J Biochem Tech (2009) 2(1):126-132
ISSN: 0974-2328

Xylitol production from lactose by biotransformation

Tomoyuki Toyoda and Kazuhisa Ohtaguchi*

Received: 28 November 2009 / Received in revised form: 3 February 2010, Accepted: 13 February 2010, Published online: 11 March 2010,

Abstract

Xylitol production from lactose was investigated by a three step biotransformations series that were: (1) breakdown of lactose to D-arabitol by *Kluyveromyces lactis* NBRC 1903, (2) oxidation of D-arabitol to D-xylulose by *Gluconobacter oxydans* NBRC 3172, and (3) reduction of D-xylulose to xylitol by *Candida shehatae* IAM 12953 in the presence of a high concentration ethanol or by cell free sorbitol dehydrogenase. Overall production rate and overall fractional yield of xylitol from lactose were 1.69 mmol L^{-1} h^{-1} and 0.141, respectively, when sorbitol dehydrogenase was utilized in the third reaction. The results of xylitol production from lactose were compared with the processes of xylitol production from D-glucose.

Keywords: Xylitol, Lactose, *Kluyveromyces lactis*, Biotransformation

Introduction

The sweetner xylitol ((*2S*,*3R*,*4R*)-Pentane-1,2,3,4,5-pentaol) is currently manufactured from D-xylose by hydrogenation on a nickel catalyst at high temperature and high pressure (Prakasham et al. 2009). The raw material D-xylose is produced by expensive laborious refining treatments for the hydrolyte of xylan in hemicellulosic materials. The annual world market for xylitol, which is priced at $4–5 per kg and used mainly in clinical and food applications, is currently $340 million (Kadam et al. 2008). Such industrial xylitol production possesses a problem of extremely low yield of xylitol on raw materials. The yield of xylitol on ligunocellulosic material is only about 10%. Creating alternative processes has been done using the available hexose D-glucose that is abundant in natural products such as amylose, amylopectin and cellulosic materials. Production of xylitol from D-glucose was first reported utilizing three biotransformations-in-series (Ohnishi and Suzuki 1969). The substrate D-glucose was first converted to D-arabitol utilizing osmophilic yeasts (Spencer and Sallans 1956) such as *Candida parapsilosis* FERM P-18006 (Utsuka et al. 2002), *Zygosaccharomyces rouxii* NRRL Y-27624 (Saha et al. 2007) or *Metschnikowia reukaufii* AJ14587 (Nozaki et al. 2003), oxidized to D-xylulose utilizing acetic acid bacteria *Gluconobacter suboxydans* (Ohmomo et al. 1983) or *Acetobacter aceti* (Ahmed and Bhowmik 2000), and then reduced to xylitol either utilizing microbial reactions (Ueng et al. 1981; Yu et al. 1995) or utilizing enzymatic reactions (Mayer et al. 2002). Preparation of D-glucose from the above natural polysaccharides is also relatively expensive and laborious, its market as industrial raw material competes with that for foods, food additives and beverages. Hence, D-glucose is relatively expensive. Recently, it was first reported that *Kluyveromyces lactis* produced D-arabitol directly from lactose (Toyoda and Ohtaguchi, 2009). Lactose is available in whey that is a by-product in the industrial effluent from cheese manufacturing. Approximately 9 kg of whey is produced in the manufacture of 1 kg of cheese. Proteins are removed from whey utilizing the ultrafiltration (UF) technique and marketed as whey protein concentrate (WPC) (Gonzalez-Siso 1996). The whey UF-permeate still contains a high concentration of lactose, which creates a high load on wastewater treatment. The conversion of lactose found in whey UF-permeate to xylitol *via* D-arabitol is attractive since whey is an ideal source for food and health care compounds. A number of osmophilic yeasts are capable of producing D-arabitol from D-glucose, however, those strains, except *Kluyveromyces* strains, are incapable of producing it from lactose. D-Glucose can be supplied by the cell free hydrolysis of lactose utilizing β-galactosidase, however, the by-product D-galactose creates a disposal problem. The present research aimed at formulating a method to convert lactose to xylitol by three biotransformations in series. Our earlier finding of the conversion of lactose to D-arabitol by *K. lactis* NBRC 1903 was extended to elevate D-arabitol production by shifting up the concentrations of *Kluyveromyces* biomass and lactose and by increasing the oxygen supply. Xylitol production from D-xylulose, which was prepared from D-arabitol utilizing *G. oxydans* NBRC3172, was performed by both microbial reaction and enzymatic reaction. An attempt to increase xylitol concentration was also made by adding ethanol into the culture of *C. shehatae* IAM

Tomoyuki Toyoda, Kazuhisa Ohtaguchi*

Department of Chemical Engineering, Tokyo Institute of Technology, Tokyo 2-12-1 Ookayama, Meguro-Ku, Tokyo 152-8552, Japan

*Tel/Fax: +81-3-5734-2113,
E-mail: ohtaguchi.k.aa@m.titech.ac.jp

12953 on D-xylulose. Different from hemicellulosic materials for xylitol production *via* D-xylose, the biotransformations presented in this study provide a more suitable process for food and medical use since the qualities of whey and whey UF permeate are same as those of dairy products.

Materials and Methods

Chemicals

Complex nutrients like Yeast Extract, Peptone and Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (YNB w/o AA and AS) were obtained from Becton, Dickinson and Co., Franklin Lakes, NJ, USA. Sorbitol dehydrogenase (SDH) and Antifoam PE-H were obtained from Wako Pure Chemical Industries, Ltd., Japan. β-NADH was purchased from Oriental Yeast Co., Ltd., Japan.

