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# Xylitol production using recombinant *Saccharomyces cerevisiae* containing multiple xylose reductase genes at chromosomal $\delta$-sequences

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**Abstract**

Xylitol production from xylose was studied using recombinant *Saccharomyces cerevisiae* 2805 containing xylose reductase genes (*XYL1*) of *Pichia stipitis* at chromosomal $\delta$-sequences. *S. cerevisiae* 2805-39-40, which contains about 40 copies of the *XYL1* gene on the chromosome, was obtained by a sequential transformation using a dominant selection marker *neo*$^r$ and an auxotrophic marker *URA3*. The multiple *XYL1* genes were stably maintained on the chromosome even after 21 and 10 days in the non-selective sequential batch and chemostat cultures, respectively, whereas *S. cerevisiae* 2805:pVTXR, which harbors the episomal plasmid pVTXR having the *XYL1* gene, showed mitotic plasmid instability and more than 95% of the cells lost the plasmid under the same culture conditions. In the first batch (3 days) of the sequential batch culture, volumetric xylitol productivity was 0.18 g $l^{-1}$ $h^{-1}$ for *S. cerevisiae* 2805-39-40, as compared to 0.21 g $l^{-1}$ $h^{-1}$ for *S. cerevisiae* 2805:pVTXR. However, the xylitol productivity of the latter started to decrease rapidly in the third batch and dropped to 0.04 g $l^{-1}$ $h^{-1}$ in the seventh batch, whereas the former maintained the stable xylitol productivity at 0.18 g $l^{-1}$ $h^{-1}$ through the entire sequential batch culture. The xylitol production level in the chemostat culture was about 8 g $l^{-1}$ for *S. cerevisiae* 2805-39-40, as compared to 2.0 g $l^{-1}$ for *S. cerevisiae* 2805:pVTXR after 10 days of cultures even though the xylitol production level of the latter was higher than that of the former for the first 5 days. The results of this experiment indicate that *S. cerevisiae* containing the multiple *XYL1* genes on the chromosome is much more efficient for the xylitol production in the long-term non-selective culture than *S. cerevisiae* harboring the episomal plasmid containing the *XYL1* gene. 

*Keywords:* Recombinant Yeast; Xylose reductase gene; Xylitol; $\delta$-Sequence; Plasmid stability

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## 1. Introduction

Xylitol, a naturally occurring five-carbon polyalcohol, is a natural sweetener with non-cariogenicity and taste of cooling sensation (Emodi, 1978; Nigam and Singh, 1995). Therefore, xylitol is being consumed as a sweetener in various food products such as chewing gums, sweets, soft drinks and ice-creams. More importantly, xylitol has been widely used as a sucrose substitute for the treatment of insulin-dependent diabetics and other diseases such as erythrocytic glucose-6-phosphate dehydrogenase deficiency (Emodi, 1978; Culbert and Wang, 1986).

Xylitol has been manufactured on an industrial scale by the chemical reduction of xylose, which was obtained by acid hydrolysis of xylan present in wood pulp from paper processing, beech and other hard wood chips, cottonseed hulls, cane sugar bagasse, rice and oat hulls and other agricultural by-products (Emodi, 1978; Nigam and Singh, 1995). However, the chemical process is not a cost-effective method for the mass production of xylitol because of its high cost for the purification of xylitol from other polyols and sugars present in the chemical reaction mixture (Nigam and Singh, 1995). Alternatively, xylitol has also been produced using natural xylose-utilizing yeasts, such as *Candida tropicalis*, *C. guillermondii*, and *C. parapsilosis* (Barbosa et al., 1988; Meyrial et al., 1991; Kim et al., 1997; Oh et al., 1998). However, the xylitol production yield is relatively low with the natural xylose-fermenting yeasts, since part of the xylitol produced is further metabolized to yield cell mass (Barbosa et al., 1988; Nigam and Singh, 1995).

*Saccharomyces cerevisiae* offers several advantages as a host for the production of many industrially important materials because large-scale fermentation technology is well developed for the yeast and the yeast is generally recognized as safe (GRAS) by the FDA (Romanos et al., 1992). Therefore, xylitol production using *S. cerevisiae* was explored by transforming the yeast with a YEp plasmid containing the *XYL1* gene (Hallborn et al., 1991, 1994; Takuma et al., 1991; Liden et al., 1996; Meinander et al., 1996), because the yeast does not have xylose reductase (XR) (Gong et al., 1981). The recombinant *S. cerevisiae* containing *XYL1* on a yeast episomal plasmid (YEp) gave close to 100% conversion of xylose to xylitol in the presence of cosubstrates, such as glucose and ethanol which are required for cell growth and maintenance (Hallborn et al., 1991, 1994). However, the high copy plasmids, YEp and YRp, are mitotically unstable under non-selective conditions (Walmsley et al., 1983; Romanos et al., 1992; Meinander and Hahn-Hägerdal, 1997). Therefore, selective synthetic media are needed for the maintenance of the plasmids during long-term cultures, such as fed-batch and chemostat cultures (Liden et al., 1996; Meinander et al., 1996; Meinander and Hahn-Hägerdal, 1997). And mitotically stable vectors, such as YIp, pYAC and pYCp, also have their own limitation for the mass production of heterologous proteins because the copy number is very low (1–2 copies $cell^{-1}$) (Romanos et al., 1992). To circumvent these problems, several researches have been focussed on multicopy integration of heterologous genes into the chromosome of *S. cerevisiae* using the $\delta$-sequences of the yeast retrotransposon Ty (Jacobs et al., 1989; Sakai et al., 1990; Mochizuki et al., 1994; Parekh et al., 1996).

