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Analysis and optimization of a two-substrate fermentation for xylitol production using *Candida tropicalis*

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Xylitol, a functional sweetener, was produced from xylose using *Candida tropicalis* ATCC 13803. A two-substrate fermentation was designed in order to increase xylitol yield and volumetric productivity. Glucose was used initially for cell growth followed by conversion of xylose to xylitol without cell growth and by-product formation after complete depletion of glucose. High glucose concentrations increased volumetric productivity by reducing conversion time due to high cell mass, but also led to production of ethanol, which, in turn, inhibited cell growth and xylitol production. Computer simulation was undertaken to optimize an initial glucose concentration using kinetic equations describing rates of cell growth and xylose bioconversion as a function of ethanol concentration. Kinetic constants involved in the equations were estimated from the experimental results. Glucose at 32 g L^{-1} was estimated to be an optimum initial glucose concentration with a final xylose concentration of 86 g L^{-1} and a volumetric productivity of 5.15 g-xylitol L^{-1} h^{-1}. The two-substrate fermentation was performed under optimum conditions to verify the computer simulation results. The experimental results were in good agreement with the predicted values of simulation with a xylitol yield of 0.81 g-xylitol g-$xylose^{-1}$ and a volumetric productivity of 5.06 g-xylitol L^{-1} h^{-1}.

Keywords: xylose; xylitol; *Candida tropicalis*; two-substrate fermentation; optimization

Introduction

Xylitol is a naturally occurring functional sweetener. The sugar alcohol is increasingly used in the food industry due to a number of advantageous properties. It has sweetening power as high as sucrose and promotes oral health and caries prevention [3]. It can be used as a sugar substitute by diabetics and glucose-6-phosphate dehydrogenase-deficient individuals since it does not require insulin and glucose-6-phosphate dehydrogenase for regulation of metabolism [13,19]. Xylitol is currently produced by chemical hydrogenation of xylose in hemicellulose hydrolyzates using Ni/Al_2O_3 as a catalyst. The product cost is high due to difficulties of purification and separation of xylitol, removal of by-products from hemicellulose hydrolyzates and a low yield of 40–50% based on xylan [6]. Biotechnological processes for xylitol production using natural xylose-fermenting yeasts, which reduce xylose to xylitol by the NAD(P)H-dependent xylose reductase (XR) have several advantages such as selective conversion of xylose to xylitol with high yield. Microorganisms employed for biotechnological production of xylitol include bacteria [7,20,21], fungi [2] and yeasts, especially, *Pachysolen tannophilus* [15], *Candida* sp such as *C. pelliculosa* [22], *C. boinidii* [14], *C. guilliermondii* [9], *C. parapsilosis* [5,16] and *C. tropicalis* [12,18]. Recently, a metabolically engineered *Saccharomyces cerevisiae* containing the xylose reductase gene, *XYL1*, was developed to produce xylitol with a very high yield close to 100% [17], but it showed a relatively lower production rate and volumetric productivity than the wild-type yeasts.

In the xylose metabolism of *Candida tropicalis*, xylose was taken up by a specific transferase and reduced to xylitol by xylose reductase (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 an oxygen supply sufficient for regeneration of NADPH and cell maintenance. Low oxygen levels also favor xylitol production because they decrease the NAD^+/NADH ratio, which favours the xylitol dehydrogenase-catalyzed reaction to xylitol accumulation by changing the equilibrium constant. In the present investigation, a two-substrate fermentation was designed to increase xylitol yield and productivity: a cell growth step using glucose followed by a bioconversion step from xylose to xylitol without cell growth by controlling the oxygen supply. Initial glucose concentrations were optimized for the two-substrate fermentation. Computer simulation was undertaken to determine an optimum initial glucose concentration utilizing kinetic equations relating ethanol concentrations to cell growth and xylose production. The optimized two-substrate fermentation obtained from computer simulation was verified experimentally.

Materials and methods

Microorganism and culture conditions

C. tropicalis ATCC 13803 was maintained at 4°C on a YPX agar plate containing (per L): 10 g yeast extract, 20 g bactopeptone, 20 g xylose and 15 g agar. The medium for

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inoculation and fermentation was the same as the maintenance medium except for carbohydrate concentrations: 20 g L^{-1} glucose and 60 g L^{-1} xylose were added to the preculture medium and 100 g L^{-1} xylose and various concentrations of glucose were added to the fermentation medium.

