# Production of Some Sugar Derivatives by Methanol Yeast

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# INTRODUCTION

Methylotrophic yeasts are any strains of yeast which can utilize C1-compound, e.g. methane or methanol, as the sole source of carbon for growth. In this study, a number of yeast strains who can grow on methanol, simply called methanol yeast, are used. In general, methanol yeasts utilize the C1-carbon source through the assimilation and dissimilation pathways (Tani, 1984).

In the assimilation pathway, the formaldehyde formed from oxidation of methanol is fixed with xylulose 5-phosphate to enter the xylulose monophosphate pathway (XMP-pathway) with formation both dihydroxyacetone and glyceraldehyde 3-phosphate (Fig. 1). These trioses will regenerate the xylulose 5-phosphate to keep the cycle in the pathway. The XMP pathway was firstly proposed by van Dijken *et al.* (1978), and was established after a novel formaldehyde-fixing enzyme, dihydroxyacetone synthase was found by Kato *et al.* (1979) and Waites and Quayle (1980).

In the dissimilation pathway, methanol is oxidized to $CO_2$ through formaldehyde and formate, which involved alcohol oxidase/catalase system in peroxisome, formaldehyde dehydrogenase, S-formylglutathione hydrolase, and formate dehydrogenase. At first, this pathway was supposed to be essensial as the energy supply for methylotrophic growth. The recent report (Sakai *et al.*, 1997) confirmed that this pathway functions mainly in detoxication of cells from formaldehyde and formate rather than to stimulate the energy generation.

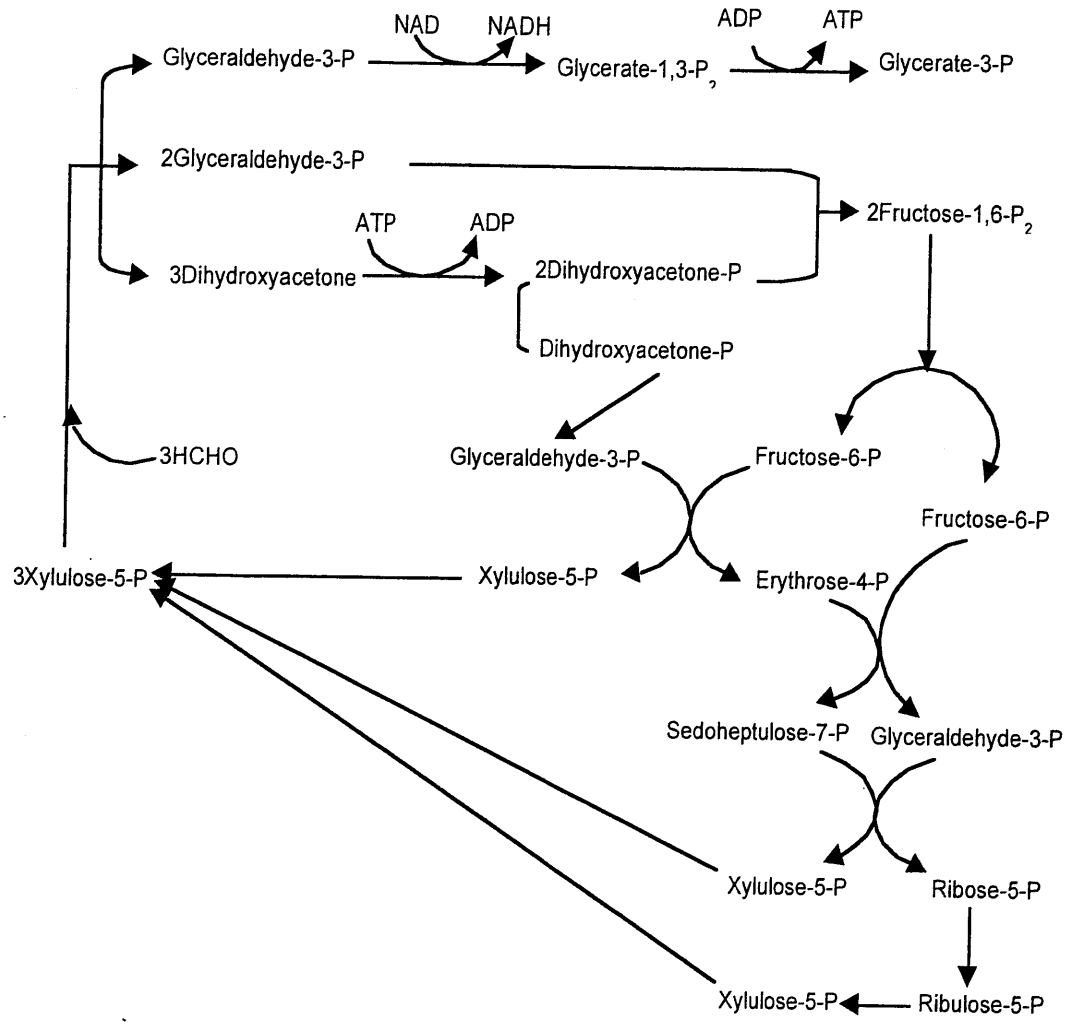


**FIG. 1. The xylulose monophosphate pathway in methanol-utilizing yeast.**

Since it was firstly isolated by Ogata *et al.* in 1969, the methanol yeast has been studied from various aspects by many groups of researcher. Processes to produce valu-able chemicals (such as, amino acids, citric acids, polyols, nucleotides and flavin co-enzymes) have been proposed using methylotrophic yeast (Tani, 1984). However, the report on sugars derivatives were limited. Polyols are interesting compounds and sim-ple derivatives of sugars, which produced by reduction of aldehyde function of sugars in concomitant with oxidation of NAD(P)H to $NAD(P)^+$.

Naturally-occurring polyols are found in small quantities, mainly in fruits and vegetables (Washuttl *et al.*, 1973). Polyols, which are used as sweetener for sucrose substitute, possess some physical and chemical properties similar to those of sucrose and have significantly less energy than sucrose. Some of its functional properties are described in Table 1. The taste of polyols varies and their sweetness ranges from the highest in xylitol to the lowest in lactitol. On the basis of sweetness per gram, xylitol was considered as being "isosweet" with sucrose. Maltitol, mannitol and sorbitol and the maltitol products were rather less sweet, with sweetness value ranging from 45 to 90% of the sweetness of sucrose. Some polyols in crystalline form are characterized by their intense cooling effect which improves taste in confectionaries. Xylitol is felt very cool because of its highly negative heat of solution (-153 kJ/g or –36.6 kcal/g). Erythritol is felt quite cool, sorbitol and mannitol produce a significant cooling effect as well, whereas maltitol, isomalt, maltidex and hydrogenated glucose syrup (HGS) do not (Dills, 1989; Bornet, 1994).

**Table 1. Functional properties of undigestible sugars.**

| Undigestible sugars | Sweetness (sucrose=1) | Taste of dry sugar | Stability | | Viscosity | Higroscopicity |
|---|---|---|---|---|---|---|
| | | | Heat ( C) | pH | | |
| Sorbitol | 0.70 | Cool | < 160 | 2-10 | Low | High |
| Xylitol | 0.90 | Very cool | < 160 | 2-10 | Very low | High |
| Mannitol | 0.50 | Cool | < 160 | 2-10 | Low | Low |
| Erythritol | 0.65 | Cool | < 160 | 2-10 | Very low | Low |
| Maltitol | 0.75 | None | < 160 | 2-10 | High | Mild |
| Isomalt (Palatinit) | 0.60 | None | < 160 | 2-10 | High | Low |
| Lactitol | 0.40 | Slightly cool | < 160 | 2-10 | Very low | Mild |
| Maltidex | 0.75 | None | < 160 | 2-10 | High | Mild |
| Fructooligosaccharides | 0.30 | = Sucrose | < 140 | > 3 | = Sucrose | Mild |

Generally, the viscosity of monosaccharide polyols are lower than sucrose, and depend on their molecular weight. The viscosity of disaccharide polyols except lactitol and HGS are higher than that of sucrose. The food product with a particular mouth-feel match to that of sucrose can be obtained by combination of these two different types of polyols or polyol with HGS. Polyols have also anticariogenicity, which is valuable for prevention of caries, especially among the children (idem, 1994; Pepper and Olinger, 1988).

Monosaccharide polyols are slowly absorbed or incompletely metabolized. Lactitol is totally undigestible, while maltitol and hydrogenated glucose syrup are partially digested by α-amylase and maltase (Patil *et al.*, 1987; Nilsson and Jagerstad, 1987). These properties are resulting in low glycemic and insulinemic responses in healty and diabetic subjects after ingestion with polyols.

Another features of polyols are used as starting materials for production of rare sugar, such as D-tagatose, L-xylulose and L-sorbose (Izumori *et al.*, 1984; Khan *et al.*, 1991; Kosseva *et al.*, 1991).

So far, polyols have been produced commercially by the chemical reduction process of the corresponding sugars. The sugars can be derived from hydrolysis of starch or sucrose (starch or sucrose pathway, Fig. 2), and wood hydrolysates as well. The process are involving hydrogenation step at high temperature and pressure (80-140°C, about 40-50 atm), and purification step with ion-exchange chromatography (fractionation) and crystalization. Therefore, the microbial production of polyols has attracted much attention because of the existing drawbacks in the purification step above (Bornet, 1994; Hyvonen and Koivistoinen, 1982; Winkelhausen and Kuzmanova, 1998). Recently, erythritol can be industrially produced through aerobic fermentation

of glucose by yeast. This process is more efficient than the chemical reduction due to uneconomical price of its precursor, dialdehyde starch (Roper *et al.*, 1993).

Recently, a considerable effort has been made on microbial production of polyols, especially xylitol. The fermentation processes have been studied with various yeasts, such as *Candida boidinii* (Tani and Vongsuvanlert, 1987; Vongsuvanlert and Tani, 1989; idem, 1988, Vandeska *et al.*, 1995), *Candida tropicalis* (Gong *et al.*, 1981, Yahashi *et al.*, 1996), *Candida* sp. L-102 (Lu *et al.*, 1995), *Pachysolen tannophilus* (Jeffries, 1983), and *Candida guillermondii* (Barbosa *et al.*, 1988, Meyrial *et al.*, 1991).

