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Estimation of Theoretical Yield for Ethanol Production from D-Xylose by Recombinant *Saccharomyces cerevisiae* Using Metabolic Pathway Synthesis Algorithm

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Abstract The metabolic pathway synthesis algorithm was applied to estimate the maximum ethanol yield from xylose in a model recombinant *Saccharomyces cerevisiae* strain containing the genes involved in xylose metabolism. The stoichiometrically independent pathways were identified by constructing a biochemical reaction network for conversion of xylose to ethanol in the recombinant *S. cerevisiae*. Two independent pathways were obtained in the xylose-assimilating recombinant *S. cerevisiae* as opposed to six independent pathways for conversion of glucose to ethanol. The maximum ethanol yield from xylose was estimated to be 0.46 g/g, which was lower than the known value of 0.51 g/g for glucose-fermenting and wild-type xylose-fermenting yeasts.

Key words: Xylose, ethanol, theoretical yield, metabolic pathway synthesis algorithm

D-Xylose metabolism has been examined to study the physiological characteristics of xylose-assimilating microorganisms. Xylose is the five-carbon sugar which constitutes approximately one-third of the total carbohydrate sugars in cellulosic materials [4, 5, 10, 14]. Genetic engineering techniques have been used to produce ethanol from xylose mostly for commercial uses. *Saccharomyces cerevisiae* has been used as a strong producer of ethanol from hexoses, however, it can not utilize xylose, since it does not possess a metabolic activity needed to convert xylose to xylulose. On the other hand, it can slowly metabolize xylulose to produce ethanol. Efforts have been made towards metabolic engineering of *S. cerevisiae* by introducing genes which are involved in the xylose metabolism of xylose-fermenting yeasts. Two enzymes, xylose reductase and xylitol dehydrogenase, to convert xylose to xylulose, were cloned and expressed in *S. cerevisiae* [9]. The recombinant *S. cerevisiae* strain was able to produce ethanol from xylose with a yield of approximately 0.3 g/g ethanol. Transaldolase and transketolase which participate in the pentose phosphate pathway were also overexpressed to prevent accumulation of sedoheptulose-7-phosphate and 6-phosphogluconate [20], a possible rate-limiting step [11]. Because of the difficulties in ethanol bioconversion by genetically engineered microorganisms, it was necessary to calculate the maximal theoretical yield based on a secure principle to make a comparison with an experimentally measured value. As the conversion of xylose to xylulose is regulated by cofactor balance and regeneration in xylose-assimilating yeasts, estimation of theoretical yield must be dealt within all pathways of related carbohydrate metabolisms. The metabolic pathway synthesis approach to estimate the yield of lysine production was used [12]. Metabolic pathway synthesis has been defined as the construction of stoichiometrically consistent routes of enzyme-catalyzed biochemical reactions which meet a certain specification [12, 18]. Metabolic pathways could be synthesized systematically by means of a formula containing two independent metabolites, substrate and product, except other intermediate metabolites. Pathways are enumerated and developed recursively by proceeding from the substrate to the product, in order to eliminate redundant reactions in the biochemical network during the production of the desired compound, and to investigate all possible pathways of substrate conversion by advancing to the product for the purpose of satisfying a set of stoichiometric constraints.

This work was undertaken to calculate a theoretical yield of xylose bioconversion to ethanol by taking into

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account both cofactor regeneration and oxidative recycling in a metabolic pathway synthesis scheme.

Construction of Biochemical Reaction Network

A xylose-assimilating recombinant *S. cerevisiae* was prepared in this study. The genes encoding xylose reductase and xylitol dehydrogenase were introduced and expressed in *S. cerevisiae* in order to be able to utilize xylose as a carbon source.

Two cases were examined: Case I was prepared for the xylose source system and Case II for the glucose source system. A biochemical reaction network was constructed for each of the two model systems as shown in Table 1. The algorithm that was used for the lysine production [19] was modified to be suitable for *S. cerevisiae* carbohydrate metabolism [13]. It has been reported that the recombinant *S. cerevisiae* strain did not possess any transhydrogenase activity, which interconverts $NADP^+$ and NADH to NADPH and NAD^+ [1, 2, 3], along with phosphoketolase activity [6, 15], therefore, reactions of transhydrogenase and phosphoketolase were not included. Another point to consider is a specificity of the xylose reductase for cofactor: The specificity of the xylose reductase for cofactor may vary depending on yeast strains, but most yeast strains prefer NADPH to NADH for utilizing xylose. Therefore, the cofactor of xylose reductase was decided to be 100% NADPH. This hypothesis, so called the dissimilar cofactor scheme, was well-supported by Schneider [16].