The yeast was cultured in 100 ml of the preculture medium at 30°C, pH 6 and 200 rpm in a shaking incubator (Vision, Seoul, Korea). Fermentations were performed at 30°C, 500 rpm and 1 vvm ($K_{La} = 1.06$ min^{-1}) in a 3.5-L fermentor (Korean Fermentation Corp, Korea) containing 1 L of the fermentation medium. The medium was maintained at pH 6 by 2 N NaOH and 2 N HCl. Initial cell density was set at 0.5–1 g L^{-1} [8].

Analytical methods

Xylose, xylitol and glucose were determined by HPLC (Knauer, Berlin, Germany) using the Carbohydrate Analysis (Waters, Milford, MA, USA) column with 85% (v/v) acetonitrile as mobile phase at a flow rate of 2 ml min^{-1}. Carbohydrates were measured by using a reflective index detector (Knauer). To monitor fermentation by-products an Aminex HPX-87C and an Aminex HPX 87H (BioRad, Hercules, CA, USA) columns were also used. Xylitol concentrations below 1 g L^{-1} were determined by using the d-sorbitol/xylitol kit (Boehringer Mannheim, Mannheim, Germany). Ethanol was measured by gas chromatography (Younglin, Seoul, Korea) using a 2HWP/10PEG20M column with N_2 as a carrier gas at a flow rate of 50 ml min^{-1} and a flame ionized detector. Temperatures of injector, detector and column were 200°C, 200°C and 150°C, respectively. Cell mass was estimated by 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 xylose consumption rate and specific xylitol production rate were defined as differences in xylose and xylitol concentrations divided by average cell mass and the time interval between the two samples of interest, respectively.

Results and discussion

Two-substrate batch culture

Efficient production of xylitol from xylose requires continuous regeneration of NADPH, a cofactor of xylose reductase. Under aerobic conditions NADPH is normally produced by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway, by isocitrate dehydrogenase in the TCA cycle and transhydrogenase, the exchanger of H^+ from NADH to $NADP^+$ in the cytosol [1,4]. All these pathways are related to energy generation for cell growth and redox balance. The best way to increase xylitol yield would be simultaneous transport of a co-substrate with xylose into the cell. The co-substrate is used primarily for NADPH regeneration while xylose is converted to xylitol without being metabolized further. To make such a scheme possible, xylose and co-substrate enter the cell at the same time without inhibition of the required transferases and do not inhibit enzymes involved in co-substrate metabolism and xylose conversion. Alcohols, hexoses or pentoses are not suitable as co-substrate owing to the inhibition of cell metabolism and high cost. Glucose, a good candidate for a co-substrate, blocks xylose transport and represses XR activity. Therefore a certain amount of xylose must be used for NADPH regeneration, which decreases xylitol yield. To increase xylitol yield, the xylose flux to cell mass has to be minimized, but provides sufficient maintenance energy and NADPH regeneration by controlling the oxygen supply rate. Microaerobic conditions might keep the NAD^+/NADH ratio and ATP levels low. Xylose flux to cell growth is restricted by the lack of cofactors necessary for xylitol dehydrogenase and xylulose kinase.

Glucose was chosen as substrate for cell growth to obtain high volumetric productivity. Since volumetric productivity is proportional to cell mass, it is necessary to increase cell mass by using glucose as an energy source. A two-substrate fermentation is established in such a way that glucose is used for cell growth and xylose is converted to xylitol with high yield.

A number of two-substrate fermentations using *C. tropicalis* were performed to see the pattern of production and utilization of by-products. Major by-products of xylose and glucose metabolism included ethanol, acetic acid and glycerol which were usually utilized again as substrates for cell growth. As shown in Figure 1, only ethanol was produced from glucose without formation of glycerol and acetic acid and was not consumed during the xylose bioconversion phase. Since the xylose flux to glycolysis and oxygen were limited, no ethanol was produced from xylose by the Crabtree effect during the xylose bioconversion phase, which is beneficial to a high xylitol yield. After complete depletion of glucose, xylose was converted to xylitol with an 81% yield.

