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Production of Xylitol by Xylitol Dehydrogenase Defective Mutant of *Pichia stipitis*

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This study was carried out to development the xylitol dehydrogenase defective mutants(XDH) from *Pichia stipitis* CBs 5776 and investigate the characteristics of xylitol fermentation by a xylitol dehydrogenase defective mutant PXM-4 in an effort to determine the optimum conditions for the high yield production of xylitol from xylose. The XDH defective mutants were screened by a xylose assimilation test. Among about several hundreds mutant screened, the best mutant PXM-4 was selected. And also gluconic acid was selected as a appropriate co-substrate for the xylitol fermentation. Since gluconic acid neither blocked xylose transport nor repressed xylose reductase expression. An increase in gluconic acid concentration reduced the rates of xylitol production and cell growth by decreasing medium pH. The optimal concentration of gluconic acid for xylitol production was determined at 20 g/l with approximately 100% xylitol conversion yield. A fed-batch cell culture resulted in 42.4 g/l xylitol concentration with 97% yield based on xylose consumed.

Introduction

Xylitol, a five-carbon sugar alcohol, is a naturally occurring sweetener present in small quantities in a wide variety of fruits, vegetables and mushroom [19]. It is also a normal metabolite in mammalian carbohydrate metabolism, including that of human [8]. The use of xylitol in the food industry is increasing due to its high sweetening power [9]. In addition, it has an anticariogenic property which does not cause acid formation [18] and can be used as sugar substitute for diabetics and glucose-6-phosphate dehydrogenase-deficient individuals since it does not require insulin and glucose-6-phosphate dehydrogenase for regulation of metabolism [18, 25].

Xylitol is currently produced by chemical hydrogenation of xylose in hemicellulose hydrolyzates using Ni/Al_2O_3 as a catalyst [16]. The product cost is high due to difficulties in separation and purification of xylitol, removal of by-products from hemicellulose hydrolyzates and the low yield of 40~50% based on xylan [9]. Biotechnological processes for xylitol production using xylose-fermenting microorganisms have several advantages such as selective conversion of xylose to xylitol with high yield. Microorganisms employed for biotechnological production of xylitol include bacteria [10], fungi [4] and yeasts, especially *Pachysolen tannophilus* [14], *Pichia stipitis* [6], *Candida pelliculosa* [20], *C. boidinii* [26, 27], *C. guilliermondii* [14, 17, 21], *C. tropicalis* [7, 11, 22] and *C. parapsilosis* [13, 21].

The xylose metabolism in yeasts starts with the reduction of xylose to xylitol catalyzed by xylose reductase (XR: EC 1.1.1.22) using cofactor NAD(P)H [3, 24]. Xylitol is subsequently oxidized to xylulose by xylitol dehydrogenase (XDH: EC 1.1.1.9) using cofactor $NAD(P)^+$ [3, 24]. 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. The first two enzymes, XR and XDH are known as key enzymes in xylitol production by yeasts. Several studies showed the existence of a correlation between key enzyme activities and xylitol production in yeasts [21, 27]. XR and XDH in most yeasts are inducible in a medium containing xylose as carbon source [1]. However, the presence of hexose, such as glucose, in the fermentation medium represses the expression of XR and XDH [5], resulting in a decrease in xylitol yield and productivity.

Xylose is an expensive substrate for the production of xylitol. Hence, it is important to increase the yield and productivity of xylitol by improving the strain and by optimizing the bioconversion processes. This study was carried out to investigate the characteristics of xylitol fermentation by a xylitol dehydrogenase defective mutant PXM-4 of *P. stipitis* CBS 5776 in an effort to determine the optimum bioconversion scheme for the high yield production of xylitol from xylose.

MATERIALS AND METHODS

Microorganism and Xylitol Fermentation

Pichia stipitis CBS 5776 and its XDH defective mutant PXM-4 as reported previously [12] were used in this study. *P. stipitis* was maintained on a YPX slant composed of 10 g/l yeast extract, 10 g/l peptone, 20 g/l xylose at 4℃. 20 g/l glucose (YPD) instead of xylose was used for the mutant PXM-4. Xylitol fermentation was conducted in a 500 ml Erlenmyer flask containing 200 ml fermentation medium composed of 5 g/l yeast extract, 5 g/l peptone and 5 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4$ and an appropriate amount of xylose and a co-substrate. Xylitol fermentation for determination of XR activity profiles were performed in a 2.5 liter fermenter (Korea Fermenter Co, Korea) at 250 rpm agitation, 1 vvm aeration and 30℃. Fed batch cultures for obtaining high cell concentration were performed in the same fermenter. The cultures were performed in batchwise with fermentation medium containing 10 g/l glucose as carbon source and switched to a fed-batch mode by feeding the mixture of glucose and yeast extract of 4 : 1 ratio.

