pYS31N	*PsXYL3* in pRS314	14
pYS32N	*PsXYL3* in pRS424	14
pYS31	*TRP1* CEN/ARS *TDH1*$_P$*-XYL3-TDH1*$_T$	This study
pYS32	*TRP1* 2μm origin *TDH1*$_P$*-XYL3-TDH1*$_T$	This study
pYS41	*TRP1* CEN/ARS *TDH1*$_P$ *-XKS1-TDH1*$_T$	This study
pYS42	*TRP1* 2μm origin *TDH1*$_P$*- XKS1-TDH1*$_T$	This study

coli DH5a (F$^-$ *recA1 endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1 gyrA relA1*) (Gibco BRL, Gaithersburg, Md.) was routinely used for gene cloning and manipulation.

Media and culture conditions. Yeast and bacterial strains were stored in 15% glycerol at –70°C. E. coli was grown in Luria-Bertani medium. Fifty micrograms of ampicillin/ml was added to the medium when required. Yeast strains were routinely cultivated at 30°C in YP medium (10 g of yeast extract/liter, 20 g of Bacto Peptone/liter) with either 20 g of glucose/liter (to constitute YPD medium), 20 g of xylose/liter (YPX-2%), or 40 g of xylose/liter (YPX-4%). YPD or YPX plus 20 g of agar/liter was used for plates. To select for yeast transformants by using the *URA3, TRP1,* or *LEU2* selectable markers, we used yeast synthetic complete (YSC) medium containing 6.7 g of yeast nitrogen base/liter without amino acids plus 20 g of glucose/liter, 20 g of agar/liter, and a mixture of appropriate nucleotides and amino acids. To select for transformants by using the Neo^r marker, we used YPD agar supplemented with 200 μg of G418 per ml (Geneticin; Sigma). Yeast cells were cultivated at 30°C in 50 ml of medium in a 125-ml Erlenmeyer flask. To screen for *XYL3* transformants, we inoculated 40 putative transformants each into 5 ml of YPX-4% medium in 15-ml sterile culture tubes and incubated them with shaking at 200 rpm at 30°C for 72 h. Cell growth and product formation were measured to identify 10 strains for further screening. The selected strains were tested again by culturing cells in 50 ml of YPX-4% medium in 125-ml Erlenmeyer flasks shaken at 200 rpm.

Enzymes, primers, and chemicals. Restriction enzymes, DNA-modifying enzymes, and other molecular reagents were obtained from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), Stratagene (La Jolla, Calif.), and Roche Biochemical (Indianapolis, Ind.). Reaction conditions were as recommended by the suppliers. All general chemicals were purchased from Sigma (St. Louis, Mo.). Sigma-Genosys (The Woodlands, Tex.) and Invitrogen (Carlsbad, Calif.) synthesized primers for PCR and sequencing.

Yeast transformation. A yeast EZ-Transformation kit (BIO 101, Vista, Calif.) or Alkali-Cation yeast kit (BIO 101) was used for all yeast transformations. Integration vectors were linearized with an appropriate enzyme prior to transformation. Transformants were selected on YSC medium containing 20 g Of glucose per liter. Amino acids were added as necessary. After transformation with plTyX3, the cells were grown for 12 h in YPD to allow for Neo^r expression, and transformants were then selected on YPD plates containing G418.

Plasmid construction. Plasmids used in this study are summarized in Table 1. The pX3 (14) plasmid was digested with *Sma*I and *BsaA*I to produce a 2.0-kbp fragment, which was then inserted into the *Sma*I site of pUC18 to produce pUC18X3. The orientation of *XYL3* in pUC-X3 was confirmed by cutting the plasmid with *Sac*I. pYS32 was constructed by inserting the 2.0-kbp *Eco*RI-*Sal*I fragment from pUC18-X3 into pYPR2831 (10). For construction of the single-copy vector, pYS31, which contains *XYL3* with the glyceraldehyde-3-phosphate dehydrogenase promoter (*TDH1*$_P$), pYS32 was digested with *Hin*dIII. The resulting 3.2-kbp *Hin*dIII-*Hin*dIII fragment was blunt ended with T4 DNA polymerase and inserted into the *Sma*I site of pRS314 (28). The plasmid pYS41 containing *XKS1* with *TDH1*$_P$ and the terminator *TDH1*$_T$, was constructed by inserting 3.3-kbp blunt-ended *Hin*dIII-*Hin*dIII fragment from pY2XK (15) into the *Sma*I site of pRS314. Because the *XKS1* gene contains a *Hin*dIII site in the open reading frame between *TDH1*$_P$ and *TDH1*$_T$, we isolated a 3.3-kbp *Hin*dIII-*Hin*dIII from pY2XK by partial digestion. To construct a vector for the tunable expression of *XYL3,* we digested pX3 with *Pst*I to produce a 2.9-kbp *Pst*I-*Pst*I fragment containing *PsXYL3.* We then inserted the 2.9-kbp *Pst*I-*Pst*I fragment into the *Pst*I site of pITy4 (21) to produce pITyX3.

