0273-2289/89/2021—0327$02.00

Construction of Yeast Xylulokinase Mutant by Recombinant DNA Techniques

Scientific Note

PANAYIOTIS E. STEVIS AND NANCY W. Y. HO*

Laboratory of Renewable Resources Engineering and Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907

ABSTRACT

A *Saccharomyces cerevisiae* xylulokinase mutant was constructed by using the cloned yeast xylulokinase gene, XYK-Sc, and the gene disruption technique. The *S. cerevisiae LEU2* gene was used to disrupt the XYK-Sc gene cloned on pLSK4 by insertion into the unique HindIII site of the gene. The disrupted gene was liberated from the remainder of the plasmid with XhoI digestion, yielding a 4.4 kb DNA fragment. Transformation of a *S. cerevisiae leu2* mutant with this fragment and selection for Leu$^+$ complementation resulted in the isolation of transformants that were unable to grow in pure xylulose medium. The ability to grow in xylulose medium and increased xylulokinase activity were obtained by transforming the mutant with a plasmid-borne wild-type XYK-Sc gene. Insertional inactivation of the chromosomal XYK-Sc gene was also demonstrated by xululokinase assays.

Index Entries: Xylulokinase gene; yeast; gene disruption, mutant, LEU2 gene, yeast transformation.

INTRODUCTION

D-glucose and D-xylose are the two major monomeric sugar components of plant biomass, which is abundantly available in nature. Most yeasts, including *Saccharomyces cerevisiae* can effectively ferment D-glucose

*Author to whom all correspondence and reprint requests should be addressed.

but cannot ferment D-xylose (*1*). Only a few yeast species, such as, *Pachysolen tannophilus, Pichia stipitis,* and *Candida shehatae* were recently found to be able to ferment D-xylose with reasonable efficiency (*2,3*). Nevertheless, all yeasts can ferment xylulose effectively (*4*). In yeasts, xylulokinase converts xylulose to xylulose-5-phosphate. The latter is then metabolized via the pentose phosphate pathway. Lachke and Jeffries recently reported that the mutants of *P. tannophilus* with enhanced D-xylose fermentation rates invariably showed 5- to 10-fold increases in their xylulokinase activities (*5*). This indicated that the enzyme might play a pivotal role in yeast xylose metabolism.

Although the capability of various yeasts to ferment or metabolize D-xylose has been characterized extensively in recent years, very little is known about the molecular mechanism(s) controlling xylose metabolism at the level of gene expression in these yeasts. Recently, we have isolated a yeast (*S. cerevisiae*) DNA fragment that can complement a number of independent isolated *E. coli* xylulokinase mutants. In order to further prove the cloned yeast DNA actually containing the yeast xylulokinase gene and to facilitate the analysis of the mechanisms that control the expression of the yeast xylulokinase gene, one needs to isolate a stable yeast xylulokinase mutant of *S. cerevisiae*. In this paper, we report on the construction of a stable yeast xylulokinase mutant by the one-step gene disruption technique (*6*). In addition to other benefits, the construction of such a mutant unequivocally proves that the cloned yeast gene is the xylulokinase gene.

MATERIALS AND METHODS

Strains

AH22 (*leu2*-3, *leu2*-112, *his4*-529) was used for transformations and mutant isolation. LSK1 corresponds to *S. cerevisiae* AH22 with the xylulokinase gene mutated (*XYK-Sc::LEU2*). SR14 is an *E. coli* xylulokinase mutant derived from GM8 (*EndoI thi* $r_k^- m_k^+$), as described by Rosenfeld et al. (*7*). *E. coli* C600 (*thr-1, leuB6, tonA21, lacY1, supE44, thi-1,* λ) *is a leuB6* mutant used for selecting the plasmid-borne *LEU2* gene of *S. cerevisiae* (*8*).

Transformations

E coli transformation was carried out according to the $CaCl_2$ procedure described by Norgard et al. (*9*). Yeast transformation was either by the spheroplast method (*10*) or by the lithium acetate method (*11*).

DNA Isolation and Manipulation

Plasmid DNA from *E. coli* was isolated according to the clear lysate method of Godson and Vapnek (*12*), and Guerry et al. (*13*). Rapid plasmid preparations were carried out according to the method of Holmes and

Quigley (*14*). Conditions for DNA digestion and ligation were according to the specifications of the supplier (BRL). Filling in was carried out with the Klenow fragment of polymerase I (Klenow polI) and 2.5 m*M* of the dNTPs at 37°C for 15 min.