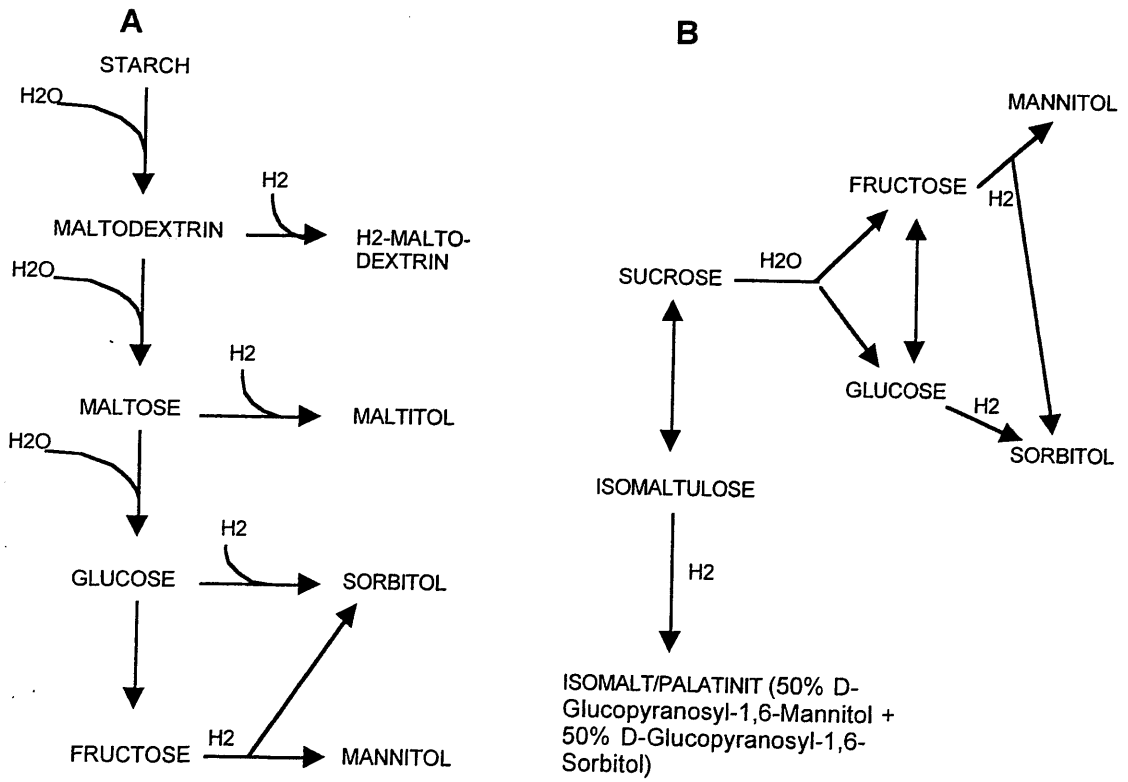


**FIG. 2. Scheme of industrial production of polyols from starch (A) or sucrose (B).**

In this study a new polyols producer was screened from a number of methanol yeasts with various sugars as substrate (Chapter I). As the result, *Hansenula polymorpha* was selected for production of xylitol from D-xylose. Optimization of culture conditions was carried out, including nitrogen sources, initial cell density, $Mg^{2+}$ concentration, aeration, and substrate concentration as well (Chapter II).

An osmolite, glycerol, was examined for its potential as non-toxic co-substrate instead of methanol, for bioconversion of substrate D-xylose at high concentration (Chapter III). This idea came from the results in Chapter II, that D-xylose utilization decreased by the increase of sugar concentration more than 100 g/*l*.

Beside the experiment with glycerol, a mutant tolerance to high osmotic pressure environtment was also sought using chemical (EMS) and physical (UV) mutagenesis. This tolerant mutant was found, but it showed bad growth and had no or very low activity for D-xylose conversion to xylitol. On the other side, a flavinogenic mutant of *C. boidinii* was also found, which secreted riboflavin during growth in YPD medium. The potential of flavinogenic microorganism for industrial production of vitamin $B_2$ has been recognized early. The early fermentation studies carried out with flavinogenic ascomycetes, *Eremothecium asbyii* (MacLaren, 1952), suggested a biosynthetic connection between purine and riboflavin. The terminal reactions sequence of riboflavin biosynthetic pathway, and formation of its co-enzyme FMN and FAD have been developed relative recently. A four carbon precursor for formation of lumazine was confirmed its origin from the pentose phosphate pool (Bacher *et al.*, 1996). Characterization of the flavinogenic mutant of *C. boidinii* is described in Chapter IV.

# CHAPTER I

## Polyol Production by Methanol Yeasts

In order to develop a biotechnological process for polyol production by methanol yeast, this preliminary study was carried out. A number of the methanol yeasts were screened for their ability to convert sugars including hexose and pentose, disaccharide, and oligosaccharide to the corresponding polyols in basal medium containing yeast extract, supplemented with methanol. Some experiment to study galactitol and ribitol production in test tube scale were also performed.

## MATERIALS AND METHODS

**Microorganisms and cultivation** Six strains of methanol-utilizing yeasts including *Candida boidinii* no. 2201 (AKU 4705), *Hansenula polymorpha* DL-1 (AKU 4327), *Hansenula ofunaensis* (AKU 4328), *Pichia pinus* (AKU 4259), *P. pastoris* (AKU 4264) and *Saccharomyces* sp. H1 (AKU 4137) were obtained from the Faculty of Agriculture, Kyoto University, Kyoto, Japan. The yeasts were maintained on YPD-agar medium containing 5 g of yeast extract (Difco Laboratories, Detroit, MI), 5 g of Polypepton® (Nihon Pharmaceuticals Co., Tokyo), 5 g of malt extract (Nihon Pharmaceuticals), 15 g of D-glucose, and 15 g of agar in 1 *l* of tap water. Basal medium for cultivation contained 4 g of $NH_4Cl$, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 4 g of yeast extract in 1 *l* of water, pH 6. Inoculum was prepared according to Vongsuvanlert *et al.*, (1989) using a 500-ml Erlenme-

yer flask containing 100 ml of basal medium supplemented with 1% glucose, and cultivated under rotary shaking at 200 rpm, 30°C for 24 h. Cells were collected by centrifugation at 5000 x g for 5 min, washed with physiological saline and resuspensed with sterile water.

**Chemicals** Sugars and polyols used were D-glucose, D-fructose, D-arabinose, D-ribose, D-xylose, D-galactose, L-sorbose, L-rhamnose, D-mannose, lactose, sucrose, maltose, sorbitol, galactitol, ribitol, iditol, xylitol, arabitol, rhamnitol, mannitol, lactitol, and maltitol. They were purchased from the usual commercial sources and were of reagent grade.

**Screening of yeasts for polyol production** Screening of polyol-producing yeast was conducted with 5 ml of the basal medium in 16.5 x165-mm test tube supplemented with 3% (w/v) sugar as substrate and 2% (v/v) methanol as co-substrate. The cultures were incubated at 30°C, 150 rpm for 3 d. Periodically, sample was drawn from each tube, and analyzed for growth and polyol formation. Some experiments to study the D-ribose and D-galactose conversion were also performed in test tubes containing the same basal medium supplemented with 3.6 % sugar.

**Analytical methods** Cell concentration were estimated turbidometrically at 660 nm, after sample dilution to an appropriate range of optical density range (0.1 – 0.5). One OD unit corresponded to 0.23 mg dry cells/ml. For screening of sugar alcohols producers, standard and sample solutions were identified by thin-layer chromatography on silica gel 60 plates (E. Merck, Darmstadt, Germany) impregnated with 20 mM sodium acetate using n-butanol-acetone-water [8:10:2, by volume] as a solvent. Separation was achieved by two-step development and the plate was sprayed with anisaldehyde-sulfuric acid reagent, and the spots were visualized by heating at 90°C for 5-10

min (Stahl, 1969). D-Xylose and xylitol were determined by HPLC with pump LC-10AD, column oven CTO-10A, system controller SCL-10A, automatic injector SIL-10A, and integrator Chromatopac CR-7A (Shimadzu Corp., Kyoto), and with a Sugar AXG column, 4.6 x 150 mm (Tosoh Corp., Tokyo), at 65°C. Buffer solution, 0.7 M boric acid pH 8.7 was used as a solvent at a flow rate of 0.4 ml/min. The active components were detected by refractive index detector (Shodex RI-71, Showa Denko).

## RESULTS AND DISCUSSION

**Screening of yeast for polyol producer** The ability of six strains of methanol yeast to convert sugars to the corresponding polyols in the presence of methanol as co-substrate was tested. The results were shown in Table 2. *C. boidinii* converted D-glucose and D-fructose to sorbitol, D-ribose to ribitol, D-xylose to xylitol, D-galactose to galactitol, and L-rhamnose to rhamnitol; *H. polymorpha* converted D-fructose, D-galactose and D-xylose to the corresponding sugar alcohols; *H. ofunaensis* converted D-fructose and D-xylose to the corresponding sugar alcohols; *P. pinus* converted D-ribose, D-galactose and D-xylose to the corresponding sugar alcohols; *P. pastoris* and *Saccharomyces* convert D-xylose and D-galactose to the corresponding sugar alcohols, respectively. *H. polymorpha* and *P. pinus* produced higher amount of xylitol and galactitol than *C. boidinii* did. *H. polymorpha* produced xylitol whether the medium contained methanol or not (Table 3). These results were quite different with the result of Vandeska *et al.* (1995), where *C. boidinii* was better xylitol producer than *Hansenula anomala.*

**TABLE 2. Polyol production by methanol yeasts.**

| Sugar | *C. boidinii* | | *H. polymorpha* | | *H. ofunaensis* | | *P. pastoris* | | *P. pinus* | | *Saccharomyces* sp. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cell mass (g/*l*) | Polyol produced* | Cell mass (g/*l*) | Polyol produced* | Cell mass (g/*l*) | Polyol produced* | Cell mass (g/*l*) | Polyol produced* | Cell mass (g/*l*) | Polyol produced* | Cell mass (g/*l*) | Polyol produced* |
| D-Glucose | 10.5 | + | 11.1 | - | 6.4 | - | 10.9 | - | 5.7 | - | 5.9 | - |
| D-Fructose | 13.5 | +++ | 11.6 | ++ | 9.8 | ++ | 10.5 | - | 9.5 | - | 10.3 | - |
| D-Arabinose | 7.4 | - | 8.3 | - | 8.4 | - | 8.0 | - | 8.1 | - | 6.6 | - |
| D-Ribose | 10.7 | + | 12.2 | - | 10.4 | - | 8.8 | - | 9.4 | + | 10.5 | - |
| D-Xylose | 11.7 | ++ | 14.8 | +++ | 11.3 | + | 9.3 | + | 10.9 | ++ | 5.1 | - |
| D-Galactose | 9.4 | + | 10.7 | ++ | 9.0 | - | 12.1 | - | 9.6 | +++ | 5.6 | + |
| L-Sorbose | 0.4 | - | 2.4 | - | 2.1 | - | 2.5 | - | 2.5 | - | 1.7 | - |
| L-Rhamnose | 3.0 | ++ | 2.1 | - | 2.4 | - | 2.7 | - | 2.3 | - | 1.6 | - |
| D-Mannose | 11.8 | - | 11.6 | - | 11.0 | - | 11.1 | - | 10.3 | - | 4.5 | - |

*) +++, strongly positive ( > 0.02 g/h · g of dry cells); ++, positive ( 0.01 - 0.02 g/h · g of dry cells);+, weakly positive (< 0.01 g/h · g of dry cells);
-, negative (not detected).

Cultivation was carried out with 5 ml of the basal medium supplemented with 3% (w/v) sugar and 2% (v/v) methanol in 16.5 x 165 mm test tubes at 30°C with shaking at 150 rpm for 3 d.

**TABLE 3. Growth and xylitol yield from D-xylose by *C. boidinii and H. polymorpha.***

| Yeast | Methanol (1%, v/v) | Cell mass (g/*l*) | D-Xylose consumed (%) | Xylitol | |
|---|---|---|---|---|---|
| | | | | (g/*l*) | Yield (g/g) |
| *C. boidinii* | + | 2.9 | 38 | 30 | 0.79 |
| | - | 3.6 | 35 | 21 | 0.60 |
| *H. polymorpha* | + | 3.0 | 53 | 43 | 0.82 |
| | - | 3.9 | 75 | 38 | 0.51 |

Fermentation was done in a 30 ml of basal medium containing 10% D-xylose with or without methanol, in a 100-ml Erlenmeyer flask.

