



Short Communication

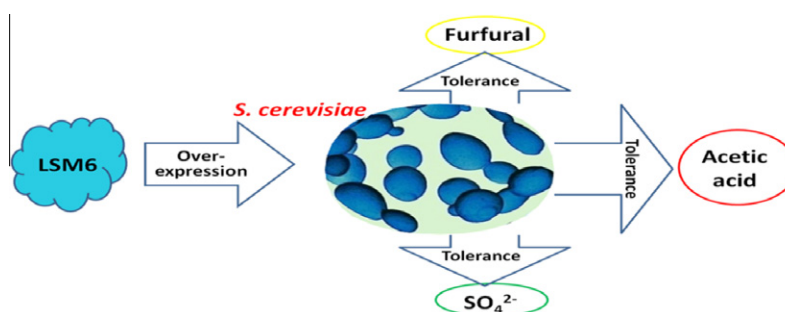
Sm-like protein enhanced tolerance of recombinant *Saccharomyces cerevisiae* to inhibitors in hemicellulosic hydrolysate

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GRAPHICAL ABSTRACT

Over-expression lsm6 gene can improve the resistance and fermentation performance of recombinant ZU-910 for the existence of acetic acid, furfural, and SO_4^{2-} .



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ABSTRACT

A current challenge of the cellulosic ethanol industry is to improve the resistance of inhibitors present in biomass hydrolysates. RNA-binding protein gene lsm6 was cloned from industrial *Saccharomyces cerevisiae* ZU-E8, which is able to ferment glucose and xylose, and transformed into ZU-E8 via expression vector pRS426. The positive transformant ZU-910 with over-expressing lsm6 was identified on the culture plates using high concentration of acetate and re-screened by fermentation test. Fermentation by the recombinants was performed in a medium containing 80 g/L xylose and 2 g/L acetic acid or 20 g/L $\text{NH}_4\text{Ac}/\text{NaAc}$. After 96 h shaking-flask fermentation, ZU-910 utilized 90.2% xylose with an ethanol yield of 26.9 g/L, which was 8.5- and 10-fold higher than ZU-E8. Further, in the corn stover hemicellulosic hydrolysate fermentation, both the xylose conversion and ethanol production by ZU-910 was larger by 50% and 40% than ZU-E8. ZU-910 has also enhanced tolerance against furfural and SO_4^{2-} .

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1. Introduction

Saccharomyces cerevisiae is a promising candidate for industrial bioethanol production due to its robustness and high ethanol productivity (Karhumaa et al., 2007). Genetic engineering has enabled *S. cerevisiae* to utilize xylose through heterologous expression of

genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis*, and over-expression of the endogenous gene for xylulokinase (XK) (Chu and Lee, 2007; Eliasson et al., 2000; Jeffries, 2006). However, efficient fermentation of hemicellulosic sugars is critical for ethanol bioconversion (Van Vleet and Jeffries, 2009).

Corn stover is regarded as the best lignocellulosic resource for large-scale ethanol production in China because (Zhao and Xia, 2010) it is a cheap, renewable feedstock, which is drought resistant. In the bioconversion of lignocellulose to ethanol, pretreatment is an essential procedure, but produces toxic compounds including

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acetic acids, furfural (Fujitomi et al., 2012; Hasunuma et al., 2011), and SO_4^{2-} during the pretreatment of biomass. The toxic inhibitor residues reduce cell growth, metabolism, and ethanol yield (van Maris et al., 2006). The xylose fermentation performance of recombinant *S. cerevisiae* strains were severely affected by the inhibitor residues especially acetic acid in the cellulose and hemicellulosic hydrolysates. The acetic acid is released during solubilization and hydrolysis of hemicellulose and is found at a high concentrations in hydrolysate (Almeida et al., 2007; Thomsen et al., 2009), with the concentrations varying depending on the type of biomass and the pretreatment method (Sanda et al., 2011). Therefore, it is necessary to improve cellular tolerance against multiple inhibit compounds for efficient bioethanol production.