In this work, we describe xylitol production from xylose using the recombinant *S. cerevisiae* containing multiple *XYL1* genes on the chromosome in the sequential batch and chemostat cultures under the non-selective condition.

## 2. Materials and methods

### 2.1. Strains, plasmids and media

*Pichia stipitis* CBS 5773 (KCTC7222, Korea) was used as a source for the *XYL1* gene (Verduyn et al., 1985) and *Escherichia coli* XL 1 Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ *F′*[*proAB*$^+$ *lacIq lacZ*ΔM15 Tn*10*(*tet*$^r$)]) (Stratagene, CA, USA) for the propagation of plasmids. *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1 can1 his 3 ura 3-52*) (Sohn et al., 1995) was used as a host strain for the xylitol production. pUC19 (New England Biolabs, MA, USA) was

used for subcloning in *E. coli*. pVT103-U (Vernet et al., 1987), an *E. coli–S. cerevisiae* shuttle vector containing a yeast 2 $\mu$m origin, an *URA3* gene, an *ADH1* promoter and an *ADH1* terminator, was obtained from Professor S.W. Nam (University of Dongeui, Korea). Plasmid pITy4 (Parekh et al., 1996), which has a Ty2 $\delta$-sequence and a neomycin-resistant gene (*neo*$^r$) gene from Tn603, was kindly given by Professor K.D. Wittrup (University of Illinois, MI, USA).

The media used for the cultivation of yeast cells were as follows: YPD medium [composed of 1% yeast extract, 2% bacto-peptone, and 2% glucose]; YPDX medium [YPD medium supplemented with 2% xylose]; SDC medium [0.67% yeast nitrogen base without amino acids (Difco, MI, USA), 2% glucose, 0.5% casamino acids (Difco)] (Wang and Silva, 1993), unless mentioned otherwise. The media were solidified with 2% agar (Difco) for plates. To prepare YPD-G418 plates, filter-sterilized G418 antibiotic (Geneticin, Gibco BRL, MD, USA) was added to the YPD-agar medium ranging from 0.1 to 8.0 g $l^{-1}$. *E. coli* strains harboring plasmids were grown in LB medium supplemented with kanamycin (100 $\mu$g $ml^{-1}$) or ampicillin (50 $\mu$g $ml^{-1}$) whenever necessary (Sambrook et al., 1989).

### 2.2. Construction of plasmids

One episomal plasmid, pVTXR and two integrating plasmids, pITyXR and pIDXR, were constructed as described below for the study of xylitol production using *S. cerevisiae* 2805. The *XYL1* gene was isolated from *P. stipitis* CBS 5773 by PCR using two primers, 5′-GGGGATCCATGCCTTCTATTAAGTTGAAC-3′ and 5′-CCCTCGAGATTAGGTTTTATTTCCTCTCT-3′ (*Bam*HI or *Xho*I site on the 5′-end of each primer is underlined). The PCR product was digested with *Bam*HI and *Xho*I and subcloned between the *Bam*HI and *Sal*I sites of pUC19, resulting in pUCXR. The 1-kb *Bam*HI–*Pst*I fragment carrying the *XYL1* gene of pUCXR was inserted between the *Bam*HI and *Pst*I sites of pVT103-U to yield pVTXR. The 1.9-kb DNA fragment, which contains the *ADH1* promoter, *XYL1* gene and *ADH1* terminator, was isolated from pVTXR after digestion with *Sph*I and then inserted into the *Sph*I site of pITy4, producing pITyXR. The 1-kb *Bgl*II fragment containing the *URA3* marker was isolated from pVT103-U and inserted into the *Bgl*II site of pITy4 to generate pITyURA, and then the 1.9-kb *Sph*I fragment of pVTXR was inserted to the *Sph*I site of pITyURA, producing pIDXR. The *XYL1* gene in plasmids, pVTXR, pITyXR, and pIDXR (Fig. 1) is under the control of the constitutive *ADH1* promoter.

### 2.3. Yeast transformation

Yeast transformation was performed by the procedure of Gietz et al. (1995) with the following modifications. The integrating plasmids were linearized with *Xho*I and *Apa*I for the target integration at the $\delta$-sequence and at the *URA3* locus on the chromosome, respectively. For the pITyXR, transformants were grown for 4 h in non-selective YPD liquid media to allow for *neo*$^r$ expression and selected on YPD-G418 plates. For the pVTXR and pIDXR, transformants were selected on the SDC plates (Wang and Silva, 1993). *S. cerevisiae* carrying the episomal plasmid pVTXR was designated 2805:pVTXR.

### 2.4. Cultivation conditions

The batch and sequential batch cultures were conducted for the evaluation of xylitol production in a 250-ml Erlenmeyer shake flask containing 100 ml of YPDX medium. All the flask cultures were performed in a rotary shaking incubator (Innova 4330, New Brunswick Scientific, NJ, USA) at 30°C and 200 rpm. For the sequential batch culture, the first flask culture was inoculated with 5 ml of seed culture, which was prepared by inoculating with a single colony and incubating in the shaking incubator for 20 h at 30°C. A total of seven identical flask cultures were carried out in series for the sequential batch culture. The inoculum size was controlled so that each flask had the same initial cell concentration (at 600 nm OD = 1.5).