Organisms

K. lactis NBRC 1903, *C. melibiosica* NBRC 10238 and *G. oxydans* NBRC 3172 were obtained from the NITE Biological Resource Center (NBRC). *C. mogii* IAM 1295, *C. shehatae* IAM 12953, *D. hansenii* IAM 12837, *Pichia farinose* IAM 12223, *P. stipitis* IAM 12952 and *Z. rouxii* IAM 12879 were obtained from the IAM Culture Collection. Yeast strains were maintained on YPD plates (111 mmol L^{-1} D-glucose, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} agar) at 4 °C. *G. oxydans* NBRC 3172 was also maintained at 4 °C on an agar plate containing 137 mmol L^{-1} mannitol, 5 g L^{-1} yeast extract, 3 g L^{-1} peptone and 20 g L^{-1} agar.

Preparation of D-arabitol from lactose

K. lactis NBRC 1903 was precultured at 30 °C in 50 mL of preculture medium (YLN medium) in a 500-mL baffled flask shaken at 200 rpm on a rotary shaker. The composition of the YLN medium was as follows: 3 g L^{-1} yeast extract, 27.8 mmol L^{-1} lactose, 37.9 mmol L^{-1} $(NH_4)_2SO_4$, 14.7 mmol L^{-1} KH_2PO_4 and 4.06 mmol L^{-1} $MgSO_4$ $7H_2O$. Inocula for the batch culture were collected at 12 h. The cells were washed once with distilled water and the initial dry cell mass concentration for the batch culture was fixed at 0.004 g L^{-1}. The D-arabitol production medium was a synthetic lactose medium (SL medium) containing 555 mmol lactose, 68.4 mmol glutamine and 3.4 g YNB w/o AA and AS per litre.

First, a series of batch culture experiments were performed in the SL medium with the initial lactose concentration (c_{S0}) of 278 mmol L^{-1}, 416 mmol L^{-1} and 610 mmol L^{-1}. Cultivation was performed at 30 °C with 2 mL of SL medium in 20-mL test tubes that were shaken at 200 rpm.

Second, *K. lactis* NBRC 1903 cells were cultivated at 37 °C in two bioreactors-in-series. The first bioreactor was a 2-L jar fermenter (EYELA Mini jar fermenter, model M-100; Tokyo Rikakikai Co., Ltd, Japan) that was equipped with a ring sparger (hole diameter, 0.5 mm; number of holes, 6). This bioreactor was used for the production of relatively large amount of *K. lactis* NBRC 1903 cells. Cells were grown on 555 mmol L^{-1} lactose in a 1 L of SL medium that was aerated by air at 1000 mL min^{-1} and agitated at 400 rpm. Foam formation was treated by adding 0.5 g L^{-1} of Antifoam PE-H. The culture was harvested at 48 h, and the cells were separated from the culture supernatant by centrifugation at 6000 rpm (50A-IV, Sakuma, Tokyo, Japan) for 10 min. Then a five-fold increase in the cell mass concentration was achieved by re-suspending the collected cells into 50 mL of supernatant. The concentrated cell suspension was fed into the second bioreactor that was a bubble column (30mm x 200mm, D x H), and aerated by air at 250 mL min^{-1} through a single-nozzle sparger (glass ball filter, particle size of 100-120 μm, Kinoshita Rika Kogyo Co., Ltd.). This bioreactor was used for the production of D-arabitol utilizing concentrated biomass of *K. lactis* NBRC 1903. Breakdown of lactose to D-arabitol was performed with addition of lactose powder to the culture at 6 h after inoculating *K. lactis* NBRC 1903 from the first bioreactor to the second bioreactor.

Preparation of D-xylulose from D-arabitol

G. oxydans NBRC 3172 cells were precultured at 30 °C in a 500-mL baffled flask that was shaken at 200 rpm for 48 h. The preculture medium (YPM medium) contained 10 g L^{-1} yeast extract, 10 g L^{-1} peptone and 274 mmol L^{-1} mannitol. Cells were harvested by centrifugation as described above and washed with distilled water. Then cells were inoculated to a 1 mL aqueous solution of 197 mmol L^{-1} D-arabitol in a 20-mL test tube. The initial cell mass concentration of *G. oxydans* NBRC 3172 was set at 6.3 g L^{-1}. Oxidation of D-arabitol to D-xylulose was performed by shaking this test tube at 200 rpm at 30 °C.

Production of xylitol from D-xylulose

Reduction of D-xylulose to xylitol was studied in yeast strain selection and in xylitol production by cells of selected yeast strain. In addition to this, D-xylitol production by cell free solution of SDH was performed. A large number of amino acid residues in the active sites were shown to be identical in xylitol dehydrogenase and in SDH, hence the conversion of xylitol to D-xylulose was monitored to evaluate the specific activity of SDH (Rutten et al. 2009).