Effects of ethanol on cell growth

Ethanol, a major by-product of glucose metabolism, inhibits cell growth and product formation. Cell growth using glucose was inhibited with increasing ethanol concentrations in the YP medium (Figure 2). The agitation rate was controlled to maintain the dissolved oxygen tension (DOT) above 20% saturation during the cell growth phase. The experimental data illustrated in Figure 2 were fitted by the Luong equation describing the relationship between ethanol concentration and specific growth rate. The three parameters involved in the Luong equation were estimated by a non-linear regression analysis:

$$\frac{1}{X_1}\frac{dX_1}{dt} = \mu_{1max}\left(1 - \left(\frac{P_1}{P_{1m}}\right)^{a}\right) \quad (1)$$

where X_1 and P_1 mean cell mass increased using glucose and ethanol concentration. The maximum specific growth rate using glucose, μ_{1max}, was estimated to be 0.56 h^{-1}. The critical ethanol concentration above which cells cannot grow (P_{1m}) and the dimensionless constant (α) were calculated as 24.4 g-ethanol L^{-1} and 0.271, respectively.

Ethanol also influenced metabolic activity and product formation. The experimental data of specific glucose consumption rate (q_{S1}) and specific ethanol production rate (q_{P1}) are presented as functions of ethanol concentration in Figure 3. The lines were drawn by a non-linear regression

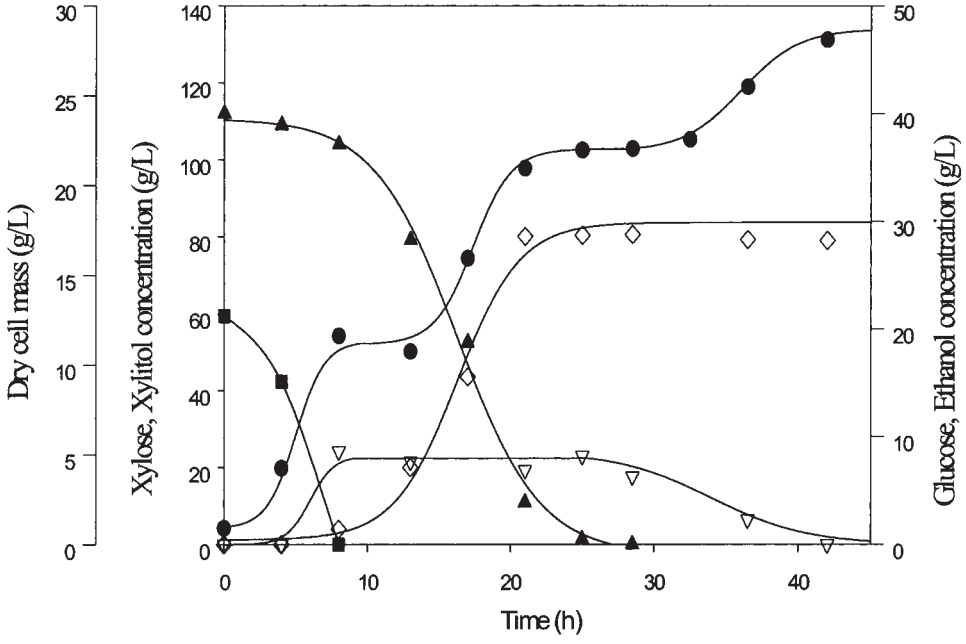

Figure 1 A two-substrate batch culture at 30°C and pH 6 with 20 g L^{-1} glucose and 100 g L^{-1} xylose. ● Dry cell mass (g L^{-1}); ■ glucose (g L^{-1}); ◇ xylitol (g L^{-1}); ▲ xylose (g L^{-1}); ▽ ethanol (g L^{-1}).

