Cloning and over-expression of NAD+-dependent xylitol dehydrogenase (XDH) in thermotolerant *Gluconobacter frateurii* THD32

Wichai Soemphol[1,], Kazunobu Matsushita[2] and Hirohide Toyama[3]*

[1]   *Species Diversity in Sub Mekong Region and Applications, Department of Science and Technology, Nong Khai campus, Khon Kaen University, Nong Khai, Thailand, 43000*

[2]   *Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan, 753-8515*

[3]   *Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan, 903-0213*

*   *Correspondent author: wichai.s@nkc.kku.ac.th*

Abstract

Gene locus of GOX0865 amplified from *Gluconobacter oxydans* IFO 12528 was expressed in *Escherichia coli*. The result revealed the high activity of NAD-dependent dehydrogenase with xylitol as a substrate indicating that it encoded for xylitol dehydrogease (XDH). The shuttle vector containing this gene was then constructed and transformed into thermotolerant *G. frateurii* THD32 of which two types of membrane-bound dehydrogenases responsible for D-sorbitol (SLDHs) oxidation was inactivated. The transformants exhibited increasing of the XDH activity almost 10 times. However, these over-expressed strains showed a slight increase of keto-hexose production from D-sorbitol and the resulting product was identified as L-sorbose. These results suggested that these strains may require the effective redox balance. On the other hand, they might prefer to accumulate L-sorbose.

Keywords: Acetic acid bacteria, *Gluconobacter*, Xylitol dehydrogenase, polyol dehydrogenase

1. Introduction

The genus of *Gluconobacter* is an attractive microorganism for industrial fermentation employed by their effective oxidative fermentation (1). These reactions are carried out by the various primary dehydrogenases on the periplasmic membrane, and connected to the simple respiratory chain system for bio-energy generation (2). Such oxidation reaction results in the rapid biotransformation and high accumulation of the valuable products outside cell. In addition, there are many cytosolic dehydrogeases catalyzing the similar reaction to those done by the former enzymes but the reaction is reversible and mostly required NAD(P) or NAD(P)H as a cofactor. Moreover, the most NAD(P) dependent dehydrogenases are also favorable catalyzing substrate at D-*xylo* configuration and result in the distinguished oxidized products(1, 3). The complete genome sequence of *G. oxydans* ATCC621H has been reported to compose of a lot of

enzymes in oxido-reductase family and still many of which are unknown function (4). The certain function of the several cytosolic dehydrogenases in *G. oxydans* IFO 12528 (synonymous to *G. oxydans* ATCC621H) have been elucidated by expressing in *E. coli.* Most NAD$^+$ dependent dehydrogenases seemed to be preferable oxidizing D-*xylo* configuration such as the biotransformation of D-sorbitol to D-fructose and xylitol to D-xylulose by XDH (1). We have been attempted to improve the production of D-fructose from D-sorbitol by *Guconobacter* with the oxidative fermentation. However, the enzymes on the membrane were only able to catalyze D-sorbitol to L-sorbose. Therefore, we assumed that enhancing of activity of NAD$^+$-dependent in cytoplasm may improve the formation of D-fructose from D-sorbitol.

2. Materials and Methods

Bacterial strains and medium. Gluconobacter strains were maintained on potato-CaCO$_3$ agar slants and cultivated as described previously (5). *Gluconobacter* strains were grown on YP medium containing 3 g of yeast extract, 3 g of polypeptone and appropriate carbon source, filled to 1 L with tap water. Bacterial growth was monitored with a Klett-Summerson photoelectric colorimeter with a red filter. *Escherichia coli* for plasmid construction and DNA sequencing was cultured in Luria-Bertani (LB) medium containing 50 µg/ml ampicillin, or 50 µg/ml kanamycin when required.

Enzyme assays. The enzyme activity of NAD-dependent dehydrogenases were measured at 25ºC following the increase of NADH at 340 nm in reaction mixture (1 ml) containing 100 µmol of substrate, 0.1 µmol NAD(P), and the appropriate amount of enzymes in 100 mM Tris-HCl buffer (pH 9.0). One unit of enzyme activity is defined as the amount of enzyme catalyzing 1.0 µmol of substrate.

Product analysis by TLC. To analyze the oxidation product from the culture medium, the supernatant was collected by ultracentrifugation at 9,000 rpm for 5 min and then analyzed by using thin layer chromatography (TLC) analysis. All of sample were spot on a silica gel plate 60 (MERK, UK) together with the standard sugars and developed with a solvent reagent containing ethyl acetate: acetate: methanol: DW (6: 1.5: 1.5: 1). After the silica plate gel was dried, phenol sulfuric reagent was spayed and incubated at 120 ºC until the spot appeared (5-10 min).