Other experiments for investigating the production of ribitol from D-ribose and galactitol from D-galactose was carried out by *C. boidinii* and *H. polymorpha,* respectively. The optimum pH for both D-ribose and D-galactose conversion was pH 8, using phosphate buffer as buffering agent (Fig.3 part A). At this condition 0.1% ribitol and 0.7% galactitol were obtained from 3 and 3.6% D-ribose and D-galactose, respectively, after 2 d of cultivation. Either at pH 5 or pH 9 showed low accumulation of galactitol, possibly due to inhibition of growth.

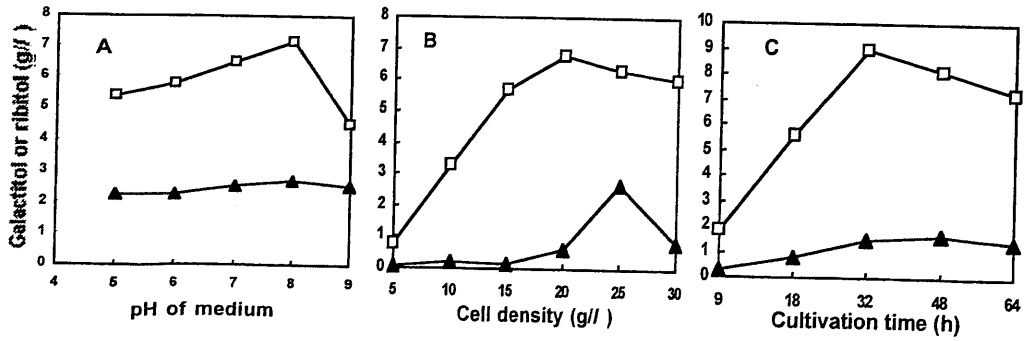


**FIG. 3. Effect of initial pH of medium (A), initial cell density (B), and cultivation periods (C).**

**□ , galactitol; ▲ , ribitol.**

The initial cell density was optimized by varying the biomass concentration from 5 to 30 g/*l* (as dry cell weight). The results were shown in Fig. 3 part B. Galactitol accumulation increased significantly by increasing the initial cell density and the maximum value was obtained when the initial cell density was 20 g/*l*. An amount of 6.8 g/*l* of galactitol was resulted from 36 g/*l* of D-galactose (yield, 0.19 g/g). For ribitol, the maximum conversion was obtained at initial cell density of 25 g/*l*, which produced 2.7 g/*l* of ribitol from 30 g/*l* of D-ribose (yield, 0.09 g/g). The ribitol yield soon decrease significantly when the initial biomass was 30 g/*l*, which mean most of the substrate was consumed for growth.

**Table 4. Effect of initial concentration of galactose and ribose on its polyol formation.**

| D-Galactose | | D-Ribose | |
|---|---|---|---|
| (g/*l* ) | Yield (g/*l* ) | (g/*l* ) | Yield (g/*l* ) |
| 18 | 3.6 | 15 | 0.2 |
| 36 | 4.5 | 30 | 1.2 |
| 54 | 4.0 | 45 | 2.2 |
| 72 | 3.6 | 60 | 4.5 |
| 90 | 3.6 | 75 | 5.4 |
| 108 | 3.2 | 90 | 6.0 |

Initial sugar concentration was also tested to find an optimum conversion conditions to polyols (Table 4). D-Ribose conversion was greatest at initial concentration of 60 g/*l* with the yield of ribitol 0.075 g/g. At initial D-ribose concentration of 75 g/*l*, ribitol concentration still increased, but the yield was lower than that of 60 g/*l* of D-ribose. For galactitol, the best yield (0.13 g/g) was obtained at initial D-galactose concentration of 36 g/*l*, which produced 4.5 g/*l* galactitol.

The greatest yield of galactitol was obtained by optimization the cultivation time (Fig. 3 part C). An amount of 9 g/*l* galactitol from 36 g/*l* D-galactose (yield, 0.25 g/g) was obtained after 36-h cultivation. In the case of ribitol, maximum conversion of D-ribose was obtained after 2 d of cultivation, which produced 1.65 g/*l* of ribitol from 30 g/*l* of D-ribose (yield, 0.06 g/g).

## SUMMARY

In this preliminary study, some potential polyol producer from methanol yeasts were found. *C. boidinii*, *H. polymorpha* and *P. pinus* were potential for production of sorbitol, xylitol, and galactitol, respectively. Either *C. boidinii* or *P. pinus* produced ribitol from D-ribose, but the yield was smaller than those of xylitol or galactitol. Among them, xylitol production from D-xylose by *H. polymorpha* showed the highest productivity, and was selected for further study.

# CHAPTER II

## Xylitol Production by a Methanol Yeast, *Hansenula polymorpha*

Among polyols, xylitol has sweetness properties more close to sucrose. Beside that, xylitol also has interesting properties such as anticariogenicity for prevention of dental caries, cooling effect for improving taste in confectionaries, low calory content, and elicit low glicemic and insulinemic response which are useful to patients with diabetes, heart deseases or cancer of gastrointestinal tract (Dills, 1989; Bornet, 1994; Hyvonen and Koivistoinen, 1982).

For application with commercial feasibility, xylitol can be produced from D-xylose derived from hemicellulose hydrolyzates of wood or agricultural waste (e.g., corn draff). These renewable resources have been considered as an inexpensive raw material for bioconversion of many useful products (Gong *et al.*, 1981; Magee and Kosaric, 1985; Kennedy and Paterson, 1989). In this chapter the optimization of xylitol production from D-xylose by *Hansenula polymorpha* is described, using methanol as co-substrate.

## MATERIALS AND METHODS

**Microorganism and medium** *H. polymorpha* used in the experiment was described in Chapter I, and maintained in YPD-agar medium. Basal medium for cultivation contained 4 g of $NH_4Cl$, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 4 g of yeast extract in 1 *l* of water, adjusted to pH 6.

**Culture conditions for xylitol production** Inoculum was prepared by modification of reported procedure (Tani and Vongsuvanlert, 1987). At the first step, the yeast cells were grown in the basal medium with 0.5 % glucose at 30°C under rotary shaking at 150 rpm for 24 h. In the second step, inoculum (0.1 volume) of the seed culture was transferred into the same basal medium supplemented with 1% (w/v) glucose and 1% (v/v) methanol, and cultivated under rotary shaking at 150 rpm and 30°C for 2 d. In other way, the seed culture was transferred into the same basal medium supplemented with 1% (v/v) methanol, and cultivated under rotary shaking at 150 rpm and 30°C until the logarithmic phase (about 30 h). The cells was collected by centrifugation, washed with saline and transferred again into the same basal medium supplemented with 1% glucose and 1% methanol, and cultivated by rotary shaking at 30°C and 150 rpm, for 2-3 d. Another 1% (v/v) methanol was added to the seed culture after the first day of cultivation from a methanol-KOH solution (50% methanol in 0.5 M KOH). Cultivation for production of xylitol was carried out at 30°C, under rotary shaking at 150 rpm in a 500-ml Erlenmeyer flask containing the basal medium and inoculum at initial cell concentration of 2.5 g/*l*. Urea was considered as a cheap nitrogen sources, and was examined for its optimum concentration. Other inorganic and organic nitrogen sources were also tested. Aeration was examined by variation of volume of the medium in a 500-ml Erlenmeyer flask, and D-xylose concentration was also examined from 22.5 to 300 g/*l*.

**Analytical Methods** Cells concentration were estimated turbidometrically at 660 nm, after sample dilution to an appropriate range of optical density range (0.1 – 0.5). One OD unit corresponded to 0.23 mg dry cells/ml. D-Xylose and xylitol were determined by HPLC with pump LC-10AD, column oven CTO-10A, system controller

SCL-10A, automatic injector SIL-10A, and integrator Chromatopac CR-7A(Shimadzu Corp., Kyoto), and with a Shodex Sugar SZ 5532 column, 4.6 x 150 mm (Showa Denko Corp., Tokyo), at 40°C. Acetonitrile–water (70:30,v/v) was used as a solvent at a flow rate of 0.8 ml/min. The active components were detected by refractive index detector (Shodex RI-71, Showa Denko).

## RESULTS AND DISCUSSION

**Nitrogen sources** Of the eleven nitrogen sources tested, urea, $(NH_4)_2SO_4$ and $NH_4NO_3$ were potential with high yield of xylitol (0.55 g/g) from 100 g/*l* D-xylose (Table 5). With urea, almost 100% of D-xylose was consumed by yeast cells, and

**TABLE 5. Effect of nitrogen sources on xylitol production by *H. polymorpha*.**

| Nitrogen Sources | Xylose consumed (%) | Cell mass (g/*l*) | Xylitol produced (g/*l*) | Xylitol yield (g/g) | Volumetric productivity (g/*l*.h) |
|---|---|---|---|---|---|
| $NH_4Cl$ | 91.0 | 4.7 | 41.4 | 0.53 | 0.43 |
| $CH_3COONH_4$ | 55.8 | 4.4 | 25.1 | 0.52 | 0.26 |
| $HCOONH_4$ | 52.7 | 4.3 | 19.8 | 0.44 | 0.21 |
| $NH_4HCO_3$ | 39.8 | 4.0 | 18.9 | 0.55 | 0.20 |
| $NH_4NO_3$ | 89.9 | 4.6 | 42.6 | 0.55 | 0.44 |
| $(NH_4)_2SO_4$ | 93.5 | 4.7 | 44.0 | 0.55 | 0.46 |
| Urea | 99.4 | 5.8 | 45.9 | 0.54 | 0.48 |
| Yeast extract | 82.6 | 4.6 | 33.2 | 0.47 | 0.35 |
| Polypeptone | 93.6 | 4.6 | 42.0 | 0.52 | 0.44 |
| Yeast nitrogen base (Difco) | 93.5 | 5.4 | 42.3 | 0.53 | 0.44 |
| Casamino acids (Nihon Pharmaceuticals) | 99.4 | 5.2 | 44.6 | 0.52 | 0.46 |

both growth and xylitol production increased in the first 4 d of cultivation. This result is similar to the result of Vandeska *et al.* (1995) with *C. boidinii* and Lu *et al.* (1995)

with *Candida* sp. L-102. With $(NH_4)_2SO_4$ and $NH_4NO_3$, about 90% of the substrate D-xylose was consumed and their xylitol yield was similar. These results are different from those of Lu *et al.*, who found that use of $NH_4NO_3$ gave greater xylitol yield and productivity than $(NH_4)_2SO_4$.

**TABLE 6. Effect of initial pH of medium on xylitol production.**

| pH | OD660 | D-Xylose used (%) | Xylitol produced (g./ ) | Xylitol Yield (g/g) | Productivity (g/*l* .h) |
|---|---|---|---|---|---|
| 4.5 | 48.0 | 79.5 | 33.5 | 0.42 | 0.35 |
| 5 | 51.2 | 81.0 | 34.0 | 0.42 | 0.35 |
| 6 | 49.2 | 72.8 | 34.7 | 0.48 | 0.36 |
| 7 | 50.8 | 84.8 | 36.2 | 0.43 | 0.38 |
| 8 | 51.1 | 94.1 | 43.2 | 0.46 | 0.45 |

**Initial pH of medium** Basal medium with urea as nitrogen source at various pH (from 4.5-8) was tested for xylitol production from D-xylose (Table 6). The greatest productivity and yield of xylitol was obtained at initial pH of 8 (0.45 g/*l*.h and 0.46 g/g, respectively). The increase of pH from 4.5 to 8 was concomitant with the increase of xylitol accumulation in the medium, which was optimum at pH 8 (43.2 g/*l*). At the same time 94% of substrate D-xylose was utilized by the yeast cells during 4 d of cultivation. This result was rather different with previous result for *C. boidinii* (Vongsuvanlert and Tani, 1989), where the optimum xylitol accumulation was obtained around pH neutral (pH 6-7). High yield of xylitol was also obtained at pH 6, but substrate utilization at pH 6 was lower than at pH 8. Cell growth was not significantly affected by variation of pH.