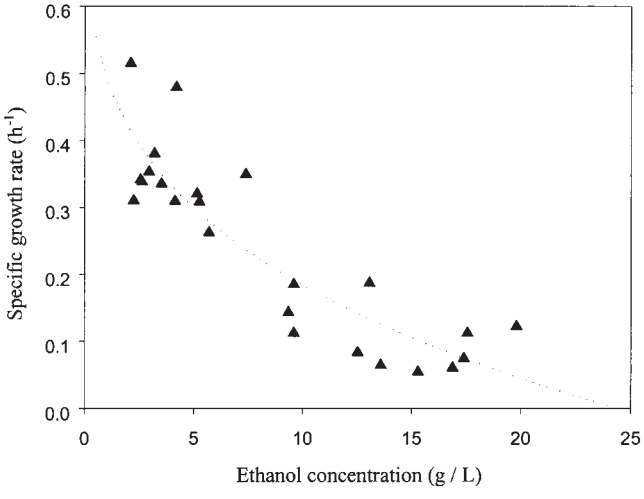

Figure 2 Effects of ethanol concentration on specific growth rate during the cell growth phase in the two-substrate fermentation. ▲ Specific growth rate (h^{-1}); · · · · regression fit of the Luong equation.

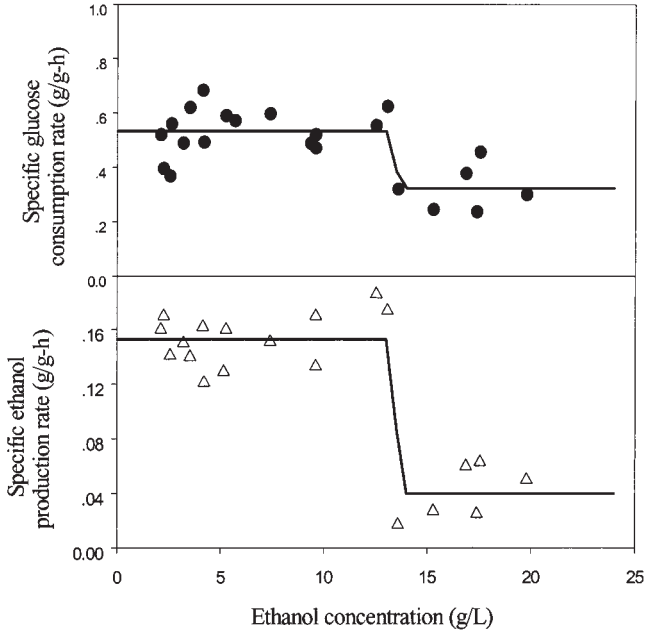

Figure 3 Effects of ethanol on glucose utilization and ethanol production during cell growth phase in the two-substrate fermentation. ● Specific glucose consumption rate (g-glucose g-$cell^{-1}$ h^{-1}); △ Specific ethanol production rate (g-ethanol g-$cell^{-1}$ h^{-1}); ———— regression fit of the logistic equations.

of the logistic equation. The specific glucose consumption rate changed from 0.53 g-glucose g-$cell^{-1}$ h^{-1} to 0.32 g-glucose g-$cell^{-1}$ h^{-1} and the specific ethanol production rate from 0.15 g-ethanol g-$cell^{-1}$ h^{-1} to 0.04 g-ethanol g-$cell^{-1}$ h^{-1}. Interestingly, both of the inflection points were the same at an ethanol concentration of 13.5 g L^{-1}. As both specific values were almost constant but changed considerably at the inflection point, the kinetic equations could be simplified as follows:

$$\frac{1}{X_1}\frac{dS_1}{dt} = -q_{S1} = 0.31 + 0.22\ U\ (P_1 - 13.5) \qquad (2)$$

(g-glucose g-$cell^{-1}$ h^{-1})

$$\frac{1}{X_1}\frac{dP_1}{dt} = q_{P1} = 0.04{+}0.11\ U\ (P_1 - 13.5) \qquad (3)$$

(g-ethanol g-$cell^{-1}$ h^{-1})

where P_1 is ethanol concentration, S_1 is glucose concentration and U ( ) is the Heaviside step function [10].