Chemostat cultures were carried out in a 2.5-l fermenter (KFC, Korea) containing 1.5 l of YPD medium under the following conditions: pH 5,

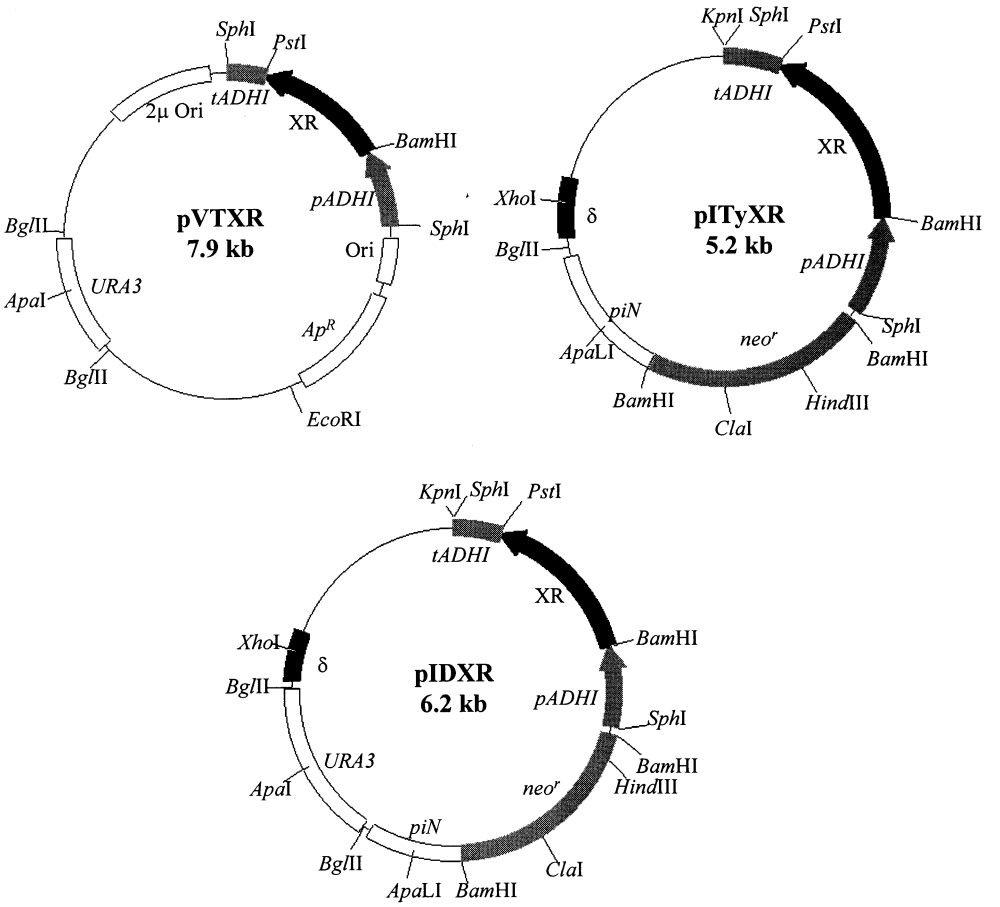


Fig. 1. Physical maps of YEp plasmid pVTXR and $\delta$-integrating plasmids, pITyXR and pIDXR. Abbreviations used: XR, xylose reductase gene (*XYL1*) of *P. stipitis*; *pADH1*, promoter of the alcohol dehydrogenase 1 gene; *tADH1*, terminator of the alcohol dehydrogenase 1 gene; *URA3*, *URA3* gene selection marker; $\delta$, $\delta$-sequence of Ty2; Ori, bacterial origin of replication; 2 $\mu$ Ori, 2 $\mu$m yeast replication sequence; *neo*$^r$, neomycin-resistant gene of Tn903; *piN*, $\pi N$ sequences of Tn903; *Ap*$^R$, $\beta$-lactamase gene. Restriction sites used are also indicated.

controlled by the addition of 10% KOH; temperature, 30°C; air flow rate at 1vvm; agitation at 200 rpm except for the first 10 h of batch-wise growing where 500 rpm was used. YPD medium containing 3% xylose was used for the continuous feeding. For the seed culture, cells were grown for 20 h in a 250-ml shake flask containing 50 ml selective SDC medium for *S. cerevisiae* 2805:pVTXR or 50 ml YPD medium for a recombinant *S. cerevisiae* containing *XYL1* genes on the chromosome. The seed culture was used to inoculate YPD medium in the fermenter. After 10 h of the initial growth in the chemostat culture, continuous nutrient feeding was initiated at the dilution rate of 0.04 $h^{-1}$, which was controlled by a Masterflex pump (Model 7520-35, Cole-Palmer, IL, USA). Samples were taken at regular time intervals during the sequential batch and chemostat cultures for the study of xylitol production, plasmid stability and XR activity.

### *2.5. Southern blots analysis and determination of the gene copy number*

The yeast genomic DNAs were prepared by the method of Hoffman and Winston (1987) and di-

gested with either *Xho*I or *Apa*LI. The digested genomic fragments were separated by electrophoresis on a 0.8% agarose gel, transferred directly onto the Nylon membrane (positively charged, Boehringer Mannheim, Mannheim, Germany) and fixed to the membrane by 2 min exposure to UV light. The 1 kb-*Bam*HI fragment containing the *neo*$^r$ gene was isolated from plasmid pIDXR and labeled by the random priming method using a Genius kit (Boehringer Mannheim). The probe could hybridize with both pITyXR and pIDXR. Prehybridization, hybridization, and subsequent nonradioactive colorimetric detection were conducted as described in the Genius protocol (Boehringer Mannheim). After Southern hybridization, the intensity of the band corresponding to the multicopy *neo*$^r$ gene in the selected transformants and the band corresponding to the single copy of the *neo*$^r$ gene in the transformant containing one copy of pIDXR was compared to estimate the copy number for the multiple integration vector in the transformants, using an image analysis system (Model GS-700, Bio-Rad, CA, USA) (Parekh et al., 1996; Wang et al., 1996). The copy number was further confirmed by adjusting the intensity of the multicopy band to be comparable to that of single copy band by 10-fold serial dilution of the genomic DNA digested with *Apa*LI (denstimetric data not shown).

