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ORIGINAL ARTICLE

Metabolic engineering of *Saccharomyces cerevisiae* for increased bioconversion of lignocellulose to ethanol

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Abstract The absence of pentose-utilizing enzymes in *Saccharomyces cerevisiae* is an obstacle for efficiently converting lignocellulosic materials to ethanol. In the present study, the genes coding xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) from *Pichia stipitis* were successfully engineered into *S. cerevisae*. As compared to the control transformant, engineering of XYL1 and XYL2 into yeasts significantly increased the microbial biomass (8.1 vs. 3.4 g/L), xylose consumption rate (0.15 vs. 0.02 g/h) and ethanol yield (6.8 vs. 3.5 g/L) after 72 h fermentation using a xylose-based medium. Interestingly, engineering of XYL1 and XYL2 into yeasts also elevated the ethanol yield from sugarcane bagasse hydrolysate (SUBH). This study not only provides an effective approach to increase the xylose utilization by yeasts, but the results also suggest that production of ethanol by this recombinant yeasts using unconventional nutrient sources, such as components in SUBH deserves further attention in the future.

Keywords Xylose · *Saccharomyces cerevisiae* · Xylose reductase · Xylitol dehydrogenase · Ethanol

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Introduction

The yeast *Saccharomyces cerevisiae* is used universally for industrial ethanol production because of its ability to produce high concentrations of ethanol and high inherent ethanol tolerance [1]. However, there are two bottlenecks for industrial ethanol production by *S. cerevisiae* from lignocellulosic materials. The one is the fermentation inhibitors (such as weak acids, furfural and phenolic compounds) produced in the hydrolytic process [2], and the other is the inability of *S. cerevisiae* to utilize pentose sugars (mainly produced from hydrolysis of hemicelluloses). As a consequence, both the ethanol yield and productivity were significantly decreased [3].

Xylose is one of the major five-carbon sugars present in lignocellulosic materials such as sugarcane bagasse and other agricultural residues. It can, however, be fermented to ethanol by bacteria, yeasts and filamentous fungi [4]. In naturally xylose-utilizing yeasts such as *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae*, the main enzymatic steps in xylose metabolism are catalyzed by xylose reductase (XYL1) and xylitol dehydrogenase (XYL2). Xylose is first oxidized by XYL1 to xylitol, which is then oxidized by XYL2 to xylulose [5]. Since the *S. cerevisiae* cannot utilize xylose, but does utilize and ferment its isomer D-xylulose, the obvious first step to allow xylose metabolism is to introduce a heterologous pathway converting xylose to xylulose [6–8].

In the present study, we have engineered two xylose metabolic genes (*XYL1* and *XYL2*) into *S. cerevisiae* from the xylose-utilizing yeast, *P. stipitis*. The influence of XYL1 and XYL2 activities on ethanol production during xylose fermentation was investigated by using strains overexpressing XYL1 and XYL2. Moreover, the ethanol production from sugarcane bagasse hydrolysate using the engineered yeast strains was also investigated.

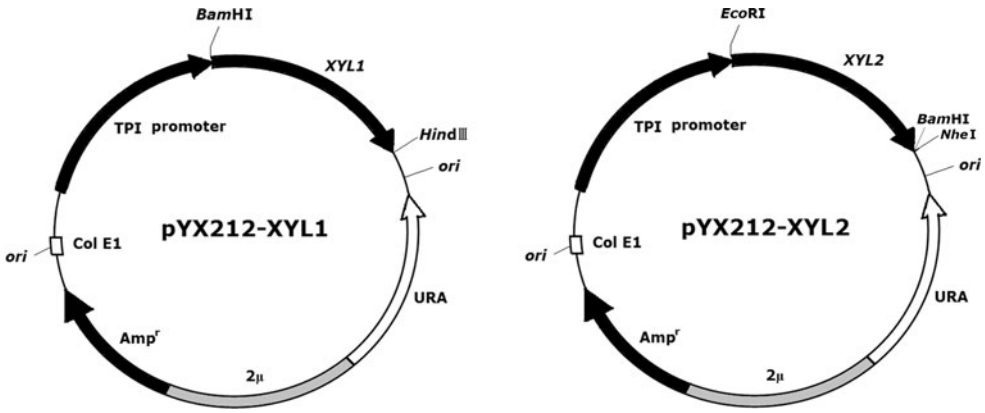

Fig. 1 Construction of recombinant plasmids

Materials and methods

Strains and media

S. cerevisiae H158 (MATα leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir^{+}) was cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or defined minimal medium (6.7 g/L yeast nitrogen base without amino acids). Glucose and xylose (30 g/L) was added to the minimal medium in fermentation experiments. *P. stipitis* CBS 6,054 was maintained on agar plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, 15 g/L agar).