The model system hypothesizes that biomass synthesis is negligible and biochemical reactions occur in the same place, therefore there is no energy consumption by mass transfer. The P/O ratio was set to be 2. Xylose reductase

Table 1. Biochemical reactions of xylose-assimilating recombinant *S. cerevisiae*.

Reaction Compartment	No	Reaction
Xylose conversion to xylulose 5-phosphate	1	XYL+NADPH → XYOH+NADP
	2	XYOH+NAD → XYLU+NADH
	3	XYLU+ATP → XYL5P+ADP
Pentose phosphate pathway	4	GLC6P+H2O+2 NADP → RIBU5P+CO2+2NADPH
	5	RIBU5P ↔ RIB5P
	6	RIBU5P ↔ XYL5P
	7	XYL5P+RIB5P ↔ SEP7P+GAP
	8	SED7P+GAP ↔ FRU6P+E4P
	9	XYL5P+E4P ↔ FRU6P+GAP
Embden-Meyerhof-Parnas pathway	0	GLC+ATP → GLC6P+ADP
	10	GLC6P ↔ FRU6P
	11	FRUC6P+ATP → 2 GAP+ADP
	12	GAP+ADP+NAD → NADH+G3P+ATP
	13	G3P ↔ PEP+H2O
	14	PEP+ADP → PYR+ATP
	15	PYR ↔ ACAL+CO2
	16	ACAL+NADH ↔ ETOH+NAD
Carboxylation reaction	17	OAA ↔ PEP+CO2
	18	PYR → OAA
Bypass through acetate	19	ACCOA+H2O ↔ ACT+COA
	20	ACT+NADH ↔ ACAL → NAD+H2O
Tricarboxylic acid cycle	21	PYR+COA+NAD → ACCOA+CO2+NADH
	22	ACCOA+OAA+H2O ↔ ISOCIT+COA
	23	ISOCIT+NAD ↔ AKG+NADH+CO2
	24	AKG+NAD ↔ SUCCOA+NADH+CO2
	25	SUCCOA+ADP ↔ SUC+COA+ATP
	26	SUC+H2O+FAD ↔ MAL+FADH
	27	MAL+NAD ↔ OAA+NADH
Glyoxalate shunt	28	ISOCIT ↔ SUC+GLYOX
	29	ACCOA+GLYOX+H2O → MAL+COA
Oxidative phosphorylation (P/O=2)	30	2 NADH+O2+4 ADP → 2 H2O+4 ATP+2 NAD
	31	2 FADH+O2+4 ADP → 2 H2O+2 ATP+2 FAD
Unaccounted ATP consumption	32	ATP → ADP

was specific only for NADPH. A steady-state assumption was also applied.

The enumeration algorithm was built and formalized by a matrix expression [12, 18]. When a certain biochemical reaction is r,

$$\text{reaction r: } a_1, A_1+a_2, A_2+\cdots+a_mA_m \rightarrow [c_1C_1, c_2C_2, \cdots, c_kC_k] \rightarrow b_1A_1,+b_2A_2+\cdots, b_mA_m$$

where A_i is the *i*th metabolite, C_i is the *i*th metabolic pathway, a_i is a coefficient of the reactant part A_i, b_i is a coefficient of the product part A_i, c_i is a coefficient of C_i, m represents the total number of metabolites that appeared in the applied biochemical network, and k is the total number of pathways that appeared in the applied biochemical network. a_i and b_i are non-negative integers, and c_i is an integer. Then, the reaction r can be expressed by a vector representation in (2m+k) order.

$$r=(a_1, a_2, \cdots, a_m, c_1, c_2, \cdots, c_k, b_1, b_2, \cdots, b_m)$$

If k reactions in the applied biochemical network are given, it is represented by [k×(2m+k)] matrix R_0 as the initial problem.