First, yeast strain selection was performed utilizing *K. lactis* NBRC 1903, *K. marxianus* ATCC 26548, *C. melibiosica* NBRC 10238, *C. mogii* NBRC 0436, *C. shehatae* IAM 12953, *Z. rouxii* IAM 12879, *D. hansenii* IAM 12837, *P. farinose* IAM 12223 and *P. stipitis* IAM 12952. This experiment was performed in the YXllN medium that contained 3 g L^{-1} yeast extract, 219 mmol L^{-1} D-xylulose, 37.9 mmol L^{-1} $(NH_4)_2SO_4$, 14.7 mmol L^{-1} KH_2PO_4 and 4.06 mmol L^{-1} $MgSO_4$ $7H_2O$. The preculture was performed aerobically at 303 K in 2 mL of YXllN medium in 20-mL test tubes that were shaken at 200 rpm for 24 h. Then 0.9 mL of preculture was centrifuged as described above and the supernatant was replaced with the fresh YXllN medium. The static cultivations were performed in micro-test tubes at 30 °C. The cultivations were static but shaken for 15 seconds at 3, 6 and 9 h.

Second, conversion of D-xylulose to xylitol was performed utilizing *C. shehatae* IAM 12953 in the presence of ethanol. The modified YX1lN medium was used for this experiment. This medium was modified from the YXllN medium by changing concentration of KH_2PO_4 to 3.8 mmol L^{-1}, and by adding 9.76 mmol L^{-1} K_2HPO_4. The method of preculturing *C. shehatae* IAM 12953 was the same as in the yeast strain selection, except for the change in the medium. Cells in preculture were harvested by centrifugation and the supernatant was replaced by the fresh modified YXllN medium. The cultivation was done in a 1.8-mL cylindrical vial. Ethanol was added to the modified YXllN medium at the initial concentration (c_{E0}) of 4.4, 169, 528 ad 1260 mmol L^{-1}. For the cultivation of *C. shehatae* IAM 12953, initial cell mass concentration was set at 4 g L^{-1}. After the preparation the vials were capped tightly. The vials were set on a reciprocal shaker and shaken at 90 rpm in the longitudinal direction.

Cell free conversion of D-xylulose to xylitol was also performed utilizing SDH. The aqueous solution of 197 mmol L^{-1} D-xylulose

was mixed with 1 mol L^{-1} phosphate buffer at the ratio of 9 to 1. SDH and β-NADH were dissolved in 0.1 mmol L^{-1} phosphate buffer (pH 7.0) and the concentrations in the reaction mixture were set at 14 g L^{-1} and 200 mmol L^{-1}, respectively. 250 μL of reaction mixture containing 112 mmol L^{-1} D-xylulose was prepared in 1.8-mL cylindrical vial. The reaction was performed at 30 °C and 90 rpm on a reciprocal shaker in the longitudinal direction. All experiments in this study were performed twice to confirm the reproducibility.

Analytical techniques

For the analysis of the concentrations of *Kluyveromyces* dry cell mass (X), substrate lactose (c_S), D-arabitol (c_A), D-xylulose (c_{Xll}) and product xylitol (c_P), the samples were removed from the culture. The optical density at 600 nm (OD_{600}) was measured by using a spectrophotometer (UV120-02, Shimadzu Corp., Kyoto, Japan). Concentrations of sugars and sugar alcohols in the filtered supernatant were analyzed by a high-performance liquid chromatograph (HPLC) (LC-10AD, Shimadzu Corp., Kyoto, Japan) and refractive index detector (RID-6A, Shimadzu Corp., Kyoto, Japan). Temperature of HPLC column (SZ5532, Showa Denko K.K., Tokyo, Japan) was set at 60 °C. The carrier was acetonitrile/H_2O (75:25) and the flow rate was 1.0 mL min^{-1}. Ethanol concentration was analyzed by a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan) with Gasukuropack 54 (GL Science, Tokyo, Japan). The temperature of the injector and the column were set at 180 °C and 150 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 60 mL min^{-1}.

Results and Discussion

Breakdown of lactose to D-arabitol utilizing growth-arrested cells of K. lactis

Batch growth curves of *K. lactis* NBRC 1903 showed the logarithmic growth phase from 0 h to 12 h, the late-logarithmic growth phase from 12 h to 48 h and the stationary phase after 48 h. Growth curves were unaffected by c_{S0}. Conversion of lactose to D-arabitol was investigated by comparing the growth-phase-fractional yield of D-arabitol ($Y_A = -\Delta c_A/\Delta c_S$) that is defined by moles D-arabitol produced in each growth phase per moles lactose consumed

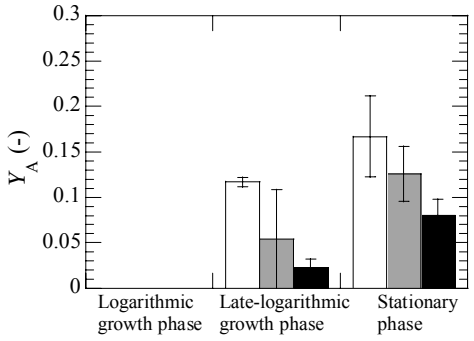

Figure 1: Effect of osmotic pressure caused by c_{S0} on the growth-phase-yield of D-arabitol on lactose (Y_A). c_{S0}: black bar; 278 mmol L^{-1}, grey bar; 416 mmol L^{-1}, white bar; 610 mmol L^{-1}.

in that growth phase. Figure 1 shows the Y_A in the logarithmic growth phase, the late-logarithmic growth phase and the stationary phase. Black, gray and white of histogram represents the c_{S0} at 278 mmol L^{-1}, 416 mmol L^{-1} and 610 mmol L^{-1}, respectively. The Y_A were extremely low in the logarithmic growth phase while the highest values were observed in the stationary phase. The rise in osmotic pressure by increasing c_{S0} was found to play an important role in D-arabitol production by cells in the late-logarithmic growth phase and stationary phase. In a preliminary experiment we observed that the increase in oxygen supply also enhanced D-arabitol production (data not shown). These observations suggest that an effective conversion of lactose to D-arabitol can be substantiated by utilizing the concentrated stationary-phase cells of *K. lactis* NBRC 1903 that are cultivated on a high concentration of lactose under highly aerobic conditions. To obtain a large amount of *K. lactis* NBRC 1903 cells in the stationary phase, the precultured cells were first cultivated until the start of the stationary phase in the first bioreactor with high aeration. Table 1 shows the initial and the final state of the culture of *K. lactis* NBRC 1903 in this reactor. The growth curve showed the beginning of the stationary phase at 48 h. During these 48 h, the cells grew for 10.2 generations.