### *2.6. Determination of enzyme activity, plasmid stability and metabolites*

Cell-free extracts were prepared by the glass bead disruption method (Ausubel et al., 1995) and NADPH-dependent XR activity in the cell-free extracts was measured by the method as described by Hallborn et al. (1991). One U of XR enzyme was defined as the amount of the enzyme catalyzing the oxidation of 1 $\mu$mol min$^{-1}$ NADPH. Protein concentration was determined by the method of bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin as a standard.

Biomass concentrations were determined by observing the optical density at 600 nm (UV-160A Spectrophotometer, Shimadzu, Kyoto, Japan). Culture samples were diluted with an appropriate amount of distilled water to obtain an OD between 0.2 and 0.7. A standard curve was then used to convert the OD to dry cell weight (DCW). One optical density unit was equal to 0.39 g l$^{-1}$ DCW. Numbers of plasmid-free cells in the cultures were determined by the patch plating method (Parker and DiBiasio, 1987). A sample was diluted, plated on YPD plates and grown for 48 h at 30°C to give 150–300 colonies on each plate. Using sterile toothpicks, about 140 colonies were transferred to SDC plates and grown for 48 h at 30°C. Percentage of plasmids-free cells was then obtained by determining the number of non-growing colonies.

Glucose, xylose, and xylitol were determined by HPLC (LC-9A, Shimadzu) using an Aminex HPX 87-H column (Bio-Rad) with a refractive index detector (Waters 410, Waters, MA, USA). The samples on the column were eluted at 65°C with 5 mM $H_2SO_4$ as a mobile phase at a flow rate of 0.6 ml min$^{-1}$.

## 3. Results

### *3.1. Construction of recombinant S. cerevisiae containing multiple XYL1 genes on the chromosome*

For the selection of the integrants containing high copies of the *XYL1* gene, we used a dominant selection marker *neo*$^r$ because auxotrophic markers are not stringent enough for the selection of multicopy integration events (Wang et al., 1996). The *neo*$^r$ gene confers resistance to the aminoglycoside antibiotic G418 and allows the copy number to be tuned by varying G418 resistance (Parekh et al., 1996). The integrating plasmid, pITyXR, which contained a $\delta$-sequence for targeting integration to the chromosomal $\delta$-sequences, has a bacterial *neo*$^r$ gene for the selection of the integrating events (Fig. 1). The numbers of transformants selected on YPD-G418 plates increased with increasing the amount of DNAs used for transformation, but decreased with increasing the G418 concentrations on the plates (data not shown). To select high copy pITyXR integrants, transformants were selected on the YPD-G418

Table 1
Xylose reductase (XR) activity from selected strains differing in the copy number and location of the inserted *XYL1* gene on their chromosomes

| Strains | Copy number | Number of insertion sites | XR activity[a] (mU $mg^{-1}$) |
|---|---|---|---|
| *S. cerevisiae* 2805-1 | 1 | 1 | 4 |
| *S. cerevisiae* 2805-39 | 30 | 1 | 10 |
| *S. cerevisiae* 2805-39-15 | 31 | 2 | 10 |
| *S. cerevisiae* 2805-39-19 | 33 | 3 | 15 |
| *S. cerevisiae* 2805-39-29 | 37 | 2 | 20 |
| *S. cerevisiae* 2805-39-31 | 38 | 2 | 21 |
| *S. cerevisiae* 2805-39-40 | 40 | 2 | 28 |

[a] XR activity was measured from the cell-free extracts of cells grown on YPDX medium for 20 h in shake flask cultures.

plates containing 8 g $l^{-1}$ of G418 and examined for the specific XR activity (data not shown). Among the transformants, *S. cerevisiae* 2805-39, which showed the highest specific XR activity, was selected and used for the further experiment. To further increase the copy number of the *XYL1* on the chromosome, *S. cerevisiae* 2805-39 was transformed with the *Xho*I-digested pIDXR containing an *URA3* gene and then transformants were selected using the *URA3* gene as an auxotrophic marker on SDC plates. $Ura^+$ transformants were randomly picked up and examined for the specific XR activity. The second transformation using the *URA3* selection marker further increase the specific XR activity, indicating that additional *XYL1* genes were integrated at the $\delta$-sequences (Table 1). About 2.8-fold increase in the XR activity was obtained with *S. cerevisiae* 2805-39-40.

To estimate the copy number of the chromosomally integrated plasmids and to determine the chromosomal integration patterns on the chromosome, the Southern blot was performed after digestion of genomic DNA with *Apa*LI, which cuts once the integrating vectors of pITyXR and pIDXR (Fig. 2A). The 1 kb-*Bam*HI $neo^r$ gene of pIDXR, which could hybridize to plasmids pITyXR and pIDXR, was used as a probe. The Southern blot analysis showed two major bands corresponding to 5.2 and 6.2 kb (Fig. 2B), which are the size of pITyXR and pIDXR, respectively. In addition to the two major bands, several lighter bands of larger sizes were also shown. The appearance of the darker bands means that the integrating plasmids were tandemly integrated at the specific $\delta$-sequences. The lighter bands represent the *Apa*LI DNA fragment which contains the $neo^r$ sequence at one end of the tandem copies (unknown fragments indicated by a vertical arrow in Fig. 2A). Therefore, the number of lighter bands indicate the number of integration sites. The copy number of the integrating plasmid, which was estimated by measuring the relative ratio of intensities of the multicopy band to the single-copy band, was in the range of 30–40 and the number of insertion sites were one to three (Fig. 2B, Table 1). *S. cerevisiae* 2805-1 containing one copy of pIDXR, which was constructed by transforming *S. cerevisiae* 2805 with the *Apa*I-linearized pIDXR and confirmed by the Southern blotting using 1 kb-*Bam*HI of pIDXR as a probe, was used as a control for the estimation of the copy number.

