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Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*

Yong-Su Jin[a,1] and Thomas W. Jeffries[a,b,c,*]

[a] *Department of Food Science, University of Wisconsin-Madison, 1605 Linden Drive, Madison, WI 53706, USA*
[b] *Department of Bacteriology, University of Wisconsin-Madison,1550 Linden Drive,Madison, WI 53706, USA*
[c] *Forest Products Laboratory, USDA Forest Service, One Gifford Pinchot Drive, Madison, WI 53705-2398, USA*

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Abstract

Metabolic pathway engineering is constrained by the thermodynamic and stoichiometric feasibility of enzymatic activities of introduced genes. Engineering of xylose metabolism in *Saccharomyces cerevisiae* has focused on introducing genes for the initial xylose assimilation steps from *Pichia stipitis*, a xylose-fermenting yeast, into *S. cerevisiae*, a yeast traditionally used in ethanol production from hexose. However, recombinant *S. cerevisiae* created in several laboratories have used xylose oxidatively rather than in the fermentative manner that this yeast metabolizes glucose. To understand the differences between glucose and engineered xylose metabolic networks, we performed a flux balance analysis (FBA) and calculated extreme pathways using a stoichiometric model that describes the biochemistry of yeast cell growth. FBA predicted that the ethanol yield from xylose exhibits a maximum under oxygen-limited conditions, and a fermentation experiment confirmed this finding. Fermentation results were largely consistent with *in silico* phenotypes based on calculated extreme pathways, which displayed several phases of metabolic phenotype with respect to oxygen availability from anaerobic to aerobic conditions. However, in contrast to the model prediction, xylitol production continued even after the optimum aeration level for ethanol production was attained. These results suggest that oxygen (or some other electron accepting system) is required to resolve the redox imbalance caused by cofactor difference between xylose reductase and xylitol dehydrogenase, and that other factors limit glycolytic flux when xylose is the sole carbon source.

Keywords: Flux balance analysis; Extreme pathways; Metabolic phenotype

1. Introduction

D-xylose is a major component of the hydrolyzate of hemicellulose from biomass. Therefore, ethanol production from xylose is essential for successful utilization of lignocellulose (Jeffries, 1985). Many bacteria, yeast, and fungi assimilate xylose, but only a few metabolize it to ethanol (Skoog and Hahn-Hägerdahl, 1988). In yeast, xylose is reduced to xylitol by NADPH-linked xylose reductase (XR), and then xylitol is oxidized to xylulose by NAD-linked xylitol dehydrogenase (XDH). *Saccharomyces cerevisiae*, traditionally used in ethanol production, is unable to utilize xylose. However, it can slowly metabolize xylulose, the ketoisomer of xylose. Thus, several research groups have tried to genetically engineer *S. cerevisiae* by introducing the genes (*XYL1* and *XYL2*) coding for XR and XDH from a xylose fermenting yeast, *P. stipitis*. The resulting strains can grow on xylose aerobically and produce ethanol under oxygen-limited conditions (Jin et al., 2000; Kotter et al., 1990; Tantirungkij et al., 1994; Toivari et al., 2001; Walfridsson et al., 1997). Ho et al. (1998) reported that overexpression of endogenous xylulokinase (*ScXKS1*) under the background of *XYL1* and *XYL2* could enhance the xylose fermentation by recombinant *S. cerevisiae*. Recently, we cloned the xylulokinase gene (*XYL3*) from *P. stipitis* (Jin et al., 2002), and were able to transfer a complete xylose pathway into *S. cerevisiae*. However, the resulting recombinant *S. cerevisiae* expressing *XYL1*, *XYL2*, and *XYL3* still prefers oxidative utilization of xylose.

Much experimental work in metabolic engineering has followed a hypothesis-driven approach. This

*Corresponding author. Forest Products Laboratory, USDA Forest Service, One Gifford Pinchot Drive, Madison, WI 53705-2398, USA.
E-mail address: twjeffri@wisc.edu (T.W. Jeffries).
[1] Present address: Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

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involves identifying a target pathway, introducing or deleting one or few genes, and determining the effects of such changes on cell metabolism (Bailey, 1991; Cameron and Tong, 1993; Stephanopoulos and Vallino, 1991). However, predicting and bringing about changes in metabolic fluxes is difficult by these methods due to the extreme complexity of metabolic networks, and the piecemeal nature of gene targeting. Therefore, more comprehensive approaches are required that consider cellular metabolism as a network or system from a global perspective. Flux balance analysis (FBA) (Delgado and Liao, 1997; Vallino and Stephanopoulos, 1993; Varma and Palsson, 1994) provides the best approach for integrating established reactions and their stoichiometric relationships into structured, quantitative metabolic models. Although it can not handle regulatory effects or unknown reactions, FBA (Varma and Palsson, 1994) employs a series of equations that describe the major known biochemical reactions. Steady-state approximations of intracellular metabolite concentrations can be used to generate a system of linear equations that relate concentrations of starting, endpoint and intermediate metabolites in the cell. These predicted values could then be compared to measured values under defined conditions. In effect FBA constitutes a complex falsifiable hypothesis, the testing of which can be used to refute starting assumptions.

