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Optimization of fed-batch fermentation for xylitol production by *Candida tropicalis*

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Xylitol, a functional sweetener, was produced from xylose by biological conversion using *Candida tropicalis* ATCC 13803. Based on a two-substrate fermentation using glucose for cell growth and xylose for xylitol production, fed-batch fermentations were undertaken to increase the final xylitol concentration. The effects of xylose and xylitol on xylitol production rate were studied to determine the optimum concentrations for fed-batch fermentation. Xylose concentration in the medium (100 g l^{-1}) and less than 200 g l^{-1} total xylose plus xylitol concentration were determined as optimum for maximum xylitol production rate and xylitol yield. Increasing the concentrations of xylose and xylitol decreased the rate and yield of xylitol production and the specific cell growth rate, probably because of an increase in osmotic stress that would interfere with xylose transport, xylitol flux to secretion to cell metabolism. The feeding rate of xylose solution during the fed-batch mode of operation was determined by using the mass balance equations and kinetic parameters involved in the equations in order to increase final xylitol concentration without affecting xylitol and productivity. The optimized fed-batch fermentation resulted in 187 g l^{-1} xylitol concentration, 0.75 g xylitol g $xylose^{-1}$ xylitol yield and 3.9 g xylitol l^{-1} h^{-1} volumetric productivity.
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Introduction

Xylitol, a natural functional sweetener, is increasingly used in the food industry due to a number of advantageous properties. Its sweetening power is as high as sucrose and it promotes oral health and caries prevention [1]. It can be used as a sugar substitute for diabetics and individuals deficient in glucose-6-phosphate dehydrogenase since it does not require insulin and glucose-6-phosphate dehydrogenase for regulation of metabolism [9,18]. Xylitol is currently produced by chemical hydrogenation of xylose with Ni/Al_2O_3 as catalyst. Product cost of the chemical process is high due to difficulty of purification and separation of xylitol, removal of by-products from hemicellulose hydrolyzates, and a low yield of 40–50% based on xylan [3]. Biotechnological processes for xylitol production using natural xylose-fermenting yeasts, which reduce xylose to xylitol by NAD(P)H-dependent xylose reductase (XR), have several advantages such as selective conversion of xylose to xylitol and a high yield. Microorganisms employed for biotechnological production of xylitol were *Candida* sp., including *C. boinidii* [14,15], *C. guilliermondii* [7,10], *C. parapsilopsis* [5,11], *C. peltata* [13] and *C. tropicalis* [2,4]. Recently, a metabolically engineered *Saccharomyces cerevisiae* containing the xylose reductase gene, *XYL1*, was developed to produce xylitol with a yield close to 100% [8,12]. However, the recombinant strains showed relatively lower production rates and volumetric productivity than the wild-type yeasts.

In the xylose metabolism of *C. tropicalis*, xylose is taken up by a specific transferase and reduced to xylitol by XR with NADPH, followed by conversion to xylulose by xylitol dehydrogenase (XDH) with NaD^+. Xylulose is then used for cell growth and NADPH regeneration through the pentose phosphate pathway, after conversion to xylulose-5-phosphate by xylulose kinase with ATP as a cofactor. To obtain a high xylitol yield, the xylose flux to xylulose has to be controlled by supplying just enough oxygen for regeneration of NADPH and cell maintenance. Low oxygen levels also favor xylitol production because of the lower NAD^+/NADH ratio, which leads the XDH-catalyzed reaction to xylitol accumulation by changing the equilibrium constant. To increase xylitol yield and productivity a two-substrate fermentation was designed: a cell growth step using glucose followed by a bioconversion step from xylose to xylitol without cell growth achieved, by controlling the oxygen supply. In particular, an initial glucose concentration was optimized by using kinetic equations related to effects of ethanol, a major by-product of glucose metabolism, on cell growth and xylose conversion in batch cultures [4].

High concentrations of xylitol, as a polyhydroxyl compound, reduce water activity or increase osmotic pressure, which could inhibit cell metabolism and interfere with the membrane transport system. In this work, fed-batch fermentations were undertaken to extend the bioconversion period for xylitol production and hence to increase the final xylitol concentration. In particular, the manner of feeding the xylose solution in the fed-batch period was determined based on the model equations for cell growth and

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xylitol production in an attempt to maintain the optimum xylose concentration at 100 g l^{-1}.

Materials and methods

Microorganism and culture conditions

Candida tropicalis ATCC 13803 was maintained at 4°C on agar plates of YPX containing (g l^{-1}): yeast extract 10 (Difco, Sparks, MD); bactopeptone 20 (Difco), and xylose 50 (Sigma, St. Louis, MO, USA); agar 15 (Difco). The medium composition for inoculation and fermentation was the same as the maintenance medium except for carbohydrate concentrations: 20 g l^{-1} of glucose and 60 g l^{-1} of xylose were added to the preculture medium, and 100 g l^{-1} of xylose and 30 g l^{-1} of glucose were added to the fermentation medium.