Preparation of crude extract and enzyme assay. *S. cerevisiae* was grown to exponential phase in YSC medium supplemented with appropriate amino acids and nucleotides and 20 g of glucose/liter or 40 g of xylose/liter. Cells were harvested by centrifugation. The pellet was washed and suspended in buffer (100 mM phosphate buffer, 1 mM EDTA, 5 mM b-mercaptoethanol [pH 7.0]). The suspended cells were mixed with glass beads (Sigma), vortexed at maximum rate in bursts of 30 to 120 s, and then cooled on ice for a similar period. This procedure was repeated for up to 10 min of vortexing with periodic microscopic examination to determine cell breakage. The crude extract collected after centrifugation Cor 10 min at 15,000 × *g* was used for the enzyme assay. Xylulokinase activity was measured according to the method of Shamanna and Sanderson (27). We used a photodiode array spectrophotometer (Hewlett-Packard, Wilmington, Del.) to monitor the reaction by absorbance at 340 nm. All assays were performed within 2 to 4 h of cell breakage. One unit of activity is defined as the amount of enzyme that phosphorylates 1 μmol of xylulose per minute at 30°C. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, Ill.).

Cell growth experiments. For growth on plates, cells were grown on YSC dropout (Leu$^-$ Trp$^-$ Ura$^-$) medium with glucose, and then the cells were harvested and washed. Cells were suspended with double-distilled H_2O to reach an optical density at 600 nm (OD_{600}) of 10. The cell suspension was serially diluted and plated on the YSC plate with glucose and xylose. The plates were incubated at 30°C until colonies grew out. For the growth rate measurement, cells were grown in 50 ml of YSC dropout medium with 40 g of xylose/liter in a 125-ml Erlenmeyer flask shaken at 200 rpm. Initial cell growth (OD_{600}, <2) was used for calculation of the specific growth rate.

TABLE 2. Xylulokinase activity in the recombinant strains

Strain	Xylulokinase sp act (U/mg) ± error forgrowthon[a]:	
	Glucose	Xylose
Control[b]	0.06 ± 0.01	0.21 ± 0.08
FPL-YSX3	0.63 ± 0.10	0.67 ± 0.11
FPL-YS31N	0.18 ± 0.01	0.30 ± 0.15
FPL-YS32N	1.53 ± 0.05	0.33 ± 0.23
FPL-YS31	6.11 ± 0.33	3.45 ± 0.66
FPL-YS32	31.35 ± 2.24	9.99 ± 0.76
FPL-YS41	0.13 ± 0.01	0.32 ± 0.04
FPL-YS42	0.77 ± 0.07	1.27 ± 0.01

[a] Values are averages of data from two independent experiments. Error represents the deviation of each value from the average.

[b] Results for the control represent the averages of the values from three strains containing control vectors (pRS314, pRS424, and pYPR2831) expressed in FPL-YS1020.

Metabolic flux calculation. Metabolic fluxes of the xylose assimilation steps were calculated from the xylose consumption rates and the xylitol and xylulose accumulation rates during initial xylose fermentation within 24 h. By assuming that no xylose, xylitol, and xylulose accumulated in the cell, metabolic fluxes were calculated by the equations $J_{XYLOSE} = J_{XYLITOL} + J_{XDH}$ and $J_{XYLULOSE} = J_{XDH} - J_{XK}$, where J_{XYLOSE}, $J_{XYLITOL}$, and $J_{XYLULOSE}$ represent the specific rates of xylose consumption, xylitol accumulation, and xylulose accumulation and J_{XDH} and J_{XK} represent the internal fluxes of the XDH and XK reactions, respectively.

Quantitative PCR for determining the copy number of *XYL3* in the cell. Genomic DNA from FPL-YSX3 and *P. stipitis* CBS6054 was isolated as described by Rose et al. (25) with modification. Genomic DNA was extracted three times by using equal volumes of phenol-chlorform-isopropyl alcohol (25:24:1). The DNA was precipitated by using one-half volume of ammonium acetate and two volumes of 100% ethanol. It was then suspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The concentration and purity of DNA was determined by using a GeneQuant photometer (Pharmacia Biotech). Quantitative PCR primers were designed to *XYL1* (5′-GATACCTTCGTCAATGGCCTTCT-3′ and 5′-TTCGACGGTGCCGAAGA-3′), *XYL2* (5′-TTCGACGGTGCCGAAGA-3′ and 5′-GATACCTTCGTCAATGGCCTTCT-3'), and *XYL3* (5′-GAAGGTGACATTGCCTCTTACTTTG-3′ and 5′-TCCGGTGAACGAGTAGATTTTACA-3′) by using Primer Express software (Applied Biosystems). Quantitative PCR was performed by using SYBR green PCR master mix (Applied Biosystems) and an AB1 PRISM 7000 sequence detection system (Applied Biosystems). Quantification was performed by using the standard curve method (6). Standard curves for *XYL1*, *XYL2*, and *XYL3* were constructed, and the copy numbers of the genes were interpolated by using these standard curves. *P. stipitis* genomic DNA (1.6, 0.4, 0.1, 0.025, and 0.00625 ng) was used to construct a standard curve for each of the three genes. Quantitative PCR conditions were as recommended by the manufacturer except that one-half of the reaction volume was used: 7.5 pmol of each primer was used in cycles of 50°C for 2 min and 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The standard curve was then used to determine equivalents of each of these genes in three dilutions of genomic DNA from FPL-YSX3. All reactions were performed in triplicate.

Statistical analyses. Statistical analysis of quantitative PCR data was performed by using Excel (Microsoft Corporation, Redmond, Wash.). For pairwise comparisons of the copy numbers, two-sided *t* tests were used to determine if the copy numbers are the same with the null hypothesis (H_o: $\mu_{XYL1} = \mu_{XYL2}$; H_o: $\mu_{XYL1} = \mu_{XYL3}$; and H_o: $\mu_{XYL2} = \mu_{XYL3}$). For the calculation of the copy number of *XYL3*, linear regression was performed with the model $Y_i = c_i x + e_i$, where *Y* and *x* represent the amounts of *XYL3* and *XYL1* in the genomic DNA, respectively, e_i corresponds to the error of the regression, and c_i indicates the copy number of *XYL3* when we assume that the copy number of XYL1 is 1.