$$R=\begin{bmatrix} a_{1,1}^{(0)} & a_{1,2}^{(0)} \cdots a_{1,m-1}^{(0)} & a_{1,m}^{(0)} & 1\ 0\cdots 0\ 0 & b_{1,1}^{(0)} & b_{1,2}^{(0)} \cdots b_{1,m-1}^{(0)} & b_{1,m}^{(0)} \\ a_{2,1}^{(0)} & a_{2,2}^{(0)} \cdots a_{2,m-1}^{(0)} & a_{2,m}^{(0)} & 0\ 1\cdots 0\ 0 & b_{2,1}^{(0)} & b_{2,2}^{(0)} \cdots b_{2,m-1}^{(0)} & b_{2,m}^{(0)} \\ \vdots & \vdots \ddots \vdots & \vdots & \vdots\ \vdots\ddots\vdots\ \vdots & \vdots & \vdots \ddots \vdots & \vdots \\ a_{k-1,1}^{(0)} & a_{k-1,2}^{(0)}\cdots a_{k-1,m-1}^{(0)} & a_{k-1,m}^{(0)} & 0\ 0\cdots 1\ 0 & b_{k-1,1}^{(0)} & b_{k-1,2}^{(0)}\cdots b_{k-1,m-1}^{(0)} & b_{k-1,m}^{(0)} \\ a_{k,1}^{(0)} & a_{k,2}^{(0)} \cdots a_{k,m-1}^{(0)} & a_{k,m}^{(0)} & 0\ 0\cdots 0\ 1 & b_{k,1}^{(0)} & b_{k,2}^{(0)} \cdots b_{k,m-1}^{(0)} & b_{k,m}^{(0)} \end{bmatrix}$$

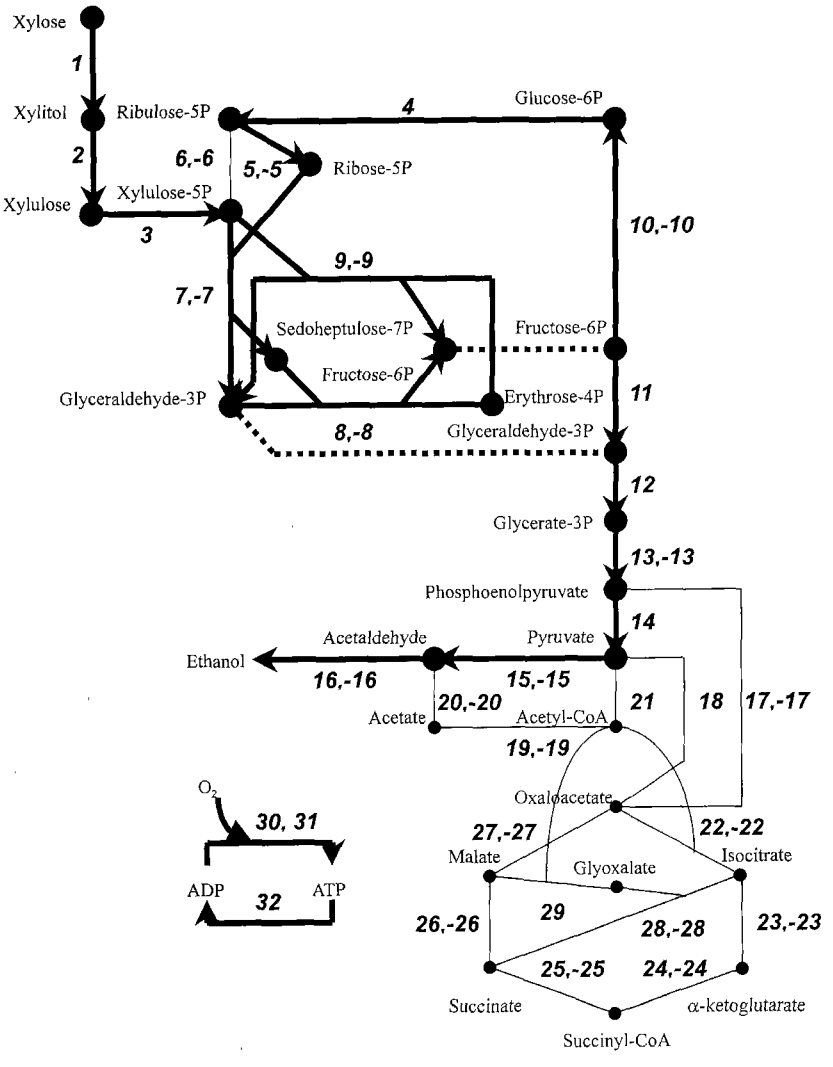

Fig. 1. The pathway of ethanol formation from xylose by metabolic pathway synthesis algorithm. Arrows indicate the direction of reactions that were numbered according to the order of Table 1.

The redundant reactions in the biochemical reaction network or linearly dependent reaction vectors were eliminated by applying simple matrix row operations. This operation was carried out by using the GCC-language programming.