Table 1 Effect of initial glucose concentration on xylitol production by *Candida tropicalis* ATCC 13803 at 30°C and pH 6

Glucose (g L^{-1})	0		20	40	60	100
	Overall fermentation	Xylitol production phase				
Ethanol (g L^{-1})	0	0	6.7	12.5	18.4	27.0
Final cell mass (g L^{-1})	26	11	28	23	22	39
Specific xylose consumption rate (g xylose g $cell^{-1}$ h^{-1})	0.57	0.57	0.51	0.33	0.24	0.19
Specific xylitol production rate (g xylitol g $cell^{-1}$ h^{-1})	0.3	0.39	0.41	0.25	0.14	0.12
Specific growth rate in xylose conversion phase (h^{-1})	–	0.01	0.03	0.01	0.01	0.02
Xylitol yield (g xylitol g $xylose^{-1}$)	0.58	0.62	0.81	0.72	0.63	0.39

Effects of ethanol on xylose bioconversion

Ethanol produced during the cell growth phase affected the conversion of xylose to xylitol. Ethanol concentrations, however, did not influence the specific growth rate due to restriction of oxygen supply (Table 1). The DOT was maintained below 10% saturation for a high xylitol yield. The specific growth rates during the xylose conversion phase were controlled around 0.02 h^{-1} in all experiments and hence could be considered as a constant:

$$\frac{1}{X_2}\frac{dX_2}{dt} = \mu = 0.02\ \mathrm{h}^{-1} \tag{4}$$

where X_2 is cell mass during xylose conversion phase.

As ethanol was a sole product from glucose metabolism and not utilized during the xylose conversion phase, an initial glucose concentration was selected as a control variable in studying the influence of ethanol concentration without extra addition to the medium. Since the rates of specific xylose consumption and specific xylitol production during the xylose bioconversion phase were not varied significantly, the average values were used for a non-linear regression analysis. The deviation of average experimental values was less than 5%. Xylose metabolism was greatly inhibited with increasing ethanol concentrations (Figure 4).

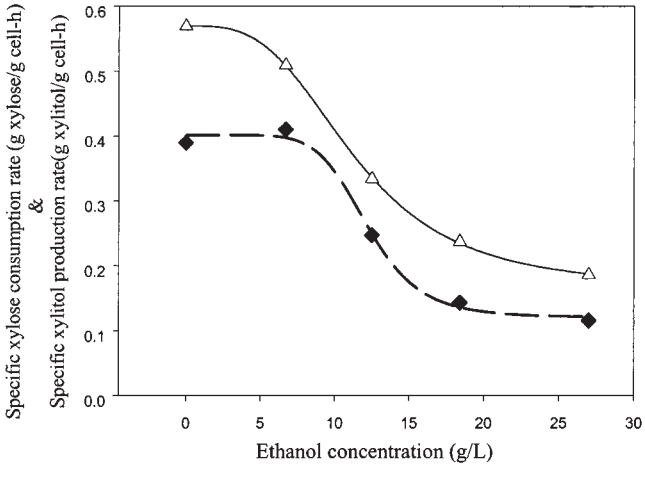

Figure 4 Effects of ethanol on xylose bioconversion phase in the two-substrate fermentation. △ Specific xylose consumption rate (g-xylose g-$cell^{-1}$ h^{-1}); ◆ Specific xylitol production rate (g xylitol g $cell^{-1}$ h^{-1}).

The effects of specific xylose consumption rate (q_{S2}) were fitted by the logistic equation (5) within the experimental ranges. A change in specific xylitol production rate (q_{P2}) was similar to that of the specific xylose consumption rate. In the experiment without glucose (and consequently no ethanol in the medium), xylose was used for cell growth till dissolved oxygen reached microaerobic conditions due to high cell mass. Therefore the specific xylitol production rate had two distinct values. One was an average value over the fermentation period and the other was a real value of xylose bioconversion without being affected by ethanol. Using the real value, the relationship between ethanol concentration and the specific xylitol production rate could be described by the logistic equation (6) as shown in Figure 4.