Analytical methods. Glucose, xylose, xylitol, xylulose, and ethanol concentrations were determined by high-performance liquid chromatography (Hewlett-Packard) with an ION 300 column (Interaction Chromatography, San Jose, Calif.). Cell growth was monitored by the OD_{600}. One unit of OD_{600} was equivalent to 0.17 g of cells/liter for *S. cerevisiae.*

RESULTS

Construction of recombinant *S. cerevisiae* expressing different levels of *XYL3* and *XKS1*. FpL-YS1020, containing single chromosome-integrated copies of *XYL1* and *XYL2* driven by $TDS1_P$, was used as the host strain for expression of *XYL3* and *XKS1*. we altered gene expression levels by changing the promoters and the plasmid copy numbers. $TDS1_P$ served as a strong promoter, and the native *XYL3* promoter was used as a weak promoter in *S. cerevisiae.* For copy number control, either a multicopy or single-copy plasmid harbored the expression cassettes (Table 2). FPL-YS1020 cells transformed with the plasmids were grown on glucose and xylose, and XK activities were measured. As expected, each transformant showed a dif-

Strains	Gene	Copy/ Promoter	Glucose (3 days)	Xylose (5days)	Growth rate on xylose (h^{-1})
FPL-YS314	Control	S			**0.130**
FPL-YS424	Control	S			0.127
FPL-YS2831	Control	M / G			0.135
FPL-YS31N	*PsXYL3*	S / N			0.123
FPL-YS32N	*PsXYL3*	M / N			0.109
FPL-YS42	*ScXKS1*	M / G			0.092
FPL-YS32	*PsXYL3*	M / G			0.063

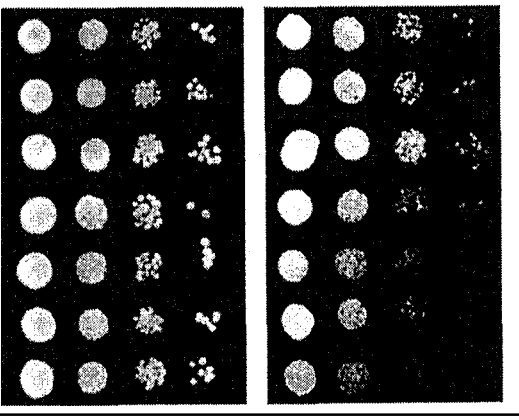

FIG. 1. Growth of recombinant *S. cerevisiae* showing different levels of xylulokinase activity. Cells were grown on YSC medium with glucose and were harvested and washed. A cell resuspension was serially diluted and spotted on YSC medium with glucose and xylose. For the calculation of growth rates on xylose, initial cell growth was monitored in 50 ml of the YSC medium with 40 g of xylose/liter in a 125-ml Erlenmeyer flask shaken at 200 rpm. S and M, single-copy and multicopy constructs, respectively. G and N, $TDH1_P$ and native promoters, respectively.