**TABLE 7. Effect of initial cell density on xylitol production.**

| Initial cells (g/*l* ) | D-Xylose used (%) | Growth (OD ) | Xylitol | | Productivity (g/*l* .h) |
|---|---|---|---|---|---|
| | | | (g/*l* ) | Yield (g/g) | |
| 2.5 | 86.7 | 40 | 33 | 0.38 | 0.34 |
| 3.5 | 76.7 | 46 | 33 | 0.43 | 0.35 |
| 5 | 77.5 | 54 | 35 | 0.45 | 0.36 |
| 7.5 | 83.5 | 63 | 35 | 0.42 | 0.36 |
| 10 | 90.5 | 77 | 40 | 0.44 | 0.41 |

**Initial cell density and $Mg^{2+}$ concentration** Initial cell density was optimized using the concentration ranging from 2.5 to 10 g/*l* in the basal medium with urea containing 100 g/*l* D-xylose (Table 7). The increase of initial cell concentration was followed by a little increase in xylitol accumulation. The greatest yield of xylitol (0.45 g/g) was obtained at initial cell concentration of 5 g/*l*. $MgSO_4 \cdot 7H_2O$ concentration was also optimized using the concentration ranging from 0.25 to 10 g/*l* in the basal medium with urea containing 90 g/*l* D-xylose. The increase of $Mg^{2+}$ concentration was concomitant with the increase of xylitol accumulated in the medium. The optimum result was obtained at $Mg^{2+}$ concentration of 1 g/*l*, and 31.5 g/*l* of xylitol was accumulated (yield, 0.38 g/g). Increasing the $Mg^{2+}$ concentration more than 1 g/*l* still increased the xylitol accumulation, but stimulated highly growth and much substrate was consumed for growth (data not shown).

**Effect of aeration** Aeration was more satisfactory from the point of view of xylitol production when 200 ml of medium in a 500-ml Erlenmeyer flask was used rather than 300 or 100 ml (Fig. 4), with shaking at 150 rpm and 30°C.

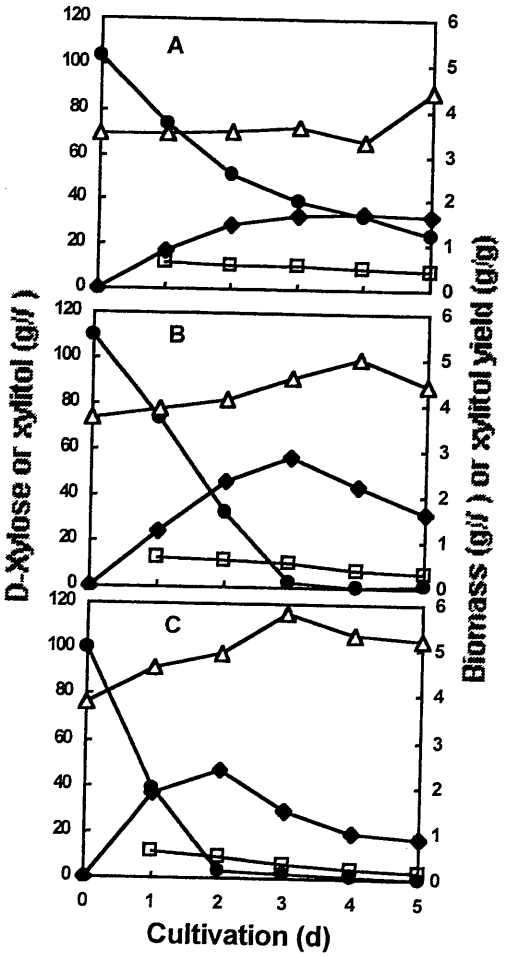


**FIG. 4. Effect of aeration level on growth and xylitol production. A, 300; B, 200; and C, 100 ml of medium, respectively, in a 500-ml Erlenmeyer flask. ● , D-xylose; ◆ , xylitol; △ , biomass; □ , xylitol-yield.**

At this condition 57 g/*l* of xylitol (yield was 0.52 g/g) was obtained from 110 g/*l* of D-xylose and 1% methanol in 3 d of cultivation. D -Xylose was utilized quickly in 100 ml and 200 ml of culture, and almost depleted after 2 and 3 d of cultivation, respectively. Xylitol accumulation peaked at this time also. That less xylitol accumulated in 100 ml of medium than in 200 ml might be accounted for by consumption of most of the substrate for growth. With 300 ml of medium, the substrate was consumed more slowly, resulting in slower growth and less xylitol accumulation. Under this condition xylitol accumulation soon reached a plateau by 3 d, and the curve did not peak.

**Effect of substrate concentration** D-Xylose utilization was greatest when its concentration was about 100 g/*l* (Fig. 5). A further increase of substrate concentration more than 10% increased the xylitol yield by decreasing the percentage of substrate consumption for growth, but the optimum harvesting time was delayed. When the initial D-xylose concentration was increased 6-fold (from 22.5 g/*l* to 135 g/*l*), xylitol produced increased 20-fold (from 2.2 g/*l* to 43.2 g/*l*) in 2 d of cultivation. The xylitol concentration decreased when the concentration of xylitol that had accumulated was equal to or higher than the concentration of D-xylose, after 36-48 h of cultivation (Fig.7, A-C). At this condition, both D-xylose and xylitol were being consumed by the yeast cells, which highly stimulate growth. At the initial D-xylose concentration of 22.5 and 95 g/*l*, xylitol accumulation curve peaked, but at concentration of 135 and 155 g/*l*, the curve did not peak. An increase of D-xylose concentration up to 300 g/*l* inhibited substrate utilization. From these results and other data, the culture conditions for xylitol production were set as follows: 200 ml of a medium in a 500-ml Erlen-

meyer flask, composed of 2.5 g of urea, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 1 g of $MgSO_4 \cdot 7H_2O$, 95-135 g of D-xylose, and 1% (v/v) methanol in 1 *l* of water pH 8.

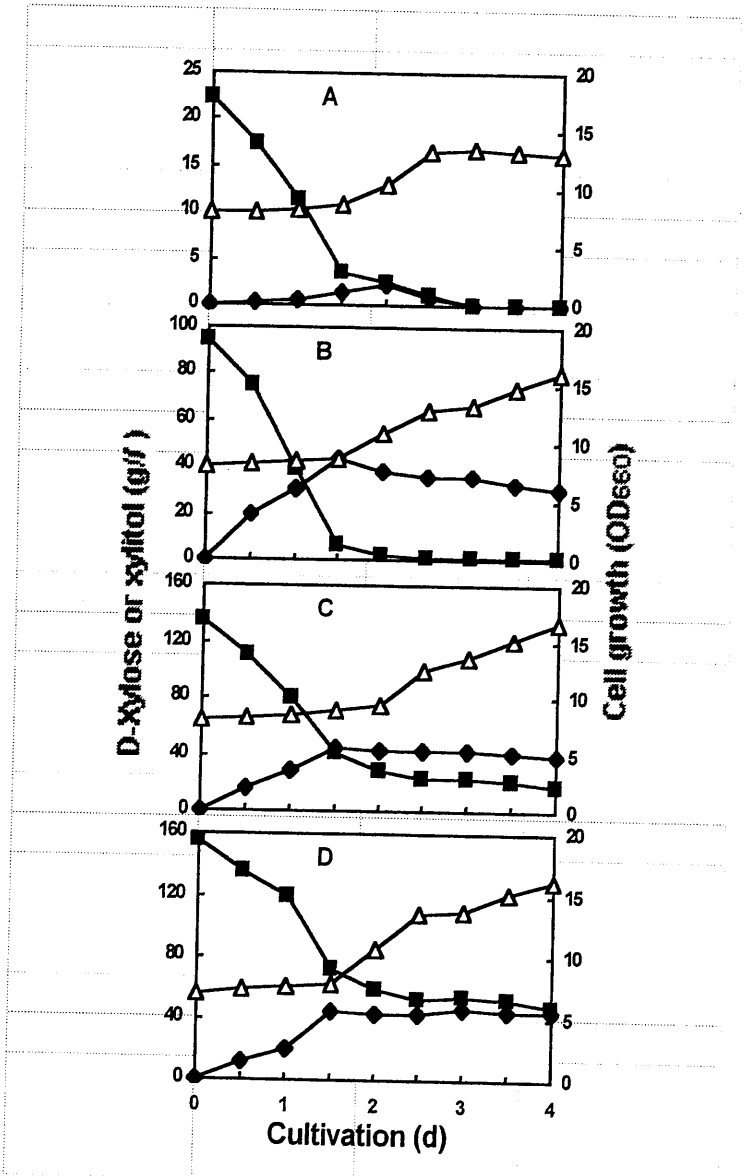


**FIG. 5. Effect of D-xylose concentration on growth and xylitol production. A, 22.5; B, 95; C,135; and D, 155 g/*l* D-xylose, respectively. ■ , D-xylose; ◆ , Xylitol; △ , $OD_{660}$.**

## SUMMARY

The optimum conditions for production of xylitol from D-xylose by *H. polymorpha* were developed in the experiments above with methanol as co-substrate. The culture medium was constructed as composed of 2.5 g of urea, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 1 g of $MgSO_4.7H_2O$, 95-135 g of D-xylose, and 1% (v/v) methanol in 1 *l* of water pH 8. The culture conditions were carried out microaerobically with 200 ml of a medium in a 500-ml Erlenmeyer flask at 30°C under rotary shaking at 150 rpm. Productivity was the greatest when inoculum at initial density of 5 g/*l* and 2.5 g/*l* of urea was used as nitrogen source, respectively. Under these conditions, 57 g/*l* xylitol was obtained from 110 g/*l* D-xylose and 1 % (v/v) methanol after 3 d of cultivation.

# CHAPTER III

## Effect of Glycerol on Xylitol Production by *Hansenula polymorpha*

Glycerol can be utilized as carbon source and energy by a number of yeasts, including some methanol yeast (Gancedo *et al.*, 1968; Babel and Hoffmann, 1982). This compound and other neutral compound with 2 or 3 carbons could penetrate the yeast cell through simple diffusion. This passive transport required less or no energy, which might be different from the transport system for sugar compound.

In the previous chapter, D-xylose was converted to xylitol effectively when D-xylose concentration was about 100 g/*l*, with 1-2 % (v/v) methanol as co-substrate. When D-xylose concentration was 125 g/*l* or higher, conversion became inefficient due to a decrease in D-xylose utilization and xylitol formation. These effects was supposed as the results of intolerance of the yeast cells to high osmotic pressure environtment at high sugar concentration. In order to solve these problems, supplement of an osmolite, glycerol, was studied to protect the cells from high osmotic pressure, and to function as co-substrate for methanol substitute.

