Biotechnology Letters Vol 6 No 10 651-656 (1984)

CONVERSION OF D-XYLOSE INTO XYLITOL BY XYLOSE REDUCTASE FROM *CANDIDA PELLICULOSA* COUPLED WITH THE OXIDOREDUCTASE SYSTEM OF METHANOGEN STRAIN HU

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SUMMARY

A preliminary test for the enzymatic conversion of D-xylose into xylitol by the intact cells of *Candida pelliculosa* (xylose reductase) coupled with the intact cells of a formate-utilizing methanogen strain HU (hydrogenase and F_{420}-NADP oxidoreductase) was conducted by using H_2 as an electron donor of $NADP^+$. In the system, NADP(H) was well regenerated *via* the methanogen cells and about 90% conversion of xylose to xylitol (*ca.* 8 g/l) could be achieved at 35°C and pH 7.5 after 24 h incubation.

INTRODUCTION

Xylitol, a five carbon sugar alcohol, is being used as sweetener in foods (Alexander, 1978) and may apply to medicinal purposes as sugar substitute for the treatment of diabetes (van Eys *et al.*, 1974). It can be produced either by chemical hydrogenation of D-xylose (Manz *et al.*, 1973) or by bacterial or yeast fermentation (Yoshitake *et al.*, 1973, Gong, *et al.*, 1981). Many yeasts possess xylose reductase which catalyzes the reduction of D-xylose to xylitol as the first step in D-xylose metabolism (Barnett, 1968, Bruinenberg *et al.*, 1983).

In this report, an enzymatic method for xylitol production from D-xylose was attempted by using the xylose reductase of *Candida pelliculosa* coupling with the oxidoreductase system of a methanogen capable of recycling NADP(H).

MATERIALS AND METHODS

Microorganism: *Candida pelliculosa* and other several yeasts stocked in

the laboratory and a formate-utilizing methanogen strain HU isolated by Nishio *et al.* (1983) were used.

Media and cultivations: The medium for yeast cultivation was as follows (per liter): xylose, 10 g; yeast extract, 5 g; $(NH_4)_2SO_4$, 4 g; KH_2PO_4, 1.2 g; $Na_2HPO_4 \cdot 2H_2O$, 0.18 g; H_3BO_3, 0.6 mg; $MnSO_4 \cdot nH_2O$, 0.3 mg; $ZnSO_4 \cdot 7H_2O$, 3.0 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; $FeCl_3 \cdot 6H_2O$, 2.5 mg and $Na_2MoO_4 \cdot H_2O$, 0.25 mg. The pH of the medium was adjusted to 5.0 with 0.5 N HCl solution before autoclaving. The inoculum was prepared by transferring a slant culture (malt-yeast extract medium) into a 500 ml Sakaguchi flask containing 100 ml of medium and incubated under shaking for 24 h. The culture (100 ml) was then transferred to a 5ℓ baffle Erlenmeyer flask containing 2ℓ of medium. The culture was incubated at 28°C with rotary shaker (160 rpm) for 3 days.

The formate-utilizing methanogen was continuously cultivated (dilution rate: 0.8 day^{-1}) on formate minimal medium in a 10ℓ jar fermentor under strictly anaerobic condition at 37°C and pH 7.2 (Eguchi *et al.*, 1983).

Preparation of cell-free extract of C. pelliculosa: The yeast cells at the end of exponential phase were harvested by centrifugation (4,000 x g: 20 min), then washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA. The cells were resuspended into 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 2 mM DTT (Bruinenberg, 1983). The suspended cells were disrupted at 35,000 psi by French Pressure Cell Press (American Inst., Co., Silver Spring, USA) and the cell debris was removed by centrifugation (20,000 x g; 15 min). The supernatant was used as the source of xylose reductase.

Preparation of methanogen cells: The cell suspension of methanogen strain HU to use as the source of hydrogenase and F_{420}-NADP oxidoreductase to generate NADPH from $NADP^+$ was prepared as described by Eguchi *et al.* (1983).

Xylitol production: The reaction was carried out in a 20 ml anaerobic tube provided with a silicone inner stopper and a screwed cap. The reaction mixture was prepared under supplying N_2 gas in the tube. Three runs of xylitol production were conducted: Run 1 consisted of NADPH, xylose reductase and xylose (reaction condition: see legend of Fig. 1) and Run 2 consisted of $NADP^+$, xylose reductase, methanogen cells, xylose and H_2 gas (reaction condition: see legend of Fig. 2). H_2 gas as an electron donor to generate $NADP^+$ to NADPH *via* methanogen was prepared as follows: after the reaction mixture was anaerobically prepared, N_2 gas in the head space of the tube (14 ml) was replaced by passing a gas mixture (H_2:N_2 = 3:1), and then the cap was screwed and another 20 ml of the gas mixture was injected to a head space with an injection syringe. In Run 3, instead of xylose reductase in Run 2, the intact cells of *C. pelliculosa* was used (reaction condition: see legend of Fig. 3). All the reactions were incubated slantingly to allow better homogenization at respective temperature and 0.5 ml of sample was withdrawn at intervals with an injection syringe.