The yeast was cultured in a 500-ml flask containing 100 ml of the preculture medium at 30°C, pH 6 and 200 rpm in a shaking incubator (Vision, Incheon, Korea). Fermentation experiments were performed at 30°C, 500 rpm and 1 vvm (K_{La}=1.06 min^{-1}) in a 3.5-l fermentor (KoBiotech, Incheon, Korea) containing 1 l of the fermentation medium. Dissolved oxygen tension (DOT) was maintained at less than 1% for efficient conversion of xylose to xylitol in the fed-batch period. pH was maintained at pH 6 by 2 N NaOH and 2 N HCI. The initial cell density was set at 0.5–1 g l^{-1}.

Analytical methods

Concentrations of xylose, xylitol and glucose were determined by HPLC (TSP, San Jose, CA) using the carbohydrate analysis column (Waters, Milford, MA) with 85% (v/v) acetonitrile as a mobile phase at a flow rate of 2 ml min^{-1}. Carbohydrates were detected using a reflective index detector (TSP). Ethanol was measured by a gas chromatograph (Younglin, Seoul, Korea) using a Carbowax 20M column with N_2 as a carrier gas at a flow rate of 50 ml min^{-1} and a flame ionization detector. Temperatures of injector, detector and column were 200, 200 and 150°C, respectively. Cell mass was estimated using the relationship between dry cell weight and optical density (OD) measured at 600 nm. One OD unit was equivalent to 0.227 g dry cell weight l^{-1}.

Specific rates of xylose consumption and xylitol production were defined as differences in xylose and xylitol concentrations divided by the average cell mass and the time interval between the two samples of interest, respectively.

Results and discussion

Effects of concentrations of xylose on xylitol production

Effects of concentrations of xylose and xylitol on xylitol production were studied to determine an optimum concentration of xylose for fed-batch fermentations. First, two-substrate batch fermentations were undertaken for different initial xylose concentrations. Xylitol yield and specific values, which are summarized in Table 1, were obtained during the bioconversion phase from xylose to xylitol. Cell densities in each experiment were almost the same because growth conditions and glucose concentration were identical. Since the specific growth rates during the bioconversion phase were less than 0.05 h^{-1}, xylose was used primarily for xylitol production and cell maintenance. Specific rates of xylose consumption and xylitol production were obtained from an early stage of the bioconversion phase where over 70% of the xylose initially added remained. A xylose concentration of 100 g l^{-1} optimal with respect to xylitol yield and production rate (Table 1) and a profile of a run starting with 100 g l^{-1} xylose is shown in Figure 1. Specific growth rates were reduced with increasing xylose concentrations, suggesting that an increase in osmotic pressure across the cell membrane by xylose in the medium might reduce the specific growth rates. For xylitol production, an optimum xylose concentration existed because high xylose concentrations help xylose uptake and consequently accumulation of xylitol in the cell to force the xylose reductase reaction toward xylose conversion to xylitol [6]. Above the optimum xylose concentration, the overall cell metabolism seemed to be inhibited by high xylose concentrations.

Table 1 Effects of xylose concentrations on xylitol production by *C. tropicalis* at 30°C and pH 6

Parameter	Xylose concentration (g l^{-1})			
	50	100	200	300
Specific growth rate (h^{-1})				
Growth phase	0.43	0.32	0.32	0.12
Xylitol production phase	0.047	0.035	0.016	0.003
Xylitol yield (g xylitol g $xylose^{-1}$)	0.59	0.81	0.77	0.46
Specific xylose consumption rate (g xylose g $cell^{-1}$ h^{-1})	0.34	0.51	0.37	0.13
Specific xylitol production rate (g xylitol g $cell^{-1}$ h^{-1})	0.19	0.41	0.27	0.05

