Expression and study of alcohol dehydrogenase from *Gluconobacter oxydans* DSM2003

การแสดงออกและการศึกษาเอนไซม์แอลกอฮอล์ดีไฮโดรจีเนสจากเชื้อกลูโคโนแบคเตอร์ออกซิดาน DSM2003

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ABSTRACT

Alcohol dehydrogenase (GoADH) from *Gluconobcter oxydans* DSM2003 was previously shown to efficiently catalyze the oxidation of ethylene glycol (EG) to glycolaldehyde, which can be used for EG bioconversion to synthesize valuable glycolic acid. In this study, the GoADH was overexpressed and characterized. The two-step purification of the GoADH enzyme could obtain >95% purity with a 40% yield. This enzyme was confirmed to oxidize EG in the presence of NAD^+ and preferred a basic condition for its catalysis. The GoADH showed several folds lower K_m^{EG} than the other reported ADHs and exhibited good stability at room temperature. The sucrose, glucose, and sorbitol cryoprotectants or organic buffers were suitable for GoADH storage and for protecting GoADH from freeze-thaw effects.

บทคัดย่อ

เอนไซม์แอลกอฮอล์ดีไฮโดรจีเนสจากเชื้อ *Gluconobcter oxydans* DSM2003 (GoADH) มีความสามารถในการเร่งปฏิกิริยาออกซิเดชั่นของเอทิลีนไกลคอลและผลิตไกลคอลแอลดีไฮด์ ซึ่งเป็นสารตั้งต้นในการผลิตกรดไกลโคลิค ในการทดลองนี้ได้ทำบริสุทธิ์เอนไซม์แบบ 2 ขั้นตอน ทำให้ได้เอนไซม์ที่มีความบริสุทธิ์มากขึ้นกว่า 95% และมีร้อยละของผลผลิตเท่ากับ 40 โดยเอนไซม์ GoADH สามารถเร่งปฏิกิริยาออกซิเดชั่นของเอทิลีนไกลคอลโดยใช้ NAD^+ เป็นสับเสตรทร่วม นอกจากนี้เอนไซม์ชนิดนี้ทำงานได้ดีในสภาวะด่าง และเมื่อเปรียบเทียบค่าคงที่ Michaelis-Menten (K_m) ของเอนไซม์ GoADH ต่อสับเสตรทเอทิลีนไกลคอลกับเอนไซม์ ADH จากแหล่งอื่นพบว่า เอนไซม์ GoADH ให้ค่า K_m ที่ต่ำกว่าหลายเท่า นอกจากนี้เอนไซม์ชนิดนี้มีความทนที่ดี จากการศึกษาคุณสมบัติการปกป้องเอนไซม์จากการแช่แข็งของน้ำตาลซูโครส, กลูโคส, ซอร์บิทอล, และบัฟเฟอร์จากธรรมชาติ พบว่าสารเหล่านี้สามารถใช้ในการเก็บและปกป้องเอนไซม์ได้

Keywords: Alcohol dehydrogenase, *Gluconobacter oxydans* DSM2003, Ethylene glycol
คำสำคัญ: แอลกอฮอล์ดีไฮโดรจีเนส เชื้อกลูโคโนแบคเตอร์ออกซิดาน DSM2003 เอทิลีนไกลคอล

