Specific Expression Patterns of *xyl1*, *xyl2*, and *xyl3* in Response to Different Sugars in *Pichia stipitis*

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The effects of two different sugars (glucose and xylose) on the expression levels and patterns of the xylose reductase (*xyl1*), xylitol dehydrogenase (*xyl2*), and xylulokinase (*xyl3*) genes were analyzed using *Pichia stipitis*. A significant increase in mRNA levels of *xyl1* was observed after 6 h growth in culture conditions using xylose as a sole carbon source, but expressions of the three genes were not influenced by normal culture media with glucose. In addition, expressions of *xyl2* and *xyl3* were not observed during the entire culture period during which xylose was added. It also was found that the expression level of *xyl1* increased as a function of the xylose concentration (40, 60, and 80 g/l) used in this study, indicating that *xyl1* expression sensitively responded to xylose in the culture media. Although the induced level of *xyl2* increased slightly after 48 h in the xylose-supplemented culture conditions, the expression of *xyl2* was not observed in the xylitol-supplemented culture conditions. Finally, considering the expression of each gene in response to glucose or xylose, the absolute expression levels of the three genes indicate that *xyl1* is induced primarily by exposure to xylose.

Keywords: Gene expression patterns, xylose, glucose, *xyl1*, *xyl2*, *xyl3*, *Pichia stipitis*

Pichia stipitis is a good candidate for developing recombinant microorganisms to produce ethanol using the five-carbon sources in xylose [5, 6–8]. The initial metabolism of xylose in *P. stipitis* is catalyzed by NAD(P)H-dependent xylose reductase (XR), which reduces xylose to xylitol; next, NAD^+-dependent xylitol dehydrogenase (XDH) oxidizes xylitol to xylulose [1–3]. Xylulose is then phosphorylated by xylulokinase (XK) to xylulose 5-phosphate, which is further metabolized through the pentose phosphate pathway and glycolysis to form ethanol [4, 8]. Numerous groups have reported that the *xyl1* and *xyl2* genes encoding XR and XDH, isolated from *P. stipitis*, are expressed in *Saccharomyces cerevisiae*, and they have proposed recombinant *S. cerevisiae* and xylose could be used for ethanol production [2, 4, 8, 10]. The xylose-fermenting *P. stipitis* is among the few organisms that use both xylose and glucose and exhibit a regulatory transition between respiratory and fermentative processes [9, 10, 14].

In the present study, the molecular responses of several sugars on *P. stipitis* were examined by studying the changes in the gene expressions of the three genes related xylose metabolism, namely *xyl1* (xylose reductase gene), *xyl2* (xylitol dehydrogenase gene), and *xyl3* (xylulokinase gene) using the method of reverse transcription polymerase chain reaction (RT–PCR). This technique is a simple and effective tool to analyze the gene expression for specific stimuli [13]. The expression level of a gene is measured by determining the mRNA amount generated by that gene *via* transcription. Through the gene expression analysis, the molecular responses of an organism exposed to specific stimuli can be determined to provide an insight into the action mode of stimuli.

In this study, to understand responses caused by exposure to glucose and xylose in *P. stipitis*, three different genes (*xyl1*, *xyl2*, and *xyl3*) were selected for analysis of their expression levels and patterns, and to quantify the expression levels and patterns of the genes in the *P. stipitis*, a

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semiquantitative RT–PCR protocol as important tools of choice for detail gene expression analysis was adopted.

MATERIALS AND METHODS

Strain, Medium, Cultivation Conditions, and Analytical Methods

Pichia stipitis CBS 5773 was used in this study and grown in YP (10 g/l yeast extract and 20 g/l bacto peptone) medium with glucose (6 g/l) or xylose (6 g/l) in 250-ml flasks at 30°C, 200 rpm. Samples were collected at the early exponential phase, mid-exponential phase, and stationary phase to monitor the levels of *xyl1*, *xyl2*, and *xyl3*. For the supplemented culture, xylose (0, 40, 60, and 80 g/l) and xylitol (0, 40, 60, and 80 g/l) were added to the culture media on the early exponential phase, which was after 6 h. Sugar and ethanol concentrations were quantified using a high-performance liquid chromatograph (Waters, Milford, MA, U.S.A.) equipped with an Rspack KC-811 (8×300 mm) column (Showa Denko, Tokyo, Japan) and a refractive-index detector (Waters 2414, Milford, MA, U.S.A.). All data points are presented as means and standard deviations, with n indicating the number of experiments.

