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Full Length Research Paper

Characterization of the recombinant group 3 alcohol dehydrogenase from *Thermotoga lettingae* TMO

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The gene encoding a thermophilic group 3 alcohol dehydrogenase from *Thermotoga lettingae* TMO was cloned and over-expressed in *Escherichia coli*. The full-length DNA sequence of TlADH was 1086-bp encoding a polypeptide of 361 amino acids. Comparative and bioinformatic analysis revealed that TlADH showed high similarity to group 3 ADHs from thermophilic and mesophilic alcohol dehydrogenases, but relatively highest similarity to *T. maritime* 1, 3-propanediol dehydrogenase. The optimal pH-values of butanol oxidation and butylaldehyde reduction of TlADH were 11.9 and 6.0, respectively. Kinetic parameters of the enzymes showed TlADH preferred $NADP^+$ to NAD^+ as a cofactor. TlADH can catalyze a range of primary alcohols oxidation, while it was inactive towards branched-chain alcohols and cyclitol. TlADH also can reduce aldehydes to corresponding alcohols, including aliphatic and aromatic aldehydes. It showed higher activity of aldehyde reduction than that of alcohol oxidation that may be relative to reactive aldehyde detoxification in cell metabolism. In the case of external ions addition we found a 5.23-fold increase in reaction activity by the adding of 1 mM $MnCl_2$. *T. lettinga* ADH was cloned and overproduced in a mesophilic heterologous expression system, and the recombinant enzyme was characterized. It will be helpful to understand more about the physiological role of group 3 alcohol dehydrogenase.

Key words: Group 3 ADH, bioinformatic analysis, recombinant expression, thermophilic, catalytic properties.

INTRODUCTION

Alcohol dehydrogenases (ADH, EC 1.1.1.1) are present in all organisms and catalyze the interconversion of alcohols and the corresponding aldehydes or ketones (Radianingtyas and Wright, 2003). Many of them display wide substrate specificities. Their abilities to catalyze the chemo-, regio- and stereoselective reduction of ketones make them useful in synthesis of chiral compounds in pharmaceutical industry (Hummel, 1997). They use NAD $(P)^+$ and/or NAD(P)H as the cofactor and are divided into three major distinct types according to molecular properties and metal content (Reid and Fewson, 1994). Group 1 ADHs which molecular sizes are approximately 350 to 900 amino acid residues usually name zinc dependent long chain ADHs for containing zinc as catalytic sites (Littlechild et al., 2004). Group 2 ADHs which molecular sizes are approximately 250 amino acid residues usually name short-chain ADHs and lack of metal (Persson et al., 1991), such as ADHs from *Lactobacillus brevis* (Niefind et al., 2003) and *L. kefir* (Weckbecker and Hummel, 2006). Group 3 ADHs, which molecular sizes vary from 385 to 900 amino acid residues, are iron-activated and show a high degree similarity among themselves but no homology to either Group 1 or group 2 ADHs (Scopes, 1983). After being firstly characterized from *Zymomonas mobilis* (Conway et al., 1987), Group 3 ADHs have been characterized not only from mesophilic bacteria such as *E. coli* (L-1,2-propanediol oxidoreductase, POR) (Conway and Ingram, 1989), *Clostridium acetobutylicum* (ADH1) (Youngleson et al., 1989), *C. acetobutylicum* (butanol dehydrogenases, BDHI and BDHII) (Walter et al., 1992), *Saccharomyces cerevisiae* (ADH4) (Williamson and

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Paquin, 1987), but also from thermophilic or hyperthermophilic bacterias, *such as Sulfolobus solfataricus* (NAD- dependent ADH) (Cannio et al., 1996), *Thermococcus* (i-ADH) (Li and Stevenson, 1997), *Pyrococcus furiosus* (i-ADH) (Ma and Adams, 1999) and *Thermococcus litoralis* (Ma et al., 1994). Their important applications in catalysis and physiological functions of microbial were successively revealed.