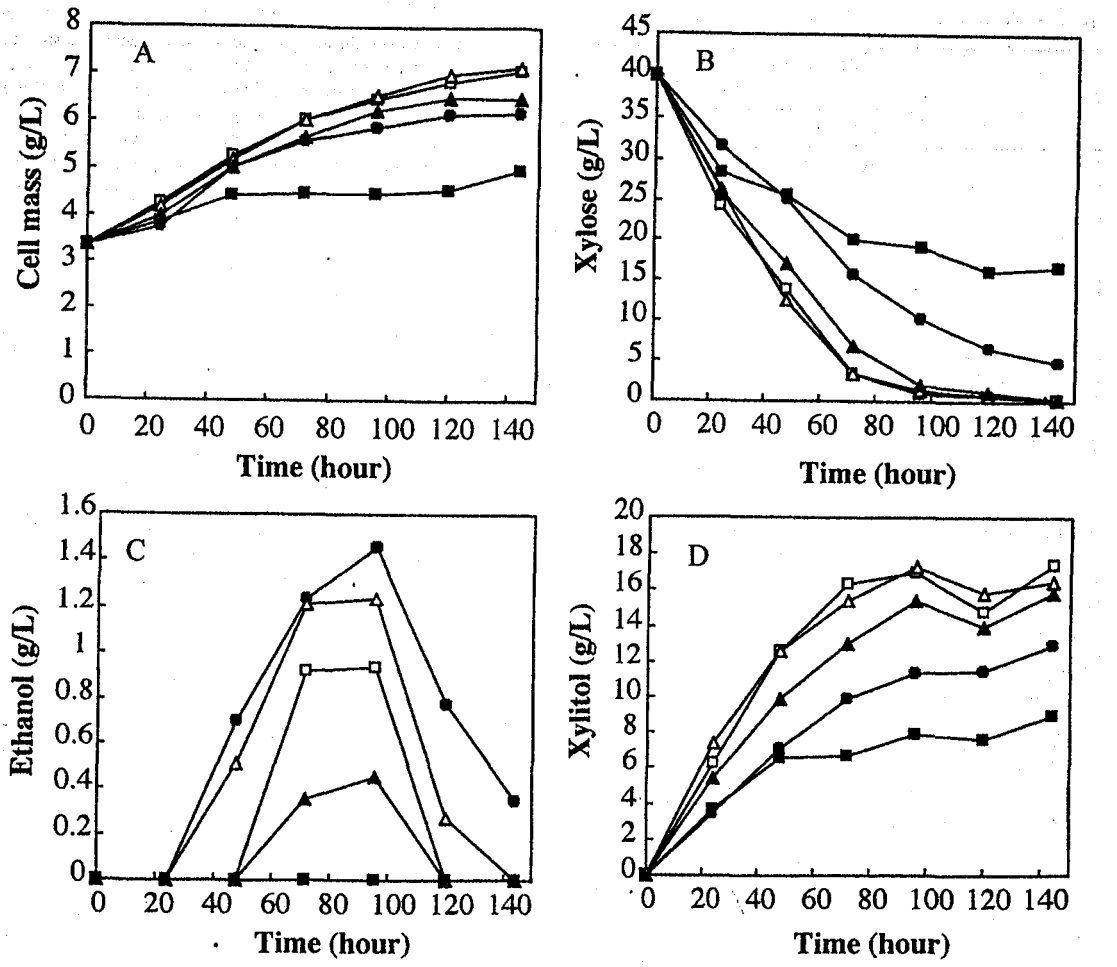

FIG. 2. Xylose fermentation by recombinant *S. cerevisiae* showing different levels of xylulokinase. Cells were cultured in 50 ml of YSC: medium with 40 g of xylose/liter in a 125-ml Erlenmeyer flask. Concentrations of cell mass (A), xylose (B), ethanol (C), and xylitol (D) are shown. Results for controls represent the averages of values from three strains containing control vectors (pRS314, pRS424, and pYPR2831). Data points are the averages of two replicate experiments. Differences between replicates were less than 10%. Symbols: ●, controls; □, FPLYS31; ■, FPL-YS32; △, FPL-YS41; ▲, FPL-YS42.

ferent level of XK activity that varied with copy number, promoter strength, and the carbon source. XK activity increased along with copy numbers and promoter strength (Table 2). FPL-YS32, containing *XYL3* under control of $TDS1_P$ in a multicopy vector, showed the highest XK activity both in the cells grown on glucose and in those grown on xylose (31.35 ± 2.24 and 9.99 ± 0.76 U/mg, respectively). Transformants containing *P. stipitis XYL3* showed higher XK activity than those containing *S. cerevisiae XKS1* although the same plasmids and promoters were used. XK activity in control strains increased when the cells were grown on xylose relative to the activity of cells grown on glucose. This suggests that expression of endogenous *XKS1* is induced by xylose (Table 2). Expression of genes driven by *TDS1*, increased significantly when cells were grown on glucose.

Effects of levels of xylulokinase activity on growth of recombinant *S. cerevisiae* strains on xylose. Growth of transformants that showed different levels of XK was tested on agar plates of YSC dropout medium with glucose or xylose as the sole carbon source (Fig, 1). The levels of XK activity did not affect growth when glucose was the carbon source. However, the FPL-YS32, FPL-YS42, and FPL-YS32N transformants, which showed higher XK activity, grew slowly on xylose. The most severe growth inhibition was observed with FPL-YS32, which showed the highest XK activity. Similar numbers of colonies were observed with various strains when grown on glucose, whereas smaller and fewer colonies were observed when high-XK strains were spotted on xylose (Fig. 1). Higher XK activity was deleterious to the cells when either of the XK genes (*XKS1* or *XYL3*) was overexpressed. Regardless of the origin, overexpression of an XK gene (*XYL3* or *XKS1*) in *S. cerevisiae* was toxic to cells when they were grown on xylose.

Effects of xylulokinase levels on xylose fermentation by recombinant *S. cerevisiae.* Transformants (FPL-YS31, FPL-YS32, FPL-YS31N, FPL-YS32N, FPL-YS41, and FPL-YS42) with the *XYL3* or *XKS1* gene under the control of either $TDH1_P$ or the native *P. stipitis* promoter in either multiple or single copy were first grown on the YSC dropout medium with 20 g of glucose/liter. Cells were harvested and inoculated again into 50 ml of YSC dropout medium with 40 g of xylose/liter in

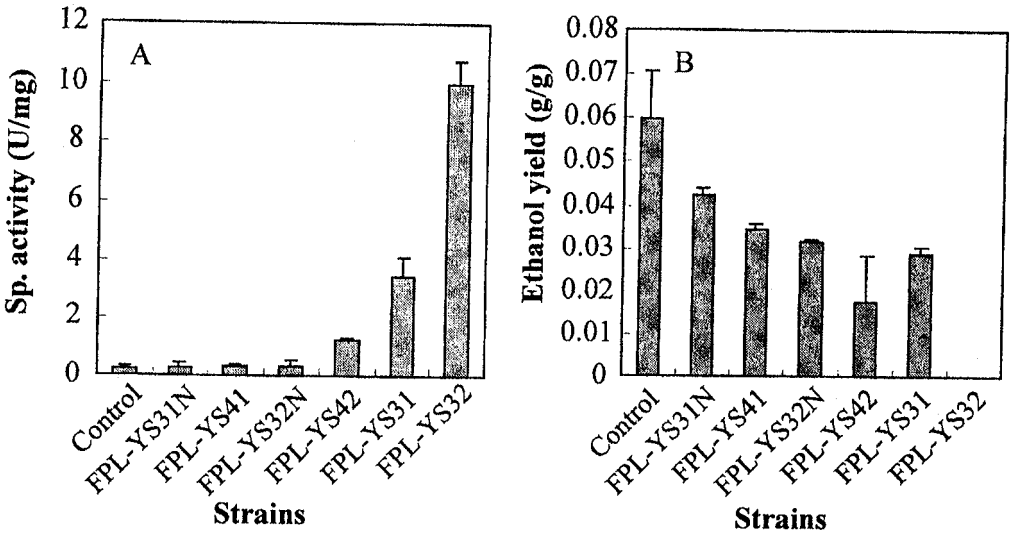

FIG. 3. Relationships between xylulokinase specific (Sp.) activity (A) and ethanol yields (B) of xylose fermentation by recombinant S. cerevisiae strains.