## MATERIALS AND METHODS

**Microorganism and medium** Methanol yeast, *Hansenula polymorpha* used in the experiment was maintained on YPD-agar medium, as described in previous chapters. The basal medium for cultivation contained 4 g of $NH_4Cl$, 1 g of $KH_2PO_4$, 1 g of

$K_2HPO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.05 g of $FeSO_4.7H_2O$, and 4 g of yeast extract in 1 *l* of water, pH 6.

**Inoculum preparation and culture conditions for xylitol production** Inoculum was prepared using one of the two procedure described in Chapter II. At first, the yeast cells were grown in the basal medium with 0.5 % glucose at 30°C under rotary shaking at 150 rpm for 24 h. Then, the seed culture was transferred into the same basal medium supplemented with 1% (v/v) methanol, and cultivated in the same manner till the logarithmic phase (about 30 h). The cells were collected by centrifugation, washed with saline and transferred again into the same basal medium supplemented with 1% glucose and 1% methanol, and cultivated in the same manner for 2-3 d. Another 1% (v/v) methanol was added to the seed culture after the first day of cultivation from a methanol-KOH solution (50% methanol in 0.5 M KOH). For glycerol grown cells, inoculum was prepared by growing the cells in 1% (w/v) glycerol instead of methanol. Cultivation for production of xylitol was carried out at 30°C, with rotary shaking at 150 rpm in a 500-ml Erlenmeyer flask containing basal medium and inoculum at initial cell concentration of 5 g/*l*.

**Analytical Methods** Cells concentration were estimated turbidometrically at 660 nm, after sample dilution to an appropriate range of optical density range (0.1 – 0.5). One OD unit corresponded to 0.23 mg dry cells/ml. D-Xylose and xylitol were determined by HPLC with pump LC-10AD, column oven CTO-10A, system controller SCL-10A, automatic injector SIL-10A, and integrator Chromatopac CR-7A(Shimadzu Corp., Kyoto), and with a Shodex Sugar SZ 5532 column, 4.6 x 150 mm (Showa Denko Corp., Tokyo), at 40°C. Acetonitrile–water (70:30,v/v) was used as a solvent at a

flow rate of 0.8 ml/min. The active components were detected by refractive index detector (Shodex RI-71, Showa Denko).

## RESULTS AND DISCUSSION

**Utilization of D-xylose and Glycerol by *H. polymorpha*** D-Xylose and glycerol utilization was observed in 16.5x165-mm test tube containing the basal medium supplemented with 15 g/*l* of single (D-xylose or glycerol) and mixed substrate (D-xylose plus glycerol). The results was shown in Fig. 6. When only D-xylose was used as carbon source, xylitol was quickly accumulated after 6 h, but soon consumed by the yeast cells together with D-xylose, and decreased to its original concentration after 25 h of cultivation. In another case, glycerol was consumed very slowly by the yeast cells.

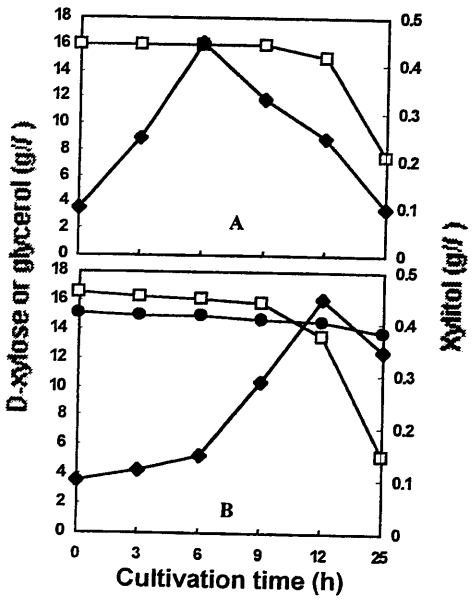


**FIG. 6. Utilization of D-xylose and glycerol by *H. polymorpha*. A, single substrate; B, mixed substrate. □, D-xylose; ◆, xylitol; ●, glycerol.**

When the two carbon source was mixed, an interesting phenomenon was observed. D-Xylose was utilized a little faster than before and xylitol accumulation was slowly, and peaked when D-xylose concentration has decreased. The xylitol was more stable at its peak than in the culture with single substrate. The existence of glycerol in the culture seem to have a protection effect against xylitol utilization. This effect was not observed when methanol was used instead of glycerol.

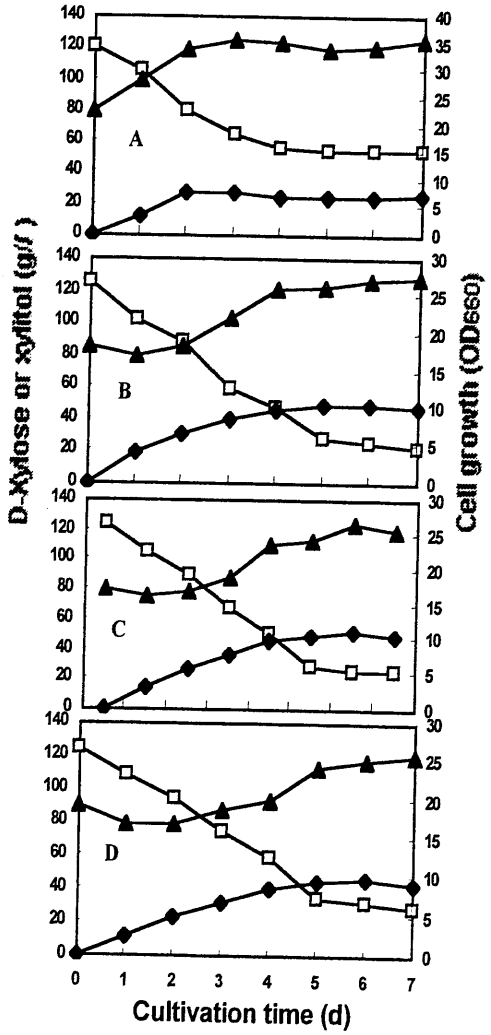


**FIG. 7. Effect of various glycerol concentrations on growth and xylitol production. A, 1%; B, 2.5%; C, 5%; and D, 7.5% (w/v) of glycerol, respectively. □, D-xylose; ♦, xylitol; ▲, OD660.**

**Effect of various glycerol concentration on xylitol production** Various concentrations of glycerol from 0.5 to 7.5% (w/v) were examined as co-substrate for xylitol production from D-xylose. Cultivation was performed in a 500-ml Erlenmeyer flask containing 200 ml of medium supplemented with 125 g/*l* D-xylose. The results were shown in Fig. 7. Glycerol at concentration of 1% showed an inhibition to xylitol accumulation, but stimulated growth earlier than those of glycerol 2.5% or higher. The similar result was obtained with 2% (w/v) glycerol ( result not shown). When glycerol concentration was 2.5%, growth was kept in the lag phase until about 3 d of cultivation, and showed an increase on day 4. During the growth inhibition, most of D-xylose utilized was converted to xylitol. Duration of growth inhibition seemed to have relation with glycerol concentration, since with 7.5% glycerol showed longer lag phase than 2.5 or 5% glycerol. In contrast, D-xylose utilization was slightly slower at 7.5% glycerol than those of 2.5 or 5% glycerol, probably due to increase of glycerol utilization by the cells or glycerol diffusion through cell membrane. The highest xylitol yield (51.6 g/*l*) was obtained in the culture from 125 g/*l* D-xylose, supplemented with 5% glycerol after 6 d of cultivation.

**Effect of glycerol or methanol as co-substrate for xylitol production** Methanol yeasts assimilate glycerol through the oxidative pathway, which involves $NAD^+$-linked glycerol dehydrogenase (Babel and Hofmann, 1982; Kong *et al.*, 1985). NADH resulting from glycerol oxidation probably is used for conversion of D-xylose to xylitol. In the previous report (Vongsuvanlert and Tani, 1989), an increase of xylitol production in the presence of 2% methanol was observed in a culture of *C. boidinii*. In this study, the increase of xylitol yield was observed at glycerol concentration of 2.5% or

higher. At the glycerol concentration of 5%, the highest yield of xylitol (58.3 g/*l*; yield, 0.62 g/g) was obtained from 125.0 g/*l* D-xylose in 4 d of cultivation (Table 8).

**TABLE 8. Effects of methanol or glycerol as co-substrate on xylitol production by *H. polymorpha*.**

| Initial xylose (g/*l*) | Initial methanol or glycerol (%, w/v) | Cultivation time (d) | Cell mass (g/*l*) | D-Xylose consumed (%) | Xylitol | |
|---|---|---|---|---|---|---|
| | | | | | (g/*l*) | Yield (g/g) |
| | Methanol | | | | | |
| 125 | 2.4 | 3 | 7.7 | 77 | 47 | 0.49 |
| | | 4 | 7.8 | 89 | 46 | 0.41 |
| 150 | 2.4 | 4 | 7.3 | 75 | 49 | 0.44 |
| | | 5 | 7.5 | 84 | 47 | 0.38 |
| | Glycerol | | | | | |
| 125 | 5 | 3 | 8.4 | 61 | 48 | 0.62 |
| | | 4 | 8.8 | 75 | 58 | 0.62 |
| 150 | 5 | 4 | 8.3 | 64 | 53 | 0.55 |
| | | 5 | 9.0 | 79 | 64 | 0.54 |

With glycerol as co-substrate, at high D-xylose concentration of 150 g/*l*, *H. polymorpha* still showed an increase of D-xylose consumption and xylitol concentration (63.7 g/*l*). These results supported the author's hypothesis that glycerol can function as cells protector against high osmotic pressure, as product (xylitol) protector against utilization by the cells, and also as co-substrate for methanol substitute.

**Effect of methanol-grown or glycerol-grown inoculum** Either methanol or glycerol can be utilized by the methanol yeast as their sole carbon and energy source (Babel and Hoffmann, 1982). Xylitol production using methanol-grown and glycerol-grown cells of *H. polymorpha* was compared (Fig. 8). The results showed that inoculation of methanol-grown cells gave higher xylitol yield and productivity than that of glycerol-grown cells. With methanol-grown cells, 63.5 g/*l* xylitol was obtained from

150 g/*l* D-xylose after 5 d of cultivation. With glycerol-grown cells, 59 g/*l* xylitol was obtained from 150 g/*l* D-xylose after 6 d of cultivation. These results are consistent with differences in the specific activity of dihydroxyacetone kinase in methanol-grown and glycerol-grown cells of *H. polymorpha* (Babel and Hoffmann, 1982; Tani and Yamada, 1987), and support my expectation that *H. polymorpha* is a good xylitol producer.

These results showed that *H. polymorpha* DL-1 is a promising yeast for xylitol production. The xylitol yield and productivity might be further improved. For commercial feasibility, fermentation could be performed by fed batch or continuous culture techniques.