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Introduction

Alcohol dehydrogenase or ADH (EC 1.1.1.1) catalyzes the oxidation of alcohol and produces aldehyde or ketone and use $NAD(P)^+$ (nicotinamide adenine dinucleotide) as a cosubstrate. The ADHs are present in a wide range of organisms. The acetic acid bacterium *Gluconobacter oxydans* (*G. oxydans*) has the ability to oxidize a wide range of carbohydrates and alcohols. The ADH from *G. oxydans* (GoADH) was recently characterized to selectively oxidize the terminal hydroxyl groups of various aliphatic and aromatic diols. The enzyme preferred to oxidize substrates that contain terminal hydroxyl groups, including 1,6-Hexanediol, 1,5-Pentanediol, 1,4-Butanediol, 1,3-Propanediol, and ethylene glycol (EG) (Zhang X et al., 2015). Interestingly, this GoADH was shown to be the most efficient at oxidizing EG to glycoaldehyde (GA) than the other alcohols (**Figure 1**) and showed a specific activity toward EG of 4.82 ± 0.23 U/mg (Zhang X et al., 2015), while other ADHs such as ADH1A, ADH2, ADH4 from human showed the specific activity of 0.17 ± 0.01, 0.074 ± 0.002 and 3.1 ± 0.2 U/mg, respectively (Lee SL et al., 2011). The GoADH belongs to zinc-dependent medium-chain alcohol dehydrogenase that requires Zn^{2+} ion cofactor. The addition of EDTA or 2-mercaptoethanol inhibited enzymatic reaction (Schweiger P et al., 2013).

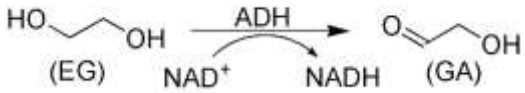

Figure 1 Oxidation of ethylene glycol to glycolaldehyde by GoADH.

The enzymes including PETase, cutinase, lipase, and esterase (Maurya A et al., 2020) has recently revealed their ability to degrade polyethylene terephthalate (PET). These enzymes can hydrolyze the ester bond, linkage in the PET molecule to release terephthalic acid (TPA) and EG. The enzymes are very worth to be used for PET plastic recycling/upcycling, in which the TPA and EG products can be used for the synthesis of virgin PET plastic or further transformed into valuable compounds. The EG, in particular, can be converted to GA using the ADH enzyme and then transformed to produce valuable glycolic acid, which is used in personal care products (Wei G et al., 2003). Moreover, the oxidation of EG by the ADH reaction may also be useful for the detection of PET because the ADH reaction produces NADH from NAD^+, in which NADH can provide the detectable signal.

As GoADH exhibits the highest efficiency in oxidizing EG, it might serve as a biocatalyst for the synthesis of valuable glycolic acid from EG obtained from the PET plastic degradation process or for detecting PET microplastics. Therefore, in this research, we studied the GoADH enzyme production and its properties. The resultant enzyme might be useful for valuable compound biosynthesis and contaminated PET microplastic detection applications.

Objectives of the study

To express, purify, and characterize GoADH in regards to activity, kinetic parameters, optimum pH, and suitable buffer for enzyme storage.

Methodology

Construction pET24b-*Goadh*.

The *Goadh* gene from *G. oxydans* DSM2003 was custom synthesized and subcloned into pET24b expression plasmid using *Hind*III and *Nde*I cut sites. The 1,038 bp digested *Goadh* gene was purified, before being ligated into cut pET24b. The ligation reaction was transformed into XL1(Blue) competent cell and spread onto LB agar plate supplemented with 34 μg/mL kanamycin. The recombinant plasmid was screened using double digestion with *Hind*III and *Nde*I, then analyzed by using agarose gel electrophoresis. The sequence was confirmed using DNA sequencing (Macrogen Inc., South Korea).

Expression and purification of GoADH

To find the optimal condition of GoADH expression, the expression was tested using auto-induction (ZY) and Luria-Bertani (LB) together with 0.15, 0.5, 1.0, and 2.0 mM IPTG concentrations. First, the pET24b-*Godah* plasmid was transformed into *E. coli* BL21(DE3) competent cells by heat shock transformation at 42°C for 1.30 min, before adding LB media and incubated at 37°C with shaking for 1.5 h. Then the transformed cells were plated onto LB agar containing 34 μg/mL kanamycin and incubated for overnight at 37°C. A single colony was inoculated into LB or autoinduction media containing 34 μg/mL kanamycin and incubated for overnight at 37°C, 220 rpm. Cells were 1% v/v inoculated into fresh media contain 34 μg/mL kanamycin and grown at 37°C, 220 rpm until OD_{600} reached around 0.6. The temperature was changed to 20°C for protein expression for 16 h for autoinduction media. While in case of LB- IPTG induction, the IPTG at specified concentration was added to allow protein expression. Cells were harvested by centrifugation at 8000 rpm, 4°C for 30 min. To analyze protein expression, cells were gently resuspended on ice-cold lysis buffer (100 mM sodium phosphate pH 7, 1 mM DTT, and 60 μM PMSF) and disrupted by sonication. The cell lysate was centrifuged at 15,000 rpm for 45 min at 4°C and supernatant and pellet parts were analyzed by SDS-PAGE.