RNA Extraction and Semiquantitative RT–PCR

To isolate the RNA in *P. stipitis*, the cell suspension was collected at 0, 6, 24, and 48 h in the glucose or xylose culture conditions, respectively. The total RNA was extracted from the harvested cells using the Trizol extraction method (Invitrogen, Belgium). Xylose and xylitol were added at the early exponential phase of culture to observe the induction effect of the xylose and xylitol on the expressions of the *xyl1*, *xyl2*, and *xyl3* genes, and the growth of *P. stipitis* at the early exponential phase that was at 6 h post induction (data not shown). Min *et al.* [11, 12] reported that most bacterial cells show sensitive responses to chemical inducers at the early exponential phase as an induction point, compared with other growth phases, without other side or synergic effects.

The total RNA concentration was evaluated by measuring the absorption at 260 nm using a Qubit fluorometer (Invitrogen Co.). The mRNA levels were determined by semiquantitative RT–PCR [15] using a TaKaRa RNA PCR Kit (TaKaRa Inc., Japan). For each experimental condition the three genes were used to analyze the expression patterns in *P. stipitis*, using the actin *(ACT1)* gene as an internal standard (Table 1). The PCR experiments were carried out using a T Gradient Thermocycler (Biometra, Germany). Thirty cycles were used to amplify the specific genes. All RNA samples without an RT step were also used for PCR to detect the presence of genomic DNA contamination. No DNA was detected at any time. The PCR products were visualized on an EtBr-stained agarose gel. The band intensities from the gel were quantified using GelScope 1.5 software (Imageline Inc., U.S.A.). PCR was carried out at three
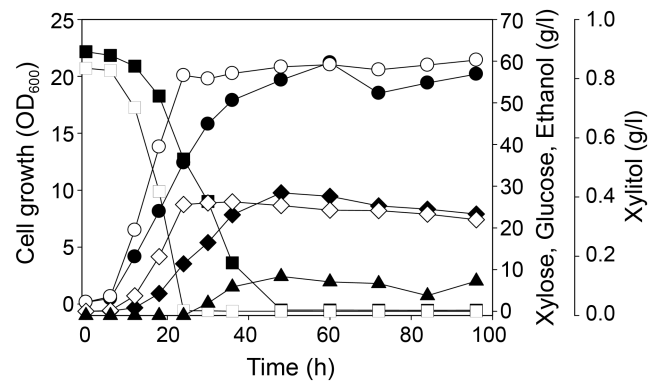
Fig. 1. Glucose and xylose consumption and ethanol production in *P. stipitis* during culture.
Cells were grown in YP media containing 60 g/l glucose (open symbols) or 60 g/l xylose (closed symbols). The circles represent cell density; the squares represent glucose and xylose concentrations; the diamonds represent ethanol concentration; the triangles represent xylitol concentration. The results show the mean values of three experiments.

different time points to confirm the reproducibility of the results. All results are reported as the mean value.

RESULTS AND DISCUSSION

Cell Growth and Ethanol Production of *P. stipitis* in Glucose and Xylose

Ethanol production by *P. stipitis* was compared in complex YP media containing either 60 g/l of glucose or 60 g/l of xylose (Fig. 1). In YP medium, 26 g/l ethanol was produced from 60 g/l glucose over 24 h; however, 28 g/l ethanol was produced from 60 g/l xylose at 48 h after using all the xylose. Although the consumption time of residual xylose and conversion time for ethanol production were significantly slower than those of glucose, the levels of ethanol production from each sugar were not different with cultivation of *P. stipitis*. However, in YP medium containing xylose, xylitol (0.04 g/l), known as the next metabolite catalyzed from xylose by XR, was continuously produced over 30 h, after which time cells began consuming the xylose. In addition, the residual xylitol concentration approached 0.1 g/l, and this level was maintained during cultivation. *P. stipitis* is a good candidate to use as a xylose-utilizing yeast because this strain used almost all available xylose to produce ethanol, whereas small amounts of xylitol were produced.

