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Short communication

High-titer-ethanol production from cellulosic hydrolysate by an engineered strain of *Saccharomyces cerevisiae* during an *in situ* removal process reducing the inhibition of ethanol on xylose metabolism

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ABSTRACT

Efficient xylose utilization is critical for the production of fuels from biomass hydrolysates. It is known that xylose catabolism is inhibited by glucose. In this study, we showed that ethanol also inhibits xylose catabolism. By introducing a xylose metabolic pathway into *Saccharomyces cerevisiae* and using evolutionary engineering, an engineered *S. cerevisiae* strain, W32N55, was obtained that can anaerobically ferment xylose to ethanol. The effect of ethanol on xylose utilization was investigated. The results showed that xylose catabolism was inhibited upon the addition of ethanol, and it resumed once ethanol was removed. Based on these results, a fermentation–pervaporation coupling process was developed. After the *in situ* removal of ethanol, 150 g/L glucose and 31 g/L xylose were consumed in 72 h, providing a total of 76 g/L ethanol and an overall total sugar yield of 0.42 g/g. We believe that this strain will be valuable to the bio-ethanol industry.

1. Introduction

Lignocellulose, one of the world's most abundant renewable resources, is the most favorable alternative feedstock for the production of fuels and chemicals [6]. Lignocellulosic biomass is generally composed of cellulose, hemicellulose, and lignin. Upon pretreatment via alkaline, acidic, and/or enzymatic hydrolyses, lignocellulose is decomposed to glucose, xylose, and a small amount of arabinose, and such treatments also result in the production of some microbial growth inhibitors [23]. To use lignocellulosic resources for biotechnological purposes, a desirable strain is expected to co-utilize glucose and xylose, and tolerate inhibitors. However, natural strains of *Saccharomyces cerevisiae* cannot utilize xylose for growth or ethanol production. Because *S. cerevisiae* possesses a metabolic pathway to convert xylulose, a metabolic product of xylose, into ethanol, many efforts have been made to introduce metabolic pathways, including the xylose reductase–xylitol dehydrogenase (XR-XDH) and xylose isomerase (XI) pathways, which convert xylose to xylulose, into *S. cerevisiae* [7,5,24]. However, the rates of xylose consumption and ethanol production in such recombinant strains are unsatisfactory. In addition, most of the previously engineered strains can only ferment xylose under aerobic or microaerobic conditions [24,15], which is not favorable for the development of the bio-ethanol industry. Therefore, evolutionary engineering is the key to the development of xylose-fermenting *S. cerevisiae* strains, and many such examples have been demonstrated [22,11]. However, few strains can co-utilize glucose and xylose in high-sugar concentration hydrolysates to produce a high ethanol yield.

In an attempt to obtain an engineered *S. cerevisiae* strain that is capable of efficiently co-utilizing glucose and xylose, a xylose metabolic pathway was introduced into a *S. cerevisiae* strain, and it was allowed to evolve under oxygen-limiting conditions. The resulting strain, W32N55, fermented xylose to ethanol under anaerobic conditions. Using this strain, we found that the consumption rate of xylose decreased significantly during the late fermentation period, when a high concentration of ethanol was obtained. These results led to the hypothesis that in addition to glucose, ethanol might inhibit xylose catabolism. To verify this, we investigated the effect of ethanol on xylose catabolism. This was followed by the development of a fermentation–pervaporation coupling process, from which the efficient production of ethanol from corn stover hydrolysates was achieved.

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Table 1
Plasmids and strains used in this study.

Strain/plasmid	Relevant genotype	Source
Plasmids		
plasmid-2 μ	plasmid-2 μ, leu, *E. coli* ori, Apr	Our lab stored
pXYL1-2 μ (LEU2)	plasmid-2 μ-xyl1, leu, *E. coli* ori, Apr	This study
pXYL2-2 μ (LEU2)	plasmid-2 μ-xyl2, leu, *E. coli* ori, Apr	This study
Xks1-2 μ (LEU2)	plasmid-2 μ-xks1, leu, *E. coli* ori, Apr	This study
pXYL12-2u(LEU2)	plasmid-2 μ- xyl1-xyl2, leu, *E. coli* ori, Apr	This study
pXYL12-XK-2u (LEU2)	plasmid-2 μ- xyl1-xyl2-xks1, leu, *E. coli* ori, Apr	This study
Strains		
S. cerevisiae W303-1B (2n)	MATa/MATα ura3-52/ura3-52; trp1Δ 2/trp1Δ2; leu2-3,112/leu2-3,112;his3-11/his3-11; ade2-1/ade2-1; can1-100/can1-100	EUROSCARF
E. coli DH5α	F-,φ80dlacZ△M15, △(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk−, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1	Takara