The GoADH was 4-L scale expressed according to the optimum condition. To purify the enzyme, the cell paste was gently resuspended on ice-cold lysis buffer (100 mM sodium phosphate pH 7, 1 mM DTT, and 60 μM PMSF), then disrupted by sonication. The cell lysate was clarified by centrifugation for 15,000 rpm for 1 h at 4 °C. The supernatant was collected and first purified using polyethylenimine (PEI) precipitation (0.5% w/v). The enzyme solution was loaded onto Ni-NTA column, which was pre-equilibrated with 50 mM sodium phosphate pH 7.4, 50 mM NaCl, and 20 mM imidazole. The column was washed with 50 mM sodium phosphate pH 7.4, 50 mM NaCl, and 50 mM imidazole. Protein was eluted using gradient buffer of 50 mM sodium phosphate pH 7.4, 50 mM NaCl, 50-500 mM imidazole.

The eluted proteins were analyzed by measuring at absorbance at 280 nm and by SDS-PAGE to confirm GoADH protein. The fractions containing GoADH were pooled and concentrated by concentrator-stirrer.

Enzyme activity assay.

The GoADH activity was measured based on the formation of NADH, which can be monitored at 340 nm. The assay reaction typically consisted of 0.5 µM ADH, 0.5 mM NAD^+, and 250 mM EG conducted in 50 mM glycine-NaOH pH 9.0. The reaction was initiated by GoADH and monitored at absorbance at 340 nm. The initial velocity was used for calculating enzyme activity. One unit is defined as amount of enzyme that produces 1 µmol of NADH per min at 25 °C.

Investigation of suitable buffer and cryoprotective agent for GoADH storage.

In order to find the suitable buffer for GoADH storage, the purified protein was dialyzed against different buffer types, including 50 mM sodium phosphate pH 7.0, 50 mM MOP pH 7.0, 50 mM MES pH 7.0, and 50 mM HEPES pH 7.0. In case of the ADH in 50 mM sodium phosphate pH 7.0 with and without 1 mM DTT, the cryoprotectants such as sucrose, glucose, sorbitol at 0.1, 0.5 and 1 M concentrations were tested. The GoADH in particular condition was subjected to repeated freeze-thaw process and enzyme activity was assayed.

Investigation of optimum pH.

To investigate effect of pH on enzyme activity, the enzyme assay was conducted in different buffers covering 6.0-10.5 pH range, including (1) 50 mM sodium phosphate for pH 6.0-7.0 (2) 50 mM Tris-HCl for pH 7.0-9.0, and (3) 50 mM glycine-NaOH for pH 9.0-10.5.

Investigation of kinetic parameters of GoADH catalysis.

In this experiment, the GoADH assay was performed by varying concentration of one substrate, while another was kept as saturated. The assay was conducted in 50 mM Tris-HCl pH 8.5 and the EG concentration was varied as 0, 4, 8, 16, 32 ,64, 128, and 256 mM and while NAD^+ was fixed at 0.5 mM. While for studying the rate-dependency on NAD^+ concentration, the NAD^+ concentration was varied as 0, 4, 8, 16, 32 ,64, 128, 256, 512, and 1024 mM and EG concentration was fixed at 250 mM.

Results

Construction pET24b-*Goadh* and expression trial.

