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Genetic engineering of yeasts to improve ethanol production from xylose

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ABSTRACT

Saccharomyces cerevisiae, expressing xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes from *Pichia stipitis*, has the ability to produce ethanol from xylose. To improve ethanol production, three recombinant strains of F102X (*fps1*Δ), F104X (*fps1*Δ *gpd2*Δ) and F106X (*fps1*Δ *gpd2*Δ *GLN1*) with *TKL1*, *TAL1*, *RKI1*, *RPE1*, *XYL1*, *XYL2* and *XKS1* overexpression were constructed in this study. Compared to the control strain FB3X, ethanol production from xylose in strains F102X, F104X and F106X was increased by 4.3, 9.5 and 17.2%, respectively. F106X produced 18.2% less glycerol from a glucose–xylose mixture medium than FB3X, whereas no glycerol production was detected in these four strains when grown on a xylose medium. In addition, the xylitol production and ethanol yield in strain F106X did not differ significantly. These results suggested that the improvement in ethanol production in strain F106X was mainly due to increased xylose consumption.

1. Introduction

Ethanol is an alcohol made from the fermentation of the carbohydrates or sugar(s) fraction of biomass materials [1,2]. In the last few years, considerable effort has been focused on ethanol production from lignocelluloses for use in gasoline blends to reduce petroleum consumption and air pollution [3–5]. Glucose and xylose are two of the major components of hydrolysates from lignocellulose. Therefore, the efficient conversion of xylose to ethanol is essential for successful utilization of lignocelluloses [6].

Saccharomyces cerevisiae, which is extensively used in the conversion of glucose into ethanol, has some advantages due to its high ethanol productivity, high tolerance to ethanol and high inhibitor tolerance [7–9]. *S. cerevisiae* is unable to utilize xylose naturally [10], but can metabolize xylulose to ethanol. Xylose can be converted into xylulose by introducing *XYL1* encoding for NADPH-linked xylose reductase (XR) and *XYL2* encoding for NAD-linked xylitol dehydrogenase (XDH) from the xylose fermenting yeast, *Pichia stipitis* [11–13]. However, the difference in cofactor preference of XR (NADPH/$NADP^+$) and XDH (NAD^+/NADH) leads to the formation of xylitol and an excess accumulation of NADH that cannot be recycled through respiration under oxygen-limited conditions. It has also been reported that overexpression of xylulose kinase, by transforming with *XKS1* or the four enzymes (*TAL1*/*TKL1*/*RPE1*/*RKI1*) in the non-oxidative pathway of *S. cerevisiae*, can improve ethanol production and decrease xylitol production [14–17]. However, the xylitol remains a major by-product. In addition, xylose can be converted into xylulose directly by xylose isomerase (XI) avoiding xylitol production. However, compared to the expression of XR and XDH, the activity of XI and the productivity of ethanol were very slow [18–21].

The ability to decrease xylitol production and improve ethanol productivity during the ethanol production from xylose is, consequently an important issue. Since XR has a higher affinity for NADPH and XDH uses NAD+ as its cofactor, changing the cofactor preference of XR and/or XDH by protein engineering is a potentially attractive method for reducing xylitol production and improving ethanol production. In addition, it has been reported that improvement in ethanol production can be achieved by partially reducing the major by-product (glycerol production) during glucose fermentation [22]. *GPD1* and *GPD2*, two abundant NAD-dependent glycerol-3-phosphate dehydrogenases, are key enzymes in glycerol synthesis by *S. cerevisiae*. Deletion of these can increase ethanol production significantly [23]. In addition, *FPS1*, a glycerol channel protein, controls glycerol excretion from the intracellular to extracellular space and its deletion also improves the ethanol production [24,25]. However, the reduction of glycerol production also leads to the NADH accumulation. Over expression of *GLN1* and/or *GLT1*, which encodes for glutamate synthase and glutamine synthesis, can convert excessive NADH into glutamate. Many papers have reported that *FPS1* deletion and/or *GPD2* deletion and/or *GPD1* deletion, associated with *GLN1* and/or *GLT1* overexpression, increases ethanol production and reduces glycerol production significantly [26–29].