Calculation of Theoretical Yield for Ethanol Production from Xylose

For Case I where xylose was employed as a starting carbon source, two independent metabolic pathways were identified in the recombinant *S. cerevisiae* for ethanol production. The synthesized pathway is at the unfeasible position based on the previous experimental results. It was reported that pyruvate decarboxylase played a major role in ethanol formation [8], and that wine yeasts normally produced less than 0.5 g/l of the acetic acid [7]. Acetic acid is formed from acetaldehyde by an oxidation reaction catalyzed by acetaldehyde dehydrogenase and the reversible reaction does not exist in yeasts *in vivo* [17]. A biochemically reliable one among the two synthesized metabolic pathways for Case I is depicted by heavy lines in Fig. 1. Interestingly, the two pathways resulted in identical stoichiometric coefficients of metabolites. The stoichiometric coefficient of H_2O had to be adjusted, since it was considered to be an excluded reactant. The net reaction of the conversion of xylose to ethanol by the metabolic pathway synthesis algorithm could be expressed as:

$$2\ C_5H_{10}O_5 + O_2 \rightarrow 3\ C_2H_5OH + 4\ CO_2 + H_2O$$

The theoretical ethanol yield was 0.46 g/g from xylose based on the above reaction stoichiometry. For Case II, however, the conversion of glucose to ethanol showed the maximal theoretical yield of 0.51 g/g, which agreed with the widely known value. This was due to the fact that cofactors were not utilized during glucose phosphorylation unlike xylose phosphorylation. In the ethanol formation pathways for Case I, an additional carbon flux from fructose 6-phosphate was continuously forced into the pentose phosphate pathway to generate the cofactor, which in turn caused a reduction of the carbon flux into the Embden-Meyerhof-Panas pathway. As a result, the theoretically obtainable ethanol yield decreased from 0.51 g/g to 0.46 g/g. Even if the reaction for xylose reductase was switched to a partially NADPH-favoring reaction, ethanol yield might have slightly increased, but could not reach 0.51 g/g.

The number of pathways and ranges of yields are summarized in Table 2. The xylose-source system Case I had two linearly independent pathways, compared with 6 pathways for the glucose-source system, suggesting that metabolisms of xylose in *S. cerevisiae* are more constrained in contrast to glucose metabolism. Consequently, it can be concluded that xylose is a weak ethanol-producing substrate for *S. cerevisiae* when compared with glucose, because the maximum ethanol yield from xylose is estimated to be lower than the theoretical value for the ethanol bioconversion from glucose. Moreover, the main merit of glucose is its diverse pathways to convert itself to ethanol under biological changes of yeast cell states, which might be due to its typical bioconversion without cofactor requirement.

Table 2. Summarized results of studies of two cases.

Case	Source	Sink	Number of pathways for ethanol production	Range of ethanol yield
I	Xylose	Ethanol	2	$0<Y_{xyl}\leq 0.46$
II	Glucose	Ethanol	6	$0<Y_{glc}\leq 0.51$

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NOMENCLATURE

ACAL : Acetaldehyde
ACCOA : Acetyl coenzyme A
ACT : Acetate
ADP : Adenosine 5'-diphosphate
AKG : α-Ketoglutarate
ATP : Adenosine 5'-triphosphate
CO_2 : Carbon dioxide
COA : Coenzyme A
E4P : Erythrose 4-phosphate
ETOH : Ethanol
FAD : Flavine adenine dinucleotide, oxidized
FADH : Flavine adenine dinucleotide, reduced
FRU6P : Fructose 6-phosphate
G3P : Glycerate 3-phosphate
GAP : Glyceraldehyde 3-phosphate
GLC : Glucose
GLC6P : Glucose 6-phosphate
GLYOX : Glyoxalate
H2O : Water
ISOCIT : Isocitrate
MAL : Malate
NAD : Nicotinamide adenine dinucleotide, oxidized
NADH : Nicotinamide adenine dinucleotide, reduced
NADP : Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH : Nicotinamide adenine dinucleotide phosphate, reduced
O2 : Oxygen
OAA : Oxaloacetate
PEP : Phosphoenolpyruvate
PYR : Pyruvate

RIB5P : Ribose 5-phosphate
RIBU5P : Ribulose 5-phosphate
SED7P : Sedoheptulose 7-phosphate
SUC : Succinate
SUCCOA: Succinyl CoA
XYL : Xylose
XYL5P : Xylulose 5-phosphate
XYLU : Xylulose
XYOH : Xylitol