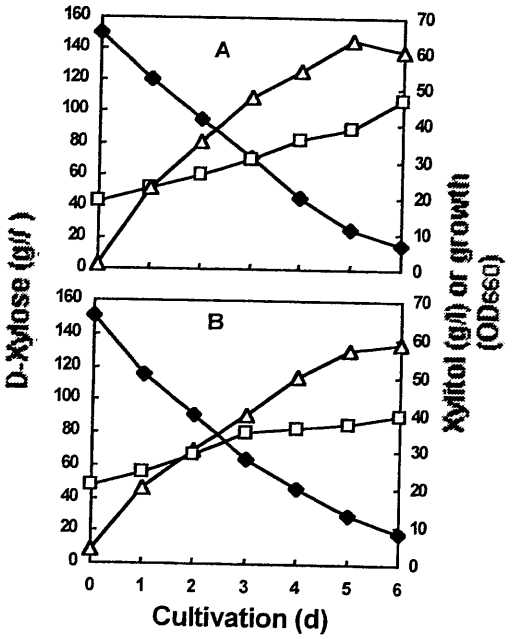


**FIG. 8. Effect of methanol-grown cells (A) and glycerol-grown cells (B) on growth and xylitol production by *H. polymorpha*. ◆ , D-xylose; △ , xylitol; □ , growth (OD660).**

## SUMMARY

A new aspect for xylitol production from D-xylose at considerable high concentration was developed using glycerol as co-substrate for methanol substitute. Glycerol at concentration of 2.5-5 % (w/v) showed a positive effect to xylitol accumulation, while time to start the growth was delayed. The highest yield of xylitol (58.3 g/*l*) was obtaied from 125 g/*l* D-xylose in 4 d of cultivation, when 5% glycerol was used as co-substrate. With 5% glycerol as co-substrate and 150 g/*l* D-xylose, *H. polymorpha* showed an increase of D-xylose consumption and xylitol accumulation. Inoculation of methanol-grown cells gave better xylitol yield and productivity than glycerol-grown cells.

# CHAPTER IV

## Characterization of a Flavinogenic Mutant of Methanol Yeast *Candida boidinii* and Its Extracellular Secretion of Riboflavin

The methanol yeast, *Candida boidinii* no. 2201, has potential for production of some valuable chemicals, such as amino acids, citric acid, sugar alcohols, nucleotides and flavin coenzyme (Tani, 1984). When glucose was used as the carbon source, expression level of the oxidative pentose phosphate pathway enzymes such as glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) were twice higher than those of methanol as a carbon source (Kato *et al.*, 1979; Sahm, 1977). Both enzymes, G6PDH and 6PGDH, are responsible for reduction of $NADP^+$ to NADPH, which is necessary for formation of amino acids, lipids, nucleotides, cell wall and other cellular components (Ihnen and Demain, 1969). Mutation in the structural gene for G6PDH phenotypically resulted in, for example, pleiotrophic effect, which is responsible for morphological changes in *Neurospora crassa* (Scott and Tatum, 1970).

In the present study, we isolated and characterized a flavinogenic mutant of *C. boidinii* no. 2201. The mutant lacked in both G6PDH and 6PGDH, and showed the increase of flavinogenic activity resulted from the increasing level of its precursors, which is synthesized through the pentose phosphate pathway. The relation between pentose phosphate precursor and the biosynthetic pathway of riboflavin has been described previously (Bacher *et al.*, 1996; see Fig. 9). The mutant cells secreted riboflavin during growth in medium with glucose and other non-fermentable carbon sources.

Some conditions for riboflavin production were also studied.

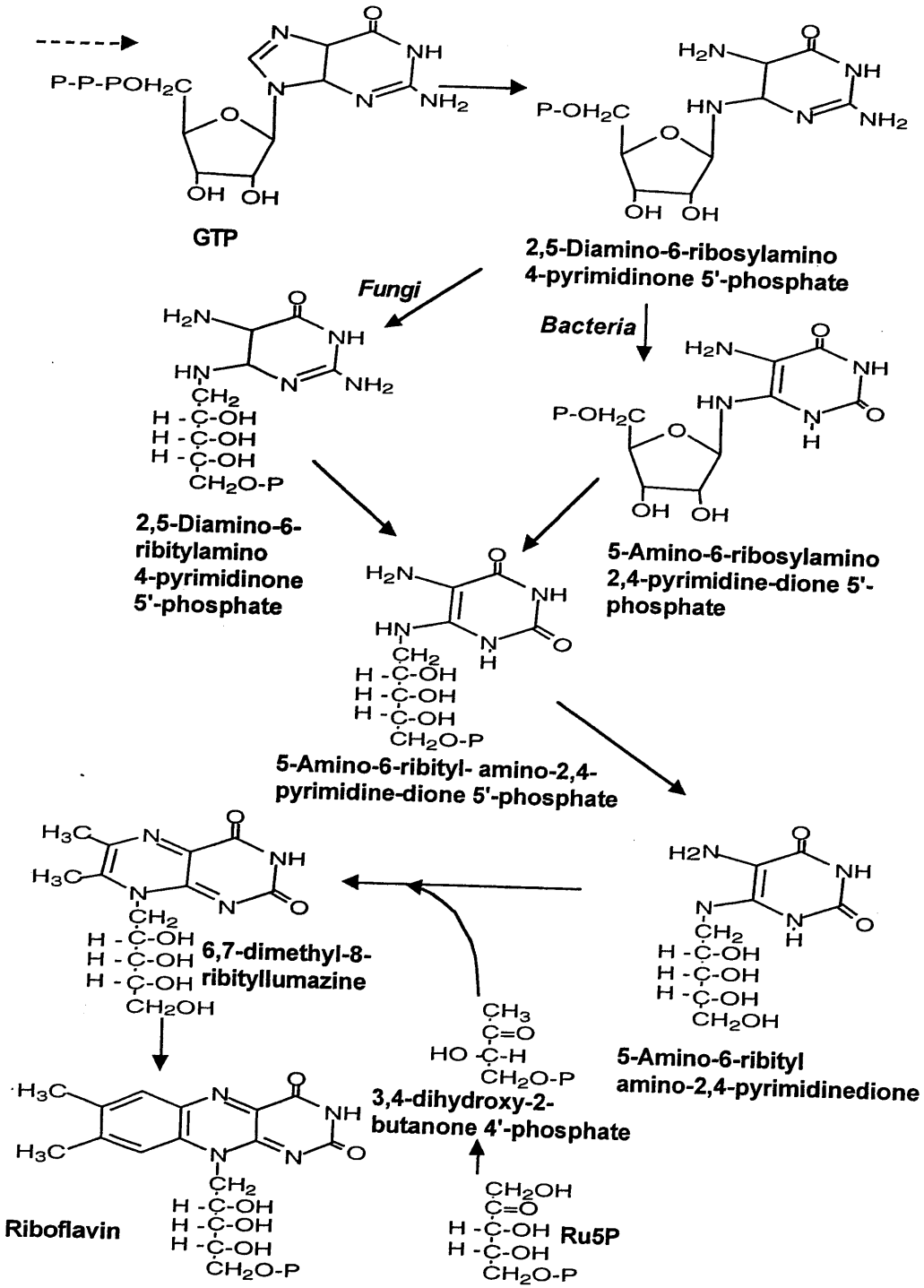


**FIG. 9. Biosynthetic pathway of riboflavin in fungi and bacteria.**

# MATERIALS AND METHODS

**Microorganism and medium** *C. boidinii* no. 2201 was obtained from Faculty of Agriculture, Kyoto University, Kyoto. Both the wild type and the mutant were maintained on YPD medium, which was composed of 10 g of yeast extract (Difco Laboratories, Detroit, MI, USA), 10 g of peptone (Polypepton®, Nihon Pharmaceutical Co., Tokyo), and 10 g of glucose in 1 *l*, pH 6.5, and solidified with 20 g of agar in 1 *l*. The basal medium for cultivation was composed of 4 g of $NH_4Cl$, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 1 g of yeast extract in 1 *l*, pH 6.5. The minimal medium was composed of 6.7 g of yeast nitrogen base without amino acid (Difco) and 20 g of glucose in 1 *l*.

**Inoculum and cultivation conditions** The seeds culture was prepared in 16x165-mm test tubes containing 5 ml of YPD medium by shaking at 30°C and 200 rpm for 24 h. The inoculum (1%, v/v) was transferred to 100 ml of basal medium supplemented with 10 g/*l* of glucose in a 500-ml Erlenmeyer flask. Cultivation was carried out under rotary shaking at 150 rpm and 30°C for 2 d or until the early stationary phase of the growth, and used to prepare cell-free extract. For riboflavin production, inoculum was prepared in YPD medium by shaking at 30°C and 150 rpm for 2 d. Riboflavin production was carried out in a 500-ml Erlenmeyer flask containing the basal medium supplemented with 30 g/*l* of sucrose and 8 g/*l* of yeast extract, and cultivated at 30°C and 150 rpm. The medium was prepared free of iron by extraction with 8-hydroxyquinoline in chloroform (Tanner *et al.*, 1945).

**Mutagenesis and isolation of mutants** Mutagenesis was performed by ethyl methanesulfonate (EMS) followed by UV irradiation to produce the most stable form of

mutation (Spencer and Spencer, 1991). It was carried out according to published reports with a little modification (Lindegren *et al.*, 1965; Matsutani *et al.*, 1992; Kong *et al.*, 1987). The mutant strains were isolated by indirect selection (Kong *et al.*, 1987).

The auxotrophy test was carried out in 16x165 mm test tubes containing 5 ml of minimal medium supplemented with all but one of the amino acids and growth factors (Spencer and Spencer, 1991), as follows (in mg/*l*): adenine · HCl (20), L-arginine (10), L-histidine (10), L-isoleucine (60), L-leucine (60), L-lysine · HCl (10), L-methionine (10), L-phenylalanine (20), L-tryptophan (20), L-tyrosine (10), L-valine (20), biotin (0.25), Ca-pantothenate (2.5), folate (0.25), inositol (40) nicotinate (1), pyridoxine · HCl (2.5), and thiamine · HCl (2.5). Solutions of these compounds were separately dissolved in water and sterilized by microfiltration.

**Preparation of cell-free extract** Cell-free extract was prepared as described previously (Tani and Yamada, 1987). Cells from the late logarithmic phase or early stationary phase were harvested by centrifugation, washed twice with 50 mM phosphate buffer (pH 7), suspended in 5-10 ml of the same buffer containing 0.5 mM dithiothreitol, and disrupted for 10 min in an ice bath by ultrasonication (20 kHz, about 300 W; Sonifier 450, Branson Sonic Power Co., Danbury, CT) at duty cycle of 30-40%. When almost all of cells were disrupted as seen under microscope, cells and other insoluble materials were precipitated by centrifugation at 14,500 x *g* for 10 min and removed.

**Enzyme assays** All assays were performed at 30°C or room temperature in quartz cuvettes using double beam spectrophotometer (UV-210, Hitachi Corp., Tokyo). One unit of enzyme activity is defined as the activity to form 1 μmol of product per min. Specific activities are given in milliunits per mg of protein.

G6PDH was measured by the formation of 6-phosphogluconate from 2 mM glucose 6-phosphate with the reduction of $NADP^+$ (Domagk and Chilla, 1975). The assay mixture contained 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM $NADP^+$, and appropriate amount of cell-free extract. The reaction was started by addition of the substrate.

6PGDH was measured by reduction of $NADP^+$ with 3 mM 6-phosphogluconate by the methods of Rippa and Signorini (Rippa and Signorini, 1975),with slight modifications. The assays mixture contained 50 mM Tris-HCl buffer (pH 8), 0.9 mM $NADP^+$, and appropriate amount of cell-free extract. The reaction was started by addition of the substrate.