Thermophilic and hyperthermophilic organisms have the abilities to thrive at high temperatures. By inference, the internal components must be able to withstand this extreme environment. These thermostable enzymes provide us with long-term functionality and novel reaction systems, such as the enzymatic vapor reaction under high temperatures (Yanai et al., 2009). More than 37 thermophilic and hyperthermophilic ADHs have been described, to better understand their applications in catalysis and physiological functions of microbial (Radianingtyas and Wright, 2003).

We focus on *T. lettingae*, a thermophilic anaerobic gram-negative bacterium by growing on methanol and acetate. In the presence of electron acceptors, such as elemental sulfur, *T. lettingae* is able to degrade methanol to CO_2 and H_2 (Balk et al., 2002). The amount of S^0 has a major effect on the activities of enzymes such as alcohol dehydrogenase, hydrogenase, and formate ferredoxin oxidoreductase (FMOR) (Ma et al., 1995). The physiological role of ADH is proposed to be in the disposal of excess reductant under S^0-limited conditions (Ying et al., 2009; Antoine et al., 1999). Thus, we isolated a putative alcohol dehydrogenase and describe the properties of the ADH in *T. lettingae* following overexpression in *E. coli*. It will be helpful to understand the internal components of *T. lettingae*.

MATERIALS AND METHODS

Cloning, expression and purification

All standard molecular biology techniques were performed essentially as described previously (Sambrook and Russell, 2001). The genomic DNA of *T. lettingae* TMO was isolated from samples according to the instruction using Power MaxTM Soil DNA Isolation Kit. The samples were collected from hot spring water and underwater soil located in Jiuqu, Guangxi, China. TlADH was amplified by polymerase chain reaction using pfu polymerase (Takara, Japan), and the genomic DNA as a template, and the primer pair (forward primer, 5′-*TCTAGA*ggcaatattttatgccca -3′ and reverse primer, 5′-*CTCGAG*ctaatctttttcaccc -3′) containing *Xba*I and *Xho*I sites (italicized), respectively. The primers designed according to the nucleotide sequence of TlADH (GeneID: 5609072). The PCR reaction was carried out by denaturing at 94°C for 5 min followed by 29 cycles of amplification (94°C for 45 s, 60°C for 45 s, and 72°C for 1 min) and by extension at 72°C for 6 min. The amplified product and were digested with *Xba*I and *Xho*I restriction enzymes and then ligated. The pET-303 vector encoded a purification tag consisting of the residues MGSDKIHHHHHH at the amino terminus of the full-length protein (Novagen, Madison, WI, USA). The constructed plasmid, pET303/TlADH, was introduced into *E. coli* BL21-codonplus, after which the transformants were cultured for 16 h in Luria-Bertan plate containing100 µg/ml of ampicillin and 30 µg/ml of chloramphenicol at 37°C. The positive clones were screened by PCR amplification and further verified by sequencing. Then the *E. coli* BL21- codon plus carrying pET303/TlADH was cultured in 1 liter LB liquid containing 100 µg/ml of ampicillin and 30 µg/ml of chloramphenicol with shaking at 150 rpm until the optical density at 600 nm reached 0.6. Expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside and cultivation was continued for an additional 5 h at 30°C.

After collection by centrifugation at 4,000×g for 10 min at 4°C, the cells were suspended in 100 mM sodium phosphate (pH 7.5), 200 mM sodium chloride, and then disrupted by ultrasonication on ice.

The resultant homogenate was centrifuged at 10,000×g for 15 min at 4°C, after which the supernatant was incubated at 50°C for 10 min, and the precipitate was removed by centrifugation at 10,000×g for 15 min at 4°C. The supernatant was mixed with Ni-NTA agarose (Qiagen, Hilden, Germany) for 1 h at 4°C and the mixture was loaded onto a chromatography column. The column was washed with buffer containing 100 mM sodium phosphate (pH 7.5), 200 mM sodium chloride, and 10 mM imidazole. A C-terminal hexahistidine-tagged TlADH was eluted from the column with the same buffer containing 500 mM imidazole. The protein was dialyzed overnight against 20 mM sodium phosphate (pH 7.5) and concentrated with polyethylene glycol (PEG) 20,000 and then freeze-dried. Enzyme powders were stored at -20°C. The purified enzymes were assayed by SDS-PAGE followed by Coomassie brilliant blue G-250 staining and the protein concentration was determined by the Bradford method (Bradford, 1976). Band intensity was quantified by Glyko Bandscan software.