The *Goadh* gene from *G. oxydans* DSM2003 was successfully constructed. The expression was tried in autoinduction and LB-IPTG (0.15 mM) media, and the results in **Figure 2** showed that GoADH could be expressed as a soluble enzyme found in supernatant fractions in both of autoinduction and LB-IPTG induction conditions. The overexpressed enzyme with an approximate size of 36.7 kDa was consistent with the expected size of the GoADH enzyme (red arrow in **Figure 2**). However, LB-IPTG induction showed a higher expression level than those of the autoinduction (**Figure 2**), therefore LB-IPTG induction was selected for GoADH production.

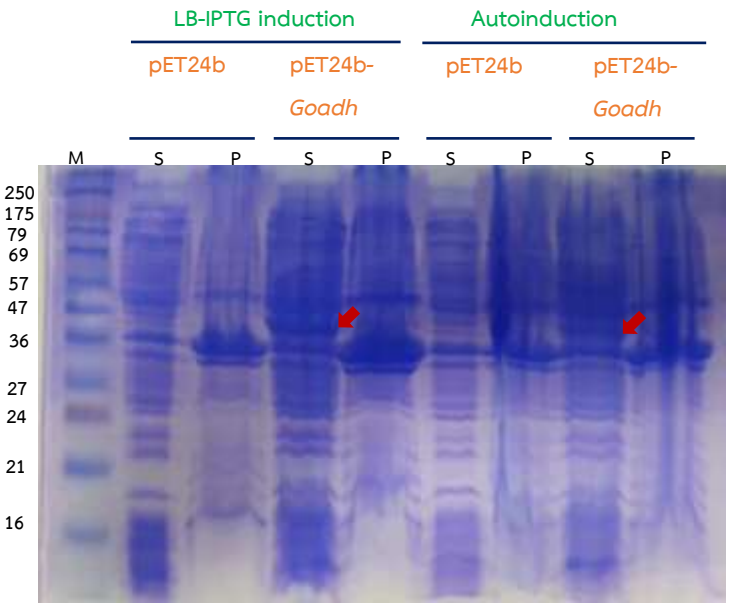

Figure 2 SDS-PAGE analysis of GoADH expression. The enzyme was expressed in *E. coli* BL21(DE3) using LB-IPTG (0.15 mM) induction and autoinduction at 20°C for 16 h. Lane M is marker, Lane S and Lane P are supernatant and pellet of *E. coli* BL21(DE3) after overexpression of GoADH, respectively. The *E. coli* BL21(DE3) harboring pET24b empty plasmid was used as control expression.

Purification of ADH.

Since, we designed GoADH to have 6xHis tagged at the C-terminus, the Ni-NTA was used to purify the GoADH enzyme. After passing through two-step purification of PEI precipitation and Ni-NTA column, more than 95 % purity of GoADH was obtained (**Figure 3**) and about 13.75 mg of GoADH from 2-L culture. The method could provide more than 240 folds of purification (**Table 1**). Although, we could a obtain high amount of eluted enzyme from Ni-column, the dialysis step to remove imidazole, the enzyme underwent precipitation and resulted in a haft of enzyme lost to yield only 40%. The enzyme was stored in 50 mM sodium phosphate pH 7.0 buffer containing 10% glycerol at -80°C.

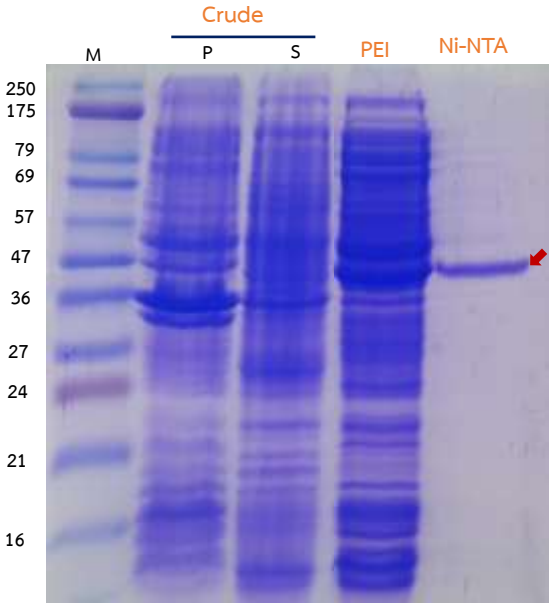

Figure 3 SDS-PAGE analysis for GoADH in each purification step. Lane M: protein marker, Lane S: crude extract, Lane P: pellet, PEI; enzyme after PEI precipitation and Lane Ni-NTA: enzyme after Ni-NTA column and dialysis.