Xylose and xylitol were determined by a high performance liquid chromatography having Shodex S801 column and differential refractometer SE-11 (Japan Spectroscopic, Co., Ltd., Tokyo). The operating condition was carried out at ambient temperature using water as eluent at a constant flow rate of 1.0 ml per min.

Determination of xylose reductase activity: Xylose reductase activity was measured spectrophotometrically at 340 nm based on the oxidation of NADPH as described by Yoshitake *et al.* (1976). One unit of enzyme was defined as the amount which caused the decrease of 1 μmole NADPH per min.

RESULTS AND DISCUSSION

Among 27 yeast strains, the 7 strains which grew well on xylose medium were tested for xylose reductase activity using cell-free extract preparations. *C. pelliculosa* gave the highest activity of xylose reductase as shown in Table 1. Therefore, *C. pelliculosa* was used through out this work.

Table 1. Screening of potent yeasts for xylose reductase producer

Strain	Xylose reductase activity (units/mg protein)
Candida pelliculosa	1.73
C. pelliculosa var. *acetaetherius*	0.09
C. utilis	0.26
Debaryomyces hansenii	0.16
Hansenula anomola Y1	0.09
Hormoascus platypoides AM 93	0.43
Pichia nakazawae	0.24

The yeasts were cultivated in the test tube (20 cm x 2.5 cm i.d.) contained 10 ml of xylose medium for 3 days at 28°C under shaking. Cell-free extracts were prepared by disruption the cells with the glass beads (Biely *et al.*, 1974).
Protein was determined by Coomassie Brilliant Blue method(Bradford, 1976).

As the xylose reductase is capable of catalyzing xylose to xylitol *via* $NADP^+$-dependent dehydrogenase, the conversion of xylose to xylitol with the cell-free extract of *C. pelliculosa* was carried out. The xylose added was stoichiometrically converted to xylitol which was equivalent to the amount of NADPH consumed as shown in Fig. 1. The accumulated xylitol was not further converted to xylulose *via* NAD^+-dependent dehydrogenase. The regeneration of NADPH from $NADP^+$ is essentially significant to economically produce xylitol from xylose. For this, the oxidoreductase system of methanogen strain HU was used to reduce $NADP^+$ to NADPH using H_2 as an electron doner (Eguchi *et al.*, 1984).

Under the coupling reaction of the resting cells of methanogen with the cell-free extract from *C. pelliculosa*, xylitol production was conducted

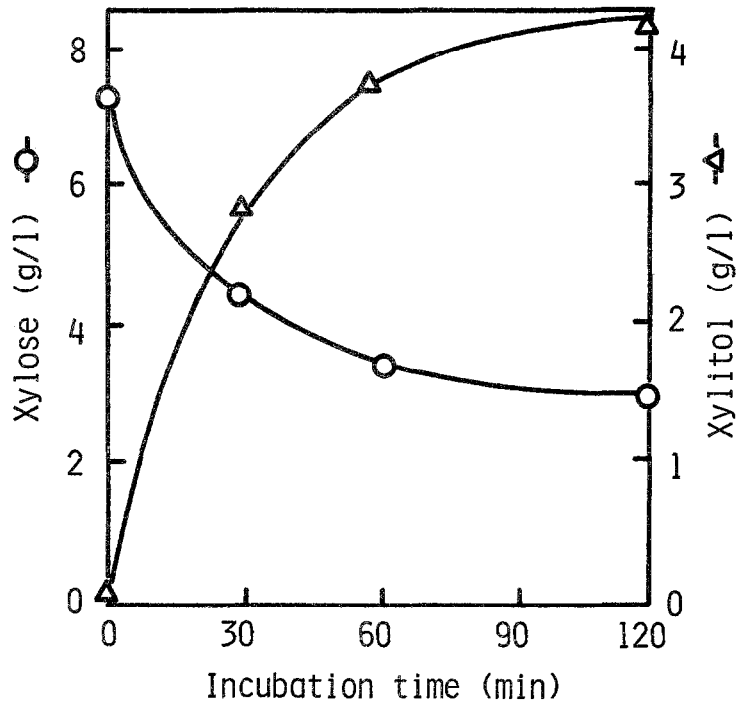

Fig. 1. Xylitol production with the cell-free extracts of *C. pelliculosa* (Run 1).