Table 1: Initial and final states of concentrations of cell mass (X), lactose (c_S) and D-arabitol (c_A)

t (h)	X (mmol L^{-1})	c_S (mmol L^{-1})	c_A (mmol L^{-1})
0	0.0045	555	0
48	5.58±0.17	284±21	70.2±8.8

The cells grown for 48 h in the aerated agitator were harvested by centrifugation and the concentrated stationary-phase cells were transferred to the second bioreactor bubble column. The value of X was shifted up to 4.8 times that of the aerated agitator at 48 h. Figure 2 shows the time courses of the culture parameters in the bubble column. Although the cells transferred from the aerated agitator to the bubble column were at the beginning of stationary

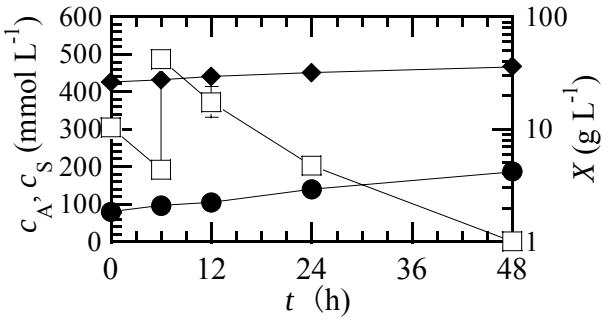

Figure 2: Time course of culture parameters of the cultivation for D-arabitol production from lactose by *K. lactis* NBRC 1903. Keys: concentrations of cell mass; X (♦), lactose; c_S (□), D-arabitol; c_A (●).

phase, a very small portion of cells started growth at 0 h. Growth arrest of cells in the aerated agitator was assumed to be caused by insufficient supply of oxygen. At 6 h in the bubble column, lactose was added to the culture in order to raise the osmotic pressure and to supply substrate. Conversion of lactose to D-arabitol was performed under highly aerobic condition in the bubble column. This conversion was found to be an autocatalytic reaction of *K. lactis* NBRC 1903 cells that grew slightly during the reaction. The concentration of biomass at the end of the reaction was 1.32 times that at 6 h. D-Arabitol production rate was almost constant at 2.16 mmol L^{-1} h^{-1} from 6 h to 48 h. The Y_A during this period was 0.220. The use of a high cell mass concentration of growth-repressed cells of *K. lactis* NBRC 1903 was found to be effective for D-arabitol production. In this paper, subscript f represents the state at the end of the reaction or at the time of the complete conversion. D-Arabitol concentration at the time of complete conversion of lactose (c_{Af}) reached 188 mmol L^{-1} which was 7.0 times higher than the one obtained in our previous work (Toyoda and Ohtaguchi 2009).

Oxidation of D-arabitol to D-xylulose utilizing stationary-phase culture of G. oxydans

D-Arabitol was successfully converted to D-xylulose when the concentrated stationary-phase cells of *G. oxydans* NBRC 3172 were inoculated to 197 mmol L^{-1} D-arabitol solution in highly aerobic condition. An extremely small amount of xylitol was detected as a by-product. Figure 3 shows the time courses of c_{XII}, c_A and c_P. The value of c_{XII}, reached its maximum of 191 mmol L^{-1} at 6 h and started to decrease. The overall fractional yield of D-xylulose in terms of D-arabitol consumption was almost 1.0 and the rate of D-xylulose production was 31.8 mmol L^{-1} h^{-1}. The conversion of D-arabitol to D-xylulose is described as follows:

$$\underset{\text{(D-arabitol)}}{C_5H_{12}O_5} \xrightarrow{G.oxydans} \underset{\text{(D-xylulose)}}{C_5H_{10}O_5} + 2H^+ + 2e^- \qquad (1)$$

It was reported that the produced electron was transported to the respiratory chain (Matsushita et al. 1994).

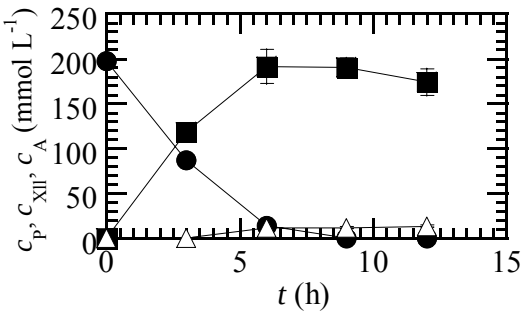

Figure 3: Time courses of culture parameters in D-xylulose production from D-arabitol by *G. oxydans* NBRC 3172. Keys: concentrations of D-xylulose; c_{XII} (■), xylitol; c_P (Δ), D-arabitol; c_A (●).

