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RESEARCH PAPER

Expression of Bacterial Xylose Isomerase in *Saccharomyces cerevisiae* under Galactose Supplemented Condition

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Abstract We constructed recombinant *Saccharomyces cerevisiae* harboring the xylose isomerase (XI) gene isolated from *Clostridium phytofermentans* to metabolize xylose and use it as a carbon and energy source. In this study, the effect of supplementation using co-substrate such as glucose or galactose on xylose utilization was studied in recombinant *S. cerevisiae*. Glucose, which is transported with high affinity by the same transport system as is xylose, was not affected by the heterologous expression of XI, thus xylose utilization was not observed in recombinant *S. cerevisiae*. However, supplemental galactose added to the recombinant *S. cerevisiae* stimulated xylose utilization as well as the expression of XI protein. Recombinant *S. cerevisiae* consumed up to 23.48 g/L of xylose when grown in media containing 40 g/L of xylose and supplemented with 20 g/L of galactose. These cells also produced 15.89 g/L of ethanol. Therefore, expression of the bacterial XI in recombinant *S. cerevisiae* was highly induced by the addition of supplemental galactose as a co-substrate with xylose, and supplemented galactose enabled the yeast strain to grow on xylose and ferment xylose to ethanol.

Keywords: xylose isomerase, galactose, xylose utilization, *Saccharomyces cerevisiae*

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1. Introduction

Saccharomyces cerevisiae is used for industrial ethanol production, and has several advantages including its high productivity and tolerance to ethanol, and also its high tolerance to various inhibitors. However, it cannot utilize pentose sugars such as arabinose, ribose, and xylose as a carbon source in feed-stocks [1-3]. Xylose utilization by *S. cerevisiae* has been achieved either by expression of the NAD(P)H-dependent xylose reductase (XR) and the NAD+-dependent xylitol dehydrogenase (XDH) isolated from *Pichia stipitis*, or by expression of genes obtained from *Piromyces sp*. E2 or *Thermus thermophilus* encoding for xylose isomerase (XI) [4-8]. Both approaches resulted in stains that could grow on xylose and ferment it into ethanol. Although expressions of XR and XHD resulted in rapid fermentation of xylose, NADPH/NAD cofactor imbalance under anaerobic conditions led to the considerable accumulation of xylitol [9]. However, XI catalyzes the reversible isomerization of xylose to xylulose, and xylulokinase (XK) converts xylulose to xylulose-5-phosphate which enters the pentose phosphate pathway (PPP). Therefore, the XI pathway instead of the XR-XDH pathway is one solution to reduce xylitol excretion. And XI requires no redox cofactors and does not produce an intracellular redox imbalance during xylose fermentation. Because xylose is converted directly into xylulose without a reaction being involved [2,10]. Numerous past attempts to express a bacterial XI with high activity in *S. cerevisiae* have been unsuccessful. On the other hand, *Clostridium phytofermentans* has the ability to ferment a broad range of feed-stock saccharides such as arabinose, fructose, galactose, glucose, maltose, mannose, ribose and xylose. In *C. phytofermentans*, XI catalyzes the reversible isomerization of xylose to xylulose. Such properties of *C. phytofermentans* define it

as a good candidate for producing a recombinant form of *S. cerevisiae* capable of ethanol production through utilization of xylose [11].

In this study, we report the cloning and expression of a bacterial xylose isomerase with high activity in *S. cerevisiae*, using a bacterial XI gene source obtained from *C. phytofermentans*. Furthermore, the ethanol was produced from xylose using galactose as an inducer, and the effect of galactose to stimulate utilization of xylose was analyzed under co-fermentation conditions with xylose and galactose. This is the first report on the utilization of xylose under galactose supplemented conditions to produce ethanol in recombinant *S. cerevisiae* harboring the bacterial XI gene isolated from *C. phytofermentans.* Ethanol production was dependent on the concentration of supplemented galactose in the medium.