Qualitative and quantitative analyses of ketohexose. For quantitative measurement of the total amount of ketohexose, resorcinol was used, as described previously (6) *Preparation of membrane and soluble fractions.* Cells cultured for appropriate period were harvested, washed and disrupted by passing through a French pressure cell press (American Instruments Co., Silver Spring, MD., U.S.A.) as described previously (6).

Protein concentration was measured by a modification of the Lowry method (7). Bovine serum albumin was used as the standard protein.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done on 12.5% (w/v) acrylamide slab gel by the method described by Laemmli (8). The following calibration proteins with the indicated molecular masses were used as references for measurement of molecular mass: phosphorylase *b* (94 kDa), bovine serum albumin (68kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa). Proteins were stained with 0.1% Coomassie Brilliant Blue R-250.

Construction of the over-expressed XDH in Gluconobacter

Gene locus GOX0865 was amplified by PCR using primers GOX0865F (5' ACACAAT-GGCCGACGCCTTA 3') and GOX0865R (5' GTCCATGCTGCGTTCGTGTT 3') designated based on the data base available. The 0.96 kb of PCR product was ligated into pGEM-T easy vector before sucloning into pBluscript SK(-) at *Eco*RI site to generate pBlue0865. Plasmid, pBlue0865 was then digested with *Eco*RI and ligated into pSA19 (9) to obtain pSA0865. The constructed plasmid was then transformed into *G. frateurii* THD32 which was genetically modified by knock out two types of membrane-bound D-sorbitol dehydrogenase (SLDH) (10) and another one was eliminated the enzyme responsible for L-sorbose assimilation (6).

3. Result and Discussion

Construction of GOX0865 in *E. coli*

The gene locus of GOX0865 was annotated as xylitol dehydrogenase belonging to the group of short-chain dehydrogenase. This gene was then amplified as described in Materials and Methods. The resulting plasmid was confirmed by expression in the *E. coli* JM109. As shown on Fig. 1, the intensity band of the proteins from the cytosolic fraction of the transformant was increased at the calculated molecular weight of 29 kDa. Consistently, the enzyme activity measured with xylitol and D-sorbitol was detectable (6.4 and 5.2 U/mg, respectively), while the host *E. coli* JM109 did not (data not shown), These result suggested that the gene locus of GOX0865 encodes for NAD-dependent xylitol dehydrogenase (XDH).
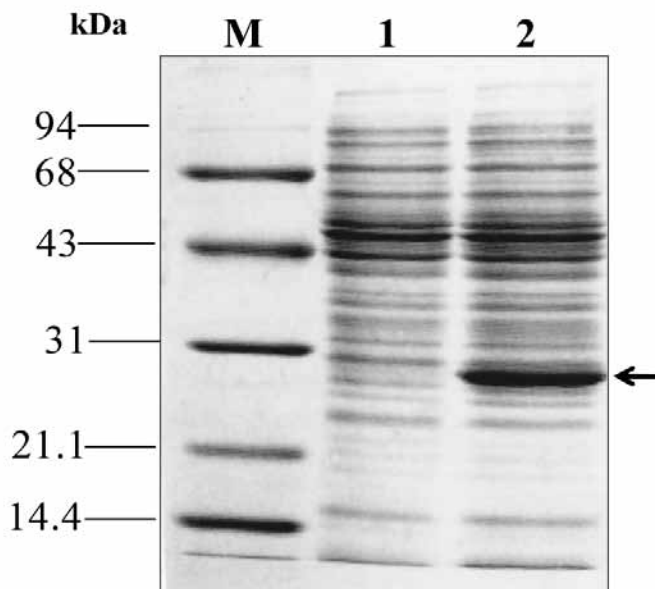

Figure 1. Expression of XDH analyzed by SDS-PAGE. 75 ug of proteins from the soluble fractions of *E. coli* JM109 (lane 1) and *E. coli* JM109 carrying pBlueo865 (lane 2) was subjected to 12.5 % (w/v) of acrylamide gel. M indicates the protein markers and the arrow indicates the expression of XDH.