### 3.2. Effect of the gene dosage of the XYL1 gene on XR activity and xylitol production

The specific XR activity of cell-free crude extracts prepared from the selected transformants and *S. cerevisiae* 2805:pVTXR was measured. Among transformants examined, *S. cerevisiae* 2805-39-40 containing about 40 copies of the *XYL1* showed the highest specific activity of XR (28 mU $mg^{-1}$), about seven times higher than that of *S. cerevisiae* 2805-1 (4 mU $mg^{-1}$) con-

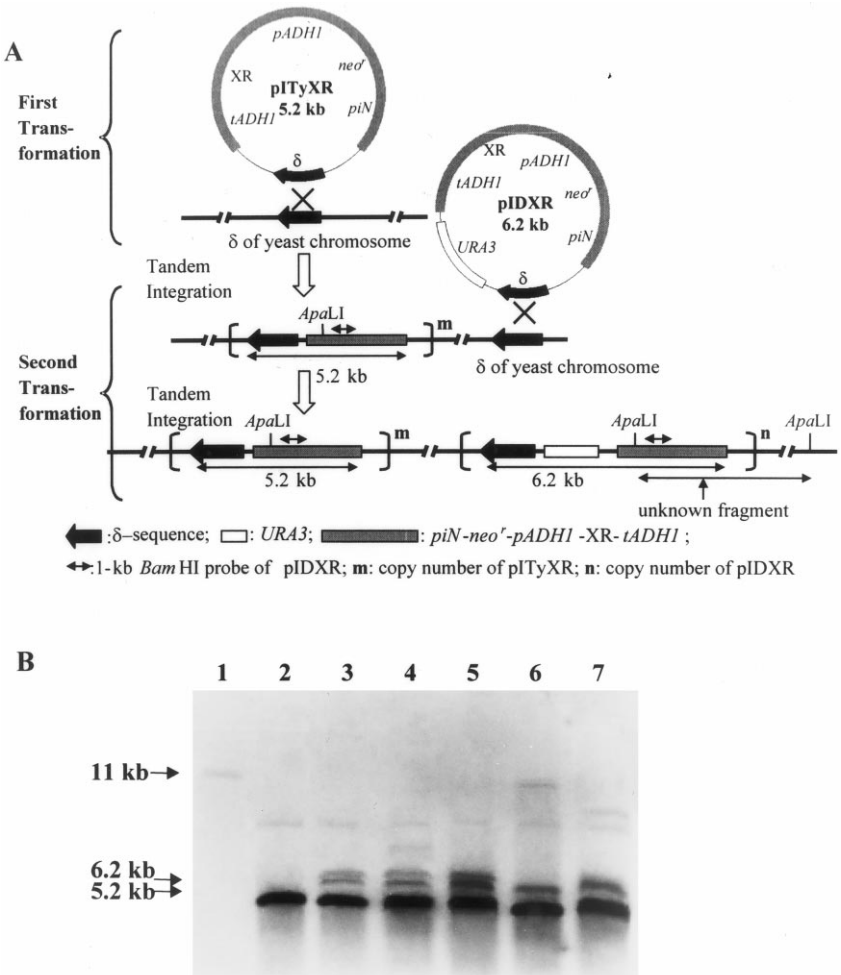


Fig. 2. Southern blot analysis of tandem integration of δ-integrating plasmids into the chromosomal δ-sequence. (A) Schematic diagram of the chromosome with tandem integration of the δ-integrating plasmids pITyXR and pIDXR into the δ-sequence target site by a homologous recombination event. (B) Southern blots analysis of *Apa*LI-digested genomic DNA from selected strains using 1 kb-*Bam*HI fragment of pIDXR as a probe. Lane 1, *S. cerevisiae* 2805-1; lane 2, *S. cerevisiae* 2805-39; lane 3, *S. cerevisiae* 2805-39-15; lane 4, *S. cerevisiae* 2805-39-19; lane 5, *S. cerevisiae* 2805-39-29; lane 6, *S. cerevisiae* 2805-39-31; lane 7, *S. cerevisiae* 2805-39-40.

taining a single copy (Table 1). Even though the level of XR activity increased with the elevated gene dosage of the *XYL1* gene, there was no linear relationship between gene dosage and specific XR activity.

The effect of copy number of the *XYL1* gene on the xylitol production was examined for four yeast strains having different numbers of the *XYL1* gene: *S. cerevisiae* 2805 (0 *XYL1* gene), *S. cerevisiae* 2805-1 (1 *XYL1*), *S. cerevisiae* 2805-39 (30 *XYL1* copies) and *S. cerevisiae* 2805-39-40 (40 *XYL1* copies). As shown in Fig. 3, the xylitol productivity was higher with increasing the copy number of the *XYL1* gene. However, *S. cerevisiae* 2805-39-40 produced only 2.5 times more xylitol than *S. cerevisiae* 2805-1 even though the XR activity of *S. cerevisiae* 2805-39-40 was seven times higher than that of *S. cerevisiae* 2805-1.