2. Materials and Methods

2.1. Strains and media

S. cerevisiae 2805 (Matα pep4:: HIS3 prb1 can1 his 3 ura 3-52) [12] was grown aerobically at 30ºC in synthetic complete (SC) medium (6.7 g/L Difco yeast nitrogen base without amino acids), supplemented with amino acids as previously described [12], and also supplemented with 20 g/L of glucose or 20 g/L of galactose as co-substrate with xylose (40 g/L). *C. phytofermentans* ATCC 700394 was grown in Chopped Meat Carbohydrate medium (BD, BBL, USA) at 37ºC using anaerobic conditions.

2.2. DNA isolation, manipulation, and transformation

Yeast strains and plasmids used in this study are listed in Table 1. The XI coding region was amplified from the chromosomal DNA of wild type *C. phytofermentans* by PCR using the primer pair, XIf (5' -ataggtacctaatgaaaaattact ttcc - 3') and XIr (5' -gtcggatccttatctaaataaaatat - 3'). The PCR product was ligated with pYES2, which is a *S. cerevisiae* - *E. coli* shuttle vector. The resulting plasmid pMBTL-JY was transferred to *S. cerevisiae* using the lithium acetate method [13].

2.3. Preparation of XI antibody

The XI coding region was amplified from the chromosomal DNA of *C. phytofermentans* wild type by PCR using the primer pair, XIAf (5' - gacggatccatgaaaaattactttcca - 3') and XIAr (5' - tctgcggccgctctaaataaaatattat - 3'), to generate a fusion protein of XI and a hexahistidine tag. The PCR product was digested and the resulting plasmid pET21a-XI transformed into *E. coli* BL21 by electroporation. The synthesis of the XI fusion protein was induced in recombinant *E. coli* BL21 (pET21a-XI) by the addition of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) after the culture had reached an optical density at 600 nm (OD 600 nm) of 0.6. The cells were grown for 2 h, harvested, and then disrupted using a Vibra Cell Sonic disruptor (Sonics & Materials Inc., USA). Purification of the fusion protein was carried out by Ni-NTA affinity chromatography according to the instructions from Qiagen (Hilden, Germany). The purified fusion protein was used directly for the production of polyclonal rabbit antibodies (Ab Frontier Inc., Daejon, Korea).

2.4. Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of the whole proteins were performed using standard protocols [13].

2.5. Fermentations

Cultures of laboratory strains were grown in 250 mL shake flasks (Erlenmeyer flasks) containing 100 mL of medium at 30ºC in a shaker at 180 rpm. Shake-flask precultures were grown until late exponential phase in SC medium supplemented with 20 g/L glucose. Cells were washed with sterile water. Cultures were inoculated at an OD 600 of ~0.6 and incubated at 30ºC with 180 rpm shaking in YPGX medium (yeast extract 20 g/L, peptone 20 g/L, galactose 20 g/L, and xylose 40 g/L), or YPDX medium (yeast extract 20 g/L, peptone 20 g/L, glucose 20 g/L, and xylose 40 g/L).

2.6. Analysis of substrates and fermentation products

Sugar and ethanol concentrations were quantified using a

Table 1. Microbial strains and plasmid used in the study

Strains or plasmid	Relevant genotype	Source or reference
Plasmids		
pYES2	Gal promoter, tCYC1, URA3	Invitrogen Co.
pMBTL-JY	pYES2 ligated with XI isolated from *C. phytofermentans*	This study
Strains		
S. cerevisiae 2805	MATa pep4::HIS prb-Δ1.6R can1 his3-20 ura3-52	(Kang *et al.,* 2000)
S. cerevisiae MBTL-M	2805 with pYES2	This study
S. cerevisiae MBTL-JY	2805 with pMBTL-JY	This study

high-performance liquid chromatograph (Waters, Milford, MA, USA) equipped with an Rspack KC-811 (8 × 300 mm) column (Showa Denko, Tokyo, Japan) and a refractive index detector (Waters 2414). The mobile phase was 4 mmol 1 L H_2SO_4 at a flow rate of 1 mL/min. The temperature of the column was maintained at 60°C. Samples were subjected to membrane filtration prior to analysis [14].