Determination of Xylose Reductase Activity

Xylose reductase activity was measured spectrophotometrically at 340 nm by the oxidation of NADPH with the cell free extract. The cells grown in fermentation medium containing xylose and a co-substrate were harvested by centrifugation at 4,000 rpm for 10 min. The cells were washed twice with 5 ml potassium phosphate buffer (0.2 mM pH 7.0), resuspended in 5 ml of the same buffer by mixing with 0.5 g glass bead (0.1 mm) and then disrupted in the Mini-BeadBeater (BioSpec Products, Inc.) for 3 min. The cell debris and glass beads were separated by centrifugation at 10,000 rpm and the supernatant was used for measurement of enzyme activity.

The reaction mixture consisted of 1 ml of 1 M potassium phosphate buffer (pH 7.0), 0.2 ml of 1 M 2-mercaptoethanol, 1 ml of enzyme solution, and 0.2 ml of 0.5 M xylose. This reaction mixture was allowed to stand for 1 min to eliminate the endogenous oxidation of NADPH. The activity of XR was determined by monitoring the oxidation of NADPH in a spectrophotometric cuvette at 340 nm at room temperature. One unit of enzyme activity was defined as the amount of enzyme able to reduce 1 μmol NADPH per min. Spcific enzyme activity was expressed as units of enzyme per mg of protein. Protein was measured by the Lowry method [15] with bovine serum albumin as a standard.

Analysis

Cell mass was estimated by using the relationship between dry cell weight and optical density (OD) measured at 620 nm. One OD unit was equivalent to 0.323 g/l dry cell weight. Xylitol and all other sugars were determined by HPLC (Waters Co, USA) using a refractive index detector and carbohydrate analysis column (Waters Co, USA) with 80% (v/v) acetonitrile as mobile phase at a flow rate of 2 ml/min. Organic acids were determined by HPLC using a UV/VIS detector at 215 nm and Aminex HPX-87H column (Bio-Rad, USA) with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Selection of XDH defective mutant

Xylose non-assimilating mutants from *P. stipitis* was produced readily by chemical mutagen. The basal growth medium containing 2 %(w/v) xylose and 0.2 %(w/v) glucose was used for isolation of mutant. Finally, after isolated colonies were simultaneously picked on basal growth medium containing glucose and xylose medium repectively, about several hundreds mutants from *P. stipitis* only to be capable of growing on xylose medium were selected as XDH defective mutants. Selected mutants were tested their xylitol productivity in the fermentation medium containing of 10g/l glucose and xylose. Mutants strains are generally good xylitol produces than their parental strains. In *P. stipitis*, all amounts of xylose were consumed but it did not produce any significant amount of xylitol. PXM-4 of *P. stipitis* is generally the best xylitol producers than their parental strains. PXM-4 yielded 100 % based on the amount of xylose consumed.

Selection of Co-substrate

P. stipitis produced ethanol from xylose as a major product in normal fermentation conditions [12, 28]. Therefore, it is necessary to use a XDH defective mutant strain of *P. stipitis* for xylitol production from xylose. Efficient conversion of xylose to xylitol by this mutant requires a good co-substrate for cell growth and redox balance, especially continuous regeneration of NAD(P)H, a cofactor of XR. Xylose and a co-substrate should enter the cell at the same time without inhibition of the required permeases and not repress the enzymes involved in co-substrate metabolism and

xylose conversion. Most of hexoses, such as glucose, block xylose transport and repress XR induction, resulting in low xylitol yield and productivity [1, 12]. For this reason, selection of an appropriate co-substrate for xylitol production by the mutant PXM-4 was investigated.