The reaction mixture (3 ml) contained: 50 mM xylose, 25 mM NADPH, 100 mM Tris-HCl buffer (pH 7.5) and the cell-free extract. The mixture contained xylose reductase 2.25 units. This was incubated at 35°C under anaerobic condition with N_2 gas.

as a function of $NADP^+$/xylose ratio provided that a xylose concentration of 55 mM (Run 2). As shown in Fig. 2, it could be confirmed that after 8 h incubation, $NADP^+$/xylose ratio over 1/30 resulted in 100% conversion of xylose to xylitol catalyzed by xylose reductase coupling with the hydrogenase and F_{420}-NADP oxidoreductase from the methanogen using H_2 as an electron donor. In Fig. 3 (Run 3), the intact cells of *C. pelliculosa* were examined, instead of using the cell-free extract, for xylitol production with the methanogen cells. Almost 100% of xylose was converted into xylitol after 24 h incubation.

This was suggested that the feasibility of using intact cells of *C. pelliculosa* to produce xylitol from xylose by coupling with the intact cells of methanogen strain HU using H_2 as an electron donor of $NADP^+$ was confirmed.

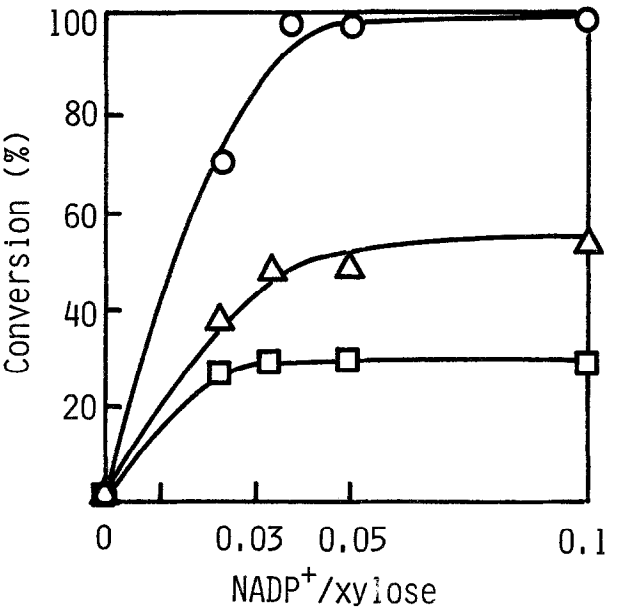

Fig. 2. Effect of $NADP^+$/xylose ratio on the production of xylitol with the cell-free extract of *C. pelliculosa* coupled with the oxidoreductase system of methanogen strain HU (Run 2).

Reaction mixture (6 ml in 20 ml tube) consisted of 2.4 ml of methanogen (17 mg dry weight), 100 mM Tris-HCl buffer (pH: 7.5), 7.5 mM methyl viologen, 1.2 mg Triton X-100, 11.9 units xylose reductase, 55 mM xylose, 1.4 to 5.5 mM $NADP^+$ and *ca.* 1 mmol H_2 in the head space of tube (14 ml).
Temperature: 30°C. Incubation time, ⊡ : 2 h; △ : 4 h; ○: 8 h.

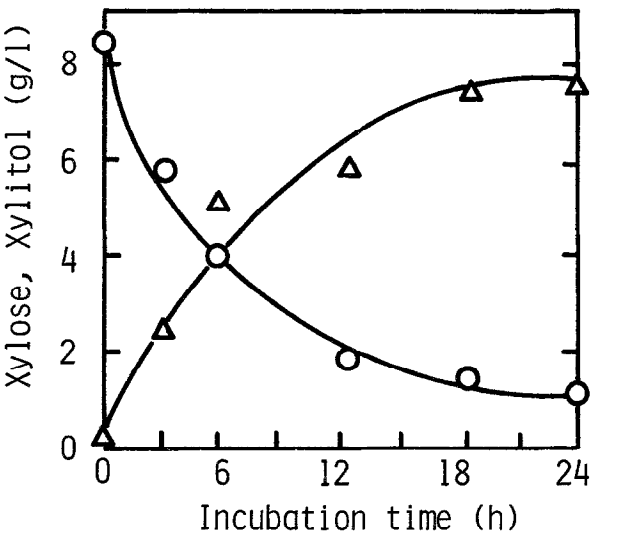

Fig. 3. The conversion of xylose to xylitol by the intact cells of *C. pelliculosa* coupled with methanogen strain HU (Run 3).

Reaction condition was the same in Fig. 2 except for using 2.38 mg dry cell of *C. pelliculosa* (0.94 unit xylose reductase per mg dry cell) and 2.75 mM of $NADP^+$.
Temperature: 35°C. ○ : xylose, △ : xylitol.

Detailed experiments will be done for optimization of xylitol production by this system.

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