Recent studies have suggested that repeated-batch fermentation (Sanda et al., 2011) and deletion of the PHO13 gene in *S. cerevisiae* improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and formic acids, and of furfural (Fujitomi et al., 2012). So far no research has reported the effect of LSM (Sm-like) protein (Cherry et al., 1998; Nielsen et al., 2007; Tharun et al., 2000) on xylose fermentation in the presence of inhibitors, only Yang (Brown et al., 2010; Yang et al., 2010) has reported the *Zymomonas mobilis* regulator hfg and the *S. cerevisiae* lsm genes effect on cellular tolerance against lignocellulosic pretreatment inhibitors. In this work, the lsm6 gene from *S. cerevisiae* ZU-E8 (which is a recombinant xylose-utilizing yeast strain with chromosomally integrated *P. stipitis* genes XYL1 and XYL2, and over-expression of the homologous gene XKS1) was cloned, and over-expression of lsm6 was successfully achieved by recombinant ZU-910.

2. Methods

2.1. Strains

S. cerevisiae ZU-E8 was used as both recipient for transformation and chromosomal DNA preparation and provider of lsm6 gene. *Escherichia coli* strain DH5 α was used for propagation of plasmids.

2.2. Cloning of lsm6 gene and construction of expression vector with P_{PGK}

Standard molecular biology techniques and manipulation of *S. cerevisiae* were used as described by Sambrook and Russell (2001).

The lsm6 gene (GeneBank: NM_001180686.1) was amplified with primers ATAGATCCATGTCCGAAAAGCTT and ATAGAGCTCCTATATTTTTGTTCA using genomic DNA isolated from ZU-E8 as a template. The PCR product was digested with *Bam*HI and *Xho*I and ligated with plasmid pRS426- P_{PGK} (Galazka et al., 2010; Ha et al., 2011) predigested with the same enzymes, resulting in expression vector pS-LSM.

2.3. Yeast transformation and transformants selection

Transformate *S. cerevisiae* ZU-E8 by electroporation after pretreatment of yeast cells with lithium acetate (LiAc) and dithiothreitol (DTT) (Thompson et al., 1998).

Positive transformants with over-expressing lsm6 was isolated on the culture plates using high concentration of acetate (NH_4Ac /NaAc) and rescreened by fermentation test. Fermentation by the recombinants was performed in fermentation medium containing 2 g/L acetic acid.

2.4. Medium

The seed medium for ZU-E8 was composed of 10 g/L glucose, 10 g/L xylose, 10 g/L yeast extract, 20 g/L peptone (adding G418

after sterilization. For transformants, add 3 g/L acetic acid additionally).

The fermentation medium for ZU-E8 was composed of 80 g/L xylose, 3 g/L yeast extract, 5 g/L peptone, 2.5 g/L KH_2PO_4 , 0.25 g/L $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 0.25 g/L CaCl_2 . The initial pH was 5.5 (For transformants, add 2 g/L acetic acid or 20 g/L NH_4Ac /NaAc additionally).

The fermentation medium contained concentrated hemicellulosic hydrolysate (Zhao and Xia, 2010) was composed of 4.8 g/L glucose, 71.8 g/L xylose, 14.3 g/L arabinose, 1.16 g/L acetic acid, 2 g/L yeast extract, 5 g/L peptone, 2.5 g/L KH_2PO_4 , 0.25 g/L $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 0.25 g/L CaCl_2 . The initial pH was 5.5.

Fermentations were performed under anaerobic conditions at 30 °C at 120 rpm in a volume of 50 ml in 100 ml Erlenmeyer flasks.

2.5. Analysis methods

Fermentation Samples were centrifuged, filtered through 0.45 μm membrane filters. Sugars, ethanol and other byproducts were analyzed using a HPLC system (Model 500, Syltech, USA) equipped with an organic acid column (IC Sep ICE-Coregel 87H3, Transgenomic, USA) and a refractive index detector (Model 6040 XR, Spectra-Physics, USA). Column temperature was fixed at 60 °C. Pure water was used as the mobile phase at a flow rate of 0.5 mL/min.

The ethanol yield on fermentable sugars = concentration of produced ethanol/initial concentration of fermentable sugars (i.e., the sum of glucose and xylose) in the hydrolysate.