Table 1 Purification table for GoADH

purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	60	2802.21	512.03	0.017	1	100
0.5 % PEI precipitation	52	2070.19	486.60	0.063	3.65	95.04
Ni-column	10	13.75	202.27	4.27	248.14	39.50

The effect of buffer types and pHs on ADH activity.

To find suitable buffer and pH for the GoADH reaction, the GoADH assay was conducted in three different buffers, 50 mM sodium phosphate pH 6.0-7.0, 50 mM Tris-HCl pH 7.0-9.0, and 50 mM glycine-NaOH pH 9.0-10.5. The results showed that, at particular pH, types of buffer showed no significant effect on GoADH activity, while the enzyme displayed the highest specific activity at pH 9.0 in both Tris-HCl (2.36±0.03 U/mg) and glycine-NaOH (2.37±0.13 U/mg) buffers (**Figure 4**). So, this indicates that Tris-HCl and Glycine-NaOH can efficiently be used for GoADH reaction and the enzyme prefers a basic condition for its catalysis. To obtain the ADH stability information, we simply stored GoADH enzyme in 50 mM glycine-NaOH pH 9.0 at room temperature and the enzyme activity was assayed at various times (0-96 h). The result showed that the ADH exhibited good stability as activity slightly decreased in the first 24-h incubation and around 50% activity reduction was observed after being incubated for 4-day at room temperature.

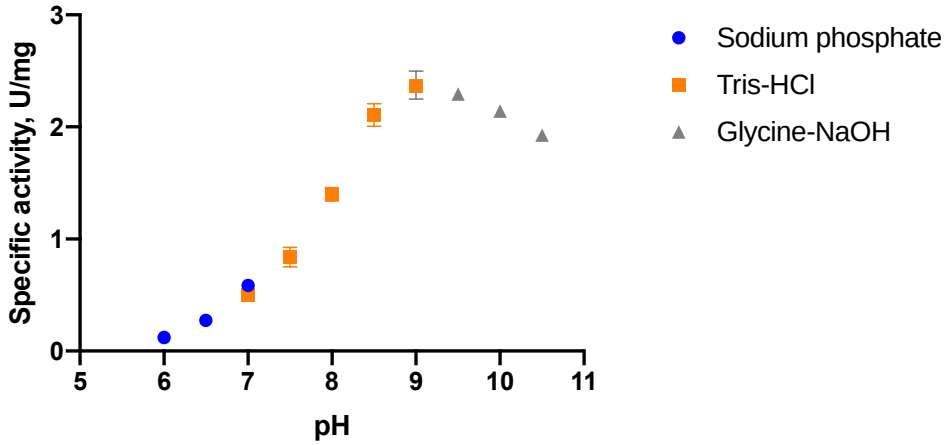

Figure 4 The specific activity of ADH in different buffer types. The ADH activity was measured based on the production of NADH. The reaction assay consisted of 0.5 mM NAD^+, 1 µM ADH, and 100 mM EG conducted in 50 mM sodium phosphate pH 6.0-7.0, 50 mM tris-HCl pH 7.0-9.0, and 50 mM glycine-NaOH pH 9.0-10.5.