Table 2: Xylitol production from D-xylulose by yeast strains in microaerobic condition

Strains	Residual D-xylulose (a) (mmol L^{-1})	Produced xylitol (b) (mmol L^{-1})	Produced D-arabitol (b) (mmol L^{-1})	$Y_{P/XII}$ (d) (-)
Kluyveromyces lactis NBRC 1903	194±7	0.92±0.03	trace	0.038
K. marxianus ATCC 26548	64.6±1.9	37.5±1.9	17.2±0.9	0.243
Candida melibiosica NBRC 10238	169±8	18.0±0.9	2.04±0.44	0.357
C. mogii NBRC 0436	179±2	12.0±0.1	3.75±0.27	0.295
C. shehatae IAM 12953	69.9±2.2	23.3±0.5	2.17±0.23	0.157
Zygosaccharomyces rouxii IAM 12879	200±1	4.73±0.05	1.05±0.01	0.247
Debaryomyces hansenii IAM 12837	190±6	4.47±0.66	2.37±0.19	0.153
Pichia farinose IAM 12223	193±3	5.06±0.40	1.12±0.02	0.196
P. stipitis IAM 12952	181±7	2.17±0.10	n. d. (c)	0.056

(a) Initial D-xylulose concentration: 219 mmol L^{-1}, (b) (Measured value) – (Initial value), (c) n. d.: not detected, (d) overall fractional yield of xylitol on D-xylulose

Yeast strain selection for microbial conversion of D-xylulose to xylitol

For the conversion of D-xylulose to xylitol, the selection of yeast strains was studied. Table 2 tabulates the final concentrations of reactant D-xylulose c_{XII}, product xylitol c_P and by-product D-arabitol c_A. The overall fractional yield of xylitol on D-xylulose ($Y_{P/XII}$) was also listed. D-Xylulose for this experiment was prepared from D-arabitol by utilizing *G. oxydans* NBRC 3172, and hence, besides 219 mmol L^{-1} D-xylulose, the YX1lN medium for xylitol production contained 2.65 mmol L^{-1} xylitol as an impurity from the D-xylulose production reaction. However, the concentration of D-arabitol in this medium was lower than the detectable level. In the xylitol production reaction, D-arabitol was detected as a by-product in the cases of all strains except *P. stipitis* IAM 12952. The highest c_P was observed in the run with *K. marxianus* ATCC 26548. The underlying premise of the selection of *K. marxianus* ATCC 26548 is the development of a very simple separation technique of xylitol and D-arabitol which are epimers in C-2, since the concentration of by-product c_A was also high (the value of c_A/c_P was 0.457) in this run. The run with *C. melibiosica* NBRC 10238 showed the highest $Y_{P/XII}$ of 0.357. This run also gave a relatively high value of c_P. Although $Y_{P/XII}$ of the run with *C. shehatae* IAM 12953 was lower than that with *C. melibiosica* NBRC 10238, *C. shehatae* IAM 12953 consumed D-xylulose well and gave a higher c_P. The run with *C. shehatae* IAM 12953 is characteristic in that the value of c_A/c_P was relatively low (c_A/c_P =0.0930). Aerobic cultivation was also performed in 20-mL test tubes for the listed nine strains. Although the consumption of D-xylulose in the aerobic cultivation was more enhanced compared to the microaerobic cultivation, both c_P and $Y_{P/XII}$ were lower than those obtained under the microaerobic conditions (data not shown). Xylitol is an upstream intermediate of the metabolic pathway from D-xylose to D-xylulose. When yeast strains are cultivated on D-xylulose, produced xylitol is not further metabolized other than returning to D-xylulose. The possibility exists that D-xylulose is converted back to xylitol since the reaction is reversible. The generation of NADH in the conversion of D-xylulose to xylitol might play an important role in maintaining the intracellular redox balance. The time course data of c_P in xylitol production by *C. shehatae* IAM 12953 were plotted against those of c_{XII} in Figure 4. Linear relation was seen in this graph. The value of $Y_{P/XII}$ was found to be constant during the reaction.

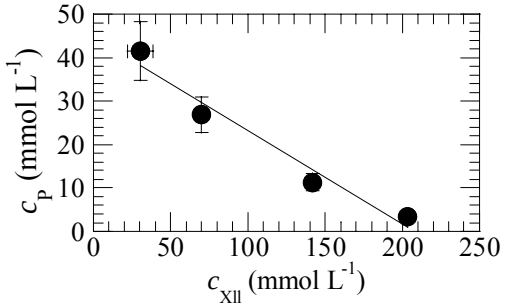

Figure 4: Relation between xylitol production (c_P) and D-xylulose consumption (c_{XII}) in the static culture *C. shehatae* IAM 12953.

Reduction of D-xylulose to xylitol utilizing C. shehatae in the presence of ethanol

Ethanol was added to the modified YX1lN medium of xylitol production to reduce the consumption of D-xylulose and NADH in the ethanol production reaction catalyzed by NADH-dependent alcohol dehydrogenase. If ethanol consumption occurs at a high ethanol concentration, the addition of ethanol also has a high

potential to generate NADH that promotes the conversion of D-xylulose to xylitol since NADH is generated in the oxidation of ethanol to acetaldehyde. Concentrated biomass of *C. shehatae* IAM 12953 from the stationary phase of preculture reduced D-xylulose to xylitol under microaerobic condition. Table 3 shows the effect of initial ethanol concentration (c_{E0}) on the parameters of xylitol production from D-xylulose. Subscript 6 represents 6 h of reaction.