Cloning of *P. stipitis* XYL1 and XYL2 genes and construction of recombinant plasmids

The DNA fragment encoding the *P. stipitis* XYL1 and XYL2 were amplified from the genomic DNA by PCR with the two specific primers (Up1: 5′-ATAAAGCTTATGC CTTCTATTAAGTTGAACTCTGG-3′, Down1: 5′-TTA GGA-TCCTTAGACGAAGATAGGAATCTTGTCCC-3′; Up2: GTCGGATCCATGACTGC-TAACCCTTCCTTGG TGTTG-3′, Down2: CATGAATTCTTACTCAGGGCCG TCAA-TGAGACACTTG-3′) supplied with *Bam*HI/*Hin-d*III and *Bam*HI/*Eco*RI restriction sites, respectively. The primers were based on the published sequences of the *P. stipitis* XYL1 (GenBank accessioin no. X59465) and XYL2 (GenBank accessioin no. AF127801) [9, 10]. The PCR reaction was performed in 25-μl reaction mixtures (0.15 μM each primer, 1 μl of template DNA [about 10 ng of genomic DNA], 12.5 μl PCR premix [Invitrogen 403061]). Denaturation, annealing and polymerization were carried out for 1 min at 94 °C, 1 min at 59.5 °C, and 1 min at 72 °C, respectively for 35 cycles. An *E. coli*/*S. cerevisiae* shuttle vector, pYX212 (Novagen), was used for protein expression. The *XYL1* and *XYL2* genes obtained from PCR amplification were gel-purified and digested with restriction enzymes before cloning into pYX212. The digested fragments were ligated to pYX212 expression vector predigested with the same restriction enzymes (Fig. 1). To multiply the recombinant plasmids, competent *E. coli* cells (DH5α) were transformed with the two plasmids by using calcium chloride and heat-shock treatment [11]. Purification of the plasmids was carried out with a Mini Plasmid Purification Kit (Omega).

Yeast transformation

Yeast transformation was performed by the electroporation methods as described by the manufacturer (Bio-Rad, USA). The positive transformants were selected from SC-Ura agar plates (20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 10 % amino acid supplement solution excluding uracil, 20 g/L agar) after incubation at 30 °C for 72 h.

Fermentation in shake flasks

After pre-cultivation of the positive transformants in 5 mL SC-Ura medium for 3 days, yeast cells were aerobically cultivated for 3 days at 30 °C in 50 mL minimal medium supplemented with glucose (10 g/L) and xylose (15 g/L) with shaking at 200 rpm. Cell pellets were harvested by centrifugation at 4 °C and washed with cold NaCl solution (0.9 %) and resuspended in 200 mL fermentation medium. The fermentation was carried out in a 500 mL flask (in triplicates) sealed with two layers of Saran wrap (under oxygen-limiting conditions) in an incubator at 150 rpm. Samples (yeast cells or culture supernatant) were collected at intervals and stored at −70 °C before analysis the enzyme activities, substrates and fermentation products.

Preparation of cell-free extract

After batch fermentation in flasks, the yeast cells were harvested by centrifugation (3,000×*g*) at 4 °C. The cell pellet was resuspended in 0.1 M sodium phosphate

(pH 7.0), containing 1 mM $MgCl_2$, 0.5 mM EDTA and 0.5 mM dithiothreitol, and vortexed together with an equal volume of glass beads (0.5 mm diameter). Cells were disrupted by vortexing six times 60 s. The samples were cooled on ice for 30 s in between the vortex steps. Cell debris and glass beads from the cell extract were separated by centrifugation and the remaining supernatant was used for enzyme determinations.