Next, we investigated the steady-state kinetic parameters of GoADH catalysis. The initial velocity was measured in the assay with varying EG concentrations (4-256 mM) and NAD^+ (4-1024 µM) (**Figure 5A** and **5B**). The calculated Michaelis-Menten constant (K_m) of EG and NAD^+ were 24.69 and 75.78 mM, respectively. The catalytic turnover (k_{cat}) was calculated to be 0.07-0.1 s^{-1}

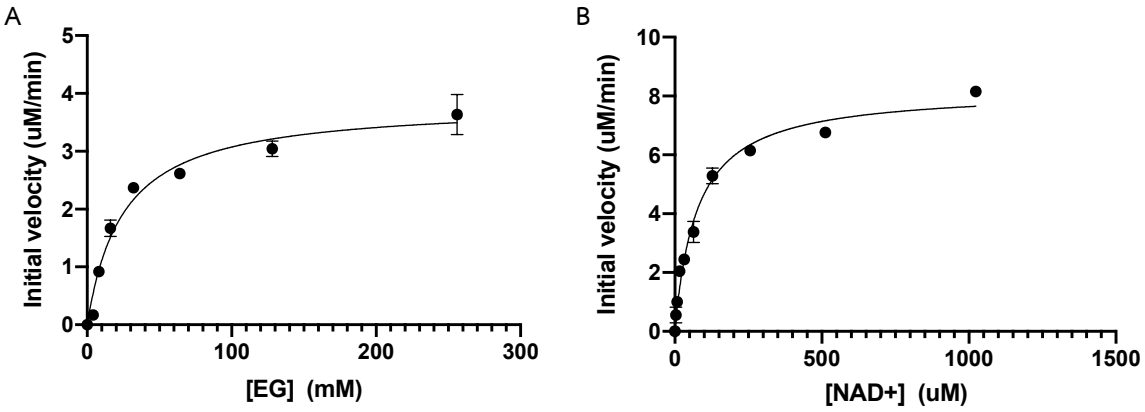

Figure 5 Direct plot of GoADH reaction. The kinetic parameters of GoADH were determined by measuring enzyme activity at different EG or NAD^+ concentrations. The kinetic parameters were calculated by nonlinear regression analysis using GraphPad Prism 8 software. The K_m for EG substrate is 24.69 mM **(A).** The K_m for NAD^+ substrate is 75.78 mM **(B).**

Study on the suitable buffer and cryoprotectant for GoADH storage.

As we could see instability of GoADH enzyme during the freeze-thaw process, next we investigated suitable cryoprotectant for GoADH storage. Although, glycerol that we used as a primary cryoprotectant was able to stabilize the GoADH, we could see the background signal of NADH production from GoADH reaction, caused by the ability of GoADH to use glycerol as a substrate. This may interfere if we further use the GoADH to detect EG from PET-degrading reaction. Thus, we try to find other cryoprotectants such as sucrose, glucose, and sorbitol that might not have a background signal from ADH reaction. The ADH in 50 mM sodium phosphate pH 7.0 with and without 1 mM DTT was added with sucrose, glucose, and sorbitol at 0.1, 0.5, 1 M. The specific activity was performed at each time of the freeze-thaw cycle and the results showed that overall specific activity of GoADH in 50 mM sodium phosphate pH 7.0 containing DTT (**Figure 6**) was slightly higher than the buffer without DTT (**Figure 7**). All sugar cryoprotectants could protect the ADH from the freeze-thaw process, while the ADH activity was almost lost after two cycles of freeze-thaw when it was stored without additive (**Figure 6 and 7, without additive**). The cryoprotectants with different concentrations had no significant effect on ADH activity. However, because of the cheaper and more activity retaining of sucrose, we decided to use 0.5 mM sucrose in 50 mM sodium phosphate pH 7.0 containing DTT condition for GoADH storage.