The fermentation efficiency = the ethanol yield on fermentable sugars/theoretical ethanol yield.

3. Results and discussion

3.1. *S. cerevisiae* lsm6 recombinant construction and selection

3.1.1. Construction of the expression plasmid with *S. cerevisiae* lsm6 gene

The lsm6 0.3 kb gene was inserted into the plasmid pRS426- P_{PGK} under the control of P_{PGK} (Fig. S1–S2).

The strong P_{PGK} of *S. cerevisiae* has been widely used for over-expression of homologous and heterologous proteins in *S. cerevisiae*. And Brown (Brown et al., 2010) has reported that the promoter of the Sm-like superfamily could be the native promoter of the LSM superfamily gene being expressed, or a heterologous promoter from a different gene. Specific examples of promoters suitable for use in expression in *S. cerevisiae* include adh1+(constitutive high

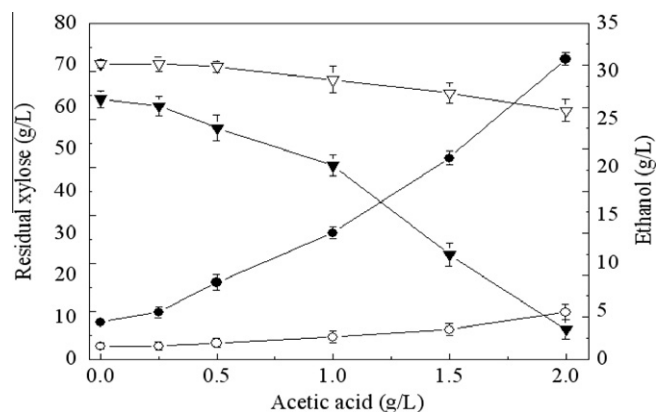


Fig. 1. Effect of acetic acid on xylose fermentation by recombinant *S. cerevisiae*. ● Residual xylose of ZU-E8; ○ Residual xylose of ZU-910; ▲ Ethanol produced by ZU-E8; △ Ethanol produced by ZU-910.

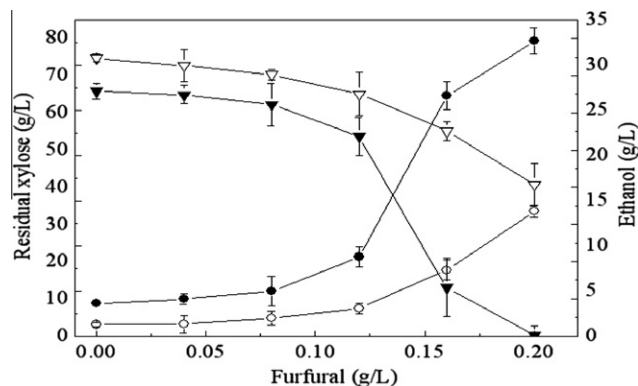


Fig. 2. Effect of furfural on xylose fermentation by recombinant *S. cerevisiae*. ● Residual xylose of ZU-E8; ○ Residual xylose of ZU-910; ▲ Ethanol produced by ZU-E8; △ Ethanol produced by ZU-910.

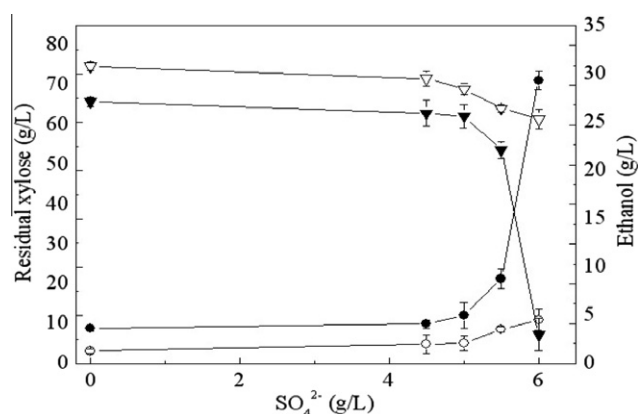


Fig. 3. Effect of SO_4^{2-} on xylose fermentation by recombinant *S. cerevisiae*. ● Residual xylose of ZU-E8; ○ Residual xylose of ZU-910; ▲ Ethanol produced by ZU-E8; △ Ethanol produced by ZU-910.