Enzyme assays

The activity of XYL1 was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm in a reaction solution with following composition: 0.1 M sodium phosphate buffer (pH 7.0), 0.2 M xylose, and 0.15 mM NADPH [5]. The activity of XYL2 was determined by the method described previously [12]. The standard assay mixture for XYL2 activity contained 50 mM $MgCl_2$ and 300 mM xylitol in 50 mM Tris–HCl (pH 9.0) buffer. All reactions were started by the addition of 0.1 mL of a 20 mM $NAD(P)^+$ solution to a final volume of 1.0 mL. One unit of enzyme activity refers to 1 μmol of $NAD(P)^+$ H produced/min. Protein concentrations were determined by the Bradford assay method using bovine serum albumin as the standard [13].

Fermentation with sugarcane bagasse hydrolysate (SUBH)

The pretreatment and enzymatic hydrolysis methods for the preparation of SUBH were described in previous reports [14, 15]. The fermentation experiments were carried out with a 5-L bioreactor (Roch Mechatronics Inc). The preparation of yeast cells was similar to the fermentation in shake flasks except that the culture volume was 500 mL. Cell pellets were harvested by centrifugation (3,000×*g*) and inoculated into 1.5 L SUBH supplemented with a nutrient solution, giving a final concentration of 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4{\cdot}7H_2O$, 1.38 g/L $NaH_2PO_4{\cdot}H_2O$, and 1 g/L yeast extract. The fermentation was maintained at 30 °C with an agitation speed at 500 rpm. Throughout the fermentation period (72 h), pH was controlled at 5.5 by automatic addition of 0.5 M NaOH and 1 M H_2SO_4. The fermentation was repeated three times and samples were collected and stored at −70 °C before analysis of the substrates and fermentation products.

Analysis of fermentation products

The cell growth was monitored spectrophotometrically at 600 nm. The glucose, xylose, xylitol, and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) method as described by Walfridsson et al. [12]. The separation was carried out with the using of an Aminex ion-exclusion HPX-87H cation-exchange column (BioRad, USA) at 65 °C with 5 mM H_2SO_4 as the mobile phase. The separated compounds were detected by a 410 differential refractometer (Waters, Milford, MA, USA).

Results

Expression of XYL1 and XYL2 genes in *S. cerevisiae*

The specific XYL1 and XYL2 activities of the cell-free extracts from recombinant yeasts transformed with different vectors are summarized in Table 1. As compared to the control strain, the strain SXYL1 exhibited the highest specific activity of XYL1, whereas the strain (SXYL2) produced more XYL2 activity. The highest specific activities of XYL1 and XYL2 were 10.41 and 13.22 U/mg, respectively. Both the activities of XYL1 and XYL2 were simultaneously elevated after the two genes were co-transformed into *S. cerevisiae*. One of the co-transformants (SXYL12) which showed the highest XYL2/XYL1 ratio (about 6/1) was selected for further investigations.

Growth, products formation and sugar utilization by recombinant yeast strains

As shown in Fig. 2a, yeast strains transformed with either *XYL1* or *XYL2* genes grew faster than the control strain. However, the highest microbial biomass was achieved by the strain SXYL12. The ethanol production profile was in good agreement with the microbial growth rate (Fig. 2b). But the highest ethanol yield (6.9 g/L) was recorded in yeast co-expressing XYL1 and XYL2. Moreover, engineering of

Table 1 XYL1 and XYL2 activities produced by different *S. cerevisiae* strains

Strain[a]	Enzyme activity (U/mg)[b]	
	XYL1	XYL2
SC	0.11 ± 0.02	0.28 ± 0.02
SXYL1	10.41 ± 1.27	0.25 ± 0.07
SXYL2	0.17 ± 0.03	13.22 ± 1.42
SXYL12	2.19 ± 0.11	12.65 ± 1.04

[a] *SC* yeasts transformed with empty vector (Control strain), *SXYL1* yeasts transformed with *XYL1* gene, *SXYL2* yeasts transformed with *XYL2* gene, *SXYL12* yeast transformed with *XYL1* and *XYL2* genes

[b] The highest specific activities were obtained from different transformants (except for SXYL12 which exhibited the highest XYL2/XYL1 ratio)