Over expression of XDH in thermotolerant *G. frateurii* THD32

The gene fragment harboring on the shuttle vector (pSA0865) was transformed into *G. frateurii* THD32 by electroporation method. After selection of the desirable transformant, it was cultured on YP medium containing 1 % D-sorbitol under shaking condition at 200 rpm for 36 h. Cells were collected and disrupted for measurement of the enzyme activity. NAD-dependent dehydrogenase activity with D-sorbitol and xylitol (Fig. 2) was then measured in soluble fractions. Among two different of transformants background, over-expression of XDH showed dramatically increasing of the enzyme activity higher than control almost 10 times. This result suggested that the XDH in *G. frateurii* THD32 was expressed successfully.
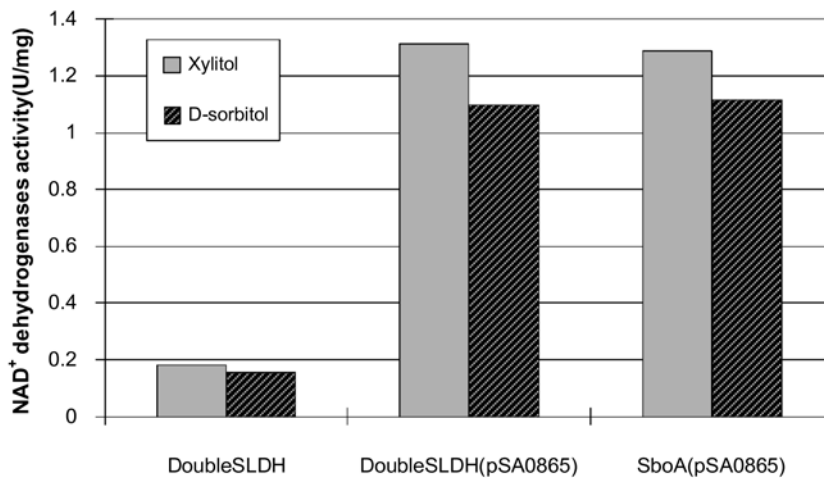

Figure 2. Comparison of XDH activity in *G. frateurii* THD32 doubleSLDH mutant harboring plasmid pSA0865. Dehydrogenases activities were measured with Xylitol and D-sorbitol as substrate with the presence of NAD^+.
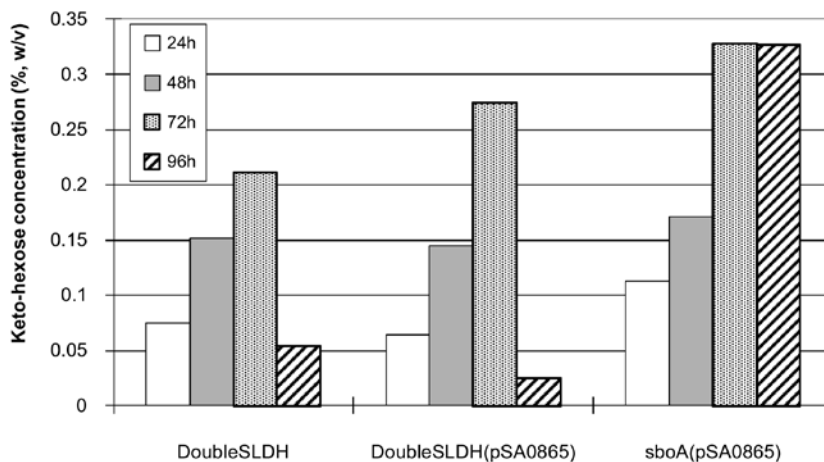

Figure 3. Keto-hexose bioformation from D-sorbitol by over-expressed XDH strains. Keto-hexose was measured by resorcinol test every 24h.
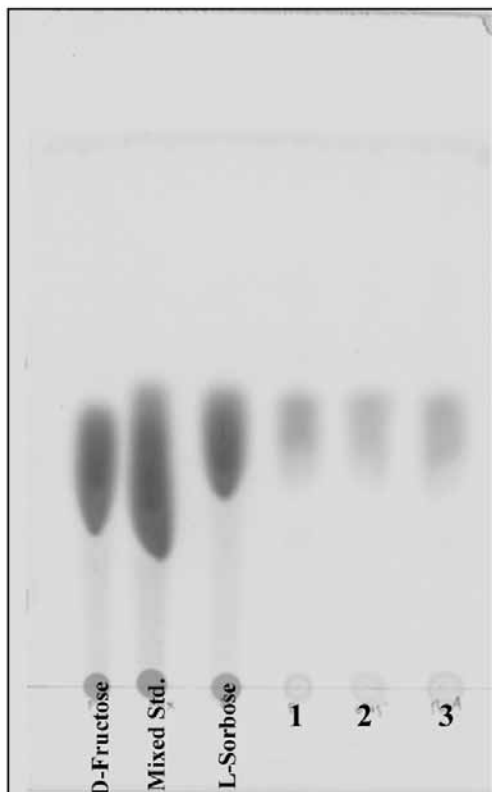
Figure 4. Identification of Keto-hexose in the culture medium by Thin-layer chromatography (TLC).
The culture supernatant from cultivation by *G. frateurii* THD32 doubleSLDH (1), doubleSLDH(pSA0865)(2), and sboA(0865)(3) were spotted on Silica Gel 60 compared with standard D-fructose, and L-sorbose.