Table 3: Effect of ethanol addition on xylitol production from D-xylulose by *C. shehatae* IAM 12953.

c_{E0} (mmol L^{-1})	4.4	169	528	1260
$c_{Xll,0}$ (mmol L^{-1})	190	163	156	165
$c_{Xll,6}$ (mmol L^{-1})	92.2±4.6	67.2±2.4	85.6±1.4	138±1
$c_{E,6}$ (mmol L^{-1})	77.5±1.0	237±1	543±2	1180±20
$c_{P,6}$ (mmol L^{-1})	15.2±0.9	22.8±1.8	20.4±2.3	10.1±2.2
$c_{A,6}$ (mmol L^{-1})	nd	nd	nd	nd
$Y_{P/Xll}$ (-)	0.155	0.238	0.290	0.374
Xylitol production rate(mmol L^{-1} h^{-1})	2.53	3.80	3.80	1.68
c_{Pf} (mmol L^{-1})*	29.5	38.8	45.2	61.7
t_f (h)**	11.7	42.9	41.1	98.2

* calculated from $Y_{P/Xll}$ ** calculated from xylitol production rate

Xylitol concentrations at 6 h of runs with different c_{E0} are compared since the yield of xylitol on D-xylulose was constant during the reaction as shown in Figure 4. The monitored parameters are the concentrations of biomass, produced xylitol ($c_{P,6}$), by-product D-arabitol ($c_{A,6}$), unreacted D-xylulose ($c_{Xll,6}$) and unreacted or produced ethanol ($c_{E,6}$). In all runs, cell growth and D-arabitol production were not observed. Ethanol was produced from D-xylulose when c_{E0} was no more than 169 mmol L^{-1}. When the ethanol concentration was low, the xylitol production reaction was found to compete with the ethanol production reaction for D-xylulose. The following equation represents the reaction at c_{E0} of 169 mmol L^{-1}:

$$\underset{\text{(D-xylulose)}}{C_5H_{10}O_5} + \text{other substrates} \xrightarrow{C.shehatae} 0.238\,\underset{\text{(xylitol)}}{C_5H_{12}O_5} + 0.707\,\underset{\text{(ethanol)}}{C_2H_5OH} + \text{other products} \quad (2)$$

An attempt to reduce the ethanol production reaction was made by increasing the initial ethanol concentration. When c_{E0} was 528 mmol L^{-1}, ethanol concentration changed little during the xylitol production reaction. The critical concentration of ethanol that inhibits ethanol production was found to be around 528 mmol L^{-1}. When c_{E0} was 1260 mmol L^{-1}, ethanol was assimilated by *C. shehatae* IAM 12953. Ethanol consumption holds promise to generate NADH that promotes the conversion of D-xylulose to xylitol. The $Y_{P/Xll}$ at 1260 mmol L^{-1} of c_{E0} was 0.374, which was 2.38 times higher than that shown in Table 2. If c_{Pf} represents the concentration of xylitol at the time of the complete conversion of D-xylulose, then the c_{Pf} of Table 3 are estimated utilizing $Y_{P/Xll}$. The xylitol production reaction is a zero order reaction, and hence the reaction rate and the time for the complete conversion of D-xylulose (t_f) are also estimated in Table 3. The attainable level of c_{Pf} is 61.7 mmol L^{-1} that can be obtained by reacting 4 g L^{-1} stationary phase cells of *C. shehatae* IAM 12953 in the modified YX1lN medium containing 165 mmol L^{-1} D-xylulose and 1260 mmol L^{-1} ethanol for 98.2 h. Figure 5 shows the relations between c_{E0} and $Y_{P/Xll}$, and between c_{E0} and the xylitol production rate. The value of $Y_{P/Xll}$ increased linearly with increasing c_{E0}. Increasing c_{E0} enhanced the xylitol production rate under conditions where ethanol is also produced while the rate decreased under the conditions where ethanol is assimilated by *C. shehatae* IAM 12953.

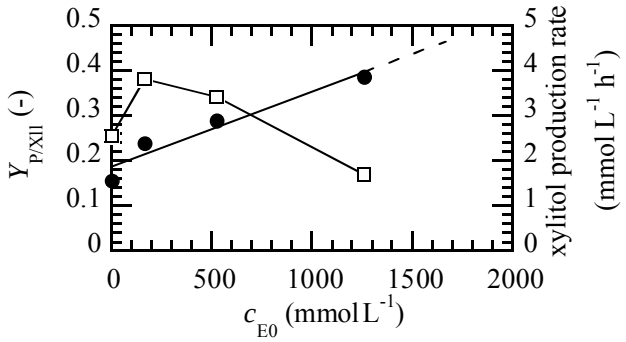

Figure 5: Effect of initial ethanol concentration (c_{E0}) on overall fractional yield of xylitol on D-xylulose $Y_{P/Xll}$ (●) and xylitol production rate (□).