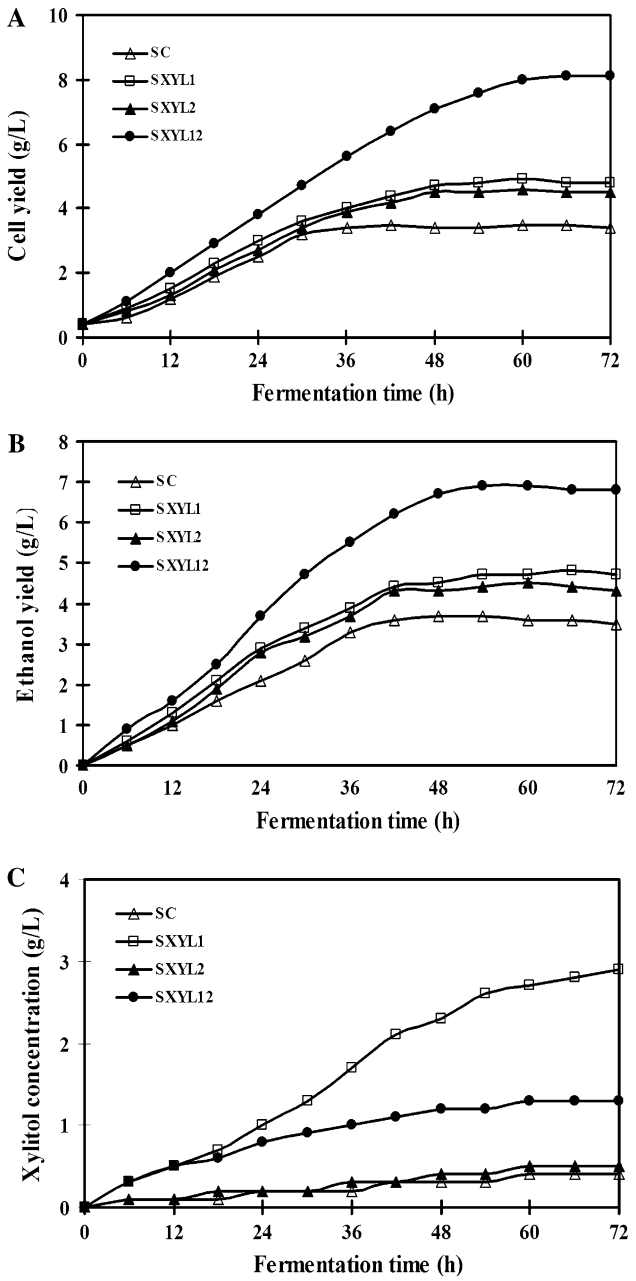

Fig. 2 Growth curves (**a**), ethanol (**b**) and xylitol (**c**) formation by different *S. cerevisiae* strains. Growth rate was monitored as OD at OD_{600}. One OD_{600} was converted to 0.21 g cells/L (dry wt). For clarity purposes, standard deviations have not been added for this figure

the *XYL1* gene into *S. cerevisiae* (SXYL1 and SXYL12) significantly increased the xylitol production (Fig. 2c). As shown in Fig. 3a, engineering of XYL1 and XYL2 into yeasts did not affect the glucose consumption rate. However, the yeast strains expressing XYL1 (SXYL1 and SXYL12) significantly increased the xylose consumption rate (Fig. 3b).