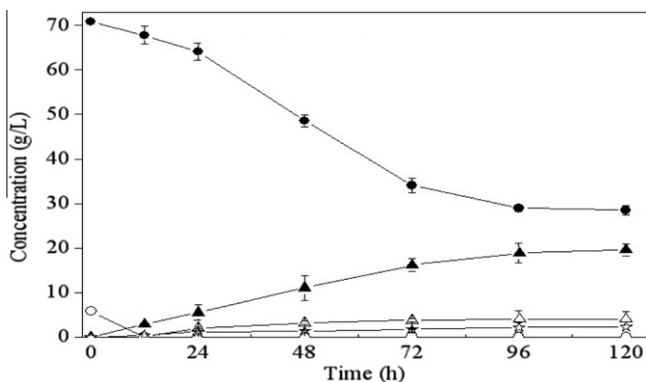


Fig. 4. Ethanol production from hemicellulose hydrolysate by ZU-E8. —○— glucose; —●— xylose; —▲— ethanol; —△— xylitol; —☆— glycerol.

expression), fbp+(carbon source responsive), a tetracycline-repressible system based on the CaMV promoter, and the nmt1+(no message in thiamine) promoter. In this study, lsm6 gene has also been inserted into vector pAUR123 (Takara) connected with adh1 promoter to construct an expression plasmid and translated into ZU-E8, but the fermentation performance tests showed that the transformants with $P_{\text{PGK-lsm6}}$ had better xylose utilization and acetic acid tolerance than the ones with $P_{\text{AHD1-lsm6}}$ (date not shown).

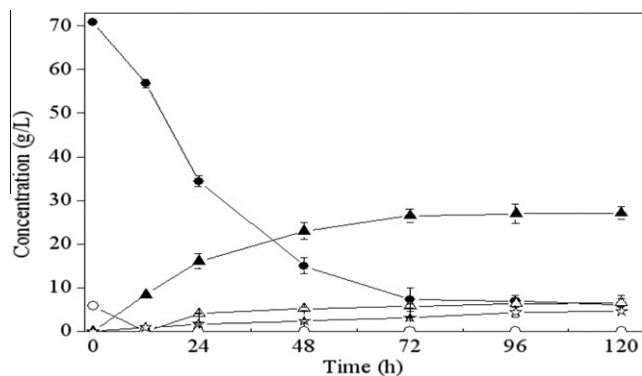


Fig. 5. Ethanol production from hemicellulose hydrolysate by ZU-910. —○— glucose; —●— xylose; —▲— ethanol; —△— xylitol; —☆— glycerol.

3.1.2. Screening of transformants

Therefore, we chose $pS\text{-LSM}$ with $P_{\text{PGK-lsm6}}$ to transform recipient ZU-E8, it was much more efficient for screening of positive transformants. And Positive transformant ZU-910 with over-expressing LSM6 was isolated on the culture plates using high concentration of acetate and rescreened by fermentation test. Fermentation by the recombinants was performed in fermentation medium containing 80 g/L xylose, 2 g/L acetic acid or 20 g/L $\text{NH}_4\text{-Ac/NaAc}$ additionally.

3.2. Effect of single factors on xylose fermentation of *S. cerevisiae* ZU-E8 and ZU-910

After cultivating cells aerobically in seed medium, the inhibitor effect on xylose fermentation with initial 80 g/L xylose as sole carbon source at 30 °C, 72 h, under oxygen-limited conditions was evaluated. Results of different concentration of acetic acid, furfural and sulfate ion by recombinant *S. cerevisiae* ZU-E8 and ZU-910 can be seen from Figs. 1–3.

It was found that, for the above three pretreatment inhibitors in hemicellulosic hydrolysate, the endurable concentration limits of the yeast ZU-E8 cell are 0.25 g/L, 0.08 g/L and 4.5 g/L, respectively. The recombinant ZU-910 showed higher xylose consumption and ethanol production rates than the original strain ZU-E8 at the same examined concentrations.