**Table 1.** PCR primers used in the analysis of *ACT1*, *xyl1*, *xyl2*, and *xyl3* expression levels.

Gene	Protein	Forward primer (5'→3')	Reverse primer (5'→3')
ACT1	Actin	TTGTTTTGGACTCTGGTGA	TCCTTTTACATACGTTCAG
xyl1	Xylose reductase	TGGAAGGCTCTTGAAAAGT	ACTTGAGATTCAACGACCC
xyl2	Xylitol dehydrogenase	TGACTGCTAACCCTTCCTT	TGTTGAGCCATTGTCTGTT
xyl3	Xylulokinase	ACTACCCCATTTGATGCTC	CCAGAGCCCATTACAGATT

Effects of Different Carbon Sources on *xyl1*, *xyl2*, and *xyl3* in *P. stipitis*

In this study, the *ACT1* gene was selected as an internal standard gene. The expression level of the *ACT1* was constant in all experimental conditions (data not shown). The effects of two different sugars (glucose and xylose) on the expression levels and patterns of the *xyl1*, *xyl2*, and *xyl3* genes were analyzed in this study because *P. stipitis* can use either glucose or xylose as carbon sources. Culture in YP with glucose resulted in normal expression levels of the three genes, indicating no response of the three genes to glucose (Fig. 2A). However, after culture in YP containing xylose for 6 h, the expression of the *xyl1* gene increased significantly in *P. stipitis*, and this increase depended upon the culture period tested (Fig. 2B). There was no expression

of either *xyl2* or *xyl3* in response to YP culture with xylose. However, *xyl1* was distinct in its expression patterns for all three concentrations of xylose (Fig. 3). There was a strong induction in the *xyl1* expression level during the xylose-supplemented conditions (Fig. 3A); however, the three different concentrations of xylitol had no discernible effect on the expressions of *xyl1*, *xyl2*, and *xyl3* in *P. stipitis*.