Assays

Yeast xylulokinase (EC 2.7.1.17) activity was assayed by measuring the disappearance of xylulose, as described by Shamanan and Sanderson (*15*).

Media

Minimal medium for yeast consisted of 0.067% Yeast Nitrogen Base (Difco Comp.) supplemented with the desired sugar and amino acids. For culturing AH22, leucine and histidine were included at 20 μg/mL. Pure xylulose was purchased from Sigma Chemical Co. Crude xylulose was prepared according to Gong et al. (*16*) 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 the same as YEPD with 3% glycerol and 2% ethanol instead of 2% dextrose.

RESULTS

Construction of Gene Disruption Vector

Plasmid pLSK4 (*17*) contains the *XYK-Sc* gene on a 2.2 kb XhoI fragment and can complement an *E. coli* xylulokinase (*xylB*) mutant, SR14. A unique HindIII site is located within the coding region of the gene. In order to create a fragment capable of achieving gene disruption, the *S. cerevisiae LEU2* gene was inserted into the HindIII site of pLSK4. Recombinants were selected by transforming C600, an *E. coli leuB* mutant, and selecting Leu^+ transformants. Disruption of the plasmid-borne *XYK-Sc* gene was verified by transforming SR14 with plasmid DNA isolated from the Leu^+ transformants and by physical analysis of the plasmids. In all cases, the Leu^+ transformants yielded plasmid DNA where the XhoI fragment increased in size from 2.2 kb to 4.4 kb and yielded a Xyl^- phenotype when transformed into SR14. The structure of the plasmid (pLSK4::*LEU2*) is shown in Fig. 1.

Isolation of Yeast Xylulokinase Mutant by Gene Disruption

S. cerevisiae AH22 (*18*) is a stable *leu2* mutant capable of growing on xylulose minimal medium supplemented with histidine and leucine (YNB-X-HL). pLSK4::*LEU2* was digested with XhoI to liberate the 4.4 kb fragment containing the *LEU2*-disrupted *XYK-Sc* gene. The fragment was