2. Materials and methods

2.1. Strain construction

Plasmids and yeast strains used in this study are listed in Table 1. The plasmid construction steps were as follows. The XR-encoding gene *xyl1* and the XDH-encoding gene *xyl2* were polymerase chain reaction (PCR)-amplified from the genomic DNA of *Pichia stipites*, and inserted into the BamHI site of plasmid-2 μ (LEU2), resulting in plasmids pXYL1-2 μ (LEU2) and pXYL2-2 μ (LEU2), respectively, in which the expression of the target genes was controlled by the GPD promoter. Similarly, the xylulokinase (XK)-encoding *xk* gene from *S. cerevisiae* was inserted into plasmid-2 μ (LEU2) under the control of GPD promoter, resulting in plasmid pXK-2 μ (LEU2). Next, a *xyl2* cassette containing a promoter, the *xyl2* gene, and a terminator was PCR-amplified from pXYL2-2 μ (LEU2) and introduced into pXYL1-2 μ (LEU2), resulting in plasmid pXYL12-2 μ (LEU2). An *xk* cassette containing a promoter, the *xk* gene, and a terminator was PCR-amplified from pXK-2 μ (LEU2) and ligated into pXYL12-2 μ (LEU2), resulting in the final expression vector pXYL12-XK-2 μ (LEU2) (Fig. S1).

S. cerevisiae strain W303-1B (2N), a diploid strain that is easy to genetically manipulate, was used as the host strain. By transforming pXYL12-XK-2 μ (LEU2) into strain W303-1B (2N), the engineered strain W32N01 was obtained and identified by PCR and enzymatic activity assays.

Minimal medium (yeast nitrogen base without amino acids, 0.67%) supplemented with 20 g/L xylose was used for strain evolution at 30 °C. First, strain W32N01 was grown under aerobic conditions on a rotary shaker at 220 rpm. The cells were cultivated until they reached the mid-exponential phase (optical density at 600 nm (OD_{600}) = 4), and then they were transferred to fresh medium. The process was repeated until the strain growth rate improved significantly. Then, the dissolved oxygen availability was reduced by gradually lowering the shaker speed from the original 220 rpm to 100 rpm. After 55 transfers (a 2% inoculum was used for each transfer), a single clone, named W3N55, was obtained from the last shake flask. W32N55 was used for ethanol fermentation in this study.

2.2. Enzyme activity assays

XR, XDH, and XK activities were measured according to the following protocol. Target strains (W32N01, W32N55, and a control strain) were cultivated in yeast-peptone-dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose) until the OD_{600} reached 7–8 under aerobic conditions. Five milliliters of broth was centrifuged at 5000*g*. The resulting cell pellets were washed with sterile water, and resuspended in 300 μl of buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8), and 1 mM Na_2EDTA). The cells were transferred to a 1.5-ml Eppendorf tube containing 0.2 g of glass beads (0.45–0.5 mm), and the cells were disrupted by vortexing in an ice bath. Cell debris was removed by centrifugation (10,000*g* for 20 min at 4 °C), which resulted in a cell-free extract that was subsequently used for enzymatic activity assays.

XR activity was measured using a multimode reader by monitoring the oxidation of NADPH at 340 nm in a reaction mixture with the following composition: 0.1 M sodium phosphate buffer (pH 7), 0.2 M xylose, and 0.15 mM NADPH. XDH activity was measured by monitoring the reduction of NAD^+ at 340 nm in a reaction mixture with the following composition: 0.1 M Tris-HCl (pH 7), 1 mM $MgCl_2$, 50 mM xylitol, and 5 mM NAD^+ [26]. XK activity was measured by monitoring the oxidation of NADH at 340 nm [20]. The specific enzyme activities were expressed as micromoles of converted substrate per milligram of protein per minute (U mg/ml).

2.3. Medium and culture conditions

YPD medium was used for cell growth. The yeast strains used for fermentation were first grown in 50 ml of YPD medium in 250-ml Erlenmeyer flasks with shaking at 220 rpm. After a 16–18-h cultivation period, cells were collected by centrifugation, washed twice with sterile water, and transferred to fresh fermentation medium for ethanol production. The initial cell density in the fermentation medium was maintained at 3 g/L dry cell weight (DCW).

Anaerobic ethanol fermentation was performed at 30 °C in a closed bottle equipped with a bubbling CO_2 outlet. Yeast extract-peptone-xylose (YPX) medium (10 g/L yeast extract, 20 g/L peptone, and 50 g/L xylose, pH 5.5) was used for xylose fermentation. To investigate the effect of ethanol on the xylose metabolism of *S. cerevisiae* strain W32N55, various concentrations of ethanol were added to the YPX medium.

A corn stover hydrolysate containing 107 g/L glucose, 35 g/L xylose, and the microbial inhibitors acetic acid (7.2 g/L) and furaldehyde (3.8 mg/L) was used to produce ethanol. The hydrolysate was kindly provided by Prof. Jianmin Xing (Institute of Process Engineering, Chinese Academy of Sciences). The biomass pretreatment and enzymatic hydrolysis protocol was similar to that described by Yang et al. [28], except that the number of washes was reduced to three. To determine whether xylose metabolism could resume after removing the ethanol, the ethanol was separated from the culture broth by evaporation using rotary evaporators when glucose was depleted, and the metabolic profile of strain W32N55 was investigated.

2.4. Ethanol fermentation–pervaporation coupling process

Cellulosic ethanol fermentation was performed at 30 °C in a 2-L fermenter using the evolved strain W32N55 and a corn