FBA has been applied to describe metabolism in *Escherichia coli* (Varma et al., 1993) and *S. cerevisiae* (van Gulik and Heijnen, 1995; Vanrolleghem et al., 1996). Recently, genomic-scale metabolic models of *E. coli* (Edwards and Palsson, 2000), *Haemophilus influenzae* (Edwards and Palsson, 1999), and *S. cerevisiae* (Forster et al., 2003) have made it possible to predict *in silico* metabolic phenotypes from genotypes. In parallel with FBA, extreme pathway analysis has been proposed to study systems properties of metabolic networks (Palsson et al., 2003; Papin et al., 2002; Price et al., 2002; Schilling et al., 2000). Extreme pathways correspond to the edges of a convex cone, which represents the feasible metabolic space constrained by a metabolic network. All possible steady-state solutions of a given metabolic network can be designated by nonnegative combinations of extreme pathways. Price et al. (Price et al., 2002) utilized extreme pathway analysis at genomic-scale to reveal unique amino acid and nucleotide metabolism in *Helicobacter pylori*.

In this study, we applied FBA and extreme pathway analysis to explore the feasible metabolic space of xylose metabolism as constrained by a stoichiometric model of *S. cerevisiae*. Metabolic phenotypes predicted from calculations of flux distributions and extreme pathways were largely in accord with corresponding experimental data sets.

2. Materials and methods

2.1. Strains and culture conditions

Strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* YSX3 (Jin et al., 2002), which contains *XYL1*, *XYL3* and *XYL3* in the chromosome, was used for xylose fermentation. Plasmid constructs for gene integration are shown in Fig. 1. *P. stipitis* UC7 (Ura$^-$; Ura3-3) (Lu et al., 1998) was used for enzymatic activity comparison. YPD (10 g/L of yeast extract, 20 g/L of bactopeptone, and 20 g/L of glucose) medium was used for inoculum preparation. YPX (10 g/L of yeast extract, 20 g/L of bactopeptone, and 40 g/L of xylose) medium was used for xylose fermentation at 30°C. Xylose fermentation was carried out in a 125 ml Erlenmeyer flask with 50 ml working volume at given agitation rates of 100, 200, 250, and 300 rpm. Oxygen transfer rates of the culture conditions were measured by the sulfite method (Cooper et al., 1944). Two different oxygen-limited conditions were generated by inoculating 5 g/L

Table 1
Plasmids and microbial strains used in this study

Name	Description	Reference
Plasmids		
pYS10	*URA3-GAPDH$_P$-XYL1-GAPDH$_t$*	Jin and Jeffries (2002)
pYS20	*LEU2-GAPDH$_P$-XYL2-GAPDH$_t$*	Jin and Jeffries (2002)
pITyX3	Ty3-*Neo-XYL3*	Jin et al. (2003)
Strains		
S. cerevisiae L2612	*MATα leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal$^+$*	Cho et al. (1999)
S. cerevisiae FPL-YSX3	Isogenic of L2612 except for *leu2*::*LEU2-XYL1*, *ura3*::*URA3-XYL2*, *Ty3*::*neo-XYL3*	Jin et al. (2003)
Pichia stipitis UC7	*ura3-3*, NRRL Y-21448	Lu et al. (1998)

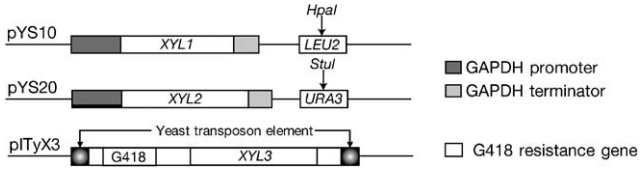

Fig. 1. Plasmids constructs for integration of *XYL1*, *XYL2*, and *XYL3*. The *LEU2* gene was targeted for site-specific integration of pYS10 containing *XYL1*. The *URA3* gene was used for integration of pYS20 containing *XYL2*. For the integration of *XYL3* yeast, yeast transposon element (Ty) was used for integration of pITyX3. All plasmids were linearized before transformation.

of cells into 50 ml of YPX medium in 125 ml Erlenmeyer flasks shaken at speeds of 100 and 200 rpm. Two different aerobic conditions were produced by inoculating 2 g/L of cells into 50 ml of YPX medium in 125 ml Erlenmeyer flasks shaken at speeds of 250 and 300 rpm.