D-Sorbitol oxidation in over-expressed strain

As mentioned above that the cytosolic enzyme including XDH could oxidized D-*xylo* configuration, therefore the oxidation of D-sorbitol would produce the product as D-fructose distinguished from the activity of dehydrogenases bounded on the membrane that catalyze D-sorbitol obeying the Bertrand-Hudson rule (1; 11). However the oxidation of D-sorbitol is mostly done by the membrane-bound enzyme to form L-sorbose and only small amount of D-sorbitol will be uptaked into cells and further catalyzed by NAD(P)-dehydrogenases (11). To enhance uptake of D-sorbitol, we then disrupted two types of membrane bound sorbitol dehydrogenase presence in *G. frateurii* THD32 and used for over expression experiment as described above. The over-expressed strain was then examined their ability in D-sorbitol oxidation by cultured on YP medium containing 3% (w/v) of D-sorbitol with shaking condition at 30ºC and culture supernatant was collected every 24 h until 96 h of cultivation. The oxidized product was measured of total keto-hexose by resorcinol test. As can be seen in Fig. 4, keto-hexose produced by these strains was very low only 10 % conversion. However, the product accumulation was found to be increased after cultivation time was longer, and the over-expressed strain, sboA (pSA0865), could produce the highest amount of keto-hexose after 72 h.

Since the resorcinol test could not distinguish the oxidized products whether it was L-sorbose or D-fructose. We then determined the oxidized products by TLC. The culture supernatant was spotted on aluminium-silica plate together with standard of L-sorbose and D-fructose. As shown in Fig. 4, the oxidized product from D-sorbitol was identified as L-sorbose not D-fructose.
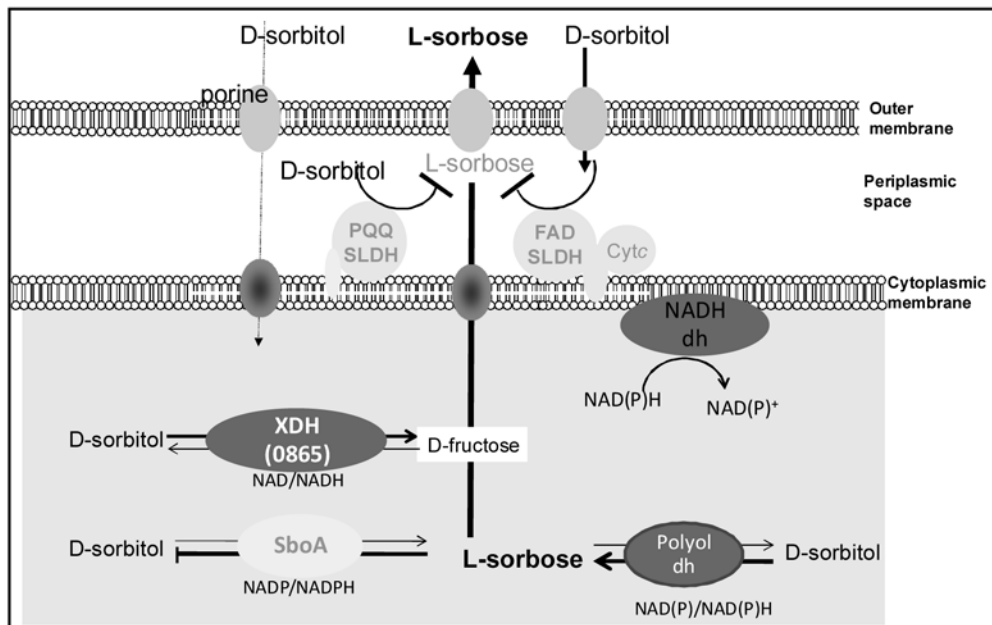
Figure 5. Speculation of D-sorbitol metabolism in *G. frateurii* THD32. The strain deficient in D-sorbitol oxidation (DoubleSLDH mutant) is only able to assimilate D-sorbitol via the specific transporter. The metabolism of D-sorbitol will be employed by the specific cytosolic dehydrogenases of which require the recycling of NAD(P)/NAD(P)H by NADH dehydrogenase and result in the formation of L-sorbose and D-fructose. Another strain was disrupted at SboA function for L-sorbose reductase might not produce or utilize L-sorbose. On the other hand, other polyol dehydrogenases presence in this strain may take a place for this reaction and produce L-sorbose from D-sorbitol.