$$\frac{1}{X_2}\frac{dS_2}{dt} = -q_{S2} = \frac{a}{1 + \left(\frac{P_1}{(P_1)_{C1}}\right)^b} + Y_{S2}$$

$$a = 0.41\ (\text{g-xylose g-cell}^{-1}\ \text{h}^{-1})$$

$$b = 3.25 \tag{5}$$

$$(P_1)_{C1} = 11.36\ (\text{g-ethanol L}^{-1})$$

$$Y_{S1} = 0.16\ (\text{g-xylose g-cell}^{-1}\ \text{h}^{-1})$$

$$\frac{1}{X_2}\frac{dP_2}{dt} = q_{P2} = \frac{a}{1 + \left(\frac{P_1}{(P_1)_{C2}}\right)^b} + Y_{P2}$$

$$a = 0.28\ (\text{g-xylose g-cell}^{-1}\ \text{h}^{-1})$$

$$b = 6.98 \tag{6}$$

$$(P_1)_{C2} = 12.19\ (\text{g-ethanol L}^{-1})$$

$$Y_{P2} = 0.12\ (\text{g-xylitol g-cell}^{-1}\ \text{h}^{-1})$$

Xylitol yield was not significantly affected by ethanol concentrations. The data are summarized in Table 1. The experimental evidence of the similarity of the two logistic equations suggested that ethanol did not inhibit certain enzymes involved in xylitol production, rather it inhibited the rate of overall cell metabolism, at least the carbon metabolism, allosterically.

Optimization of an initial glucose concentration

Computer simulation was performed to optimize an initial glucose concentration for maximum volumetric productivity (Q_p) using the kinetic equations obtained experimentally. Volumetric productivity was simply defined as final xylitol concentration (P_{2f}) divided by total fermentation time (t_{total}), which was the sum of cell growth time (t_1) and xylose bioconversion time (t_2).

$$Q_p = \frac{P_{2f}}{t_{total}} = \frac{P_{2f}}{t_1 + t_2} \tag{7}$$

The switching time between the two phases and the lag period were ignored. In the cell growth phase, a specific growth rate was considered as a function of ethanol concentration since glucose was not a limiting substrate above 5 g L^{-1} of glucose and 100 g L^{-1} of xylose in the medium. For glucose concentrations (S_1) ranging from 5 g L^{-1} to 100 g L^{-1}, a numerical analysis of the balance equations (1) to (6) was done by the 5th Runge-Kutta method [11] to calculate volumetric productivity with the following initial conditions that X_i (initial cell mass) = 1.0 g L^{-1}, S_{2i} (initial xylose concentration) = 100 g L^{-1}, P_{1i} (ethanol) = 0 g L^{-1} and P_{2i} (xylitol) = 0 g L^{-1}. Cell mass and ethanol concentration in the cell growth phase were illustrated in Figure 5a. Figure 5b depicts the results of the xylose bioconversion phase. Cell mass during xylose bioconversion was calculated to be less than 13 g $L^{-1}$of cell. A maximum final xylitol concentration of 87.0 g L^{-1} was obtained at an initial glucose concentration of 34 g L^{-1}. The shortest total fermentation time was achieved at 23 g L^{-1} of glucose concentration with an overall fermentation time of 16.2 h. The optimum initial glucose concentration to maximize volumetric productivity was estimated to be 32 g L^{-1} with a final xylitol concentration of 86 g L^{-1} (a xylitol yield of 0.86 g-xylitol g-xylose^{-1}), a total fermentation time of 16.8 h and volumetric productivity of 5.15 g-xylitol L^{-1} h^{-1}.

Simulation results were experimentally verified in the two-substrate fermentation under optimum conditions. As shown in Figure 6, the xylose bioconversion phase was clearly separated from the cell growth phase. After depletion of glucose, xylose was converted to xylitol and ethanol produced from glucose was not utilized during xylose metabolism. A xylitol yield of 0.81 g-xylitol g-xylose^{-1} and a volumetric productivity of 5.06 g-xylitol L^{-1} h^{-1} were obtained, which was in good agreement with the predicted values of simulation.

Conclusion

Xylitol is a value-added material produced from xylose by hydrogenation. This study was undertaken to produce xylitol by biological hydrogenation of xylose with high yield and productivity. A two-substrate fermentation was designed to improve both xylitol yield and volumetric productivity. Xylitol was produced in a growth-associated manner since it was an intermediate of the major carbon metabolic pathway for cell growth. But control of oxygen supply in the two-substrate fermentation changed the product formation pattern. Xylitol was produced from xylose in a nongrowth-associated manner using cell mass as catalyst without cell growth or by-product formation. A number