Experiments were conducted in a 500 ml Erlenmyer flask containing 200 ml fermentation medium with 20 g/l xylose and 20 g/l various co-substrates in a shaking incubator for 5 days. The results are summarized in Table 1. Cell growth was favorable in a medium with glucose or galactose as a co-substrate while glycerol, gluconic and acetic acids were good co-substrates for the production of xylitol. Since glycerol, acetic and lactic acids were not completely consumed in 5 days of fermentation these co-substrates were found to be not appropriate carbon sources for the mutant PXM-4. *P. stipitis* could not use mannitol and sorbitol as a carbon source either. Specific XR activities at 10 h of fermentation were low in the presence of glucose and galactose. As shown in Fig. 1, cell growth in a glucose medium rapidly increased in 24 h fermentation and then slowly decreased. But specific XR activity increased up to 3 days of fermentation due to depletion of glucose beyond 10 h of fermentation and then rapidly decreased. Xylose was continuously converted to xylitol during the fermentation period. But xylose was not completely converted to xylitol owing to the deficiency of an energy source. These results suggested that glucose repressed XR induction in the mutant PXM-4. Therefore, gluconic acid was selected as the best co-substrate for xylitol production by the mutant PXM-4.

Table 1. Comparison of xylitol production and XR activity of mutant PXM-4 in the medium containing 20 g/l of xylose and 20 g/l of various co-substrates

Co-substrate	Dry Cell Weight (g/l)	Residual Xylose (g/l)	Residual co-substrate (g/l)	Xylitol (g/l)	Xylitol Yield (%)	Specific XR Activity (unit/mg-protein)	
						10 h	24 h
Glucose	4.8	10.2	0	9.5	98	15	205
Galactose	4.7	10.0	0	10.3	100	31	211
Mannitol	0.4	20.0	20.0	0	0	ND[a]	ND
Sorbitol	0.4	20.0	20.0	0	0	ND	ND
Glycerol	2.5	6.0	17.4	13.2	97	107	167
Acetic acid	2.1	10.2	15.9	9.7	99	177	236
Gluconate	2.5	1.5	0	18.4	100	181	245
Lactic acid	1.5	17.1	16.7	3.0	100	46	59

[a] not detected.

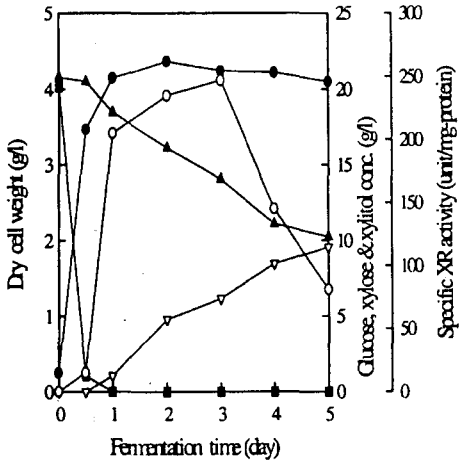

Fig. 1. Profile of cell growth(●) and glucose(■), xylose(▲) and xylitol(▽) concentration and specific XR activity(○) during the batch fermentation of mutant PXM4.

XR Activity

As the first step in the metabolism of xylose is the transport of xylose across the cell membrane which could be a limiting factor in the overall rate of xylose utilization, xylose transport and XR induction in the presence of gluconic acid as a co-substrate were investigated.

To examine the xylose transport and the profiles of specific XR activity in the mutant PXM-4, batch fermentations with 10 g/l gluconic acid and 10 g/l xylose were performed in a fermenter under 1 vvm aeration and 250 rpm agitation at 30℃. As shown in Fig. 2, both xylose and gluconic acid were consumed simultaneously and intracellular xylose was detected in the presence of gluconic acid in the medium. And specific XR activity was constantly sustained during the xylose and gluconic acid consumption period. It was clear from these results that gluconic acid neither

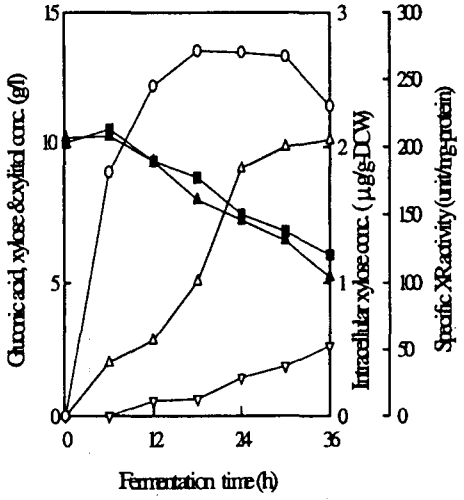

Fig. 2. Profile of cell gluconicacid(●), xylose(▲), xylitol(▽) andintracellular xylose(△) concentration and XR activity(○) during the batch fermentation of mutant PXM4.

inhibited xylose transport nor repressed XR expression. But xylose continuously accumulated inside the cell during the bioconversion period although the specific XR activity was maintained at a level high enough for the conversion of xylose to xylitol. This could be attributed to the fact the xylose conversion required continuous regeneration of the cofactor NADPH from the gluconic acid metabolism.