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Table 1
Yeast strains used in this study.

Strain	Genotype	Source
YC-DM	MATa/α	Angel Yeast Co., Ltd.
W303-1A	*MATa leu2-3, 112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100*	[36]
YC-HP	*MATa*	Ma.P
CBS6054	*Pichia stipitis* CBS 6054	[33]
YB3	*MATa ura3*	This work
H102	*MATa ura3 fps1Δ*	This work
H104	*MATa ura3 fps1Δ gpd2Δ*	This work
H106	*MATa ura3 fps1Δ gpd2Δ* P_{PGK}*-GLN1*	This work
FB3X	*MATa AXYL1/PXYL2/PXKS1/TKL1/TAL1/RKI1/RPE1*	This work
F102X	*MATa fps1Δ AXYL1/PXYL2/PXKS1/TKL1/TAL1/RKI1/RPE1*	This work
F104X	*MATa fps1Δ gpd2Δ AXYL1/PXYL2/PXKS1/TKL1/TAL1/RKI1/RPE1*	This work
F106X	*MATa fps1Δ gpd2Δ* P_{PGK}*-GLN1 AXYL1/PXYL2/PXKS1/TKL1/TAL1/RKI1/RPE1*	This work

In the present study, the *XYL1* and *XYL2* genes from *P. stipitis* encoding for xylose reductase and xylitol dehydrogenase (under control of the *ADH1* and the *PGK1* promoter respectively) and the endogenous *XKS1* gene, encoding for xylulokinase (XK) (under control of the *PGK1* promoter) were integrated into the chromosome of our recombinant strains. We studied the role of *FPS1* and/or *GPD2* and/or *GLN1* with overexpression of *XYL1*, *XYL2* and *XKS1* in the conversion of xylose to ethanol and found that *FPS1* deletion, *GPD2* deletion and *GLN1* overexpression improved ethanol production and xylose consumption rates significantly during the fermentation of xylose and a glucose–xylose mixture.

2. Methods

2.1. Yeast strains, plasmids and media

The *S. cerevisiae* haploid strain YC-HP (*MATα*) used in this study was derived from the diploid industrial strains YC-DM (Angel Yeast Co., Ltd., Hubei Province, China). Templates used in PCR were from *S. cerevisiae* W303-1A and *P. stipitis* CBS 6054 genome. Standard techniques for nucleic acid manipulations were used. Strains and plasmids used in this study are listed in Tables 1 and 2, respectively. All the primers used in this study are listed in Table 3. YNB without amino acids (Difco) supplemented with 2% glucose and amino acids according to the strains demands and 5-fluoroorotic acid medium (5-FOA) were used for yeast growth and selection of transformants [30].

2.2. Construction of YEp-3X and pUC18-G2U3-3X plasmids

For the construction of plasmid YEp-3X (YEplac195-*XYL1*/*XYL2*/*XKS1*), three plasmids pEGFP-N1-AXYL1, pUC18-PXYL2 and YEplac195-PXKS1 were first constructed. In this YEp-3X plasmid, *XYL1* was under the control of an *ADH1* strong promoter, both *XYL2* and *XKS1* was under the control of a *PGK1* strong promoter.

The plasmid pEGFP-N1-AXYL1 was constructed as follows: (1) the first 665 bp of *ADH1* promoter was PCR amplified with primers ADH1-U and ADH1-D. The resulting PCR product was digested by *BamH*I-*Sma*I and then ligated with pEGFP-N1, which resulted in the plasmid pEGFP-N1-*ADH1p* being formed; (2) the *ADH1* terminator was amplified by primer pairs of ADH1T-U and ADH1T-D. The PCR product was digested by *Hind*III-*Xho*I and then inserted into the same digestion sites of pEGFP-N1-*ADH1p* to form plasmid pEGFP-N1-*ADH1p*-*ADH1t*; (3) Primers XYL1-U and XYL1-D were used to amplify the ORF of *XYL1*, which was inserted into the *Sma*I-*Hind*III site of pEGFP-N1-*ADH1p*-*ADH1t* to form pEGFP-N1-AXYL1.