Measurements of ADH activity

The alcohol dehydrogenase(TlADH) activities were measured by following the reduction of butylaldehyde in coupled reaction of converting NADPH to $NADP^+$ or the oxidation of butanol in coupled reaction of converting $NADP^+$ to NADPH at 50°C. The change in absorbance of NADPH was monitored at 340 nm using an ultraviolet-visible spectrophotometer with a temperature-controlled curette holder (Shimadzu Co., Kyoto, Japan). One unit (U) activity was defined as the amount of ADH required for the consumption or formation of 1µmol of NADPH per min at 50°C. The standard reaction mixture for the reductive reaction contained 100 mM phosphate buffer (pH 6.0), 1 mM NADPH, 1 mM DTT, 10 mM butyraldehyde and enzyme solution in a total volume of 1 mL. The standard reaction mixture for the oxidative reaction included 100 mM Gly-NaOH buffer (pH 11.9), 1 mM $NADP^+$, 1 mM DTT, 10 mM butanol and enzyme solution in a final volume of 1.0 mL. The same reaction mixtures except that substrate (alcohol or aldehyde) was removed, were assayed at the same condition that is for decreasing in interference from instability of the coenzyme.

For the test of substrate specificity, various alcohols replaced of butanol in the standard oxidative reaction while various aldehydes replaced of butylaldehyde in the standard reductive reaction.

For the study of kinetics, a range of butanol and 1, 3-propanediol concentrations from 1 to 50 mM were assayed under the standard oxidative reaction. Five concentrations of $NADP^+$ and NAD^+, ranging from 0.025 to 0.1 mM were used to determine reaction rates under the standard reaction.

Each activity assay was repeated at least triple. Apparent values of Michaelis constant (Km) and maximum velocity (Vmax) were calculated by the double reciprocal Lineweaver-Burk plots of the reaction rate. All the reactions followed Michaelis–Menten-type kinetics.

Optimum pH and temperature

The optimal pH of butanol-dependent oxidation of recombinant

Table 1. Summary of the purification process of TlADH from 1 litre of *E. coli* BL21-CodonPlus harboring pET/TlADH. The enzyme activities were measured with butanol as the substrate in the standard oxidative reaction.

Step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)
Crude cell extract	451.9	3272	7.2	100
Ni- NTA affinity chromatography	89.4	1888	21.1	57.7

TlADH was determined at 50°C using a set of 100 mM buffers: Phosphate buffers (pH 6.4, 6.9, 7.4), Tris-HCl (pH 7.4, 7.9, 8.4, 8.9, 9.4) and Glycine-NaOH (pH 9.4, 9.9, 10.4, 10.9, 11.4, 11.9). The reaction mixture, in a total volume of 1 mL, contained 100 mM buffer, 1 mM $NADP^+$, 1 mM dithiothreitol (DTT), 10 mM butanol and enzyme. The optimal pH of butylaldehyde-dependent reduction of recombinant TlADH was determined using the same set of buffers. The reaction mixture contained 100 mM buffer, 1 mM NADPH, 1 mM DTT, 10 mM butylaldehyde and enzyme. The effect of temperature on TlADH activity was measured using butanol-dependent oxidative reaction couple with $NADP^+$ reduction at temperatures ranging from 20 to 100°C. Meanwhile, non-substrate solution was analyzed as a control experiment.

Thermal stability

For the effects of temperature on the stability of TlADH, the enzyme (3.0 mg/mL) containing 1.0 mM DTT was incubated in 100 mM Tris-HCl (pH 7.4) for 1 h interval at 85 and 95°C. The residual activity in an aliquot of the enzyme solution (60 ug/mL) was then assayed by butanol-dependent oxidative reaction at 50°C. In the control experiment, the same concentration of untreated enzyme was analyzed in parallel.