Glucose-6-phosphate isomerase was measured by formation of glucose 6-phosphate from 3 mM fructose 6-phosphate (Bergmeyer, 1975). The assay mixture contained 50 mM triethanolamine · HCl buffer (pH 7.6), 5 mM $MgCl_2$, 0.2 mM $NADP^+$, 5 μg/ml of baker's yeast G6PDH (150 units/mg, Nacalai Tesque, Kyoto), and appropriate amount of cell-free extract. The reaction was started by addition of substrate as sodium salt.

Other enzyme activities including transketolase and transaldolase (Kato et al., 1982; Van Dijken et al., 1978), dihydroxyacetone synthase (Kato et al., 1982), dihydroxyacetone kinase and glycerol kinase (Tani and Yamada, 1987), 6-phosphofructokinase (Van Dijken et al., 1978; Lobo and Maitra, 1983), and D-ribose-5-phosphate ketol isomerase (Wood, 1975) were assayed according to the published methods.

**Analytical procedures** Protein concentration was determined spectrophotometrically by protein-dye binding method (Bradford, 1976), using commercial protein assay kit (Amersham-Pharmacia Biotech K.K., Tokyo). Bovine serum albumin was used as

the standard. Cell growth was determined turbidometrically at 660 nm. One $OD_{660}$ unit was equal to about 0.19 mg/ml (as dry cell weight). Pentose or pentose phosphate accumulation was identified by HPLC (Shimadzu Corp., Kyoto) equipped with the UV-visible detector (Shimadzu SPD-10A) and refractive index detector (Shodex RI-71, Showa Denko, Tokyo). Pentose phosphate was separated in a reverse phase column (TSK ODS 80™, 6 x 150 mm; Tosoh Corp., Tokyo), with acetonitrile-water (70:30, v/v) as the solvent at a flow rate of 0.8 ml/min. Riboflavin was identified and quantified by HPLC with the same column as above, and detected with fluorescence detector (Shimadzu RF-10A), with excitation at 450 nm and emission at 530 nm.

## RESULTS AND DISCUSSION

**Isolation of mutants** Some mutant strains of *C. boidinii* were isolated which could not grow on medium with dihydroxyacetone unlike the wild type, which can grow on either dihydroxyacetone or glycerol. One of the mutants had a yellow phenotype and excreted riboflavin (as it was identified by spectrofluorometry and HPLC) during the growth on YPD medium. As far as the author knows, this is the first report for flavinogenic strain of *C. boidinii.*

**Growth characteristics of the mutant** This mutant strain showed a higher growth than the wild type during incubation for 24 h at lower temperature (20°C) and higher temperature (37, and 42°C) on YPD-agar medium (Table 9). The mutant showed positive growth in YPD medium and in the media where glucose was replaced with glycerol, sorbitol, xylitol, D-xylose, and D-ribose, but showed no growth or very low growth on dihydroxyacetone, methanol, or gluconate at the same concentration. In further

**TABLE 9. Growth of *C. boidinii* on agar medium at different temperatures.**

| Strain | Medium* | 20°C | 30° | 37 C | 42°C |
|---|---|---|---|---|---|
| Wild type | YPD | - | + | - | - |
| | YPGly | - | + | - | - |
| Mutant (yellow color) | YPD | + | + | ++ | + |
| | YPGly | + | + | ++ | + |
| Mutant (pale-white color) | YPD | + | + | ++ | + |
| | YPGly | + | + | ++ | ++ |

* YPGly medium contained glycerol instead of glucose in YPD.

++, good growth; +, moderate growth; -, no growth (after 24 h).

growth test in the basal medium supplemented with 1 or 5 g/*l* of yeast extract, the mutant showed lacked the ability to utilize methanol, gluconate, and dihydroxyacetone. These evidences suggested that flavinogenic activity in the mutant seemed to have no relation to alcohol oxidase activity, which is different from the previous report (Tani, 1984; Shimizu et al., 1977). The properties of the mutant were rather similar to those of the mutant strain of *Corynebacterium glutamicum* which was deficient in G6PDH (Ihnen and Demain, 1969), or transketolase mutant of *C. glutamicum* which failed to grow on gluconate (Ikeda et al., 1998).

**TABLE 10. Enzymes of the oxidative pentose phosphate pathway in *C. boidinii* grown on different carbon sources.**

| | | Specific activity (mU/mg protein) grown on | | |
|---|---|---|---|---|
| Strain | Enzyme | Glucose | Glycerol | Methanol |
| Wild type | G6PDH | 390 | 510 | 410 |
| | 6PGDH | 280 | 320 | 300 |
| Mutant | G6PDH | 1.5 | 4.9 | 6.7 |
| | 6PGDH | 30 | 36 | 46 |

**Defect of dehydrogenase activities of the oxidative pentose phosphate pathway**

The lower activity of both G6PDH and 6PGDH were detected in cell-free extract of the mutant than those of wild type of *C. boidinii* when cultured in basal medium with 2 g/*l* of yeast extract (Table 10). Similar result was observed with the crude enzyme from basal medium with 8 g/*l* of yeast extract, where the mutant cells showed high growth. These data are similar to those for pentose phosphate pathway mutants of *Saccharomyces cerevisiae* (Lobo and Maitra, 1982). As the both enzymes are the initial and key enzymes of the oxidative pentose phosphate pathway, these data give evidence for disfunction of oxidative pathway of pentose phosphate. It should be followed by switching its carbon metabolism to other alternative pathway, to meet the requirement for growth and energy. The most possible pathway is the non-oxidative pathway of pentose phosphate, since glucose 6-phosphate is standing at the intersection of the two pathway. This possibility was supported by the activity of glucose-6-phosphate isomerase at a quite normal level and the activity of transaldolase and transketolase in the mutant at higher level than the wild type (Table 11).

**TABLE 11. Enzymes of the non-oxidative pentose phosphate pathway in *C. boidinii* grown on different carbon sources.**

| Strain | Enzyme | Specific activity (mU/mg protein) grown on | | | |
|---|---|---|---|---|---|
| | | Glucose | Glycerol | Methanol | Ethanol |
| Wild type | Glucose-6-phosphate isomerase | 320 | 370 | 370 | 430 |
| | Transaldolase | 110 | 42 | 190 | 5.8 |
| | Transketolase | 3.6 | 19 | 29 | 19 |
| | Ribose-5-phosphate isomerase | 180 | 29 | - | 330 |
| Mutant | Glucose-6-phosphate isomerase | 210 | 230 | 190 | 160 |
| | Transaldolase | 280 | 120 | 920 | 1600 |
| | Transketolase | 31 | 47 | 51 | 50 |
| | Ribose-5-phosphate isomerase | 4800 | 370 | - | 28000 |

**Amino acids requirement and pentose phosphate accumulation** The result from the auxotrophy test showed that the mutant failed to grow in the absence of L-tryptophan and L-leucine, inositol, and nicotinate. The author supposed that the tryptophan requirement was the result from defect in any enzymes in the tryptophan pathway, but not in the L-tyrosine and L-phenylalanine pathway. Other reason is the lower degree of competitiveness of tryptophan pathway than the purine biosynthetic pathway to use phosphoribosyl pyrophosphate as its precursor. In these cases, almost all of erythrose 4-phosphate formed was used for synthesis of pentose phosphate in an alternative pathway catalyzed by transaldolase and transketolase, as described by bold arrow in Fig. 10. This hypothesis was supported by the high activities of transketolase and transaldolase in the mutant shown before (Table 11). Accumulation of ribose 5-phosphate and ribulose 5-phosphate in culture of the mutant was detected by HPLC. The existence of ribose 5-phosphate was also detected by enzymatic assay for transketolase. Accumulation of ribulose 5-phosphate was relevant with the higher activity of ribose-5-phos-phate isomerase (EC 5.3.1.6) of the mutant than in the wild type.

**Identification and production of riboflavin** Accumulation of extracellular riboflavin was observed by its yellow fluorescence and riboflavin was identified by spectrofluorometry and by HPLC with fluorescence detector. With exitation at 450 nm, crude extract of the mutant culture showed similar fluorescence spectrum to the authentic riboflavin with a peak at 519 nm. Among various carbon sources, the optimum growth of the mutant in concomitance with high accumulation of riboflavin, was observed in the basal medium with 8 g/*l* of yeast extract and 20 g/*l* of sucrose. These results were different from those obtained with *Pachysolen tannophylus* (Vanetti and

Aquarone, 1995), where D-xylose or glucose was found as the best carbon source for riboflavin synthesis.

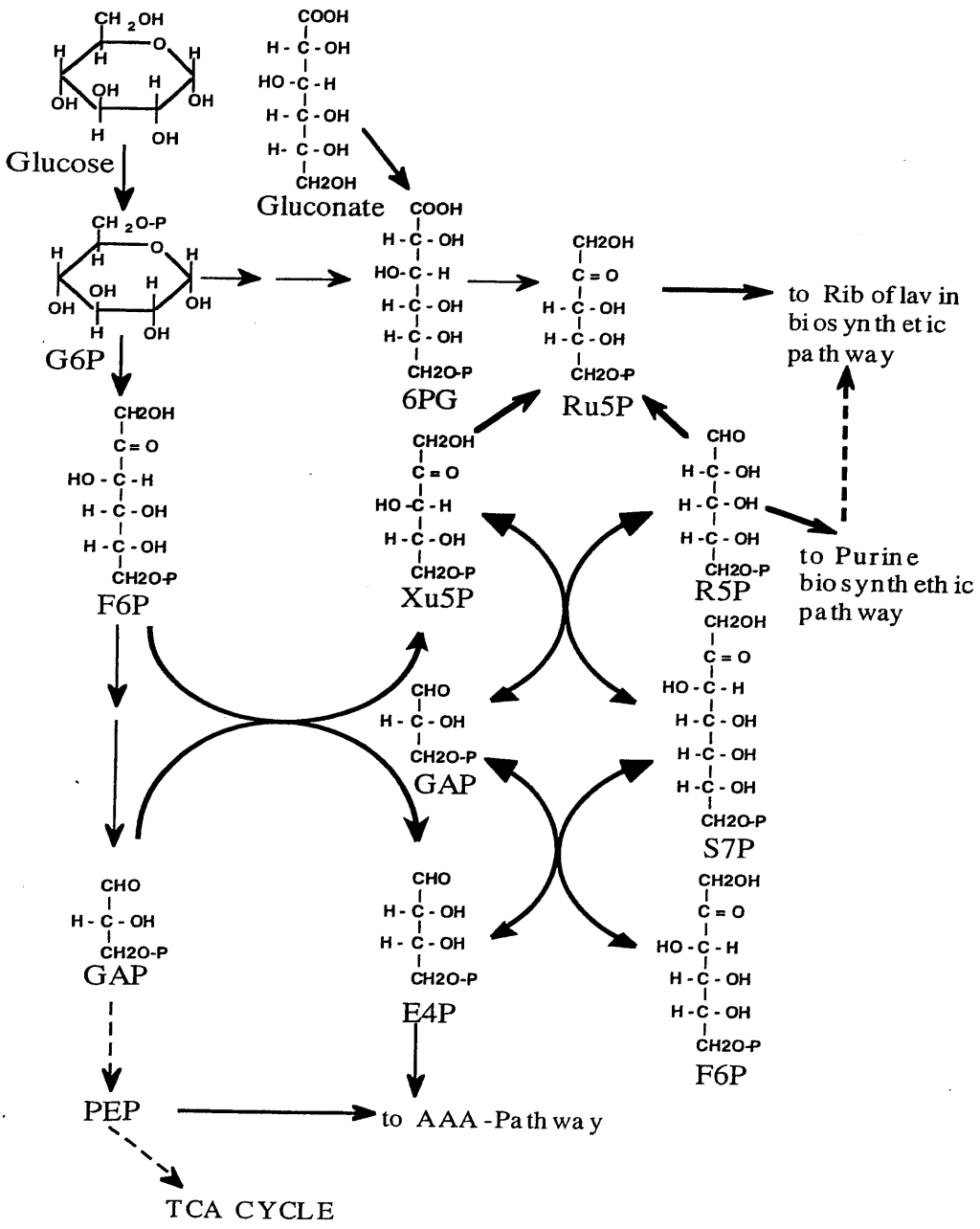


**FIG. 10. Pentose phosphate pathway and other related biosynthetic pathway.**
**Bold arrows show a direction of flux of carbon toward the pentose phosphate pool.**

**TABLE 12. Effect of Fe[II] ion concentration on growth and riboflavin synthesis.**

| $Fe(NH_4)_2(SO_4)_2$ (mM) | Biomass (g/*l*) | Riboflavin (mg/*l*) |
|---|---|---|
| 0.010 | 5.6 | 28 |
| 0.045 | 4.2 | 37 |
| 0.089 | 2.6 | 10 |
| 0.270 | 4.2 | 3.3 |
| 0.450 | 4.6 | 3.0 |
| 1.780 | 6.2 | 2.3 |

Cultivation was for 14 d.