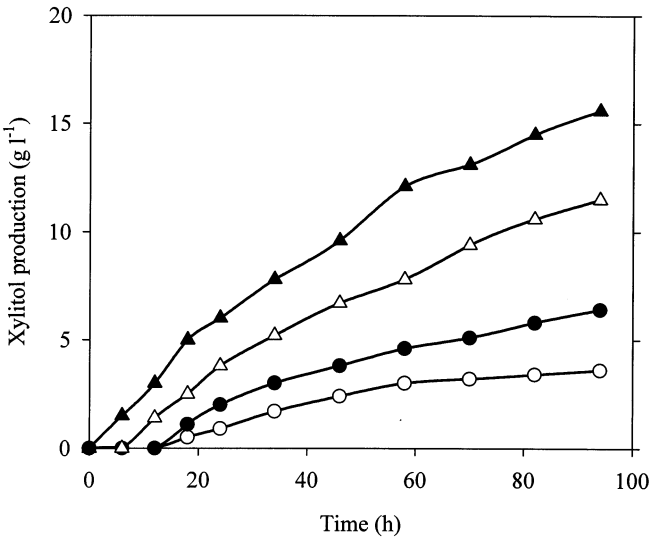


Fig. 3. Xylitol production in shake flask cultures with *S. cerevisiae* 2805 without *XYL1* gene (○), *S. cerevisiae* 2805-1 containing one copy of *XYL1* (●), *S. cerevisiae* 2805-39 containing 30 copies of *XYL1* (△), and *S. cerevisiae* 2805-39-40 containing 40 copies of *XYL1* (▲).

### *3.3. Xylitol production and mitotic plasmid stability in the sequential batch culture*

Sequential batch cultures were performed using *S. cerevisiae* 2805-1, *S. cerevisiae* 2805-39-40 and *S. cerevisiae* 2805:pVTXR to compare the xylitol production and mitotic stability between the chromosomally integrated plasmid and the episomal plasmid pVTXR during the long-term culture. The three strains were grown for 21 days in 250-ml Erlenmeyer shake flasks containing 100 ml of non-selective YPDX medium by repeatedly transferring the culture into fresh YPDX medium at 3-day intervals (Fig. 4). Seven serial batch cultures were done during 21 days of the sequential batch culture. In the first batch (3 days) of the sequential batch culture, *S. cerevisiae* 2805-39-40 produced about 10% less xylitol than that produced by *S. cerevisiae* 2805:pVTXR, but the amount of xylitol produced by two strains was nearly identical in the third batch. At the end of the sequential batch culture (21 days, the seventh batch), *S. cerevisiae* 2805-39-40 produced about four times more xylitol than 2805:pVTXR. The volumetric xylitol productivity of *S. cerevisiae* 2805-39-40 was about 0.18 g $l^{-1}$ $h^{-1}$ throughout the culture, whereas that of *S. cerevisiae* 2805:pVTXR was 0.21 g $l^{-1}$ $h^{-1}$ in the first and second batches and decreased to 0.04 g $l^{-1}$ $h^{-1}$ in the seventh batch.

To find a reason for the decrease in the xylitol production with *S. cerevisiae* 2805:pVTXR, the number of $Ura^+$ cells in the culture was determined to estimate the percentage of plasmid-free cells by the patch plating method (Fig. 5C). At the same time, the mitotic plasmid stability was also examined for *S. cerevisiae* 2805-39-40 by the quantitative Southern blot analysis of the genomic DNA taken at regular time intervals (Fig. 5B). The number of $Ura^+$ cells harboring the YEp vector started to decrease after 8 days, and became undetectable after 21 days in the non-selective YPDX medium (Fig. 5C). However, there was no decrease in the copy number of the chromosomally integrated plasmids for *S. cerevisiae* 2805-39-40 even after 21 days of growth (over 336 generations) in the same culture condition. These results indicate that the plasmids integrated at the chromosomal $\delta$-sequences were stably maintained during the mitotic growth in the culture. Another strain, *S. cerevisiae* 2805-1 containing a single copy of the *XYL1* gene showed the constant volumetric xylitol productivity of about 0.07 g $l^{-1}$ $h^{-1}$ throughout the cultures (Fig. 4), indicating that the *URA3*-integrated pIDXR was also stably maintained on the chromosome even after 21 days of the culture.

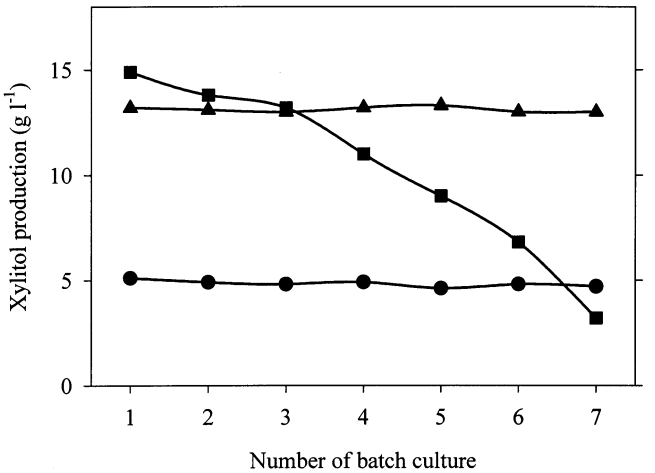


Fig. 4. Xylitol production in each stage of the sequential batch culture with *S. cerevisiae* 2805-1 (●), *S. cerevisiae* 2805-39-40 (▲), and *S. cerevisiae* 2805:pVTXR (■).