Batch Fermentation

To investigate the kinetics of cell growth and xylitol production and the pattern of XR expression in the mutant PXM-4, batch fermentations with 25 g/l gluconic acid and 50 g/l xylose were performed in a fermentation under 1 vvm aeration and 250 rpm agitation at 30℃. As shown in Fig. 3, cell growth continuously proceeded up to 6 days fermentation, reaching a maximum concentration of 4.7 g/l. Xylose and gluconic acid were consumed simultaneously up to 5 days fermentation. Specific XR activity greatly increased in 2 days fermentation and maintained at 206 to 224 unit/mg-protein until 5 days fermentation. These results indicated that xylose transport and XR production were not inhibited by gluconic acid. Xylitol continuously accumulated in the medium until 6 day fermentation, reaching a final concentration of 34.6 g/l. The volumetric productivity and yield of xylitol were 0.29 g/l-h and 99%, respectively. Low productivity may be improved by using the feb-batch fermentation for high cell density culture.

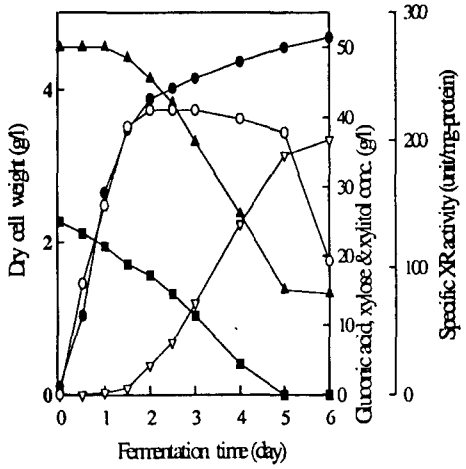

Fig. 3. Profile of cell growth(●) and gluconicn(■), xylose(▲) and xylitol(▽) concentration and specific XR activity(○) during the batch fermentation of mutant PXM4.

Fed-batch Fermentation

Fed-batch operation is useful because of easy control in the concentration of either product or substrate. To enhance xylitol productivity intermittent fed-batch fermentation was conducted to obtained high cell concentration and consequently high xylitol productivity. Glucose solution of 120 g/l was added into the fermenter when the glucose concentration in the culture broth fell below 5

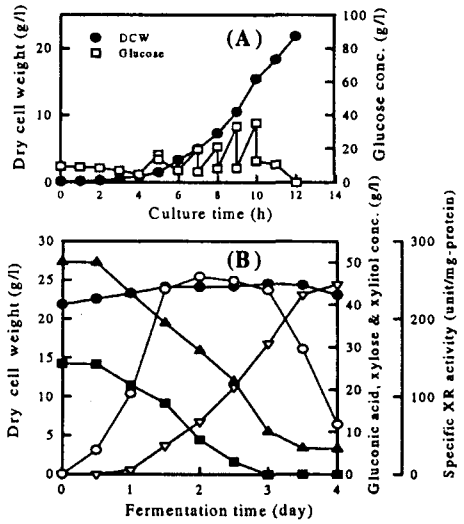

Fig. 4. Profile of cell growth(●) and gluconicn acid(■), xylose(▲) and xylitol(▽) concentration and specific XR activity(○) during the batch fermentation of mutant PXM4. Figure A represents intermittent fed-batch culture for cell growth and of zylose and 25 g/l of gluconic acid.

g/l in order to adjust the glucose concentration from 20 to 30 g/l. As shown in Fig. 4A, the cell concentration increased continuously by the feeding of glucose and the final cell concentration reached 22.6 g/l in the intermittent fed-batch culture. Then xylitol bioconversion stage started by adding 50 g/l xylose and 25 g/l gluconic acid into the culture broth (Fig. 4B). The cell concentration in the fermenter was maintained at a constant level, 22 to 24 g/l, during the xylitol fermentation period. A maximum concentration of xylitol was 42.4 g/l in 4 days fermentation with the xylitol yield of 97%. The volumetric productivity increased to 0.44 g/l-h, corresponding to a two-fold enhancement compared with the previous batch culture.

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