2.2. Crude extract preparation and enzyme assays

S. cerevisiae YSX3 was grown to exponential phase in YPD medium, and *P. stipitis* UC7 was grown to exponential phase in YPX medium. Cells were harvested by centrifugation. The cell pellet was washed and suspended in cell breaking buffer (50 mM phosphate buffer, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.0). The suspended cells were mixed with glass beads (Sigma, St. Louis, MO) and vortexed 10 times at the maximum for 1 min. To prevent thermal denaturation of enzyme, vortexing was performed at 4°C in a cold chamber and the cell–glass mixture was cooled down to 4°C on ice between vortexes. Cell debris was removed by centrifugation at 13,000*g* for 10 min. XR (EC 1.1.1.21) activity was measured in the reaction mixture with the following composition: 50 mM phosphate buffer, pH 6.0, 100 mM xylose, 0.4 mM NADPH. XDH (EC 1.1.1.9) activity was measured in a reaction mixture containing 50 mM Tris–HCl buffer, pH 8.5, 4 mM NAD^+, 5 mM $MgCl_2$, and 100 mM xylitol. Xylulokinase (EC 2.7.1.17) activity was measured according to the method of Shamanna and Sanderson (1979). We used a photodiode array spectrophotometer (Hewlett Packard, Wilmington, DE) to monitor the rate of NADPH oxidation, NAD^+ reduction, and NADH oxidation in the reaction by absorbance at wavelength of 340 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes 1 μmol of substrate per minute at 30°C. Protein concentration was determined by the BCA method (Pierce, Rockford, IL).

2.3. Analytical methods

Glucose, xylose, xylitol, xylulose and ethanol concentrations were determined by HPLC (HP, Wilmington, DE) with an ION 300 column (Interaction Chromatography, San Jose, CA). Cell growth was monitored by optical density at 600 nm (OD_{600}). One unit at 600 nm was determined to be equivalent to 0.167 g DCW/L.

2.4. Stoichiometric model of xylose metabolism in recombinant S. cerevisiae

A stoichiometric model of xylose metabolism in the recombinant *S. cerevisiae* was developed using biochemical textbooks (Berg et al., 2002; Nelson et al., 2000) and the *S. cerevisiae* genome sequence database [http://www.yeastgenome.org]. In order to simplify analysis, only primary carbohydrate metabolism (glycolysis, pentose phosphate pathway, TCA cycle, and gluconeogenesis) and energy metabolism (fermentation and respiration) were considered (Fig. 2 and Table 2). Compartmentation is usually considered as a critical factor for controlling the redox balance in the cytosol (van Dijken and Scheffers, 1986). However, *S. cerevisiae* has a symmetrical electron transport chain unlike mammalian mitochondria. Thus *S. cerevisiae* mitochondria lack energy conservation at site I. As a result, localization of reducing cofactors plays a minor role in *S. cerevisiae* (Verduyn et al., 1991). Therefore, compartmentation was neglected in the stoichiometric model. Requirements of biosynthetic precursors and cofactors for cell growth were calculated from the chemical composition (Vanrolleghem et al., 1996) of *S. cerevisiae* and the amino acid composition (Cook, 1958) of the cellular proteins in *S. cerevisiae*. The nucleotide residue composition of total RNA in *S. cerevisiae* was taken from Mounolou (1975). We assumed that 4.3 ATP molecules (4 ATP for amino acid incorporation and 0.3 for proofreading and mRNA synthesis) are required for the addition of one amino acid into a peptide chain (Neidhardt et al., 1990). For RNA synthesis, we assumed that 2.4 ATP molecules are required for the incorporation of one nucleotide (2 ATP for synthesis and 0.4 ATP for processing, Neidhardt et al., 1990). Active transport systems require a proton-motive force to transport nutrients through the cell membrane. The proton-motive force is generated by plasma membrane H^+-ATPase at the expense of ATP. Therefore, we assumed that ammonium transport requires 1 ATP molecule per ammonium ion, and sulfate ion transport requires 3 ATP molecules per sulfate ion (Verduyn et al., 1991). Table 3 summarizes calculated requirements of metabolite precursors and cofactors for cell growth. The calculated ATP requirement for growth is 37.04 mmol ATP/g cell ($Y_{ATP} = 26.9$ g cell/ATP), which is comparable to the value of 35.60 mmol ATP/g cell ($Y_{ATP} = 28.3$ g cell/ATP) calculated by Verduyn et al. (1991). However, previous investigations it have found that the theoretical Y_{ATP} value is significantly higher than the experimental value (van Gulik and Heijnen, 1995; Vanrolleghem et al., 1996; Verduyn et al., 1991). This discrepancy occurs because it is impossible to calculate the ATP consumption responsible for the maintenance of concentration and electrical potential gradients and futile cycles. To compensate for these phenomena, we employed a maintenance factor k, expressed in mmol ATP/g cell, which incorporates the incalculable ATP consumption in the cell. A value of 16.21 mmol ATP/g cell was used for k in accordance with Vanrolleghem et al. (1996).