### *3.4. Xylitol production and mitotic plasmid stability in the chemostat culture*

The glucose-limited chemostat culture was performed for *S. cerevisiae* 2805-39-40 and *S. cerevisiae* 2805:pVTXR in YPDX medium. Xylitol production of *S. cerevisiae* 2805-39-40 increased nearly linearly for the first 36 h, reaching the maximum production level of about 12 g $l^{-1}$ of xylitol, and then remained at about 8 g $l^{-1}$ for the rest of the culture (Fig. 6A). For *S. cerevisiae* 2805:pVTXR, the maximum level of xylitol production was 16 g $l^{-1}$ of xylitol (Fig. 6B), but the xylitol production started to decrease after 36 h of the chemostat culture, eventually down to 2.0 g $l^{-1}$ after 240 h.

The xylitol production level was in good agreement with the plasmid stability. The number of $Ura^+$ cells harboring the YEp vector started to decrease slowly after 36 h and then rapidly after 72 h of the culture. At the end of the culture (after 240 h), only 4% of cells maintained the $Ura^+$ plasmid (Fig. 6C). However, no decrease in the number of $Ura^+$ cells was observed for *S. cerevisiae* 2805-39-40 even after 240 h of growth in YPDX medium. This result was further confirmed by time-courses analysis of the specific activity of XR of the strains (data not shown).

## 4. Discussion

The production of xylitol from xylose was studied using recombinant *S. cerevisiae* containing multiple *XYL1* genes of *P. stipitis* integrated at the chromosomal $\delta$-sequences both in the sequential batch and chemostat cultures. Through a sequential transformation with two $\delta$-sequence-mediated integrating vectors which have a $neo^r$ marker or an *URA3* marker, transformants containing about 40 copies of the *XYL1* gene were obtained (Fig. 2B).

The multiple tandem integration occurred only at a few sites (1–3 sites) as confirmed by Southern blot analysis (Fig. 2B), even though about 100 copies of $\delta$-sequences are randomly distributed on the chromosome (Cameron et al., 1979; Sakai et al., 1990). A similar observation was reported by Wang et al. (1996). This indicates that the target sites of $\delta$-mediated homologous recombination are selected on a particular region of a chromosome as hot-spots. It seems that the first integration of a plasmid at a $\delta$-sequence may accelerate the next integration event at the same site because more $\delta$-sequences are available around the first target site, making the first integration region as a hot-spot.

Specific XR activity was roughly proportional to the copy number of *XYL1*, but much less to

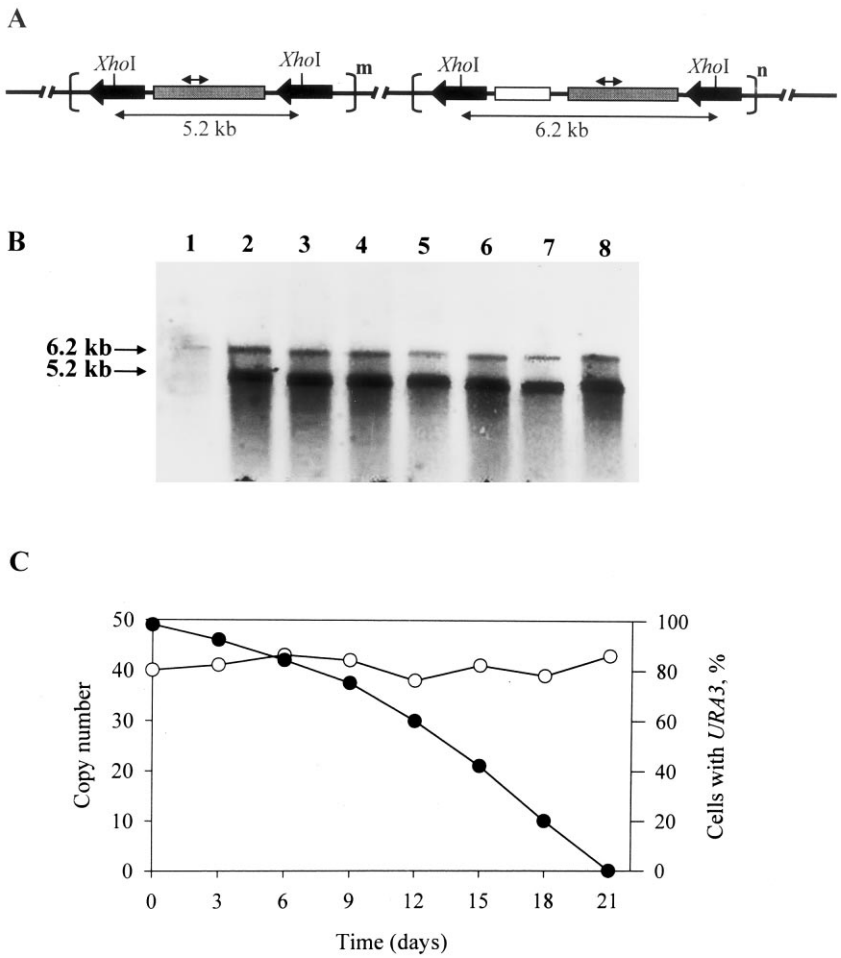


Fig. 5. Mitotic stability analysis of $\delta$-integrating plasmids, pITyXR and pIDXR, and YEp plasmid pVTXR during the sequential batch culture. (A) Schematic diagram of the chromosome with multiple tandem integration of pITyXR and pIDXR. (B) Southern blot analysis of *S. cerevisiae* 2805-39-40 genomic DNA using 1 kb-*Bam*HI fragment of *neo*$^r$ of pIDXR as a probe. Lane 1, *Apa*I-digested genomic DNA from *S. cerevisiae* 2805-1; lanes 2-8, *Xho*I-digested genomic DNA from *S. cerevisiae* 2805-39-40 taken at 3-day intervals for the period of 21 days during the sequential batch culture. (C) Number (○) of $\delta$-integrating plasmids, pITyXR and pIDXR, on the chromosome of *S. cerevisiae* 2805-39-40 and percentage of Ura$^+$ cells (●) of *S. cerevisiae* 2805:pVTXR during the sequential batch culture. Copy number was estimated by quantitative densitometric analysis of the Southern blot result.