125-ml Erlenmeyer flasks shaken at 200 rpm. Figure 2 shows the profiles of cell mass, xylose consumption, ethanol production, and xylitol production of FPL-YS31, FPL-YS32, FPL-YS41, FPL-YS42, and control strains during xylose fermentation. As shown in the previous section, FPL-YS32 did not grow as well as the other strains when cultivated in xylose liquid medium. However, in contrast to the plate experiments, FPL-YS31, FPL-YS41, and FPL-YS42 grew slightly better than the control strains when each was cultivated in xylose liquid medium. The slowest grower, FPL-YS32, also consumed xylose slowest and did not produce ethanol at all. FPL-YS31, FPL-YS41, and FPL-YS42 consumed xylose faster but accumulated more xylitol than control strains. Ethanol production decreased with increasing XK activity in recombinant ***S. cerevisiae.*** Ethanol yields from xylose are presented along with enzymatic activities in the transformants (Fig. 3). The results showed an inverse relationship between ethanol yield and XK activity during xylose fermentation.

Tunable expression of *XYL3* in the recombinant *S. cerevisiae* FPL-YS1020. We found that overexpression of XK was deleterious to the cell when xylose was the sole carbon source. However, previous studies (8, 16, 33) reported that overexpression of *S. cerevisiae XKS1* enhances xylose fermentation by recombinant *S. cerevisiae* containing *XYL1* and *XYL2.* These results, combined with an observed xylulose accumulation during xylose fermentation with recombinant *S. cerevisiae* express-

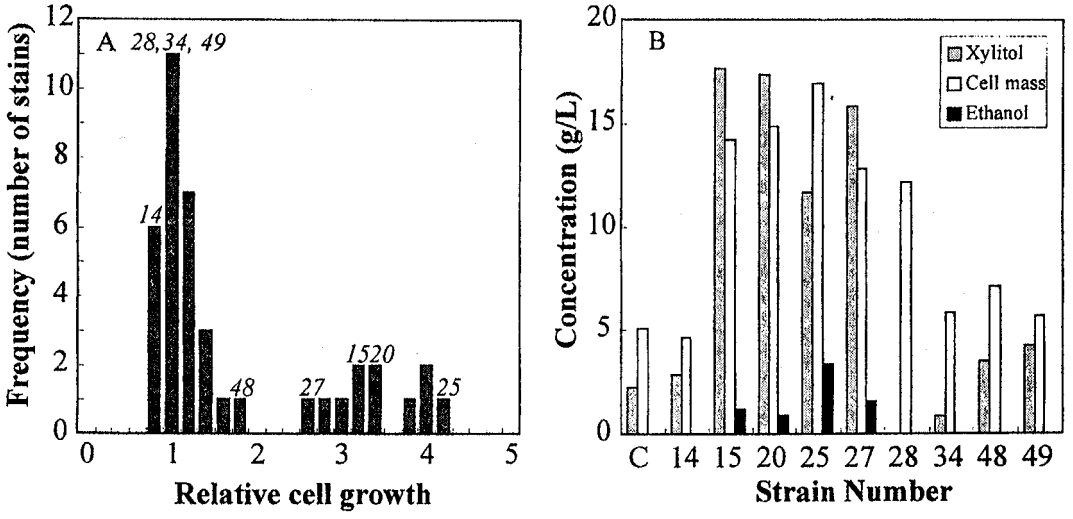

FIG. 4. Screening of Neo^r strains obtained after transformation with the pITyX3 vector, (A) Relative cell growth of 40 transformants after introduction of *XYL3* by a tunable expression vector. Each strain was precultured in YPD medium, and similar amounts of cells were inoculated into tubes of YPX medium in single trials. Relative cell growth was calculated by dividing the cell density of each transformant's culture by the cell density of the parental strain (FPL-YS1020) culture after 3 days. The numbers above columns in the histogram indicate the strain numbers in panel B. (B) Fermentation activities of the selected nine strains along with those of the parental strain (FPL-YS1020, shown in the columns designated C). Cells were cultivated in 50 ml of YPX-4% medium in a 125-ml Erlenmeyer flask shaken at 200 rpm.