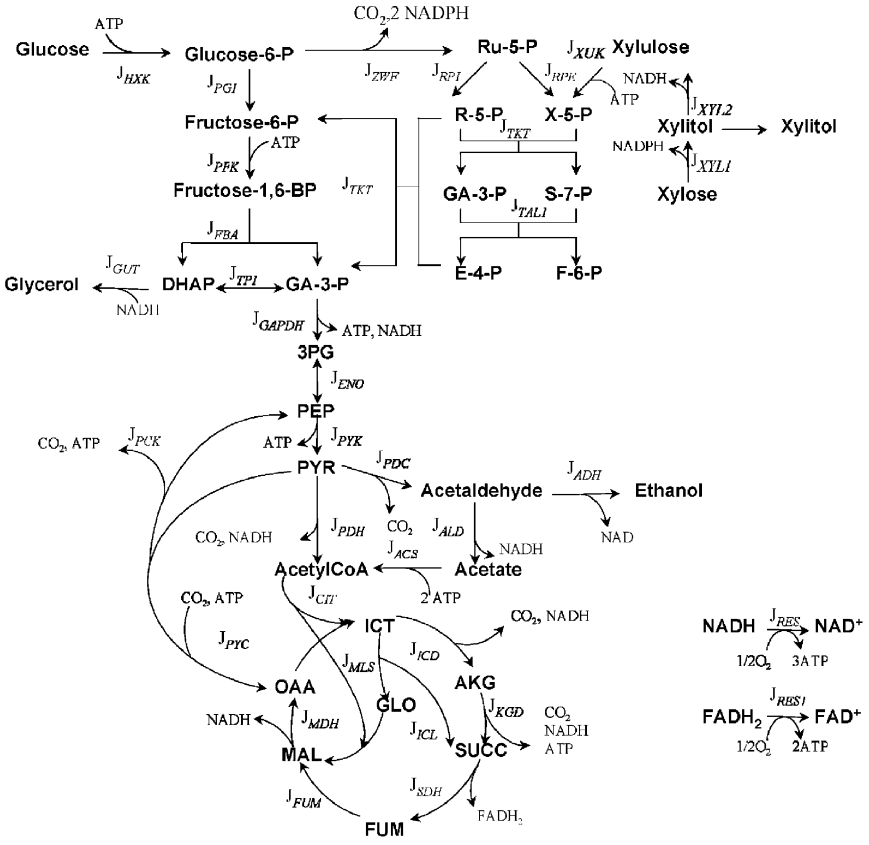

Fig. 2. Metabolic network of xylose metabolism in the recombinant *S. cerevisiae*.

Table 2
Summary of stoichiometric model used in this study

Parameters	Description
Included pathways	Glycolysis, pentose phosphate pathway, gluconeogenesis, TCA cycle, xylose assimilation pathway
Number of metabolites	35
Number of reactions	56
Compartmentation	Not considered
P/O ratio	1.0
Energy requirement for biomass	37.04 mmol ATP/g cell
Maintenance factor	16.21 mmol ATP/g cell

2.5. Exploring methods for feasible metabolic space with given stoichiometric matrix

In general, dynamic mass balances around metabolite can be written as

$$\frac{dX}{dt} = S \cdot V - b, \tag{1}$$

where X is the vector of metabolite concentrations, S is the stoichiometric matrix, V is the vector of reaction rates, and b is the vector of substrate consumption, product formation and cell growth rates. Since the relaxation time for the metabolic reaction is much shorter than the relaxation times for cell growth, substrate consumption and product formation rates, metabolites concentrations in the cell can be considered to be at pseudo-steady state. This reduces Eq. (1) to

$$S \cdot V = b. \tag{2}$$

The number of metabolic fluxes is usually greater than the number of mass balance equations. Therefore, more than one solution exists and the number of solutions is determined by the degrees of freedom of the system. The solution space (feasible metabolic domain) can be explored using linear programming (LP) with an objective function. Various objective functions can be considered that maximize ATP (Majewski and Domack, 1990), metabolite production (Varma et al., 1993), and cell growth (Edwards et al., 2001). Here, we tried to mimic the evolutionary driving force of the cell by defining the objective function as

$$\text{Maximize } V_{\text{growth}}, \quad \text{where } V_{growth} = \sum_{all\ m} d_m \cdot X_m. \tag{3}$$

We can formulate a cell growth reaction (Table 3) that converts all precursors into cell mass by Eq. (3), where d_m represents the biomass composition of each metabolite X_m.