The plasmid pUC18-PXYL2 was constructed as follows: (1) *PGK1* promoter was amplified by primers PGK1X2-U and PGK1X2-D. The PCR product was digested by *Pst*I-*BamH*I and ligated with pUC18 to form pUC18-PGK1X2; (2) Primers PGK1X2T-U and PGK1X2T-D were used to amplify *PGK1* terminator, this fragment was digested by *Xba*I-*BamH*I and then ligated with pUC18-PGK1X2 to create plasmid pUC18-PGK1X2-PGK1X2T; (3) *XYL2* ORF was obtained by PCR with primers XYL2-U and XYL2-D, which was digested by *Sal*I-*Xba*I and then ligated with pUC18-PGK1X2-PGK1X2T to form pUC18-PXYL2 plasmid.

The plasmid YEplac195-PXKS1 was constructed as follows: (1) Primers PGK1XK1-U and PGK1XK1-D were used to amplify the promoter of *PGK1*. This PCR product was digested by *Hind*III-*Sph*I and ligated with YEplac195 to form YEplac195-PGK1XK1 plasmid; (2) *PGK1* terminator was amplified by PCR with primers PGK1XK1T-U and PGK1XK1T-D, which was digested by *Sal*I-*Eco*RI and then inserted into the same sites of YEplac195-PGK1XK1 to create YEplac195-PGK1XK1-PGK1XK1T; (3) Primers XKS1-U and XKS1-D was used to PCR amplify the ORF of *XKS1*, and this PCR product was digested by *Sph*I-*Sal*I and then ligated with YEplac195-PGK1XK1-PGK1XK1T to build plasmid YEplac195-PXKS1.

To construct plasmid YEp-3X, pEGFP-N1-AXYL1 and pUC18-PXYL2 were digested by *BamH*I-*Xho*I and *Pst*I-*BamH*I, respectively, and then the resulting 2.0 kb and 2.2 kb fragments were inserted into the corresponding restriction sites of plasmid YEplac195-PXKS1 to form plasmid YEp-3X.

The plasmid of pUC18-G2U3-3X was constructed as follows: (1) GEA2T fragment was obtained by PCR amplification with primers GEA2T-U and GEA2T-D, and this PCR product was digested by *Hind*III-*Xba*I and ligated with pUC18 to form plasmid pUC18-G2; (2) Primers of URA3-U and URA3-D was used to amplify part of *URA3* gene, which was digested by *Xba*I-*Eco*RI and then ligated with pUC18-G2 to build plasmid pUC18-G2U3; (3) YEp-3X plasmid was digested with *Spe*I and *Xho*I, and the resulted 7.2 kb fragment was ligated to the *Xba*I-*Sal*I sites of pUC18-G2U3 to form the final plasmid pUC18-G2U3-3X. The map of pUC18-G2U3-3X plasmid was shown in Fig. 1.

Table 2
Plasmids used in this study.

Plasmids	Marker and description	Source
pJJ242-ura3	*AMP*	[34]
pUC18-RKUR	*AMP*	[35]
pUC18-*PGK1-GLN1*	*AMP*	This work
pGU-3X	*AMP, XYL1/XYL2/XKS1*	This work
pUC-TTRR	*AMP, TAL1/TKL1/RKI1/RPE1*	This work

2.3. Construction of plasmid to integrate the four genes in the PPP

The four genes (*TAL1*/*TKL1*/*RKI1*/*RPE1*) were integrated into the non-functional site of chromosome VII (+18,248 bp). The methods to construct pUC18-*TAL1*/*TKL1*/*RKI1*/*RPE1* (pUC-TTRR) plasmid were described as follows.

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