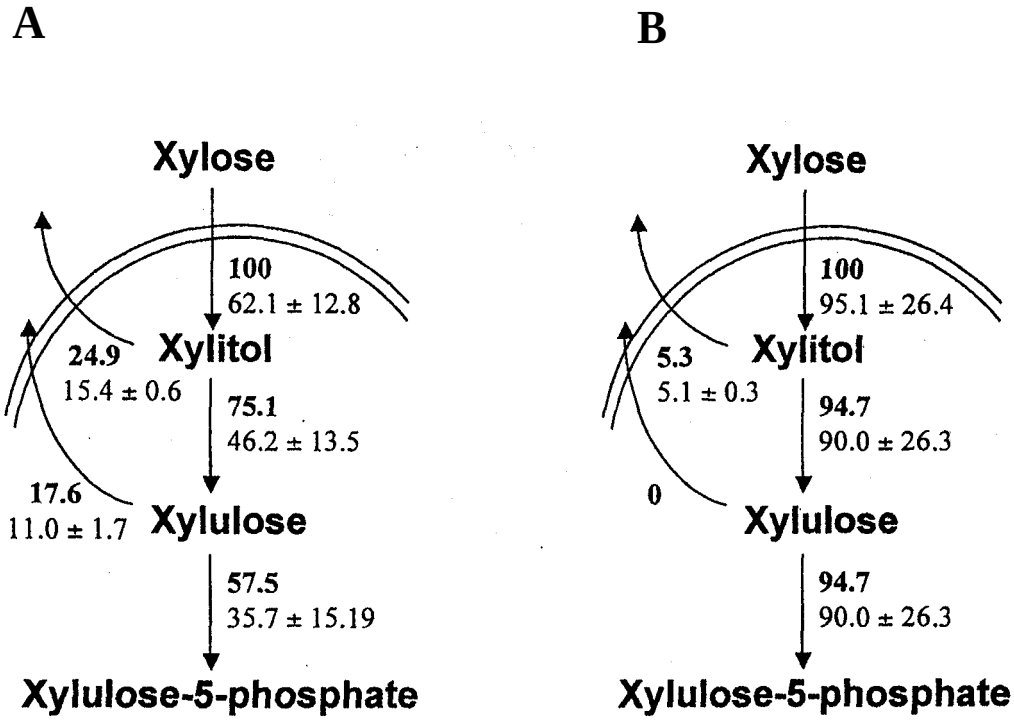

FIG. 5. Metabolic flux distributions in recombinant S. cerevisiae FPL-YS1020 (A) and FPL-YSX3 (B) during xylose fermentation. Fluxes were calculated from the initial xylose consumption rates and xylitol and xylulose accumulation rates of three independent batch fermentations in YP with 40 g of xylose/liter at the oxygen transfer rate of 4.3 mM O_2/h. Fluxes were represented as the averages ± standard deviations in μmol (g of cells)$^{-1}$. Bold numbers indicate the fluxes normalized with respect to the xylose uptake flux.

ing only *XYL1* and *XYL2* (13), suggested that growth is inhibited if the expression level of XK is too high and that if it is too low, cells cannot maintain flux for efficient xylose assimilation. Therefore, we attempted to bypass these problems by optimizing the expression level of *XYL3*.

To this end, we employed a tunable expression vector (21), which uses a yeast transposon (Ty, d) element (2) in the chromosome as an insertion site. It is possible to obtain transformants with multiple integration of the vector because more than several hundred copies of Ty are present in the yeast genome (17). Multiple integrations could give rise to higher levels of gene expression because expression is essentially a linear function of copy number. *XYL3* was introduced into FPL-YS1020 by using pITyX3, which contains a Ty3 element, the G418 resistance gene (*neo*), and *XYL3*. pITyX3 was linearized by cutting with *Xho*I and transformed into FPL-YS1020. Putative transformants were selected on YPD containing 300 μg of G418/ml. pITyX3 could integrate into the *S. cerevisiae* chromosome at multiple sites. The location and copy number of pITyX3 in the chromosome could affect the expression of XYL3, so we tested 40 independent transformants in single trials for growth on xylose. Each showed a different growth characteristic. Figure 4A shows the growth of the various transformants as a histogram in which the relative cell growth is plotted versus the frequency of its occurrence. The fastest-growing strain–alongwith eight other strains–were examined in a fermentation trial. As expected, each transformant also showed significantly different ethanol production from xylose (Fig. 4B). The best strain (no. 25) was selected on the basis of its product yield and was named FPL-YSX3. XK activity in FPL-YSX3 was found to be much higher than that in the parental strain, FPL-YS1020 (Table 2).

Copy number determination of *XYL3* in FPL-YSX3 strain. Quantitative PCR allows the analysis of gene copy number from a small amount of DNA and offers a wide dynamic range of quantification with high accuracy (1, 6, 11). Ingham et al. (11) found 95% overall correlation between Southern blot and quantitative PCR data during determination of the copy num-

TABLE 3. Comparison of xylose consumption and xylitol and ethanol production among *S. cerevisiae* strains[a]

Strain	Xylose consumed (g/liter)	Xylitol formed (g/liter)	Ethanol formed (g/iter)	Xylitol yield (g/g)	Ethanol yield (g/g)
L2612	0.37 ± 0.18[b]	0.27 ± 0.03	0	0.74 ± 0.18	0
FPL-YS10	0.61 ± 0.12	0.33 ± 0.01	0	0.55 ± 0.09	0
FPL-YS1020	7.9 ± 0.58	3.93 ± 0.30	0	0.50 ± 0.07	0
FPL-YSX3	16.91 ± 0.44	4.56 ± 0.03	1.94 ± 0.05	0.27 ± 0.01	0.12 ± 0.01

Results show grams of xylose, xylitol, or ethanol per liter of medium and grams of xylitol or ethanol per gram of xylose.
Displayed values are the averages ± standard deviations of the results of three independent replicate experiments.

bers of genes in transgenic plants. We determined the copy numbers of *XYZ1*, *XYL2*, and *XYL3* in FPL-YSX3 by using quantitative PCR. Because *XYL1* and *XYL2* were inserted into FPL-YSX3 by site-specific integration, a single copy per haploid genome can be assumed. By comparing the number of copies of *XYL3* present in 0.2, 0.1, and 0.05 ng of DNA to the number of copies of *XYL1* and XYL2 present, the number of copies of XYL3 per genome was calculated. For a standard curve, genomic DNA of *P. stipitis* was used because it contains exactly the same genes present in FPL-YSX3, and each of them is present in a single copy. For *XYL1* and *XYL2*, 0.275 ± 0.77 and 0.207 ± 0.082 equivalent per genome were found, respectively. The copy numbers of *XYL1* and *XYL2* were similar ($P > 0.05$). In contrast, XYL3 was found to have 1.095 ± 0.348 equivalents per genome, which was significantly different from *XYL1* and *XYL2* ($P < 0.001$ for both). Because *XYL1* and XYL2 are each present in a single copy in the haploid genome, the copy number of XYL3 was calculated to be in the interval of 3.67 ± 1.30 copies per genome with a 95% confidence.