The null space from the equation with positive constraint (2) can be transformed into a convex cone for which the edges represent extreme pathways (Schilling et al., 2000). To transform the null space into a convex space, all reversible reactions were decomposed into their forward reaction and reverse reactions. By this, all metabolic fluxes become positive. Thus, the feasible metabolic space constrained by Eq. (2) can be represented using a convex basis, i.e. an extreme pathway (Eq. (4))

$$v = \sum \omega_i \cdot \mathrm{EP}_i, \quad 0 \leqslant \omega_i \leqslant \infty, \tag{4}$$

where v represents the feasible metabolic space in the convex system, and ω_i and EP_i symbolize weight and extreme pathway, respectively. All possible metabolic flux distributions that can maintain intracellular metabolites at steady state are confined to the convex cone. The extreme pathways at the edge of the cone, are unique and systemically independent, i.e. a given extreme pathway cannot be generated from a combination of other extreme pathways (Schilling et al., 2000). Therefore, we can characterize the limitations and production capabilities of metabolic networks by identifying extreme pathways.

3. Results

3.1. Enzymatic assays of XR, XDH, and XK in the recombinant S. cerevisiae YSX3

Enzymatic activities of XR, XDH, and XK in the recombinant *S. cerevisiae* YSX3 were measured in order to confirm the integrity of the construct. The crude extract from the YSX3 strain showed significant activity of XR, XDH, and XK whereas host strain L2612 (Cho et al., 1999) did not show detectable enzymatic activity (Table 4). The YSX3 strain contained about 38% XR activity, 78% XDH activity, and 50% XK activity relative to *P. stipitis* UC7 grown on xylose.

Table 3
Requirement of precursor metabolites and cofactors for cell growth

Precursor metabolite	Amount required (μmol/g cells)	Precursor metabolite	Amount required (μmol/g cells)
G6P	−2346	ACCOA	−628
R5P	−725	AKG	−1027
E4P	−564	OAA	−1032
TP	−77	NADH	3350
3PG	−790	NADPH	−9147
PEP	−972	ATP	−37,041
PYR	−1131		

− sign in front of number means consumption and + sign means production during cell growth.

3.2. Xylose fermentation by recombinant S. cerevisiae YSX3 expressing XYL1, XYL2, XYL3

We tested growth of recombinant *S. cerevisiae* YSX3 in YP medium with 40 g/L of xylose. The YSX3 grew better on xylose in proportion with increases in aeration rate (Fig. 3). Specific growth rates (calculated from the growth within 24 h) doubled from 0.031 to 0.063 h^{-1} at 100 and 300 rpm. Xylose consumption also increased drastically with increases in aeration. Specific xylose consumption rates were 0.100, 0.118, 0.141, and 0.147 g xylose/(g cell h) at 100, 200, 250, and 300 rpm, respectively. These results suggest that xylose metabolism by recombinant *S. cerevisiae* also depends on aeration rates as in *P. stipitis*. Semi-quantitative relationships between xylose fermentation and aeration rates were calculated from four different aeration conditions. In addition, initial xylose consumption, cell growth, xylitol production, and ethanol production of YSX3 strain at different aeration conditions were monitored. Cell growth yields from xylose increased directly with levels of aeration. However, xylitol yields decreased with increased aeration. Maximum ethanol yields were observed under respiro-fermentative conditions (Fig. 4A).

3.3. Metabolic capacity of the constructed stoichiometric network

The capability of the metabolic network to produce metabolic energy (ATP) and cofactors (NADH and NADPH) was investigated by maximizing production from glucose and xylose (Table 5). Maximum yields of ATP were 16.0 mol ATP/mol glucose and 13.0 mol ATP/mol xylose, respectively. NADH production capability was higher on xylose, whereas NADPH production capability was higher on glucose. This is due to the cofactor difference between XR and XDH in the xylose assimilation pathway. As a result, the energy charge ([ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP]), catabolic reduction charge ([NADH]/[NADH] + [NAD]), and anabolic reduction charge ([NADPH]/[NADPH] + [NADP]) should be different during glucose and xylose metabolism in yeast. Both energy and cofactor levels play important roles in controlling metabolic fluxes in the cell (Nelson et al., 2000). This suggests that the control and regulation of xylose metabolism would be entirely distinct from glucose metabolism in yeast (Table 5).