This study compared the gene expression patterns induced by different sugars (glucose, xylose, and xylitol as an intermediate metabolite) by transcriptional analysis of three genes selected from a xylose-using yeast, *P. stipitis*. The effects of glucose, xylose, and xylitol in *P. stipitis* were elucidated from the transcriptional level responses. The *xyl1* gene used in this study provided considerable information on the effect of xylose; however, some of these sugars require further transcriptional analysis for specific genes
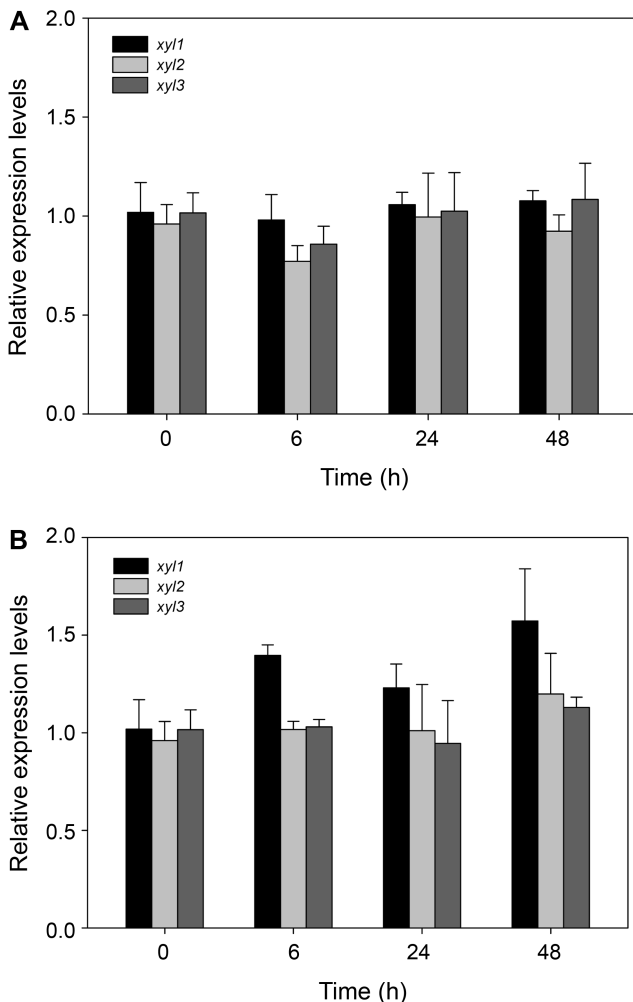
Fig. 2. Fold induction in the expression levels of *xyl1*, *xyl2*, and *xyl3* in *P. stipitis* according to the different sugars added to the culture.
Either (**A**) 60 g/l glucose or (**B**) 60 g/l xylose was added as the sole carbon source to cultured *P. stipitis*. All data correspond to expression levels relative to that of the *ACT1* gene.
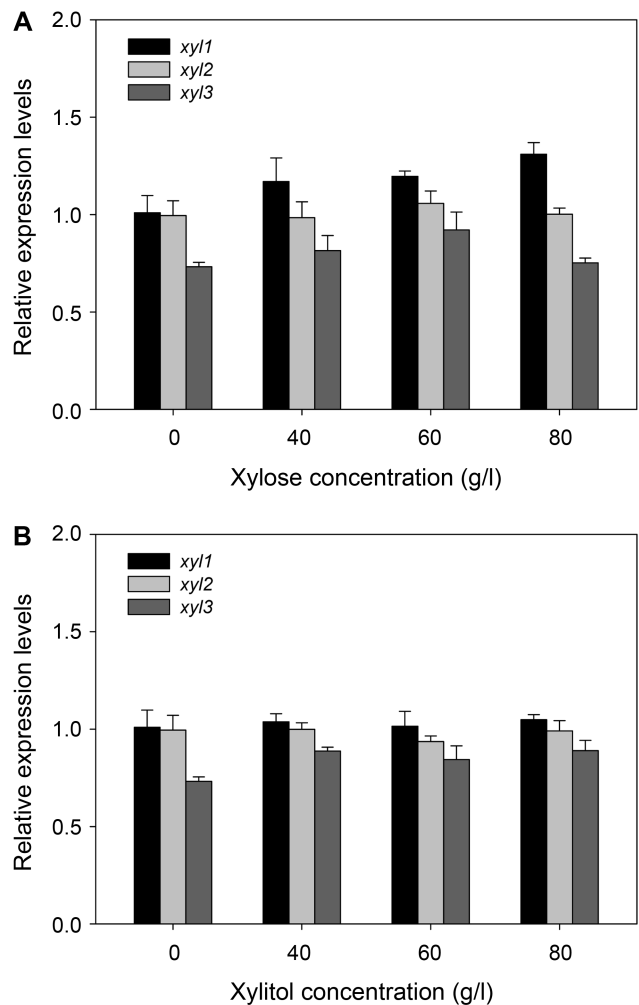
Fig. 3. Fold induction in the expression levels of *xyl1*, *xyl2*, and *xyl3* in *P. stipitis* according to the concentration of (**A**) xylose and (**B**) xylitol.
All data correspond to expression levels relative to that of the *ACT1* gene.

produced in response to other metabolic pathways in order to more accurately analyze them and their effects on *P. stipitis*. This study briefly analyzed the molecular responses of *xyl1*, *xyl2*, and *xyl3* in *P. stipitis*, and focused on its mRNA responsive features.

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