Comparison of metabolic fluxes in FPL-YS1020 and FPL-YSX3. Fluxes of metabolites in the xylose assimilation steps were calculated from the xylose consumption rates and the xylitol and xylulose accumulation rates during xylose fermentation by FPL-YS1020 and FPL-YSX3. Metabolic flux distributions changed drastically after the introduction of XYL3. Xylose consumption increased 1.7-fold and xylitol accumulation decreased threefold after the expression of *XYL3* in FPL-YS1020. Moreover, xylulose accumulation was not observed (Fig. 5). This result shows that an appropriate level of XK is indispensable for efficient xylose utilization by recombinant *S. cerevisiae.*

Comparison of xylose fermentation by recombinant *S. cerevisiae* in YP medium with xylose. We compared xylose fermentation by L2612, FPL-YS10, FPL-YS1020, and FPL-YSX3. L2612 was the parental strain used for further engineering of xylose metabolism (4). FPL-YS10 has only *XYL1*, and FPL-YS1020 contains *XYL1* and *XYL2* (13). FPL-YSX3 contains *XYL1*, *XYL2*, and *XYL3*. YP medium with 20 g of xylose/liter was used for the fermentation experiment. As shown in Table 3, the parental strain FPL-YS10 did not consume significant amounts of xylose (less than 1 g/liter). FPL-YS1020 consumed 8 g of xylose/liter, but one-half of the consumed xylose was converted into xylitol (3.93 g/liter). Ethanol production by FPL-YS1020 was not significant (less than 1 g/liter). However, FPL-YSX3 consumed xylose much faster than other strains and produced ethanol with a yield of 0.12 g of ethanol/g of xylose. FPL-YSX3 still accumulated xylitol as a by-product. However, the xylitol yield was much lower than with FPL-YS1020 (0.27 compared to 0.50 g of xylitol/g of xylose). These results clearly showed that an appropriate low level of *XYL3* expression increases xylose uptake and ethanol production but decreases xylitol accumulation during xylose fermentation by recombinant *S. cerevisiae.*

Xylose fermentation by FPL-YSX3 in minimal medium with xylose. FPL-YSX3 was transformed with the control vector (pYPR2831) for the comparison of growth and ethanol production from YSC medium with xylose. The cells were grown on YSC dropout medium with 20 g of glucose/liter and inoculated again into 50 ml of YSC dropout medium with 40 g of xylose/liter in 125-ml Erlenmeyer flasks shaken at 200 rpm

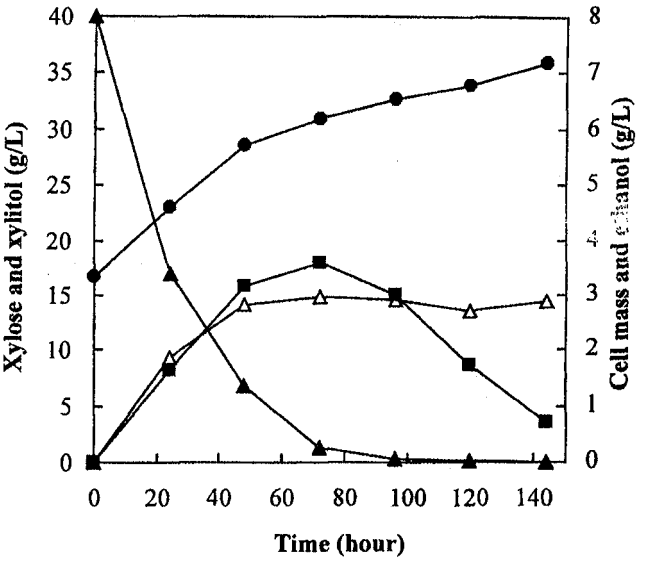

FIG. 6. Xylose fermentation by recombinant *S. cerevisiae* FPL-YSX3(pYPR2831) in YSC medium with 40 g of xylose/liter. Data points are the averages of the results of two replicate experiments. Differences between the two replicates were less than 10%. Symbols: ●, cell mass; ■, ethanol; ▲, xylose; △, xylitol.

(Fig. 6). The FPL-YSX3 transformant grew better on xylose and consumed xylose much faster than other strains (cf. Fig. 2 and 6). Maximum ethanol concentration was 3.4 g/liter, which was more than a twofold increase compared to results for control strains that did not contain *XYL3*. However, xylitol was still a major by-product (13 g/liter).

DISCUSSION

The inhibition of cell growth and ethanol production observed when cells express high levels of XK is similar to substrate-accelerated death (31), which is observed when an *S. cerevisiae tps1* mutant is cultivated on glucose (32). *TPS1* encodes trehalose-6-phosphate (Tre6P) synthase, so a *tps1* disruptant cannot synthesize Tre6P, which is a potent inhibitor of hexokinase (9). As a result, hexokinase in a *tps1* background phosphorylates glucose without control of Tre6P, thereby resulting in excess flux of glucose into glycolysis. Overexpression of XK in *S. cerevisiae* could be inhibitory for several reasons (Fig. 7). First, *S. cerevisiae* might not possess a guard system that prevents excessive ATP consumption in the presence of XK because this yeast has not evolved to utilize xylose. Rapid ATP depletion would then inhibit the cells because ATP is necessary for other cellular activities. Second, the PPP capacity in *S. cerevisiae* might be not enough to maintain metabolic flux at a steady state for ATP synthesis when XK is overexpressed. Excess XK activity could then result in accumulation of X5P and depletion of ATP. Third, it is possible that X5P itself is toxic to the cell, The toxicity resulting from excess accumulation of sugar phosphate was previously reported for galactose metabolism (20). Regardless of the exact mechanism, previous research by Toivari et al. supports the hypothesis that overexpression of XK is toxic, because levels of X5P were significantly