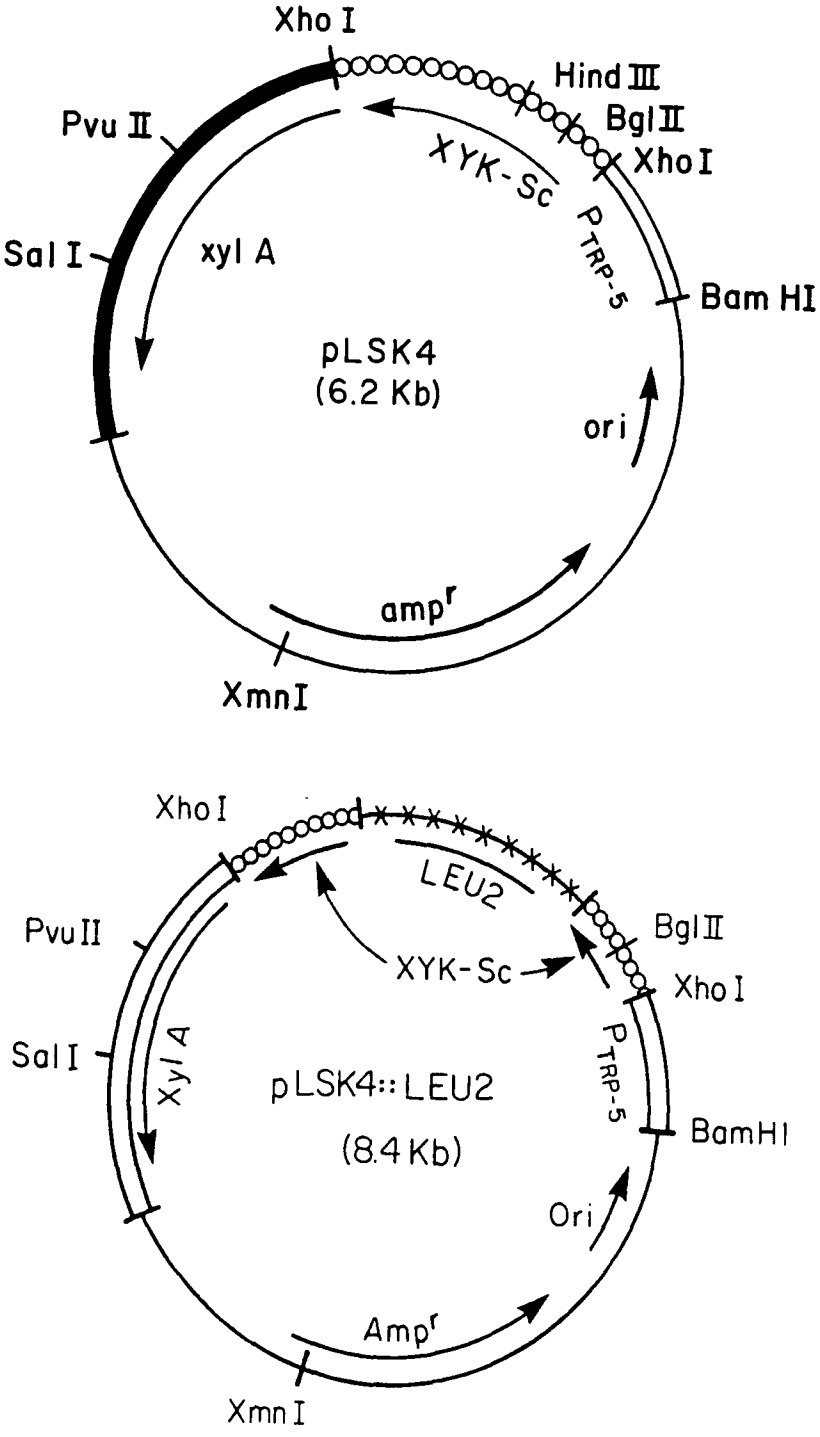

Fig. 1. Structure of pLSK4 and pLSK4::LEU2. The single line region represents pUC9 sequences while all the other relevant regions are clearly designated or described in the text, along with the construction of pLSK4::LEU2.

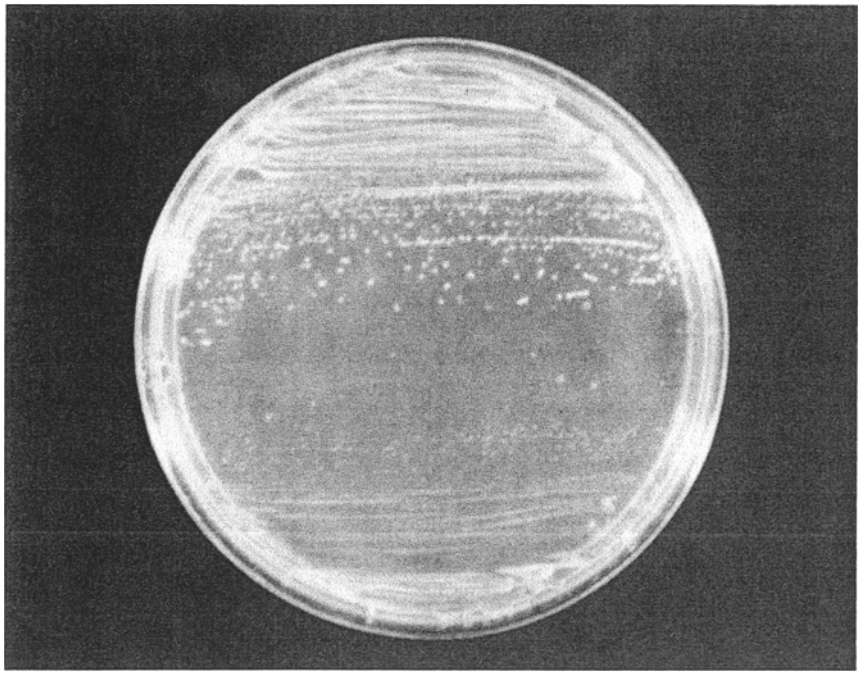

Fig. 2. Comparison of the colony size of the mutant and the wild type. LSK1 and AH22 were streaked on an agar plate containing YNB-X-HL. **Top:** AH22 with wild-type XYK-Sc forms larger colonies on the plate. **Bottom:** LSK1 with defective XYK-Sc gene forms smaller colonies on the plate.

used for the transformation of AH22 via the lithium acetate procedure while selecting the Leu$^+$ transformants on glucose minimal medium supplemented with histidine (YNB-G-H). A total of five Leu$^+$ transformants were recovered and used for further analysis.