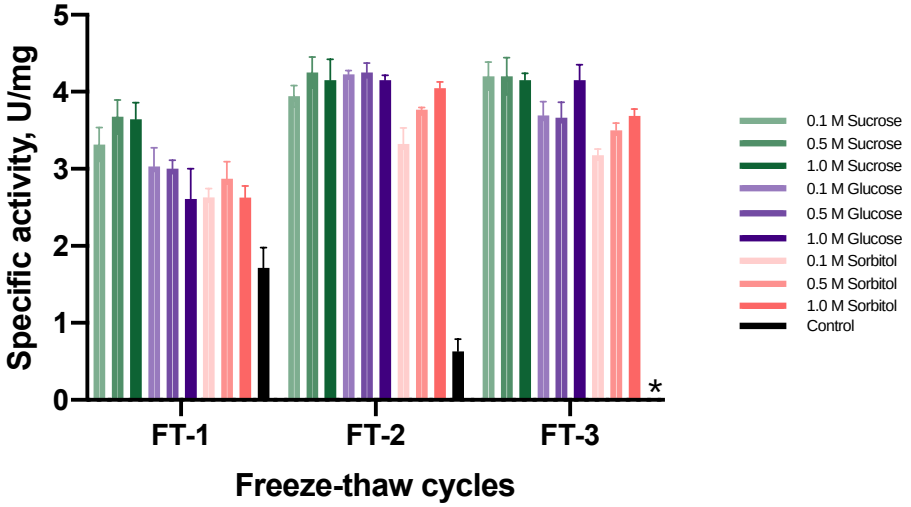

Figure 6 The specific activity of GoADH in different storage conditions with 1 mM DTT during cycle of freeze-thaw process. The various cryoprotectants at different concentrations were added into GoADH enzyme in 50 mM sodium phosphate pH 7.0 containing 1 mM DTT. The enzyme was freeze at -20 °C and thaw in ice, the GoADH activity was measured using 0.5 mM NAD^+, 1 µM ADH, 250 mM EG, conducted in 50 mM glycine-NaOH pH 9.0 buffer and the reaction was monitored absorbance 340 nm. Control is the enzyme that without cryoprotectant. Asterisk is not detectable.

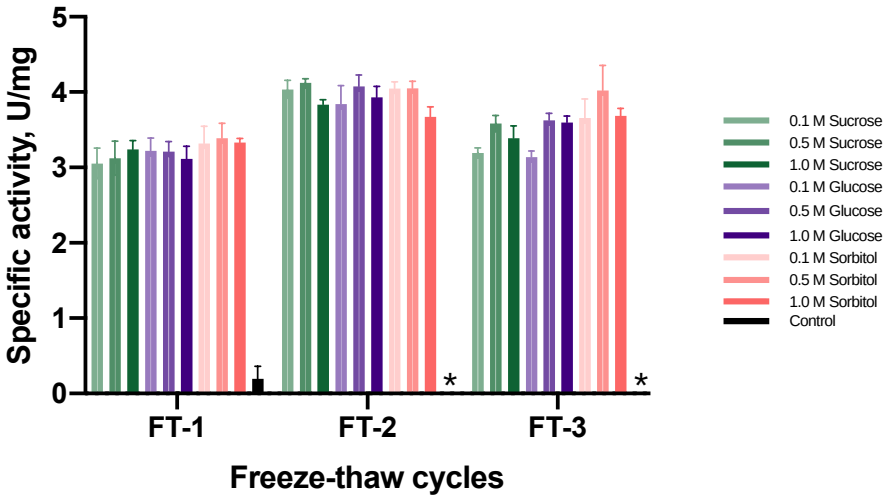

Figure 7 The specific activity of ADH in different storage conditions without DTT during cycle of freeze-thaw process. The various cryoprotectants in different concentrations were added into ADH enzyme in 50 mM sodium phosphate pH 7.0. During freeze-thaw process, the ADH activity was measured using 0.5 mM NAD^+, 1 µM ADH, 250 mM EG, and 50 mM glycine-NaOH pH 9.0 condition and the reaction was monitored absorbance 340 nm. Control is the enzyme that without cryoprotectant proceed freeze-thaw cycle in 50 mM sodium phosphate pH 7.0. Asterisk is not detectable.

The freeze-thaw protection effect of organic buffers, including MOP, MES, and HEPES for GoADH was also investigated by dialyzing GoADH in 50 mM MOP, MES, and HEPES pH 7.0. The results showed that all buffers exhibited good cryoprotectant for GoADH (**Figure 8**) as no significant change of GoADH activity was observed during freeze-thaw process. However, those buffers affected the specific activity of GoADH compared to the phosphate buffer. Among them, the 50 mM HEPES pH 7.0 provided the highest specific activity. Thus, 50 mM HEPE pH 7.0 is the best organic buffer for GoADH storage.