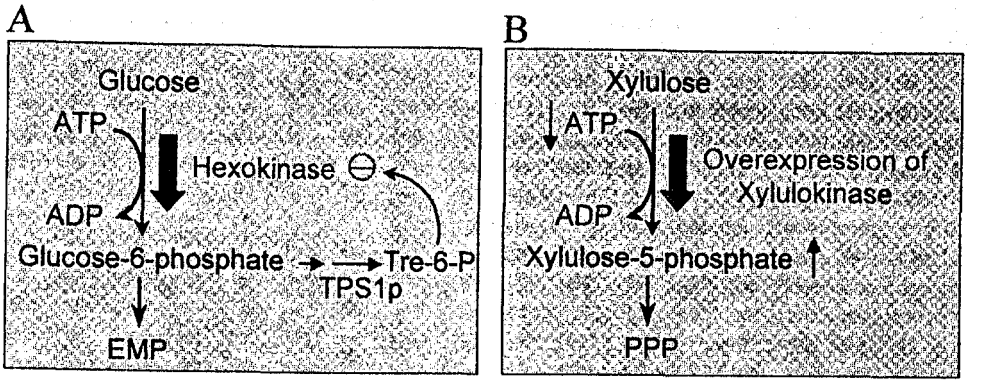

FIG. 7. Shown are schematic diagrams of substrate-accelerated cell death in *TPS1* disruptant (A) and the homologous phenotype in xylose metabolism when xylulokinase is overexpressed (B).

higher and levels of ATP were lower in an XK-overexpressing strain compared to parental strains (33).

The inhibitory effect of higher XK activity correlated closely with the growth rate under fully aerobic conditions. However, in the fermentative trials with low aeration, only cells with the highest XK activity showed significant growth inhibition (Fig. 2A). Both ATP demand and xylulose supply would be maximized under aerobic conditions. But under oxygen-limited conditions, where growth rates were about one-tenth of those observed under the fully aerobic condition, the ATP demand would be much lower, and the inhibitory effect would not be as strong. Alternatively, the initial oxidoreductase step for conversion of xylose into xylulose could be limited by oxygen availability. The supply of xylulose to deplete ATP would then be limited under fermentative conditions.

A direct comparison of XK activities observed in other studies can help resolve some of the apparent conflicts. Rodriguez-Pena et al. (24) first noted the toxicity of XK overexpression with cells grown on D-xylulose. These researchers did not report XK activity, but they used a multicopy vector with a strong promoter. Johansson et al. (16) reported XK activities of 28 and 36 U/mg in H158-pXks and CEN.PK-pXks from cells grown in defined media on a glucose-xylose sugar mixture. These activities should be compared to activities that we observed with cells grown on glucose because Johansson et al. prepared cell extracts from early batch cultures in which glucose was probably still present. Johansson et al. found XK overexpression to be deleterious, as characterized by the severe inhibition of xylose consumption: approximately 7 g of xylose/liter was consumed within 125 h, whereas the parental strain consumed 40 g of xylose/liter within the same period. In contrast, Toivari et al. (33) and Ho et al. (8) reported much lower XK activities (7 nkat/mg [i.e., 0.42 U/mg] and 0.1 U/mg, respectively) in their recombinant *S. cerevisiae* than in FPL-YS32, H158-pXks (16), and CEN.PK-pXks (16). However, these values are still higher than the reported native XK activity. Both groups of researchers observed that overexpression of *XKS1* significantly enhances xylose fermentation rather than causing toxic effects, which were observed with *XKS1* in FPL-YS32, H158-pXks (16), and CEN.PK-pXks (16).

Heterologous XK expression should be modulated because its effect on cell physiology can vary with the expression level. However, it is very difficult to define the optimum level a priori. The appropriate level of XK might vary by intrinsic strain-specific properties, such as XR and XDH activity, PPP capacity, and respiration capacity, because it is related to cellular levels of X5P and ATP. Moreover, the optimum XK level can also be affected by extrinsic factors such as aeration, which mainly controls generation of ATP. Therefore, it is almost impossible to define and maintain an optimal XK level in the cell by forward engineering.

Instead, we used an empirical approach to select the strain that grew best on xylose from a collection of transformants. The Ty vector integration copy number ranges from 1 to 30 after a single transformation; the level of amplification is stable over 50 generations in the absence of antibiotics, and the chromosomal context around the integration site affects gene expression (21). Each transformant in our study showed a different growth characteristic on xylose (Fig. 4A), as would be expected from multiple random integration events. Perhaps XK activity in the transformant could not reach high levels because the native *P. stipitis* promoter was used to drive *XYL3* expression (14). Our activity and copy number results indicate that this promoter is not very effective in *S. cerevisiae.*

Our results show the toxic effects of overexpression of XK genes (*XKS1* and *XYL3*) during xylose fermentation by recombinant *S. cerevisiae.* These results suggest that levels of introduced enzyme activity should be designed in concert with the capacity of the surrounding metabolic network. Since numerous intrinsic and extrinsic factors affect the flow of metabolites in cells, we think that reverse engineering of metabolism, as shown in our study, would be a practical approach for successful optimization of metabolic pathways.

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