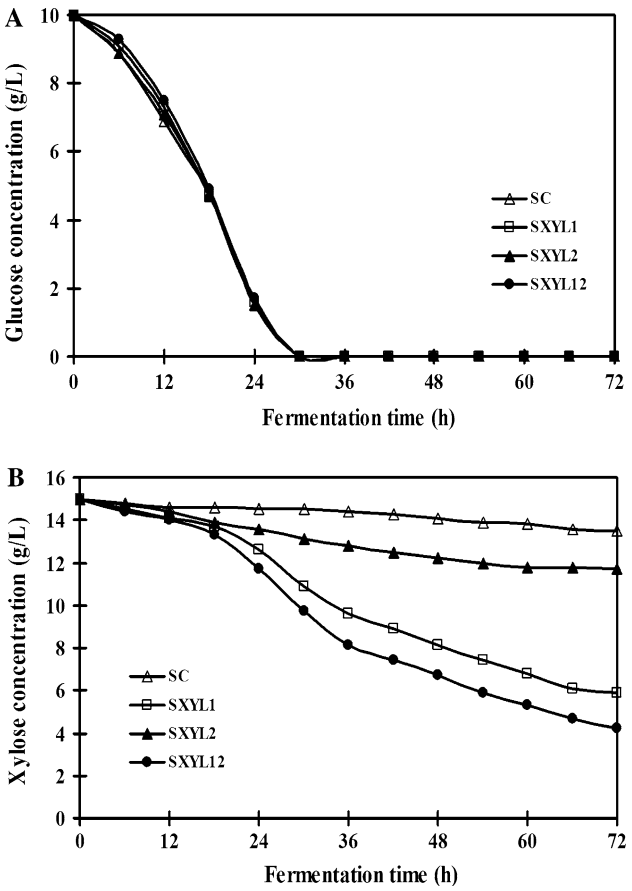

Fig. 3 Glucose (**a**) and xylsoe (**b**) utilization by different *S. cerevisiae* strains

Fermentation with sugarcane bagasse hydrolysate (SUBH)

As shown in Table 2, co-expressing of XYL1 and XYL2 in yeasts significantly increased ethanol ($P < 0.05$) and xylitol (<0.01) yield from SUBH. After 72 h fermentation, the glucose and mannose were both depleted by the two recombinant yeast strains. During the fermentation, the strain XYL12 consumed more xylose than H158 ($P < 0.05$), whereas the strain H158 tended to utilize more galactose (Table 2). No significant difference was observed for arabinose utilization between the two yeast strains ($P > 0.05$).

Discussion

Ethanol is the most widely used liquid biofuel and is fermented from sugars, starches or lignocellulosic materials [16]. Although the production of ethanol by fermentation of sugars has already been commercially established, the conversion technologies for producing of ethanol from

Table 2 Products formation and substrates consumption by different yeast strains during the fermentation with SUBH

Products formation/consumption	SUBH composition	SUBH after 72 h fermentation	
		S. cerevisiae H158	SXYL12
Biomass (g/L)[a]	NS	13.11 ± 0.67	15.94 ± 1.22[#]
Ethanol (g/L)	NS	5.26 ± 0.45	7.12 ± 0.88*
Xylitol (g/L)	0.25 ± 0.03	0.31 ± 0.03	1.58 ± 0.13**
Glucose (g/L)	13.85 ± 1.24	NS	NS
Xylose (g/L)	10.03 ± 1.07	9.11 ± 1.04	4.25 ± 0.41**
Arabinose (g/L)	1.74 ± 0.11	1.62 ± 0.13	1.65 ± 0.16
Galactose (g/L)	0.61 ± 0.07	0.21 ± 0.03	0.49 ± 0.06*
Mannose (g/L)	0.48 ± 0.05	NS	NS

[a] For all batch fermentations in bioreactor, the SUBH was given a yeast inoculum of 1 g/L (DW)

NS undetected

* $P < 0.05$; ** $P < 0.01$; [#] $0.05 < P < 0.10$

Table 3 Ethanol yields (g ethanol/g sugar) in cultures with glucose and xylose by recombinant *S. cerevisiae* strains

Strain	Fermentation conditions	Ethanol yield	Ref.
SXYL12	10 g/L glucose + 15 g/L xylose	0.34	This study
TMB3001	10 g/L glucose + 10 g/L xylose	0.29	[18]
TMB3001	20 g/L glucose + 20 g/L xylose	0.31	[18]
TMB3001	50 g/L glucose + 50 g/L xylose	0.16	[18]
CBP, CR4	20 g/L glucose + 50 g/L xylose	0.34	[19]
CBP, CR5	20 g/L glucose + 50 g/L xylose	0.28	[19]
H2490	3 g/L glucose + 27 g/L xylose	0.28	[20]
RWB202	20 g/L glucose + 10 g/L xylose	0.39	[21]
A4	50 g/L glucose + 50 g/L xylose	0.27	[22]
A6	50 g/L glucose + 50 g/L xylose	0.27	[22]

lignocelluloses are still under development and have not been demonstrated commercially [17]. Since the pentose sugars (i.e. xylose) comprise a high percentage of the available sugars in lignocellulosic materials [4], its fermentation is essential for the economic conversion of lignocellulose to ethanol. In this study, we have successfully engineered two xylose metabolic genes (*XYL1* and *XYL2*) into *S. cerevisiae* from *P. stipitis*. As a result, both the ethanol yield and xylose consumption rate were significantly elevated.