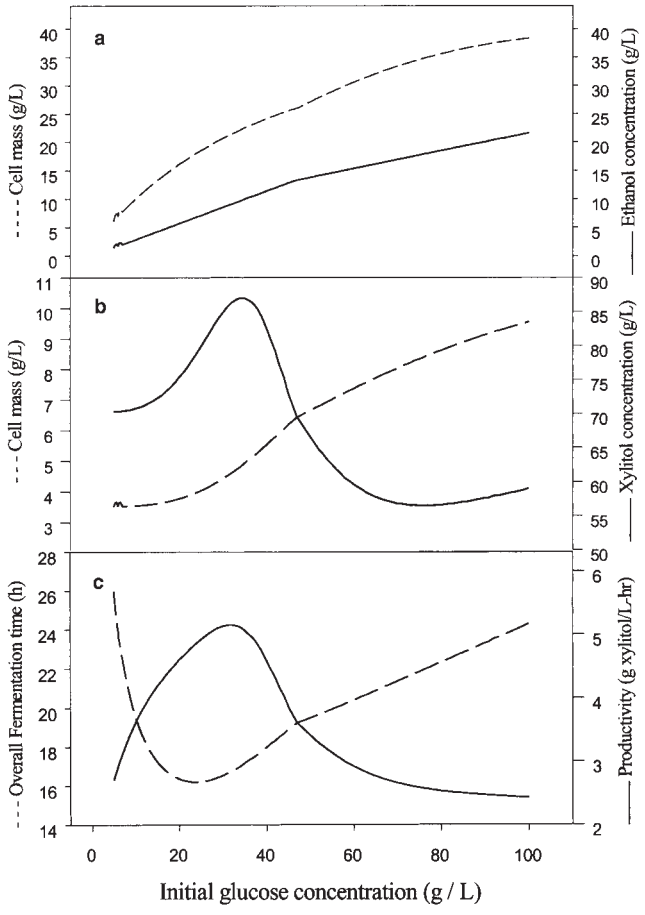

Figure 5 Computer simulation for determination of optimum glucose concentration. (a) Cell growth phase: · · · · cell mass (g L^{-1}); ———— ethanol (g L^{-1}). (b) Xylose bioconversion phase: ———— xylitol (g L^{-1}); · · · · cell growth (g L^{-1}). (c) Overall results: ———— volumetric productivity (g xylitol L^{-1} h^{-1}); · · · · overall fermentation time (h).

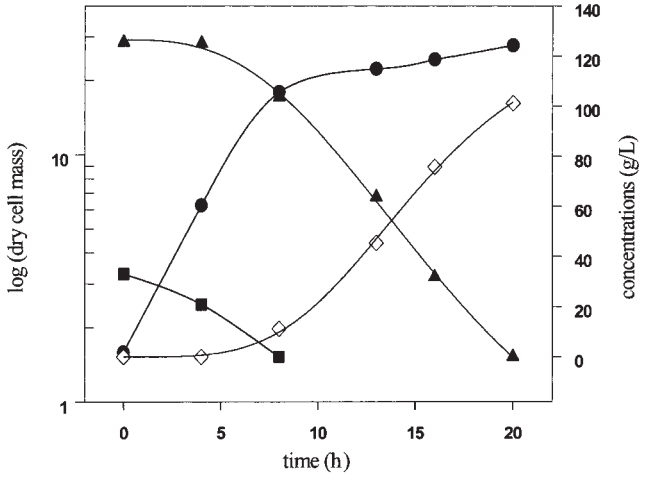

Figure 6 Results of the two-substrate batch culture under optimized conditions. ● Cell mass (g L^{-1}); ■ glucose (g L^{-1}); ▲ xylose (g L^{-1}); ◇ xylitol (g L^{-1}); ▽ ethanol (g L^{-1}).

of batch fermentations were done to analyze the effects of ethanol, a by-product of the cell growth phase using glucose, on cell growth and xylose bioconversion. An initial glucose concentration was optimized by computer simulation in order to maximize volumetric productivity. The experiments performed to verify the computer simulation results showed good agreement with the estimated values which were 1.4 times higher in xylitol yield and 1.85 times higher in volumetric productivity compared with those of the experiments done without glucose under the same conditions. A yield of 0.81 g-xylitol g-xylose^{-1} is equivalent to 90% of the theoretical xylitol yield from xylose.

Acknowledgements

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