A quantitative relationship between xylitol production and xylitol concentration was characterized in the fed-batch mode of operation to provide a ground for optimization of fed-batch fermentations. Xylitol, a polyhydroxyl compound, could inhibit xylitol production by increasing the osmotic pressure of the growth medium. Xylose concentration in the medium was maintained at around 100 g l^{-1} by controlled feeding of concentrated (800 g l^{-1}) xylose solution. The optimum xylose concentration for xylitol production was determined from earlier experiments. The specific xylose consumption rate (open triangle) and the specific xylitol production rate (closed diamond) decreased in a stepwise manner with respect to an increase in xylitol concentrations. As shown in Figure 2, a specific xylose consumption rate of 0.72 g xylose g $cell^{-1}$ h^{-1} and a specific xylitol production rate of 0.61 g xylitol g $cell^{-1}$ h^{-1} were obtained for up to 110 g l^{-1} xylitol. But increasing the xylitol concentration by the continuous conversion of xylose for 58 h fermentation time reduced the specific values to 0.38 g xylose g $cell^{-1}$ h^{-1} specific xylose consumption rate and 0.27 g xylitol g $cell^{-1}$ h^{-1} specific xylitol production rate. Despite the decline of xylitol production rates, xylitol yield (open circles and dashed line) in this period was not changed significantly, indicating that the increase of xylitol in the medium might block secretion of xylitol rather than the conversion of xylose to xylitol. Such xylitol-mediated inhibition of xylose conversion was also observed in batch cultures (data not shown). Xylitol, like other metabolites such as alcohol or organic acid, is secreted by diffusion or passive transport using the concentration gradient across the membrane. Therefore, an intracellular xylitol concentration higher than the concentration in the medium must be maintained for continuous production of xylitol from xylose. It was reported for *S. cerevisiae* that high osmotic pressure caused by high xylitol concentration in the medium inhibited glucose phosphotranferase,

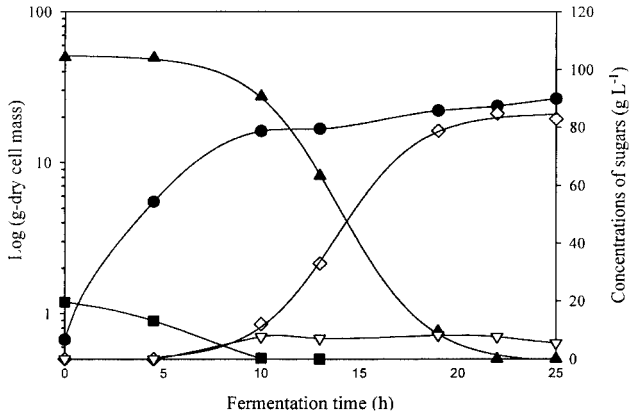

Figure 1 Profiles of two-substrate batch fermentation at 30°C and pH 6. ● Log (g dry cell mass); ▲ xylose (g l^{-1}); ◇ xylitol (g l^{-1}); ■ glucose (g l^{-1}); ▽ethanol (g l^{-1}).

the membrane protein for glucose transport [16]. Also, the xylose transport system might be inhibited by high osmotic pressure through changes in membrane protein or membrane fluidity. Addition of nystatin, a membrane-poring agent, enhanced the xylitol production rate and cell growth by helping secretion of intracellular xylitol to the medium [17].

The xylitol yield was reduced considerably in the latter period of the fermentation (Figure 2); 15 g l^{-1} xylitol was produced for 28.5 h, compared to the first high xylitol production period, 94.5 g l^{-1} of xylitol for 15.5 h. During the latter period, the intracellular xylitol flux may have been driven to further cell metabolism instead of secretion to the medium by the high xylitol concentration in the medium and the requirement of maintenance energy to overcome the high osmotic stress.

Optimization of fed-batch fermentation

A fed-batch fermentation was optimized to increase the final xylitol concentration. The conditions were established after taking into account the experimental results obtained previously.

First, during the xylose bioconversion phase, xylose concentration (S_2) in the medium was controlled at 100 g l^{-1}. Second, the specific growth rate during the xylose conversion phase (μ) is maintained at less than 0.02 h^{-1} by controlling the oxygen supply rate. The feeding rate of xylose solution was estimated from Eq. (1), which could be derived from the mass balance equations for fed-batch fermentations

$$F = \frac{q_{s2}}{(S_f - S_2)} V \cdot X = \frac{q_{s2}}{(S_f - S_2)} V_0 \cdot S_0 \cdot \exp(\mu \cdot t) \qquad (1)$$

where F is the flow rate of xylose solution, q_{s2} is the specific xylose consumption rate, S_f is the xylose concentration of the feeding solution, S_2 is the xylose concentration in the fermentation medium, V is the volume of medium, X is the dry cell mass concentration, and t is the fermentation time. F indicates the supply rate of xylose for complete conversion to xylitol with maximum rate and yield without accumulation of xylose in the medium. F was simply the function of fermentation time because S_f, V_0, S_0, S_2, q_{s2} and μ were constant. The flow rate was adjusted in the feedback control mode by using q_{s2} calculated in each sampling term to maintain the xylose concentration more accurately. Figure 3 shows the experimental results of the optimized fed-batch fermentation