3. Results

3.1. Ethanol fermentation from xylose

Batch fermentations using 40 g/L of xylose supplemented with 20 g/L of glucose showed that recombinant *S. cerevisiae* MBTL-JY was able to grow on only glucose and did not utilize xylose during the fermentations. As shown in Fig. 1A, the recombinant strain rapidly consumed the glucose and produced ethanol from glucose. Next, media containing 40 g/L of xylose but supplemented with 20 g/L of galactose was analyzed for xylose utilization. Addition of galactose strongly stimulated the consumption of xylose during the fermentations. Comparing to the glucose supplemented condition, the galactose was slowly consumed, but xylose was simultaneously consumed with galactose. As a result, 23.48 g/L of xylose was consumed and 15.89 g/L of ethanol was produced. The overall ethanol concentration, volumetric ethanol production rate, and ethanol yield to carbon recovery yield (conversion ratio of 51.14 g ethanol from 100 g sugar was defined as theoretical 100% carbon recovery yield) after 159 h of fermentation were 15.89 g/L of ethanol production, 0.148 g/L/h of volumetric xylose consumption and 73.5% of the carbon recovery (Table 2). The ethanol yield in the co-fermentation from xylose/galactose with MBTL-JY was 73.5% of the carbon recovery. In addition, the yield of ethanol produced by the supplement of galactose was 83.3%. Therefore, when excluding the ethanol produced from the xylose in the culture medium in which the xylose and galactose were simultaneously added, the carbon recovery generated due to the xylose was 67.3%.

3.2. Galactose induces XI protein level

Glucose is reported to stimulate the utilization of xylose [15-17]. This result prompted us to test whether supplemental glucose increases the utilization of xylose mediated by XI protein expression. As shown in Fig. 2, glucose did not affect the expression of XI protein from recombinant *S. cerevisiae* MBTL-JY, showing patterns consistent with fermentation results in Fig. 1. The same absence of XI protein expression was found after addition of xylose (40 g/L). To determine whether supplemental galactose mediates the expression of XI in *S. cerevisiae* MBTL-JY, we used 20 g/L

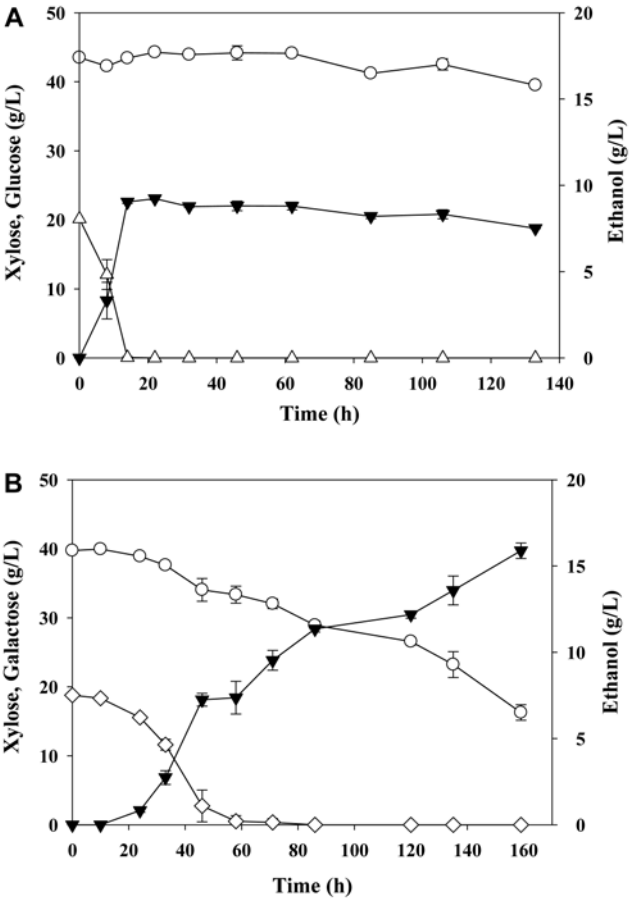

Fig. 1. Sugar consumption and ethanol formation during batch fermentation by recombinant *S. cerevisiae*. Shown are graphs of batch fermentation of strain MBTL-JY. (A) The strain was grown in YPDX (20 g/L glucose, 40 g/L xylose) medium. (B) The strain was grown in YPGX (20 g/L galactose, 40 g/L xylose) medium. Open circle represents residual xylose; open triangle represents residual glucose; open diamond represents residual galactose; filled triangle down represents ethanol. Shown are results of a typical experiment.