REFERENCES

1. Anderlund, M., T. L. Nissen, J. Nielsen, J. Villadsen, J. Rydstrom, B. Hahn-Hägerdal, and M. C. Kielland-Brant. 1999. Expression of the *Escherichia coli pntA* and *pntB* genes, encoding nicotinamide nucleotide transhydrogenase, in *Saccharomyces cerevisiae* and its effect on product formation during anaerobic glucose fermentation. *Appl. Environ. Microbiol.* **65:** 2333–2340.
2. Bruinenberg, P. M., J. P. van Dijken, and W. A. Scheffers. 1983. An enzymic analysis of NADPH production and consumption in *Candida utilis* CBS 621. *J. Gen. Microbiol.* **129:** 965–971.
3. Bruinenberg, P. M., R. Jonker, J. P. van Dijken, and W. A. Scheffers. 1985. Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066. Evidence for the absence of transhydrogenase activity in yeasts. *Arch. Microbiol.* **142:** 302–306.
4. Chandrakant, P. and V. S. Bisaria. 1998. Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Crit. Rev. Biotechnol.* **18:** 295–331.
5. Cho, K. M. and Y. J. Yoo. 1999. Novel SSF process for ethanol production from microcrystalline cellulose using the δ-integrated recombinant yeast, *Saccharomyces cerevisiae* L2612δGC. *J. Microbiol. Biotechnol.* **9:** 340–345.
6. Evans, C. T. and C. Ratledge. 1984. Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: The key to efficient xylose metabolism. *Arch. Microbiol.* **139:** 48–52.
7. Henschke, P. A. 1997. Wine yeast, pp. 527–567. *In* F. K. Zimmermann, and K. D. Entian (eds.), *Yeast Sugar Metabolism: Biochemstry, Genetics, Biotechnology, and Applications.* Technomic Publishing Company, Lancaster, U.S.A.
8. Hohmann, S. 1997. Pyruvate decarboxylase, pp. 187–211. *In* F. K. Zimmermann, and K. D. Entian (eds.), *Yeast Sugar Metabolism: Biochemstry, Genetics, Biotechnology, and Applications.* Technomic Publishing Company, Lancaster, U.S.A.
9. Jin, Y.-S., T.-H. Lee, Y.-D. Choi, Y.-W. Ryu, and J.-H. Seo. 2000. Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae* containing genes for xylose reductase and xylitol dehydrogenase from *Pichia stipitis*. *J. Microbiol. Biotechnol.* **10:** 564–567.
10. Kim, J.-S., K.-K. Oh, S.-W. Kim, Y.-S. Jeong, and S.-C. Kim. 1999. Ethanol production from lignocellulosic biomass by simultaneous saccharification and fermentation employing the reuse of yeast and enzyme. *J. Microbiol. Biotechnol.* **9:** 297–302.
11. Köter, P. and M. Ciriacy. 1993. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **38:** 776–783.
12. Mavrovouniotis, M. L., G. Stephanopoulos, and G, Stephanopoulos. 1990. Computer-aided synthesis of biochemical pathways. *Biotechnol. Bioeng.* **36:** 1119–1132.
13. Ogata, H., S. Goto, K. Sato, W. Fujibuchi, H. Bono, and M. Kanehisa. 1999. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **27:** 29–34.
14. Pack, S. P., K. M. Cho, H. S. Kang, and Y. J. Yoo. 1998. Development of cellobiose-utilizing recombinant yeast for ethanol production from cellulose hydrolyzate. *J. Microbiol. Biotechnol.* **8:** 441–448.
15. Ratledge, C. and E. Holdsworth. 1985. Properties of a pentulose-5-phosphate phosphoketolase from yeast grown on xylose. *Appl. Microbiol. Biotechnol.* **22:** 217–221.
16. Schneider, H. 1989. Conversion of pentose to ethanol by yeast and fungi. *Crit. Rev. Biotechnol.* **9:** 1–40.
17. Steensma, H. Y. 1997. From pyruvate to acetyl-coenzyme A and oxaloacetate, pp. 339–357. *In* F. K. Zimmermann, and K. D. Entian (eds.), *Yeast Sugar Metabolism: Biochemstry, Genetics, Biotechnology, and Applications.* Technomic Publishing Company, Lancaster, U.S.A.
18. Stephanopoulos, G., A. Aristidou, and J. Nielsen. 1998. *Metabolic Engineering*, pp. 285–308. Academic Press, San Diego, California, U.S.A.
19. Vallino, J. J. and G. Stephanopoulos. 1990. Flux determination in cellular bioreaction network: Applications to lysine fermentations, pp. 205–219. *In* S. K. Sikidar, M. Bier, and P. Todd (eds.), *Frontiers in Bioprocessing.* CRC Press, Boca Raton, U.S.A.
20. Walfridsson, M., J. Hallborn, M. Penttilä, S. Keränen, and B. Hahn-Hägerdal. 1995. Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the *TKL1* and *TAL1* genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. *Appl. Environ. Microbiol.* **61:** 4184–4190.