Since pLSK4::*LEU2* contains no yeast replicon and the ends of the 4.4 kb XhoI fragment are homologous to the chromosomal *XYK-Sc* gene, the most likely event would be integration of the 4.4 kb XhoI fragment at the chromosomal *XYK-Sc* locus. Prolonged growth of the transformants on nonselective medium did not yield Leu$^-$ segregants, indicating that a stable integration had occurred.

To determine if the presumptive integration event had any effect on xylulose utilization, the Leu$^+$ transformants and the original AH22 strain were streaked on YNB-X-HL (with crude xylulose) and the size of the colonies was compared. The colonies of the Leu$^+$ transformants were found to be 2–3 times smaller than the AH22 colonies (Fig. 2). The Leu$^+$ transformants and the original AH22 strain were found to form identical size of colonies on the YNB-G-HL plate (data not shown). The limited growth of the Leu$^+$ transformants was presumably a result of impurities in the crude xylulose that can serve as a carbon source for the mutant for limited

Table 1
Final Cell Density and Xylulokinase Activity of Yeast Strains and Transformants

Strain	OD[a]	Xylulokinase specific activity[b]
blank	0	
AH22[pUKm8]	.226	195
LSK1[pUKm8]	.0014	ND
LSK1[pLSK10]	.066	89

[a]For growth analysis in YNB-X-HL liquid medium with pure xylulose, the cultures were first grown in YNB-glycerol (3%), ethanol (2%)-HL, then transferred to YNB-X-HL (1/10 dilution). Cell density was measured after 4 d incubation at 30°C and corresponds to optical density at 660 nm. Blank corresponds to YNB-X-HL medium without inoculation and was used to establish a baseline reading.

[b]For xylulokinase assays, cells were grown in YEPGE containing 1% xylulose and 0.5% xylose. Xylulokinase specific activity corresponds to nanomoles xylulose consumed per min per mg protein.

ND = not detected.

growth. When pure xylulose was employed for growth analysis in liquid minimal medium the mutant was unable to grow, whereas the wild-type strain (AH22) exhibited good growth (Table 1). The xylulokinase mutant was designated as LSK1.

LSK1 was further proven to be the yeast xylulokinase mutant by complementation with pLSK10, a yeast–*E. coli* shuttle plasmid containing the cloned yeast XYK-Sc gene. pLSK10 is a derivative of YEp13 (*19*). LSK1 transformants harboring pLSK10, LSK1 (pLSK10), can grow in yeast minimal medium with pure xylulose as the sole carbon source (Table 1). LSK1 (pSLK10) also form larger colonies on YNB-X-HL plates than the mutant LSK1 (Fig. 3). LSK1 to be a xylulokinase mutant was further demonstrated by the analysis of the xylulokinase activity as shown in Table 1.

CONCLUSION

We have demonstrated that a yeast xylulokinase mutant can be constructed by using the cloned yeast xylulokinase gene and the gene disruption methodology. The *S. cerevisiae LEU2* gene was used to disrupt the plasmid-borne *XYK-Sc* gene in pLSK4 by insertion into the unique HindIII site. The disrupted gene was liberated from the remainder of the plasmid with XhoI digestion, yielding a 4.4 kb DNA fragment. Transformation of an *S. cerevisiae leu2* mutant with this fragment and selection for Leu$^+$ by complementation resulted in the isolation of transformants which were unable to grow in pure xylulose medium. The ability to grow in xylulose medium and increased xylulokinase activity were obtained by transforming the mutant with a plasmid-borne wild-type *XYK-Sc* gene. Insertional inactivation of the chromosomal *XYK-Sc* gene was also demonstrated by