3.4. Prediction of ethanol yields with respect to oxygen availability

Oxygen availability is one of the critical factors in yeast sugar metabolism. Although glucose concentration also acts as an effector, oxygen availability is the major controller for switching modes of energy

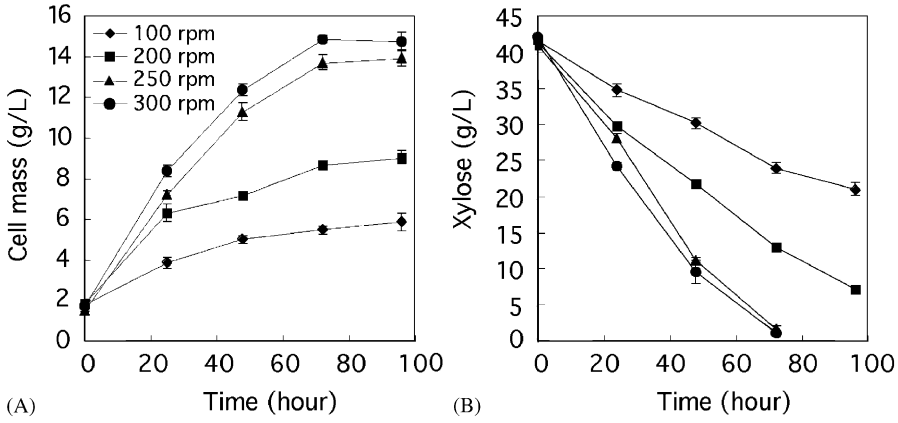

Fig. 3. Growth and xylose consumption by recombinant *S. cerevisiae* YSX3 under various aeration conditions. (A) Growth on YPX with 40 g/L of xylose at 100, 200, 250, and 300 rpm, (B) Xylose consumption at 100, 200, 250, and 300 rpm. Data points represent the averages of values from triple replicate experiments and error bar shows standard deviation.

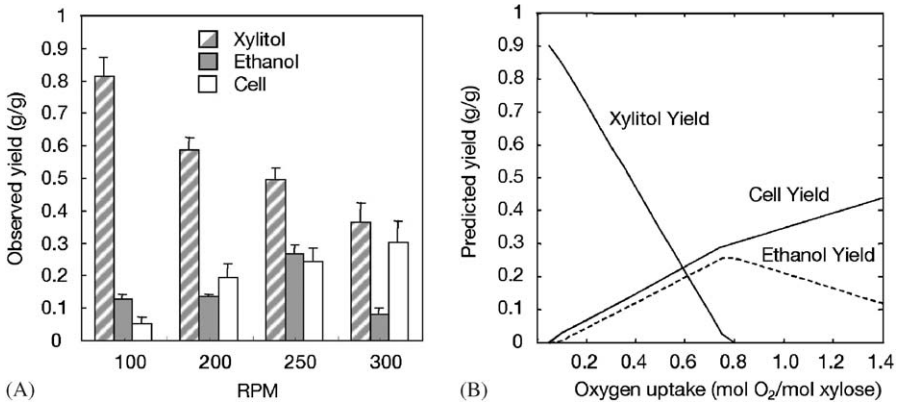

Fig. 4. Cell mass, xylitol, and ethanol yield from xylose under various aeration conditions. (A) Experimental yields by recombinant *S. cerevisiae* YSX3 from various aeration conditions, (B) predicted yields from FBA. Data points in (A) represent the averages of values from triple replicate experiments and error bar shows standard deviation.

Table 4
In vitro enzyme activities in crude extract from cell cultures of yeast strains

Strains	Specific enzyme activity (U/mg of total soluble protein)[a]		
	Xylose reductase	Xylitol dehydrogenase	Xylulokinase
S. cerevisiae L2612	ND[b]	ND	ND
S. cerevisiae YSX3	0.333	0.101	0.264
P. stipitis UC7	0.881	0.129	0.529

[a] Data are the average of at least two experiments and standard deviations were less than 10% for all assays.
[b] ND, not detected.