Table 2. Xylose consumption and product formation in recombinant *S. cerevisiae* during fermentation of 20 g/L galactose and 40 g/L xylose

Galactose consumed (g/L)	Xylose consumed (g/L)	Product		
		Ethanol (g)	Volumetric xylose consumed (g/L/h)	Volumetric ethanol product rate from xylose (g/L/h)
18.77	23.48 ± 1.12	15.89 ± 0.45	0.148 ± 0.007	0.051 ± 0.007

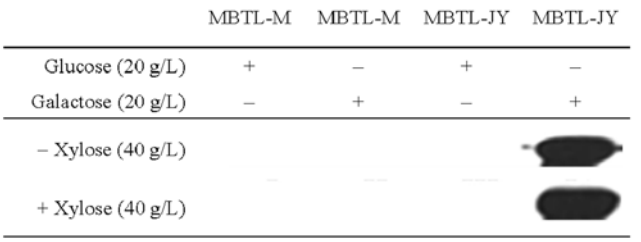

Fig. 2. Western blot of the xylose isomerase from *S. cerevisiae* MBTL-JY. Expression of XI induced by glucose (20 g/L) or galactose (20 g/L) as co-substrate with xylose (40 g/L). Expression of XI was only added galactose. Symbols: +, supplement of sugar; -, absence of sugar.

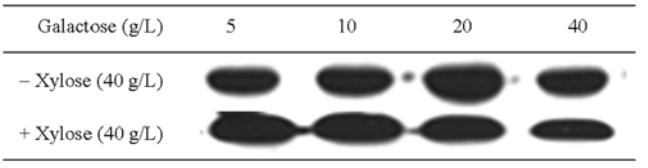

Fig. 3. Effect of galactose on expression of XI in recombinant *S. cerevisiae* MBTL-JY. XI expression of MBTL-JY under galactose supplemented conditions. Shown is graph of highest expression of XI protein for xylose utilization was found using culture media containing 20 g/L of galactose. Symbols: +, supplement of sugar; -, absence of sugar.

of galactose to induce XI expression. Galactose is well known to convert xylose as a co-substrate [18]. As shown in Fig. 2, supplemental galactose significantly increased XI expression, suggesting that the galactose induces the expression of XI by stimulating the Gal promoter in *S. cerevisiae* MBTL-JY. Transport of xylose into the cell is the first metabolic step of xylose conversion. Usually, xylose is taken up by the facilitated diffusion hexose transport system in *S. cerevisiae*, which also transports glucose, fructose, galactose, and mannose [18,19]. In our study, a mixture of xylose and galactose may stimulate the transport of xylose, leading to expression of XI and finally, increase the production of ethanol from xylose.

3.3. Optimal galactose concentration for xylose utilization

To optimize the concentration of galactose for xylose utilization, concentrations of 0, 5, 10, 20, and 40 g/L of galactose were selected and added to *S. cerevisiae* MBTL-JY. As shown in Fig. 3 the highest expression of XI protein for xylose utilization was found using culture media containing 20 g/L of galactose. However, a mixture of galactose and xylose did not significantly affect the increment of XI protein (Fig. 3). In addition, the levels of xylose consumption and ethanol production were also consistent with XI protein expression (Fig. 4). Therefore, 20 g/L of galactose as a co-substrate was the optimal concentration to utilize xylose and produce ethanol from xylose.

