

Xylulokinase Activity in Various Yeasts Including *Saccharomyces cerevisiae* Containing the Cloned Xylulokinase Gene

Scientific Note

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ABSTRACT

D-Xylose is a major constituent of hemicellulose, which makes up 20–30% of renewable biomass in nature. D-Xylose can be fermented by most yeasts, including *Saccharomyces cerevisiae*, by a two-stage process. In this process, xylose is first converted to xylulose in vitro by the enzyme xylose (glucose) isomerase, and the latter sugar is then fermented by yeast to ethanol. With the availability of an inexpensive source of xylose isomerase produced by recombinant *E. coli*, this process of fermenting xylose to ethanol can become quite effective. In this paper, we report that yeast xylose and xylulose fermentation can be further improved by cloning and overexpression of the xylulokinase gene. For instance, the level of xylulokinase activity in *S. cerevisiae* can be increased 230fold by cloning its xylulokinase gene on a high copy-number plasmid, coupled with fusion of the gene with an effective promoter. The resulting genetically-engineered yeasts can ferment xylose and xylulose more than twice as fast as the parent yeast.

Index Entries: Xylulokinase activity; yeast; cloning; overexpression; high copy-number plasmids; xylose fermentation.

INTRODUCTION

D-Glucose and D-xylose are the two major monomeric sugar components of plant biomass, which is abundantly available in nature. Many

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yeasts, particularly *Saccharomyces cerevisiae* can ferment glucose to ethanol very effectively, and most of these yeasts can also ferment xylose to ethanol by a two-stage process (1,2). In this process, xylose is first converted to xylulose in vitro by the enzyme xylose isomerase, followed by fermenting the latter sugar molecules with yeasts to ethanol. With careful monitoring of various parameters, the two-stage process for the conversion of xylose to ethanol has proven to be practical, even with the use of commercial xylose isomerase currently available (3). Recently, the *E. coli* xylose isomerase gene has been cloned in *E. coli*, and the latter has been genetically engineered to produce 50 times more of its xylose isomerase activity than the parent strain (4–6). With the use of inexpensive xylose isomerase produced by recombinant *E. coli*, the above described process will be more effective for converting xylose to ethanol.

Xylulokinase is a key enzyme for yeast xylose and xylulose fermentation. Some yeasts, including *S. cerevisiae*, contain very low levels of xylulokinase activity, and those yeasts are also invariably not so effective in fermenting xylulose. In this paper, the relationship between the level of xylulokinase activity in yeasts and the effectiveness of the yeasts in fermenting xylulose has been examined. Furthermore, in this paper, preliminary results on the possible improving of yeast xylulose fermentation by cloning and overexpression of its xylulokinase gene will also be presented.

MATERIALS AND METHODS

Strains

LSK1, a derivative of *S. cerevisiae* AH22 (leu2–3, leu2–112, his4–529), is a xylulokinase mutant that we constructed by gene disruption technique (7). SR14 is an *E. coli* xylulokinase mutant derived from GM8 (*end A1*, *thi*, *r_K⁺m_K⁺*) as described by Rosenfeld et al. (8).

Transformations

E. coli transformation was carried out according to the CaCl_2 procedure described by Norgard et al. (9). Yeast transformation was by the spheroplast method (10).

DNA Isolation and Manipulation

Plasmid DNA from *E. coli* was isolated according to the clear lysate method of Godson and Vapnek (11) and Guerry et al. (12). Rapid plasmid preparations were carried out according to the method of Holmes and Quigley (13). Conditions for DNA digestion and ligation were according to the specifications of the supplier (BRL). Yeast xylulokinase (EC 2.7.1.17)

Table 1
Comparison of Xylulokinase Activity and Ethanol Production
of Various Yeasts

Stain	Xylulokinase ^a activity, Δ OD 540 nm	EtOH, % produced
<i>Candida shehatae</i>	0.871	1.02 ^b
<i>Pachysolen tannophilus</i>	0.084	0.42 ^c
<i>Schizosaccharomyces pombe</i>	0.914	1.93 ^b
<i>Saccharomyces cerevisiae</i>	0.170	0.75 ^b
<i>Pichia stipitis</i>	0.849	1.27 ^b

^aFor xylulokinase assays, cells were grown in YEPD (5 mL) for 2 d then washed with water and transferred to YEPGE with xylose (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol, and 2% xylose).