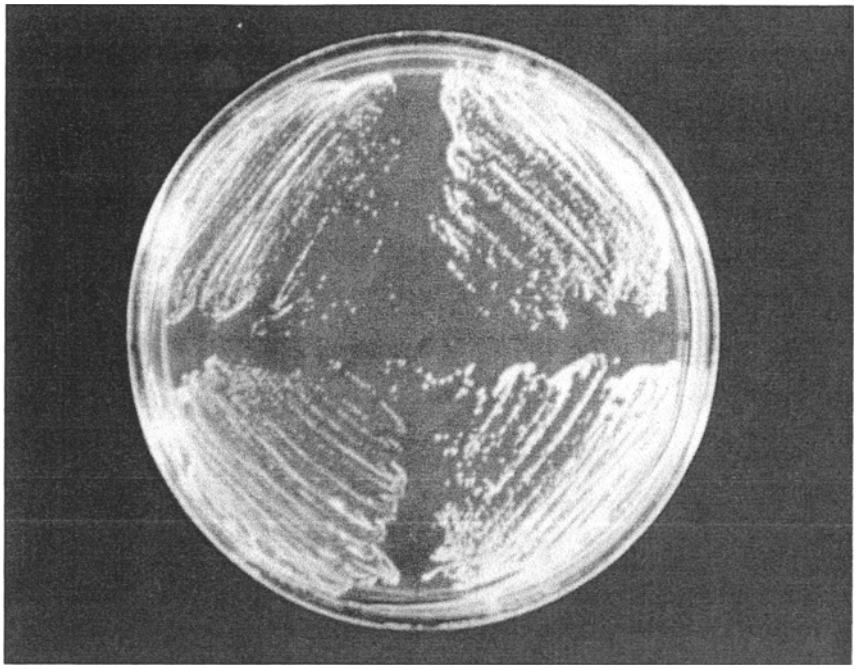

Fig. 3. Comparison of the colony size of the mutant and the transformant. LSK1 and LSK1 (pLSK10) were streaked on YNAB-X-HL. **Left:** LSK1 forms smaller colonies on the plate. **Right:** LSK1 (pLSK10) forms larger colonies on the same plate.

xylulokinase assays. A full paper on the same subject will be published elsewhere.

The availability of a *S. cerevisiae* xylulokinase mutant and the cloned *XYK-Sc* gene provides an opportunity to study the role of the enzyme in xylose and xylulose catabolism in yeast. This information will be useful in designing strategies to improve yeast growth rates as well as the production of ethanol from xylose.

ACKNOWLEDGMENTS

This work was supported in part by Solar Energy Research Institute subcontract XX-8-17245.

REFERENCES

1. Barnett, J. A. (1976), *Adv. Carbohydr. Chem. Biochem.* **32,** 125–234.
2. Slininger, P. J., Bothaster, R. J., Van Cauwenberge, I. E., and Kurtzman, C. P. (1982), *Biotechnol. Bioeng.* **24,** 371–384.

3. du Preez, J. C., et al. (1985), *Enzym. Microb. Technol.* **8,** 360–364.
4. Jeffries, T. W. (1982), *Biotechnol. Bioeng. Symp.* **17,** 315–324.
5. Lachke, A. H. and Jeffries, J. W. (1986), *Enzyme Microb. Technol.* **8,** 353–359.
6. Rothstein, R. S. (1983), *Methods Enzymol.* **101,** 202–211.
7. Rosenfeld, S. A., Stevis, P. E. and Ho, N. W. Y. (1984), *Mol. Gen. Genet.* **194,** 410–415.
8. Andreadis, A., Hsu, Y.-P., Kohlhaw, G. B., and Schimmel, P. (1982), *Cell* **31,** 319–325.
9. Norgard, M. V., Keem, K., and Monahan, J. J. (1978), *Gene* **31,** 279–292.
10. Hinnen, A., Hicks, J. B., and Fink, G. R. (1978), *Proc. Natl. Acad. Sci. USA* **75,** 1929–1933.
11. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983), *J. Bacteriol.* **153,** 163–168.
12. Godson, G. N. and Vapnek, D. (1973), *Biochem. Biophys. Acta.* **299,** 516–520.
13. Guerry, P., LeBlane, D. J., and Falkow, S. (1979), *J. Bacteriol.* **116,** 1064–1066.
14. Holmes, D. S. and Quigley, M. (1981). *Anal. Biochem.* **114,** 193–197.
15. Shamanan, D. R. and Sanderson, M. E. (1979), *J. Bacteriol.* **139,** 64–70.
16. Gong, C. S., Chen, L. F., Flickinger, M. C., Chiang, L. C., and Tsao, G. T. (1981), *Appl. Environ. Microbiol.* **41,** 430–436.
17. Chang, S.-F. and Ho, N. W. Y. (1988), *Appl. Biochem. Biotech.* **16,** 316–318.
18. Broach, J. R. (1983), *Methods Enzymol.* **101,** 307–325.
19. Broach, J. R., Strathern, J. N., and Hicks, J. B. (1979), *Gene* **8,** 121–133.

Session 3
Bioengineering Research