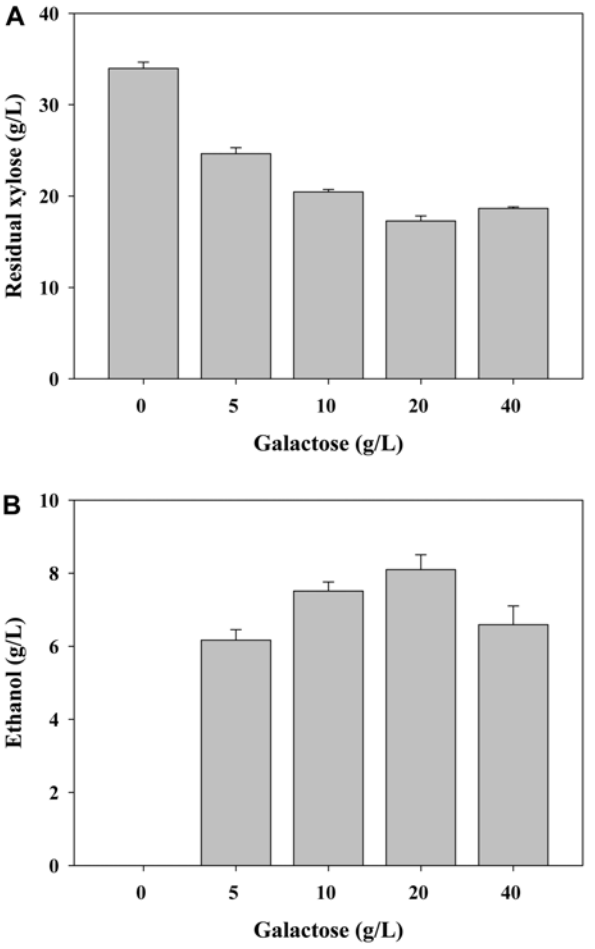

Fig. 4. Xylose consumption and ethanol formation during batch fermentation of *S. cerevisiae* MBTL-JY under galactose supplemented conditions. Comparison of xylose consumption of MBTL-JY (A) and ethanol production (B) depending on the galactose concentration. Here, 40 g/L of xylose was used.

4. Discussion

This study constructed a recombinant *S. cerevisiae* MBTL-JY harboring the bacterial XI gene isolated from *C. phytofermentans* for the bioconversion of xylose to ethanol. *S. cerevisiae* MBTL-JY did not show any activity for xylose utilization. However, when galactose was supplemented into the medium, XI protein was highly expressed through induction of the Gal promoter, leading to a high level of xylose consumption and subsequent ethanol production. A controllable expression system, in which the galactose-regulated promoter GAL1 served as powerful promoter, was employed in this study to achieve a high level of XI gene expression in *S. cerevisiae*. Transcription of GAL1 genes is strongly repressed by the presence of glucose and is positively regulated by the galactose-dependent Gal4 activator, which binds specific sequences in its promoters. When galactose is present in a

medium, the expression level of protein can increase ~1,000-fold [8,20]. We expressed the bacterial XI gene using the system and it resulted in a volumetric ethanol product rate of 0.051 g/L/h, which was similar to results for improvement of xylose fermentation in industrial yeast strains. Moreover, co-fermentation of xylose and galactose increased the rate of xylose utilization compared with pure xylose fermentation. Enhanced xylose uptake in the presence of galactose has previously been reported [1,15,18]. However, glucose and xylose are consumed simultaneously only under conditions of limited glucose, which has have attributed to competition for the transport system [21,22]. Therefore, the different transporter systems for glucose and galactose were suggested to enhance the availability of xylose for consumption in the presence of galactose.

This is the first report of xylose utilization by *S. cerevisiae* harboring the bacterial XI gene, through media supplementation using galactose as a co-substrate. Therefore, further studies to improve the performance of the yeast are expected to produce higher ethanol yields through the application of industrial strains.