The highest xylitol production rate of 3.80 mmol L^{-1} h^{-1} was observed at the c_{E0} of 169 mmol L^{-1}. If a linear relation between c_{E0} and $Y_{P/Xll}$ is assumed, extrapolation of the data suggests that $Y_{P/Xll}$ can be increased to 0.464 at the c_{E0} of 1670 mmol L^{-1}. This condition gave the following equation for xylitol production:

$$\underset{\text{(D-xylulose)}}{C_5H_{10}O_5} + 6.60\,\underset{\text{(ethanol)}}{C_2H_5OH} + \text{other substrates} \xrightarrow{C.shehatae} 0.464\,\underset{\text{(xylitol)}}{C_5H_{12}O_5} + \text{other products} \quad (3)$$

Table 4: Conversion of D-xylulose (c_{Xll}) to xylitol (c_P) by sorbitol dehydrogenase

t (h)	c_{Xll} (mmol L^{-1})	c_P (mmol L^{-1})
0	112	nd
6	75.9±0.253	36.7±2.4

n. d.: not detected

Table 5: Comparison of xylitol production from D-glucose with that from lactose

Raw material	Number of steps	Microorganisms and enzyme	Overall reaction time (h)	Over all production rate (mmol L^{-1} h^{-1})	Reference
D-Glucose	3	*Debaryomyces hansenii* ATCC 20212 *Acetobacter suboxydans* ATCC 621 *Candida guilliermondii* var. soya ATCC 20216	211	0.561	Ohnishi and Suzuki, 1969
	1	Recombinant *S. cerevisiae*	100	0.0197	Toivari et al. 2007
	1	*Asaia ethanolifaciens* sp. nov. FERM BP-6751	120	0.290	Mihara et al. 2002
Lactose	3	*Kluyveromyces lactis* NBRC 1903 *Gluconobacter oxydans* NBRC 3172 *Candida shehatae* IAM 12953	140	0.519	This work
	3	*Kluyveromyces lactis* NBRC 1903 *Gluconobacter oxydans* NBRC 3172 Sorbitol dehydrogenase	113	1.69	This work

Reduction of D-xylulose to xylitol utilizing sorbitol dehydrogenase

A cell free enzymatic reaction was also performed utilizing SDH. Table 4 shows the results of the run in which 112 mmol L^{-1} D-xylulose was treated by SDH. The concentration of produced xylitol and unreacted D-xylulose at 3 h were 36.7 mmol L^{-1} and 75.3 mmol L^{-1}, respectively. This reaction was confirmed to proceed with the theoretical yield. The enzyme activity was 4280 U g^{-1}. The complete conversion of 112 mmol L^{-1} D-xylulose to 112 mmol L^{-1} xylitol can be achieved in 9.3 h of reaction. The equation of conversion of D-xylulose to xylitol utilizing SDH is described as

$$\underset{\text{(D-xylulose)}}{C_5H_{10}O_5} + NADH + H^+ \xrightarrow{SDH} \underset{\text{(xylitol)}}{C_5H_{12}O_5} + NAD^+ \qquad (4)$$

The regeneration of coenzyme is required in an industrial process to reduce the production cost, however, the substrate and product of coupled reaction are limited in propriety. For instance, the appearance of acetaldehyde produced from ethanol utilizing NAD^+-dependant alcohol dehydrogenase is not preferable for food or medical use. The separation of xylitol from acetaldehyde creates an economic problem.

Reaction path synthesis for xylitol production from lactose

Figure 6 shows the overview of the possible reaction paths to xylitol from lactose. The new technique that is developed in this work is represented by three bold arcs leading from lactose to xylitol *via* D-xylulose and D-arabitol. This series reaction is characteristic in that lactose is directly converted to D-arabitol utilizing the culture of *K. lactis* NBRC 1903. Hence this process does not require cell free *β*-galactosidase treatment of lactose to D-glucose. The other hallmark of the process is the achievement of high conversion of D-xylulose to xylitol. If stationary-phase cells of *C. shehatae* IAM 12953 are utilized as the biocatalyst for this conversion, then the addition of 1670 mmol L^{-1} ethanol has a promise to produce xylitol with the overall fractional yield in this reaction of 0.464. If SDH is utilized as the biocatalyst for this conversion, then the overall fractional yield in this reaction of 1.0 is achieved. The overall fractional yield of three series reactions from lactose to xylitol is 0.0547 when biocatalysts *K. lactis* NBRC 1903, *G. oxydans* NBRC 3172 and *C. shehatae* IAM 12953 are employed, while it is 0.141 when *C. shehatae* IAM 12953 is replaced by SDH.

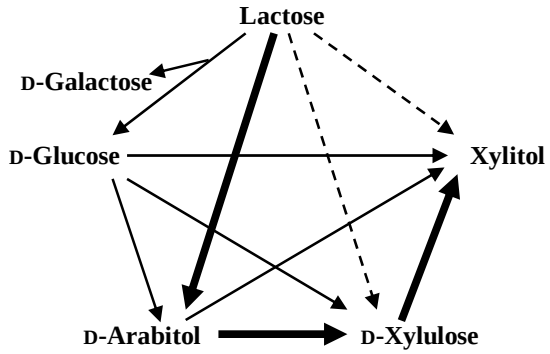

Figure 6: Overview of production of xylitol from lactose by biotechnological transformations. Solid lime, reported reaction (bold line, this work; thin line, previous works shown in Table 5 and Introduction); broken line, unreported reaction.

Production of xylitol from lactose is compared with that from D-glucose in Table 5. The overall production rate of xylitol from lactose in this work was 1.69 mmol L^{-1} h^{-1}, which is the highest among the processes shown in Table 5. Lactose as a raw material appears very attractive since it is abundant in cheese whey. There are single reactions from D-glucose to xylitol utilizing *A. ethanolifaciens* sp. nov. FERM BP-6751 (Mihara et al. 2002) or recombinant *S. cerevisiae* (Toivari et al. 2007). The application of such reactions has potential to reduce operation costs, however, the overall xylitol production rates of those reactions are presently lower than that of the series reaction presented in this study.