After 72 h, 93.4% xylose was utilized with an ethanol production of 29.1 g/L by ZU-910, were 2.6 and 1.5 times than that of ZU-E8 under the 2 g/L acetic acid condition, respectively. When acetic acid concentration rose to 2 g/L, the ZU-910 xylose utilization was 87.2% with an ethanol production of 25.9 g/L, were 8.2 and 9.5 times as that of ZU-E8.

Furfural had stronger inhibitory effect on the yeast ZU-E8 and ZU-910 xylose fermentation than that of acetic acid. As concentration of furfural below 0.08 g/L, ZU-910 xylose utilization was greater than 93%, and ethanol yield was above 0.37. As furfural concentration was higher than 0.08 g/L, ZU-E8 fermentation would be significant inhibited, the inhibitory appeared effect on ZU-910 while the furfural above 0.12 g/L. When furfural concentration increased to 0.2 g/L, xylose fermentation of ZU-E8 was completely inhibited, meanwhile, ZU-910 xylose utilization and ethanol yield were 58.1% and 0.36 respectively, and were 59.9% and 87.8% of the condition without inhibitors.

SO_4^{2-} concentration below 4.5 g/L would have less affect on ZU-E8 and ZU-910. ZU-E8 fermentation would be significant inhibited as SO_4^{2-} was higher than 5 g/L, and be completely inhibited at 6 g/L. But at the same condition of 6 g/L SO_4^{2-} in the medium, ZU-910 xylose utilization was 87.5% with ethanol production 25.3 g/L,

were 8 and 8.4 times as that of ZU-E8 in the same condition, and the ethanol yield of ZU-910 was 0.36.

Single-factor inhibitor experimental results show that compared with the initial xylose fermentation yeast ZU-E8, over-expression *lsm6* gene can indeed improve the resistance and fermentation performance of recombinant yeast ZU-910 for the existence of acetic acid, furfural, and SO_4^{2-} .

3.3. Fermentation performance of hemicellulose hydrolysate

Concentrated hemicellulosic hydrolysate from ammonia/dilute sulfuric acid pretreatment of corn stover was used for fermentation.

Differences between fermentation of hemicellulosic hydrolysate by ZU-E8 and ZU-910 were obvious (Figs. 4 and 5). Glucose was totally utilized within 12 h by both of the two strains, but ZU-E8 consumed xylose slowly because of the presence of acetic acid. After 120 h fermentation, only 59.8% xylose was utilized with an ethanol production of 19.6 g/L by ZU-E8. Whereas ZU-910 nearly finished the fermentation at 96 h, its xylose utilization was 90.2% with an ethanol production of 27 g/L, were 1.5 and 1.4 times as that of ZU-E8, respectively.

Recombinant *S. cerevisiae* are candidate microorganism in cellulose fuel ethanol industry. Previous studies did some efforts to enhance the recombinant yeast ability to overcome the presence of several inhibitors in production of cellulosic ethanol, and have made some meaningful results. But most of the recombinant yeasts were laboratory strains, which were very different from industrial strains in genetic background and metabolic regulations. Furthermore, few studies have examined the Sm-like family of RNA-binding proteins in the role of improving the strain sensitive of inhibitors. In this study, industrial *S. cerevisiae* strain was used as host to over-express *lsm6* gene which is coordinating a broad array of responses including multiple stress responses. These results confirmed that regulator *lsm6* did contribute to improve strain tolerance against multiple inhibitors in hemicellulosic hydrolysate.

4. Conclusions

This is the first report about Sm-like protein enhanced the fermentation performance of industrial recombinant *S. cerevisiae* in hemicellulosic hydrolysate. In the tests, the *S. cerevisiae* LSM6 protein plays an important role in resisting major relevant inhibitors industrially. The good fermentation performance of recombinant yeast ZU-910 with over-expressing *lsm6* gene in the hemicellulosic hydrolysate will have important industrial applications and prospects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2012.08.104>.

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