4. Conclusion

In this study, we have been attempted to improve the activity of the cytosolic dehydrogenase for producing the alternative valuable product from D-sorbitol oxidation. In fact, most of D-sorbitol is oxidized to L-sorbose and rapidly accumulated in the culture medium. We constructed the mutant deficient in membrane-bound SLDHs indicating that the oxidation of D-sorbitol should carry out definitely from enzymes in cytoplasm. Those cytosolic enzymes are able to catalyzed D-sorbitol to either D-fructose or L-sorbose (1). The results present herein suggest that even we could over-express the XDH activity for biotransformation of D-fructose from D-sorbitol, but this enzyme also requires the sufficient cofactor binding for substrate-product turn-over. The correlation in redox balance should be concerned. The presence of other NAD(P) D-sorbitol dehydrogenases might be another key factor since the accumulation of L-sorbose was increased (see in Fig. 5) in the strain defective in NADPH L-sorbose reductase (SboA) which is responsible for assimilation of L-sorbose (6). Moreover, this mutation may trigger the metabolic pathway to another type of NADP-depentdent D-sorbitol dehydrogenase presence in this strain (12). Moreover, these results also give us the important consideration in the natural characteristics of *Gluconobacter* strains that they might prefer to accumulate L-sorbose for maintaining their suitable habitat.

5. Acknowledgements

6. References

(1)   Adachi, O., Moonmangmee, D., Shinagawa, E., Toyama, H., Yamada, M., Matsushita, K. New quinoproteins in oxidative fermentation. Biochem. Biophys. Acta. 2003; 1647: 10-7.

(2)   Matsushita, K., Toyama, H., Adachi, O. Respiratory chains and bioenergetics of acetic acid bacteria. Advanced Microbial Physiology. 1994; 36: 247-301.

(3)   Cheng, H., Li, Z., Jiang, N., Deng, Z. Cloning, Purification and Characterization of an NAD-dependent D-arabitol dehydrogenase from acetic acid bacterium, *Acetobacter suboxydans*. Protein J. 2009; 28: 263-72.

(4)   Prust, C., Hoffmeister, M., Liesegang, M., Liesegang, H. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol. 2005; 23: 195-200.

(5)   Ameyama, M., E. Shinagawa, E., Matsushita, K., Adachi, O. D-Fructose dehydrogenase of *Gluconobacter industrius*: purification, characterization, and application to enzymatic microdetermination of D-fructose. J. Bacteriol. 1981; 145: 814-23.

(6)   Soemphol, W., Toyama, H., Moonmangmee, D., Adachi, O., Matsushita, K. L-Sorbose reductase and its transcriptional regulator involved in L-sorbose utilization of *Gluconobacter frateurii*. J. Bacteriol. 2007; 189: 4800-8.

(7)   Dulley, J. R., Grieve, P. A. A simple technique for eliminating interference by detergentsin the Lowry method of protein determination. Anal. Biochem. 1975; 64: 136-41.

(8)   Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 1970; 227: 680-5.

(9)   Tonouchi, N., Tsuchida, T., Yoshinaga, F., Horinouchi, S., Beppa, T. A host-vector system for a cellulose-producing *Acetobacter* strain. Biosci. Biotechnol. Biochem. 1994; 59: 1899-901.

(10)   Soemphol, W., Adachi, O., Matsushita, K. & Toyama, H. Distinct physiological roles of two membrane-bound dehydrogenases responsible for D-sorbitol oxidation in *Gluconobacter frateurii*. Biosci. Biotechnol. Biochem. 2008; 72: 842-50.

(11)   Toyama, H., Soemphol, W., Moonmangmee, D., Adachi, O. & Matsushita, K. Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. Biosci. Biotechnol. Biochem. 2005; 69: 1120-9.

(12)   Adachi, O., Toyama, H., Theeragool, G., Lotong, N., and Matsushita, K. Crystallization and Properties of NAD-Dependent D-Sorbitol Dehydrogenase from *Gluconobacter suboxydans* IFO 3257 Biosci. Biotechnol. Biochem., 1999; 63: 1589-95.