Table 5
Comparison of maximum ATP, NADH, and NADPH production

Metabolites	Yield (mol/mol)	
	Glucose	Xylose
ATP	16.00	13.00
NADH	10.00	8.50
NADPH	11.37	9.00

generation from fermentation to respiration. Ethanol yields from glucose and xylose with respect to oxygen consumption were calculated by optimizing the growth with the stoichiometric model. Normalized flux distributions during glucose and xylose metabolism with respect to oxygen consumption were also calculated. The maximum ethanol yield (0.51 g/g) from glucose was predicted under anaerobic conditions. Ethanol yields

from glucose decreased with increasing oxygen consumption because more carbon flux was directed into the TCA cycle at the pyruvate branch point. However, the maximum ethanol yield (0.26 g/g) from xylose was predicted under respiro-fermentative conditions. Xylitol accumulation was predicted under oxygen-limited conditions. FBA cannot simulate xylose metabolism under anaerobic conditions, which has only recently been demonstrated in mutants of *S. cerevisiae* expressing *XYL1*, *XYL2* and the *S. cerevisiae* gene for xylulokinase, *XKS1* (Sonderegger and Sauer, 2003). The product formation patterns of xylose fermentation predicted by FBA were largely consistent with fermentation experiments at different aeration conditions (Fig. 4). One significant disagreement between the model and the experimental results was that in the fermentation trial, xylitol production persisted under high aeration conditions.

3.5. Calculation of extreme pathways underlying metabolic network of glucose and xylose metabolism in recombinant S. cerevisiae

Extreme pathways were calculated from the given metabolic network. Glucose, xylose, xylitol, ethanol, O_2, CO_2, and biomass were regarded as extra-cellular metabolites, and levels of the remaining metabolites were assumed to be at steady state. Extra-cellular metabolites were set as allowable inputs and outputs for calculation of the extreme pathways. There were 43 extreme pathways in glucose metabolism, which represent the allowable phenotype from the given metabolic network. However, 18 of the extreme pathways did not show any net stoichiometric reaction because of futile cycles and reaction cycling. Most extreme pathways resulted in very similar net reactions because of redundancy in the metabolic network. From those extreme pathways, we were able to find phenotype-defining extreme pathways representing anaerobic ethanol fermentation with a theoretical yield of 0.51 g ethanol/g glucose and aerobic cell growth on glucose with a yield of 0.55 g DW/g glucose.

We found 72 extreme pathways in xylose metabolism and 17 of them also did not show any net reaction. Unlike glucose metabolism, all extreme pathways from xylose metabolism contain oxygen in their net reactions. This suggests that xylose cannot be metabolized under anaerobic conditions with the given reaction network. The extreme pathway corresponding to the net reaction for the maximum ethanol yield (0.46 g ethanol/g xylose) without cell growth was calculated:

$$\text{Xylose} + 0.50\ O_2 = 1.50\ \text{ethanol} + 2.00\ CO_2.$$

In this case 0.5 mol of oxygen consumption is required for 1 mol of xylose fermentation, which is consistent with previous report (Lee et al., 2001). This oxygen requirement is likely due to a redox imbalance caused by the cofactor difference between XR and XDH. If the XR and XDH reaction stoichiometry is replaced with xylose isomerase (XI) in the reaction network, the following extreme pathway is obtained:

$$3\ \text{xylose} = 5\ \text{ethanol} + 5\ CO_2.$$

This represents anaerobic xylose fermentation. In this case, maximum theoretical ethanol yield from xylose will then become 0.51 g ethanol/g xylose without cell growth. However, if we consider cell growth, the maximum ethanol yield becomes 0.26 g ethanol/g xylose. This finding is consistent with aforementioned results predicted by FBA. This confirms that optimal solution lies on the edge of convex cone defined by extreme pathways (Schilling et al., 2000).

4. Discussion

In this study, we investigated the effects of aeration on xylose metabolism in recombinant *S. cerevisiae* harboring *XYL1*, *XYL2*, and *XYL3* genes from *P. stipitis*, in conjunction with FBA. Xylose metabolism by the recombinant *S. cerevisiae* was directly dependent on aeration conditions, which is consistent with previous reports (Toivari et al., 2001). Ethanol, xylitol, and biomass mass yields from xylose were describable as functions of aeration. Xylitol and cell mass yield were simple linear functions of aeration: xylitol yield decreased with increasing aeration, and cell mass yield increased with increasing aeration. However, ethanol yield was maximized at 250 rpm. Tantirungkij et al. (1994) also reported this optimum oxygen-limited condition for maximum ethanol production by recombinant *S. cerevisiae*. Those results suggest that recombinant *S. cerevisiae* like *P. stipitis* also requires optimal aeration conditions for ethanol production from xylose (Grootjen et al., 1990). Oxygen is likely to play two important roles in xylose utilization in recombinant *S. cerevisiae*. The first is to resolve the redox imbalance by accepting electrons from NADH through the electron transport chain. Thus, xylitol production decreases with increasing aeration. The second is to induce ethanol oxidation by increasing respiration activity, because xylose does not repress respiration in the same manner as glucose (Jin, 2002). These two roles are apparently antagonistic. Therefore, optimal aeration conditions exist for ethanol production during xylose metabolism by recombinant *S. cerevisiae*.