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References

1. Hahn-Hägerdal, B., K. Karhumaa, C. Fonseca, I. Spencer-Martins, and M. F. Gorwa-Grauslund (2007) Towards industrial pentose-fermenting yeast strains. *Appl. Microbiol. Biotechnol.* 74: 937-953.
2. Matsushika, A., H. Inoue, T. Kodaki, and S. Sawayama (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: Current state and perspectives. *Appl. Microbiol. Biotechnol.* 84: 37-53.
3. Van Vleet, J. H. and T. W. Jeffries (2009) Yeast metabolic engineering for hemicellulosic ethanol production. *Curr. Opin. Biotechnol.* 20: 300-306.
4. Walfridsson, M., M. Bao, M. Anderlund, G. Lilius, L. Bülow, and B. Hahn-Hägerdal (1996) Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus* xylA gene, which expresses an active xylose (glucose) isomerase. *Appl. Environ. Microbiol.* 62: 4648-4651.
5. Kuyper, M., H. R. Harhangi, A. K. Stave, A. A. Winkler, M. S. Jetten, W. T. Laat, J. J. Ridder, H. J. Op den Camp, J. P. van Dijken, and J. T. Pronk (2003) High-level functional expression of a fungal xylose isomerase: The key to efficient ethanolic fermentation of xylose by Saccharomyces cerevisiae? *FEMS Yeast Res.* 4: 69-78.
6. Eliasson, A., C. Christensson, C. F. Wahlbom, and B. Hahn-Hagerdal (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. *Appl. Environ. Microbiol.* 66: 3381-3386.
7. Zaldivar, J., J. Nielsen, and L. Olsson (2001) Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol.* 56: 17-34.
8. Chen, Z., Z. Li, and N. Yu (2010) Expression and secretion of a single-chain sweet protein, monellin, in *Saccharomyces cerevisiae* by an alpha-factor signal peptide. *Biotechnol. Lett.* 33: 721-725.
9. Kotter, P., R. Amore, C. P. Hollenberg, and M. Ciriacy (1990) Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Curr. Genet.* 18: 493-500.
10. Brat, D., E. Boles, and B. Wiedemann (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 75: 2304-2311.
11. Warnick, T. A., B. A. Methe, and S. B. Leschine (2002) *Clostridium phytofermentans* sp nov., a cellulolytic mesophile from forest soil. *Int. J. Syst. Evol. Microbiol*. 52: 1155-1160.
12. Chung, B. H., D. J. Seo, and S. W. Nam (1999) High-level secretory production of recombinant human lipocortin-I by *Saccharomyces cerevisiae*. *Proc. Biochem*. 35: 97-101.
13. Sambrook, J., E. F. Fritsch, and T. Maniatis (2001) *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
14. Choi, G. W., S. K. Moon, H. W. Kang, J. Min, and B. W. Chung (2009) Simultaneous saccharification and fermentation of sludge-containing cassava mash for batch and repeated batch production of bioethanol by *Saccharomyces cerevisiae* CHFY0321. *J. Chem. Technol. Biotechnol.* 84: 547-553.
15. Han, J. H., J. Y. Park, H. W. Kang, G. W. Choi, B. W. Chung, and J. Min (2010) Specific Expression patterns of xyl1, xyl2, and xyl3 in response to different sugars in *Pichia stipitis*. *J. Microbiol. Biotechnol*. 20: 946-949.
16. Jeffries, T. W. and Y. S. Jin (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl. Microbiol. Biotechnol.* 63: 495-509.
17. Jeffries, T. W. (2006) Engineering yeasts for xylose metabolism. *Curr. Opin. Biotech.* 17: 320-326.
18. Garcia Sanchez, R., B. Hahn-Hägerdal, and M. F. Gorwa-Grauslund (2010) Cross-reactions between engineered xylose and galactose pathways in recombinant *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* 3: 19.
19. Ho, N., W. Z. Chen, and A. P. Brainard (1998) Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* 64:1852-1859.
20. Maya, D., M. J. Quintero, M. D. Munoz-Centeno, and S. Chavez (2008) Systems for applied gene control in *Saccharomyces cerevisiae*. *Biotechnol. Lett*. 30: 979-987.
21. Boles, E. and C. P. Hollenberg (1997) The molecular genetics of hexose transport in yeasts. *FEMS Microbiol. Rev.* 21: 85-111.
22. Hamacher, T., J. Becker, M. Gárdonyi, B. Hahn-Hägerdal, and E. Boles (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiol.* 148: 2783-2788.