^bData obtained from S. Lastick et al. (18).

^cCalculated from data reported by Toivola et al. (19).

activity was assayed by measuring the disappearance of xylulose, as described by Shamanan and Sanderson (14).

Chemicals and Media

Pure xylulose was purchased from Sigma Chemical Co. (St. Louis, MO). Crude xylulose was prepared according to Gong et al. (15) and consisted of a mixture of xylulose and xylose, usually around 2:1, respectively. YEPD consists of 1% Yeast Extract, 2% Bacto Peptone, and 2% Dextrose. YEPGE is YEPD with 3% glycerol and 2% ethanol instead of dextrose.

RESULTS AND DISCUSSION

Analysis of Xylulokinase Activity in Various Yeasts

The xylulokinase activity of *S. cerevisiae*, *Schizosaccharomyces pombe*, *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* were analyzed and compared with their effectiveness in producing alcohol during xylose fermentation, as shown in Table 1. *S. cerevisiae* was chosen because it is a superior yeast for fermenting glucose. *Sch. pombe* was chosen because it is one of the best known xylulose fermenting yeasts. *P. stipitis*, *C. shehatae*, and *Pa. tannophilus* were chosen because they are the three most studied yeasts that can directly ferment xylose. The results indicated that yeast that can effectively ferment xylulose, such as *Sch. pombe*, always contain a high level of xylulokinase activity, and those containing very low levels of

xylulokinase activities, such as *S. cerevisiae* and *Pa. tannophilus*, are always ineffective in fermenting xylose to ethanol.

Enhancement of Xylulokinase Activity in *S. cerevisiae* by Cloning the Xylulokinase Gene on Various Plasmids

Recently, we have succeeded in cloning and expression of the xylulokinase gene from *S. cerevisiae* in *E. coli* (16,17). The gene has been fused to different promoters and subcloned on high and low copy-number yeast-*E. coli* shuttle vectors. A total of five different plasmids, pLSK10, pLSK15, pLSK20, pLSK25, and pLSK30, containing the cloned yeast xylulokinase gene were constructed. The promoters to which the xylulokinase gene is fused to and the copy-number of the recombinant plasmids harboring the xylulokinase gene are listed in Table 2. All five recombinant shuttle vectors contain the geneticin (Kanamycin) resistance gene and use it as the selection marker. Each of the five recombinant vectors were used to transform *S. cerevisiae* xylulokinase mutant LSK1 (7) to geneticin resistance. The xylulokinase activities of the yeast transformants harboring the various recombinant plasmids [LSK1 (pLSK10), LSK1 (pLSK15)...and LSK1 (pLSK30)] have been analyzed, and the results are also shown in Table 2. These results demonstrate that the level of xylulokinase activity in *S. cerevisiae* can be markedly increased by cloning and overexpression of its xylulokinase gene.

Improvement of Yeast Xylulose Fermentation by Genetically-Engineered Yeasts Containing the Cloned Xylulokinase Gene

Preliminary analysis of the effectiveness of the yeast transformants harboring each of the five recombinants in fermenting xylulose has also been carried out, and the results are also listed in Table 2. Xylose fermentation was carried out by growing the cells in YEPD for 2 d. The same amount of cells (~ 10 mL) for each transformant was transferred to a 12 mL presterilized plastic tube (with cap). The cells were washed with water and resuspended with 5 mL YEP containing approximately 7% xylulose and 3.75% xylose (crude product). The cells were incubated at 30°C, and 0.5 mL medium was removed from each tube each day for analysis. The ethanol contents of the samples were analyzed by GC.