the xylitol productivity (Table 1, Fig. 3). Four-fold increase in XR specific activity enhanced only 1.1-fold in xylitol productivity. A similar result was also reported in the fed-batch cultures by Meinander and Hahn-Hägerdal (1997) who reported that a 20-fold increase in XR expression level resulted in a less than 2-fold increase in xylitol productivity. The discrepancy between specific XR activity and xylitol production indicates that the generation of reduced cofactors NAD(P)H or transport of xylose into the cells may be one of the important rate controlling factors for the xylitol production (Hallborn et al., 1994; Meinander et al., 1996; Meinander and Hahn-Hägerdal, 1997).

The chromosomally integrated *XYL1* genes were very stably inherited during the growth in rich medium even after 21 days (over 336 generations) in the sequential batch culture and after 10 days (over 160 generations) in the chemostat cul-

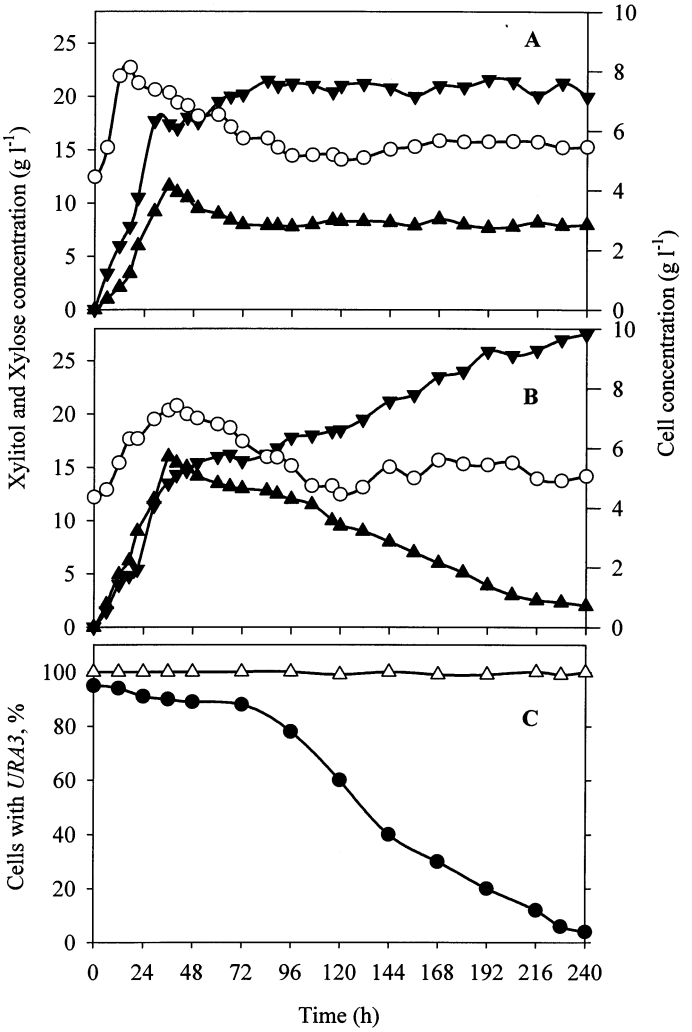


Fig. 6. Xylitol production and plasmid stability by *S. cerevisiae* 2805-39-40 and *S. cerevisiae* 2805:pVTXR during the chemostat culture at the dilution rate of 0.04 $h^{-1}$. YPD medium containing 3% xylose was used for the chemostat feed. (A) and (B) Xylitol production by *S. cerevisiae* 2805-39-40 and *S. cerevisiae* 2805:pVTXR, respectively. ▲, xylitol; ▼, xylose; ○, cell mass. (C) Percentage of $Ura^+$ cells in *S. cerevisiae* 2805-39-40 (△) and *S. cerevisiae* 2805:pVTXR (●) during the chemostat culture.

ture. Mitotic loss of inserted sequences by $\delta-\delta$ recombination or gene conversion events have been reported (Kupiec and Petes, 1988; Wang et al., 1996). However, our result revealed that plasmids integrated at $\delta$-sequences of the chromosome were mitotically stable, as reported by Sakai et al. (1990). In contrast, the YEp vector showed mitotic unstability after 130 generations in the sequential batch culture and after 65 generations in the chemostat culture, respectively. Yeast episomal plasmids (YEp), based upon the naturally occurring yeast 2 $\mu$m DNA, are commonly employed for the introduction of heterologous genes into the yeast. These vectors generally display copy numbers between 50 and 200 (Broach, 1983), but are only stable with selective pressures (Walmsley et al., 1983). However, the conditions used in industrial fermentation, i.e. a long-term culturing of the host cells in poorly defined media without selection pressures, make YEp vectors less suitable for the large-scale production.

In the sequential batch and chemostat culture, the xylitol production was very closely related to the plasmid stability in the cells. The plasmid instability causes population heterogeneity and clonal variability in cells, which may complicate medium optimization (Elliott et al., 1989) and is one of the major obstacles in the application of yeast strains for the production of recombinant proteins and alcohols (Hirata et al., 1992; Romanos et al., 1992).

In this work, we showed that *S. cerevisiae* containing the multiple *XYL1* genes on the chromosome is much more efficient for the xylitol production in the long-term non-selective culture than *S. cerevisiae* harboring the episomal plasmid containing the *XYL1* gene.

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