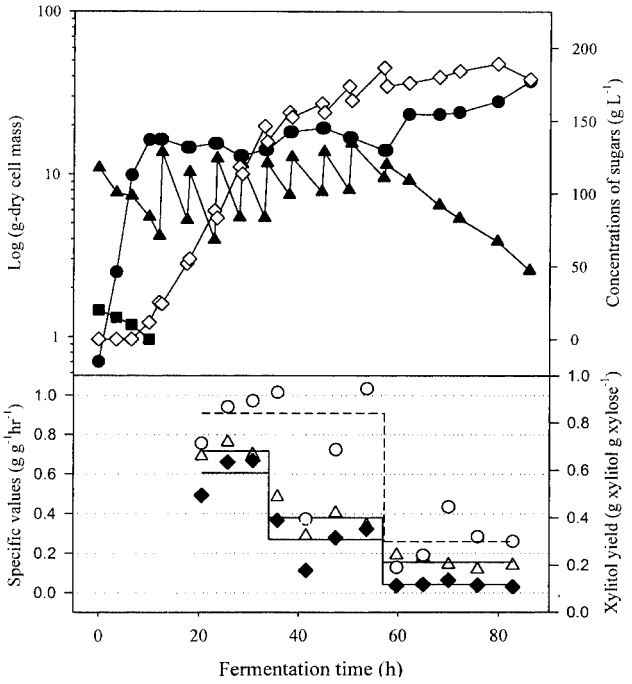

Figure 2 Effects of xylitol concentration on xylitol production in fed-batch fermentation at 30°C and pH 6. ● Log (g dry cell mass); ▲ xylose (g l^{-1}); ◇ xylitol (g l^{-1}); ■ glucose (g l^{-1}); △ specific xylose consumption rate (g xylose g cell^{-1} h^{-1}); ◆ specific xylitol production rate (g xylitol g cell^{-1} h^{-1}); ○ xylitol yield (g xylitol g xylose^{-1}).

and the xylose feeding rate as a function of fermentation time. The fermentation was run batchwise up to 14 h to achieve a high cell mass concentration. The fed-batch mode of operation was initiated by feeding the xylose solution when glucose was completely exhausted. During the fed-batch period, the specific growth rate

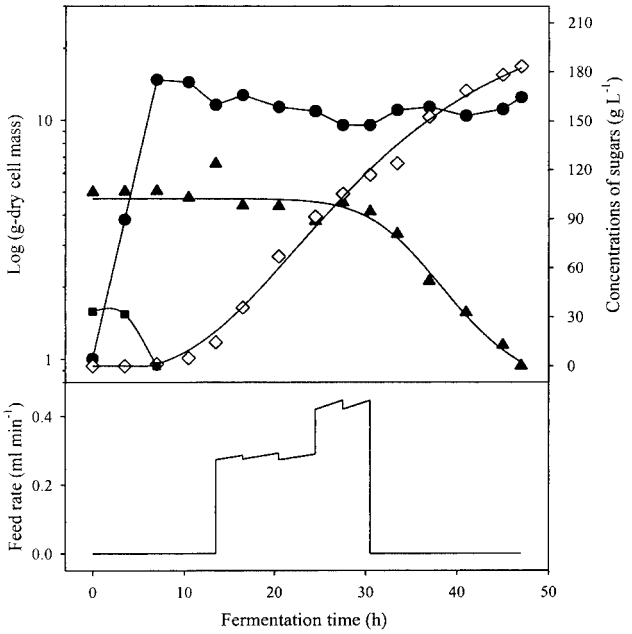

Figure 3 Results of optimized fed-batch fermentation for xylitol production. ● Log (g dry cell mass); ▲ xylose (g l^{-1}); ◇ xylitol (g l^{-1}); ■ glucose (g l^{-1}); — feeding rate of xylose solution (ml/min).

was controlled at less than 0.01 h^{-1} and the xylose concentration in the medium was maintained at 100 g l^{-1}. Microaerobic conditions (DOT 1% or below) were maintained for efficient production of xylitol from xylose. The feeding of xylose solution was stopped when 119 g l^{-1} of xylitol was produced. The fermentation continued to consume xylose present in the medium. To conclude, the xylitol yield of 0.75 g xylitol g $xylose^{-1}$ and the volumetric productivity of 3.9 g xylitol l^{-1} h^{-1} were obtained with a final xylitol concentration of 187 g l^{-1}. Vandeska *et al* [14] studied xylitol production in a fed-batch fermentation of *C. boidinii* and reported a xylitol yield of 0.68 g xylitol g $xylose^{-1}$, volumetric productivity of 0.46 g xylitol l^{-1} h^{-1} and a final xylitol concentration of 59.3 g l^{-1}. The experimental data obtained in this study were much improved by controlling the concentrations of glucose and xylose in the xylose conversion period. Such a remarkable improvement in fermentation performance was due mainly to quantitative analysis of the effects of environmental factors on xylitol production and implementation of the experimental results in an optimized fed-batch fermentation process. Research is in progress to make metabolic flux analysis of xylose metabolism at a molecular level in an effort to determine a critical step in xylitol production.

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