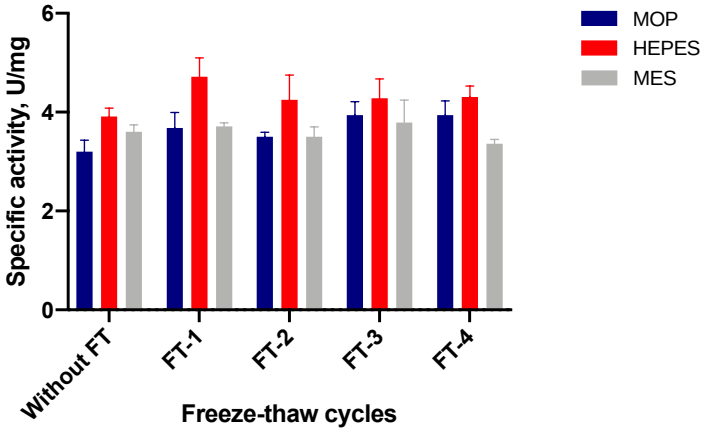

Figure 8 The specific activity of ADH in different organic buffers during cycle of freeze-thaw process. The enzyme was stored in 50 mM MOP, MES, and HEPES pH 7.0. During freeze-thaw process, the ADH activity was measured using 0.5 mM NAD^+, 1 µM ADH, 250 mM EG, and 50 mM glycine-NaOH pH 9.0 and the reaction was monitored absorbance 340 nm.

Discussion and Conclusions

In this study, the *adh gene* from *G. oxydans* DSM2003 was cloned and expressed in *E. coli* BL21 (DE3). The GoADH could express in both autoinduction and LB-IPTG induction conditions. However, IPTG induction showed higher expression level than the autoinduction. After purification using Ni-column, the enzyme showed very high purity (>95% purity). However, protein yield is relatively low (13.75 mg/ 2-L culture) due to enzyme instability and precipitation during dialysis to remove imidazole. In order to reduce enzyme precipitation, we tried to include additives, i.e. glycerol and NaCl, to stabilize the enzyme. However, none of them succeeded. It was speculated to be an incompatibility between the enzyme and imidazole, leading to enzyme instability. Therefore, further investigation with other chromatographic methods for GoADH purification may improve GoADH production yield. The effects of buffer type and pH indicated the preference in basic condition (Tris-HCl and Glycine-NaOH) for enzyme catalysis. This is consistent with the proposed mechanism of zinc-dependent medium-chain ADH (Zhang X et al., 2015; Vidal LS et al., 2018), in that the reaction first proceeds via deprotonation of hydroxyl proton to generate an alkoxide intermediate, which requires Zn^{2+} to stabilize. Electron delocalization and hydride transfer then occur, resulting in NAD(P)H and ketone products. So, in basic condition, deprotonation of hydroxyl proton is easier and more efficiently drives the reaction. The GoADH showed K_m^{EG} of 24.69 mM, which is several folds lower than the K_m of human ADHs, ADH1A K_m^{EG} = 440±40 mM, ADH2 = 420±20 mM, and ADH4 = 2600±300 mM (Lee SL et al., 2011). This result is consistent with the highest specific activity of GoADH toward EG substrate. The preliminary test on enzyme stability also showed promising stability of the enzyme at room temperature. Thus, GoADH is considered to be the suitable enzyme for application in EG bioconversion and PET plastic detection as previously mentioned. Sucrose, glucose, and sorbitol were shown to be suitable cryoprotectants for GoADH. These sugars can protect proteins or enzymes by preventing molecule mobility and protein structure change. A replacement of hydrogen bonds that form between water and protein to sugar with protein can maintain the native form of protein during repeated freeze-thaw process (Mensink MA et al., 2017). Moreover, the organic buffers can also be used for GoADH storage. In conclusion, we succeeded in producing GoADH for bioconversion and detection applications in the future.

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