Metabolic flux analyses of xylose metabolism in *S. cerevisiae* were presented previously (Pitkanen et al., 2003; Wahlbom et al., 2001). They investigated the levels of intracellular fluxes in recombinant *S. cerevisiae* consuming a glucose/xylose mixture under anaerobic conditions. Here, we examined the relationships between

xylose fermentation and oxygen consumption under different aeration conditions by growing recombinant *S. cerevisiae* on xylose as a sole carbon source. The *in silico* phenotype predicted by FBA was consistent with our experimental results. Predicted patterns of product formation under different aeration conditions largely agreed with the experimental data. In particular, optimum ethanol yield was predicted by FBA as shown by fermentation experiments. However, there was also a discrepancy between the model and experimental data. In fermentation experiments, xylitol production did not stop at the aerobic conditions that resulted in optimal ethanol production as predicted by FBA. The existing regulatory or metabolic network in *S. cerevisiae* might not be optimized to support efficient xylose assimilation since wild type *S. cerevisiae* does not utilize xylose as a carbon source. Therefore, the recombinant *S. cerevisiae* strains might have grown on xylose in a sub-optimal mode rather than an optimal mode, the latter of which was attempted in our simulation. As a similar case, Ibarra et al. (2002) showed that wild type *E. coli* grew sub-optimally on glycerol but its growth rate approached the optimal growth rate predicted *in silico* model after serial subcultures on glycerol. This suggests that serial subculture of recombinant *S. cerevisiae* on xylose could reduce xylitol accumulation. One sub-optimal growth condition could be an inappropriate regulatory response to xylose as a carbon source. Because xylose accumulation continued to increase even after ethanol production reached a maximum and began to decrease, this means that redox imbalance alone cannot explain xylitol accumulation. Rather, the glycolytic flux from xylitol to pyruvate must be insufficient. During glucose metabolism by *Saccharomyces*, glycolysis increases and fermentative reactions leading to ethanol exceed respiration. In the recombinant cells on xylose this does not appear to be the case.

Extreme pathways calculated from the metabolic network suggest that conversion of xylose into ethanol by yeast requires an electron acceptor such as oxygen, because of the redox the imbalance caused by cofactor difference between XR and XDH. However, electron acceptors other than oxygen should support xylose fermentation by resolving this imbalance. It was recently reported that furfural, 5-hydroxymethyl furfural, and acetoin act as external electron acceptors during anaerobic fermentation of xylose in recombinant *S. cerevisiae* (Wahlbom et al., 2001). Adding those electron acceptors decreased xylitol production, which is consistent with our observation. Alternatively, unknown biochemical reactions that can resolve the redox balance might exist in xylose-fermenting yeasts. For instance, *Pachysolen tannophilus* generated acetic acid from xylose, even while producing ethanol under anaerobic conditions (Jeffries, 1983). If the phosphoketolase pathway existed in yeast, it would be possible to ferment xylose under anaerobic conditions, because this pathway bypasses the glyceraldehyde-3-phosphate reaction that competes with XDH for NAD^+. Recent studies have shown that mutants of recombinant *S. cerevisiae* that grow on xylose under strict anaerobic conditions can be isolated in a long-term chemostat experiments (Sonderegger and Sauer, 2003). However, the evolved strains accumulated significant amount of xylitol during xylose fermentation. This suggests that redox imbalance is still a barrier for efficient xylose fermentation in the strains.

As suggested in our flux analysis, one way to bypass the redox imbalance problem for efficient xylose fermentation is to express XI in *S. cerevisiae*. There were several attempts to express bacterial XI in *S. cerevisiae* but the results were not promising because of folding problems and low activities (Gardonyi and Hahn-Hägerdal, 2003; Sarthy et al., 1987; Walfridsson et al., 1996). Lately, Kuyper et al. (2003) reported that heterologous expression of the XI from anaerobic fungus, *Piromyces* sp. enabled *S. cerevisiae* to ferment xylose. Although recombinant *S. cerevisiae* expressing eukaryotic XI still have problems like slow xylose assimilation, xylitol and xylulose accumulation, this research will provide another direction for metabolic engineering of xylose fermentation in yeast.

5. Conclusions

The results show that recombinant *S. cerevisiae* YSX3 expressing *XYL1*, *XYL2*, and *XYL3* uses xylose in an oxidative manner. We found that the YSX3 strain, like *P. stipitis* needed optimal aeration conditions for ethanol production from xylose. *In silico* phenotypes predicted by FBA were consistent with experimental results, showing that aeration is critical to xylose fermentation by recombinant *S. cerevisiae*. Extreme pathways calculated from the metabolic network revealed that, unlike glucose metabolism, xylose metabolism requires oxygen due to the redox imbalance caused by cofactor difference between XR and XDH.

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