Complete conversion of D-arabitol to xylitol can be performed as shown in this work and in previous works (Ahmed and Bhowmik 2000; Mayer et al. 2002; Suzuki et al. 2002). Hence the conversion of lactose to xylitol is limited by the low conversion of lactose to D-arabitol. Economic aspects are also important in xylitol production. Current market prices of SDH and xylitol dehydrogenase, which catalyze the conversion of D-xylulose to xylitol, make them unsuitable for industrial purpose. The production of SDH or xylitol dehydrogenase by recombinant microorganisms seems to settle the problem. Although xylitol yield on D-xylulose is lower in microbial conversion than in enzyme conversion, the achievement of the overall fractional yield of 0.464 in the culture of *C. shehatae* IAM 12953 by adding ethanol is highlighted. More data will be needed before the industrial implications of these findings become eminent.

Conclusion

A novel biotechnological transformation from lactose to xylitol was developed. Lactose was converted to D-arabitol by the growth-arrested cells of *K. lactis* NBRC 1903 in the first step. This reaction was followed by conversion of D-arabitol to D-xylulose by *G. oxydans* NBRC 3172, and by xylitol production from D-xylulose by *C. shehatae* IAM 12953 or sorbitol dehydrogenase. This study adds useful information for production of value-added products from lactose.

Acknowledgement

This research was supported in part by the Food Science Institute Foundation in Japan.

References

Ahmed Z, Bhowmik PK (2000) Studies on the Oxidation of Ribitol and D-Arabitol by *Acetobacter aceti* IFO 3281. Pakistan J Biol Sci 3:1492-1495

Gonzalez-Siso MI (1996) The Biotechnological Utilization of Cheese Whey: A Review. Bioresour Technol 57:1–11

Kadam KL, Chin CY, Brown LW (2008) Flexible Biorefinery for Producing Fermentation Sugars, Lignin and Pulp from Corn Stover. J Ind Microbiol Biotechnol 35:331–341

Matsushita K, Toyama H, Adachi O (1994) Respiratory Chains and Bioenergetics of Acetic Acid Bacteria. Adv Microb Phys 36:247-301

Mayer G, Kulbe KD, Nidetzky B (2002) Utilization of Xylitol Dehydrogenase in a Combined Microbial Enzymatic Process for Production of Xylitol from Glucose. Appl Biochem Biotech 98-100:577-589

Mihara Y, Takeuchi S, Jojima Y, Tonouchi N, Fudou R, Yokozeki K (2002) Microorganisms and Method for Producing Xylitol or D-Xylulose. US patent 6,335,177

Nozaki H, Suzuki S, Tsuyoshi N, Yokozeki K (2003) Production of D-Arabitol by *Metschnikowia reukaufii* AJ14787. Biosci Biotechnol Biochem 67:1923-1929

Ohmomo S, Tozawa Y, Ueda K (1983) Biotransformation of D-Arabitol to D-Xylulose by *Gluconobacter suboxydans*

Immobilized within κ-Carrageenan. J Ferment Technol 61:373-378

Ohnishi H, Suzuki T (1969) Microbial Production of Xylitol from Glucose. Appl Microbiol 18:1031-1035

Prakasham RS, Rao RS, Hobbs PJ (2009) Current Trends in Biotechnological Production of Xylitol and Future Prospects. Curr Trends Biotechnol Pharm 3:8-36

Rutten L, Ribot C, Trejo-Aguilar B, Wosten H.AB, de Vries R.P (2009) A Single Amino Acid Change (Y318F) in the L-Arabitol Dehydrogenase (LadA) from *Aspergilus niger* Results in a Significant Increase in Affinity for D-Sorbitol. BMC Microbiol 9:166-172

Saha BC, Sakakibara Y, Cotta MA (2007) Production of D-Arabitol by a Newly Isolated *Zygosaccharomyces rouxii*. J Ind Microbiol Biotechnol 34:519-523

Spencer JFT, Sallans HR (1956) Production of Polyhydric Alcohols by Osmophilic Yeasts. Can J Microbiol 2:72-79

Suzuki S, Sugiyama M, Mihara Y, Hashiguchi K, Yokozeki K (2002) Novel Enzymatic Method for the Production of Xylitol from D-arabitol by *Gluconobacter oxydans*. Biosci Biotechnol Biochem 66:2614-2620

Toivari MH, Ruohonen L, Miasnikov AN, Richard P, Penttilä M (2007) Metabolic Engineering of *Saccharomyces cerevisiae* for Conversion of D-Glucose to Xylitol and Other Five-Carbon Sugars and Sugar Alcohols. Appl Environ Microbial 73:5471-5476

Toyoda T, Ohtaguchi K (2009) Selection of *Kluyveromyces* Yeasts for the Production of D-Arabitol from Lactose. J Chem Eng Japan 42:508-511

Ueng PP, Hunter CA, Gong C, Tsao GT (1981) D-Xylulose Fermentation in Yeasts. Biotechnol Lett 3:315-320

Utsuka Y, Naganuma T, Nishimura K, Kojima T, Kondo T, Bito Y (2002) Method for Manufacturing D-Arabitol and Mircoorganism Used in the Same, Japanese Patent Disclosure 2002-191388

Yu S, Jeppsson H, Hahn-Haigerdal B (1995) Xylulose Fermentation by *Saccharomyces cerevisiae* and Xylose-fermenting Yeast Strains. Appl Microbiol Biotechnol 44: 314-320