Iron ion concentration was crucial to flavinogenesis in *C. boidinii*. The concentration of $Fe(II)^{2+}$ (as Mohr's salt, $Fe(NH_4)_2(SO_4)_2$) in the basal medium supplemented with 8 g/*l* of yeast extract was optimized (Table 12). The optimum concentration was about 0.045 mM (2.5 μg/ml), which gave a slight decrease of growth, but the greatest riboflavin synthesis. High concentration of the $Fe(II)^{2+}$ ion larger than 0.089 mM gave drastical decrease in riboflavin synthesis. The same result was observed in the culture with minimal medium supplemented with trace elements, instead of yeast extract. At the ferroes ion concentration of 0.01 mM cell growth reached the stationary phase after 4 d of cultivation, but riboflavin accumulation kept increasing during the stationary phase and reached 37 mg/*l* after 14 d of cultivation. The increase of the ferrous ion concentration to 0.1 or 1.0 mM gave increase in cell growth significantly, but decrease in riboflavin synthesis to almost the same level (2-3 mg/*l*).

The aeration level of culture for riboflavin production was examined using a 500-ml Erlenmeyer flask containing 50, 100, 200, or 300 ml of medium (Fig. 11). The

highest riboflavin synthesis was given by culture under microaerobic condition (with 300 ml of medium). Under this condition cells grew slowly, but the riboflavin was

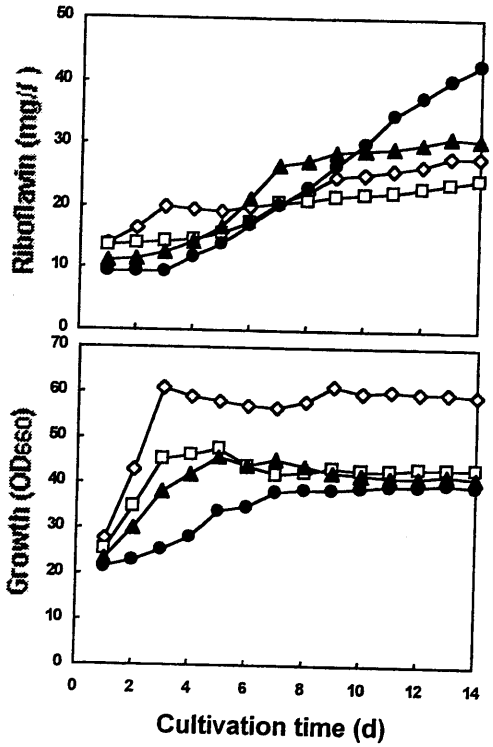


**FIG. 11. Effect of aeration on growth and riboflavin synthesis by flavinogenic mutant of *C. boidinii*. ◇ , 50 ml; □ , 100 ml; ▲ , 200 ml; and ● ,300 ml of medium in a 500-ml Erlenmeyer flasks.**

accumulated logarithmically after the stationary phase and reached the yield of 42.3 mg/*l* after 14 d of fermentation. At the high level of aeration (50 ml of medium in a 500-ml Erlenmeyer flask) cells grew rapidly and accumulated 19.8 mg/*l* of riboflavin after 3 d of fermentation, when the growth reached the stationary phase and riboflavin accumulation was almost to the maximum.

Temperature of fermentation was examined by culturing the mutant in 200 ml of the medium in a 500-ml Erlenmeyer flasks at 25, 30, 37, and 40 °C. The optimum temperature for both growth and flavinogenesis of *C. boidinii* mutant was 25°C, with

90 mg/*l* of riboflavin accumulation (Fig. 12). These results are different from those with *C. guillermondii* (Straube and Fritsche, 1973), where growth and flavinogenesis were optimum at 30°C. The mutant showed the characteristics like those of temperature-sensitive mutants, which grew better than the wild type at 20°C (Table 9).

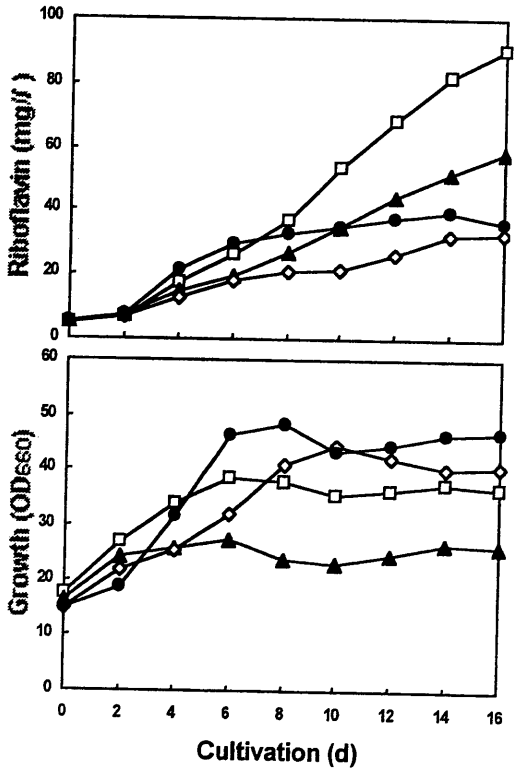


**FIG. 12. Effect of temperature on growth and riboflavin synthesis.**

**□ , 25°C; ▲ , 30°C; ◇ , 37°C; and ● , 40°C.**

This result was coincident with the faster growth in broth culture at 25°C than at 30°C or 37°C. The mutant grew faster at 40°C than at 25°C, but riboflavin accumulation was lower at 40°C than at 25°C.

## SUMMARY

A flavinogenic mutant was derived from *C. boidinii* by mutagenesis. The mutant was smaller in size than the wild type, showed no growth in a minimal medium, and required L-tryptophan, L-leucine, inositol, and nicotinate for growth. The mutant was defective in the oxidative pathway of pentose phosphate, lacking in G6PDH and 6PGDH. The specific ac-tivities of transaldolase and transketolase of the mutant were significantly higher than those of the wild type. This physiological condition might direct the flux of carbon source to the non-oxidative pathway with high formation of pentose phosphates, resulting in the increase of riboflavin synthesis. From optimi-zation of some factors affecting flavinogenesis, an amount of riboflavin (90 mg/*l*) was obtained under microaerobic conditions at 25°C.

# CONCLUSION

In this study, a biotechnological process for production of sugar derivatives by methanol yeasts was developed. A number of methanol yeasts were screened for their ability to convert sugars to the corresponding polyols, in basal medium supplemented with yeast extract and methanol (1-2%, v/v). Some potential polyol producers were found, namely *Candida boidinii* no. 2201, *Hansenula polymorpha* DL1, and *Pichia pinus*, which produced large amounts of sorbitol, xylitol, and galactitol, respectively, from the corresponding sugars. From these results, xylitol production by *H. polymorpha* was chosen for further study in larger scale, as it has some interesting properties for application.

Optimization of culture conditions was carried out by examining the fermentation parameters, such as nitrogen source, pH, initial cell density, aeration, and initial concentration of the substrate, D-xylose. The results from these experiments gave an optimum composition of medium for production culture of xylitol. The culture was microaerobic, with 200 ml of the medium in a 500-ml Erlenmeyer flask. The medium was composed of 2.5 g of urea, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 1 g of $MgSO_4.7H_2O$, about 100 g of D-xylose, and 1% (v/v) methanol in 1 *l* of water pH 8. Productivity was greatest when inoculum at initial density of 5 g/*l* (as dry cell eight) was used. Under these conditions, 57 g of xylitol was obtained from 110 g of D-xylose and 1 % (v/v) methanol in 1 *l* after 3 d of cultivation.

D-Xylose was converted to xylitol optimally at the concentration of about 100 g/*l*, with 1-2 % (v/v) methanol as the co-substrate. When D-xylose concentration was 125

g/*l* or higher, fermentation became inefficient due to a decrease in D-xylose utilization. It was supposed that these effects were resulted from intolerance of the yeast cells to the high-osmotic pressure environtment by the high concentration of the sugar. In order to solve these problems, an osmolite, glycerol was examined to protect the cells from high osmotic pressure, and to function as the co-substrate for methanol substitute.

Different patterns for D-xylose utilization and xylitol accumulation were found in the culture with single substrate (D-xylose or glycerol) and culture with mixed substrate (D-xylose+glycerol). In the culture with mixed substrate, accumulation of xylitol was slow, and peak when the substrate D-xylose has decreased, while the culture with single substrate was the opposite. Positive effect of glycerol on D-xylose conversion took place when the medium containing 125 or 150 g/*l* of D-xylose was supplemented with 25 or 50 g/*l* of glycerol. Glycerol concentration of 75 g/*l* or higher seemed to give delay in starting of the growth (lag phase), and did not stimulate accumulation of xylitol. At the glycerol concentration of 5%, the highest yield of xylitol (58.3 g/*l*; yield, 0.62 g/g) was obtained from 125 g/*l* of D-xylose in 4 d of cultivation. At the same glycerol concentration as above, *H. polymorpha* showed an increase of D-xylose consumption and xylitol accumulation (63.7 g/*l*) with high concentration of D-xylose (150 g/*l*). These results supported the author's expectation to the glycerol effects on D-xylose fermentation.

A flavinogenic mutant of *Candida boidinii* was isolated during the mutagenesis study to find a mutant resistant to high osmotic pressure. As it was a new strain of *Candida boidinii* and had interesting properties, the mutant was further characterized. The mutant was smaller in size than the wild type, showed no growth in a minimal medium, and required L-tryptophan, L-leucine, inositol, and nicotinate for growth.

The mutant was defective in the oxidative pathway of pentose phosphate, lacking in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The specific activities of transaldolase, transketolase, and ribose 5-phosphate isomerase of the mutant were significantly higher than those of the wild type. This physiological condition might direct the flux of carbon to the non-oxidative pathway with high formation of pentose phosphates, resulting in the increase of riboflavin synthesis.

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