The yeast strain co-expressing XYL1 and XYL2 (SXYL12) consumed more xylose than other engineered strains (SC, SXYL1 and SXYL2) after 72 h fermentation with a xylose-containing medium. The highest ethanol yield obtained in shake flasks for the most efficient recombinant yeast strain (SXYL12) was 6.8 g/L, which is much higher than a reported yeast strain transformed only with the *XYL1* gene [1]. As shown in Table 3, there are many reports about the ethanol yield in cultures with glucose and xylose by recombinant yeast strains. However, most of them showed quite low production levels, except in one study a higher level was obtained [21]. Actually, it is difficult to compare the production levels, since the carbon sources (glucose/xylsoe) in different studies are variable, and optimization is likely to further improve the production efficiency. It is note worthy that the xylose consumption rate in the early stage (before 24 h) was not significantly improved (Fig. 3b). This is probably due to the fact that yeast maintains a strict hierarchy in terms of sugar utilization and glucose is at the top [23]. In the present study, the glucose consumption rate was not affected by the engineering of XYL1 or XYL2, and free glucose in the fermentation medium was depleted after 30 h fermentation.

The xylitol is an intermediate metabolite and is produced in the first enzymatic step during xylose metabolism. Previous study indicated that most xylose-utilizing fungi produce considerable amounts of xylitol from xylose, and only species containing also the NADH-dependent XYL1 activity are capable of producing ethanol from it [4, 24]. We observed that yeast transformed with *XYL1* gene (SXYL1 and SXYL12) significantly elevated the xylitol concentration. As compared to SXYL12, the strain SXYL1 produced more xylitol during the fermentation (Fig. 2c). This is due to the highest XYL1 activity (10.41 U/mg) produced in SXYL1. Moreover, the absence of XYL2 activity may also result in the accumulating of xylitol. In most naturally xylose-utilizing fungi, xylitol formation is a consequence of the inability of the cells to oxidize reduced cofactors (i.e. NADH) in the absence of oxygen [25]. The intracellular redox imbalance facilitates the unfavorable

excretion of xylitol and reduced the substrates for ethanol formation [26]. Since the ratio of XYL1 to XYL2 plays an important role in maintaining yeast cellular redox balance [1], and a higher level of XYL2 is believed to be necessary to drive the xylose toward central metabolism (ethanol formation) [27, 28], the strain SXYL12 showing the highest XYL2/XYL1 ratio (about 6/1) has therefore, been selected for further investigation.

The ethanol production from lignocellulosic materials such as the sugarcane bagasse hydrolysate was investigated. As compared to the control strain (H158), the SXYL12 consumed more xylose ($P < 0.01$) and produced more ethanol ($P < 0.05$) from SUBH. To our astonishment, the strain H158 consumed more galactose than SXYL12 during the fermentation. It is a well known fact that of the many carbon sources, yeast prefers glucoses and enzymes of the galactose metabolic pathway are not expressed in the presence of glucose [29]. Carbon sources such as raffinose and glycerol neither induce nor repress the ability of galactose to activate the *GAL* gene [30]. In this study, the free glucose was completely depleted during the fermentation. Coupled with its inability of *S. cerevisiae* H158 to efficiently utilize the xylose, the expression of *GAL* may be activated in the presence of free galactose. Moreover, our results also validated the existence of a strict hierarchy for sugar utilization in yeasts and the xylose cannot prevent the galactose utilization after the free glucose is completely depleted.

In summary, the engineering of exogenous xylose-metabolizing genes into *S. cerevisiae* not only elevated the xylose consumption rate, but also increased the yield of ethanol from a synthetic medium. Interestingly, engineering of these genes in yeasts also increased the yield of ethanol from lignocellulosic materials, such as the SUBH. This study not only provides an attractive approach to increase the xylose utilization by yeasts, but the results also suggest that production of ethanol by this recombinant yeasts using unconventional nutrient sources, such as components in SUBH deserves further attention in the future.

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