Our results demonstrate that *S. cerevisiae* transformants with enhanced xylulokinase activity can also ferment xylulose with substantially-improved efficiencies. With rich medium, the engineered yeasts, containing different recombinant vectors, can produce ethanol 30-130% faster than the parent yeast. The engineered yeasts can also grow 30-50% faster than the parent yeast. We have not yet measured the final yields of ethanol produced during xylulose fermentation by the yeasts harboring the various plasmids containing the cloned xylulokinase gene described here. How-

Table 2
The Effect of Cloned Xylulokinase Gene on the Growth, Xylulokinase Activity, and Ethanol Production of the Host Yeast

Strain	Copy number	Promoter	Growth, ^a OD 600 nm	% Increase in growth	Xylulokinase Specific Activity ^b	% EtOH produced from 5% xylulose
LSK1 (pLSK10)	10	Original ^c	0.747	35	3.8	1.3 ^d
LSK1 (pLSK15)	10	Yeast TRP-5	0.833	50	166.2	0.70 ^e
LSK1 (pLSK20)	100	<i>E. coli</i> lacZ	0.791	43	123.0	1.50
LSK1 (pLSK25)	100	Original ^c	0.746	35	32.2	2.0
LSK1 (pLSK30)	100	Yeast TRP-5			363.0	1.80
AH22	1	Original	0.553		1.4	1.20
LSK1	0		0.000		0.5	1.3
						1.1
						0.8
						0.75
						1.0
						0.8

^a Adjusted values (assuming no growth for LSK1).

^b For xylulokinase assay, the cells were cultured as described in footnote *a* of Table 1. Xylulokinase specific activity is defined as nmol of xylulose converted/min/mg of protein.

^c The cloned gene may not contain the intact natural promoter for XYK-Sc.

^d Twenty-four hour samples.

^e Forty-eight hour samples.

^f Seventy-two hour samples.

ever, owing to the fact that these yeasts produced ethanol with much improved rates, we predict that they should produce more ethanol and less byproducts (such as xylitol) than ordinary yeast.

The xylulokinase activity of various genetically-engineered yeasts listed in Table 2 was analyzed when these yeasts were actively replicating under the conditions described in footnote *b*. However, the xylulokinase gene cloned under different promoters may not be expressed to the same extent during yeast xylulose fermentation. This may possibly explain, in part, why yeast harboring pLSK30 produced the highest xylulokinase activity during active growth, but did not produce the highest amount of ethanol during xylulose fermentation. Since there is no cloned strong promoters known to be effective under xylulose fermentation and since it is difficult to predict the effectiveness of various promoters during xylulose fermentation, this is why we chose to clone the gene under different known promoters in order to select those effective ones under xylulose fermentation.

We have also carried out preliminary studies on the stability of these plasmids during yeast xylulose fermentation. We found that all plasmids can be stably maintained in the presence of antibiotic geneticin. However, all the high copy-number plasmid pLSK20, pLSK25, and pLSK30 can be maintained in yeast without the presence of the antibiotic.

CONCLUSION

Our preliminary results have demonstrated that the levels of xylulokinase activity in different yeasts vary considerably, and they have a direct effect on the efficiency of yeast xylulose fermentation. By using *S. cerevisiae* as a model, we have shown that the xylulokinase activity in a yeast, which originally only contains a very low level of xylulokinase activity, can be markedly enhanced by cloning and overexpression of its xylulokinase gene. Such genetically-engineered yeasts seem also to ferment xylulose much more effectively. Currently, research is in progress in our laboratory to study whether the xylulokinase activity and the efficiency of xylose fermentation in yeast can be further improved by additional manipulations of the cloned xylulokinase genes. Research is also in progress to study whether the xylulokinase activity and the efficiency of xylulose fermentation in those yeasts, such as *Sch. pombe*, which already contain high levels of xylulokinase activities, can be further improved by cloning and overexpression of their xylulokinase genes.

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