Ions, chelator and reducing agents effects

In order to determine the effect of the metal ions, the chelator and the reducing agents on the enzyme activity, the enzyme samples (0.9 mg /mL) incubated with these compounds in 100 mM Tris-HCl (pH 7.4) for 1 h at 4°C. The buffer, Tris-HCl (pH 7.4), used for improving solubility of cations over those at alkaline pHs. Metal ions including $ZnCl_2$, $CoCl_2$, $MgCl_2$, $CuCl_2$, $CaCl_2$ (1 mM), ethylenediaminetetraacetic acid (EDTA, 1 mM), DTT (2 mM) and mercaptoethanol (2 mM) was respectively added into the ADH assay mixture. The pre-incubated enzyme samples were diluted to 0.045 mg /mL with glycine-NaOH (pH 11.9) and the activity was analyzed by butanol-dependent oxidative reaction. Meanwhile the enzyme sample without any treatment was used as an assay control.

Comparative and bioinformatic analysis

The homologues of the deduced amino acid sequence of TlADH were identified using the BLAST program with the default parameters (Altschul et al., 1997). Sequence alignments and similarity comparisons were initially conducted by the ClustalW method with default parameters (Thompson et al., 1994) and the final alignment was manually performed with the multiple sequence editor MegAlign (DNAStar, Madison, WI, USA). A phylogenetic tree was constructed using MEGA version 3 from CLUSTAL W alignments (Kumar et al., 2004). The neighbor-joining method was used to construct the tree (Saitou and Nei, 1987). Amino acid composition, theoretical isoelectric point and molecular weight were calculated using the ProtParam program at the ExPASy Proteomics Server with standard parameters (Gasteiger et al., 2005).

RESULTS AND DISCUSSION

Cloning, expression and purification

The full length sequence of alcohol dehydrogenase from *T. lettingae* TMO was 1086-bp encoding a 361-amino-acid polypeptide with a calculated molecular mass of 40.0 kDa and a putative isoelectric point of 6.23. The expression vector carrying the TlADH gene was constructed by ligating the PCR-amplified gene into the plasmid pET-303/CT-His; the resulting plasmid, pET/TlADH, was transformed into *E. coli* BL21-codonplus to yield a high-level expression of recombinant protein by IPTG-induction. The percentage of TlADH in crude cell extract was approximately 68.1% that was quantified by Bandscan analysis software. The recombinant protein was purified to homogeneity using a Ni-NTA affinity chromatography. The details of the purification results were shown in Table 1 and Figure 1. The purified recombinant TlADH kept 57.7% yield activity and 21.1U mg^{-1}.

Comparative and bioinformatic analysis

The deduced amino acid sequence of TlADH was used for searching for homologous sequences in the GenBank and protein databases with the BLAST program. Significant similarities were found in the type III (Group 3) ADH family such as *T. maritime* 1, 3-propanediol dehydrogenase (46.9% identities and 59.7% positives) (Ying et al., 2007), *Thermococcus* sp. ES1 iron alcohol dehydrogenase (Ying et al., 2009), *Thermococcus* Strain AN1 ADH (Li and Stevenson, 1997), *Z. mobilis* ADH2 (Conway et al., 1987), *S. cerevisiae* ADH4 (Williamson and Paquin, 1987), *E. coli* POR (Conway and Ingram, 1989), *C. acetobutylicum* BDHI and BDHII (Walter et al., 1992), and *B. methanolicus* methanol dehydrogenase (MDH) (de Vries et al., 1992); Thus, the BLAST analysis results indicated that TlADH belonged to the group 3 alcohol dehydrogenase family. The multiple alignment analysis results also demonstrated that the TlADH was higher homology with the *T. maritime* 1, 3-propanediol dehydrogenase (Figure 2). The sequence HXMXHXLGAXYXXPHG (letters in boldface represent the three highly conserved histidine residues) only exist

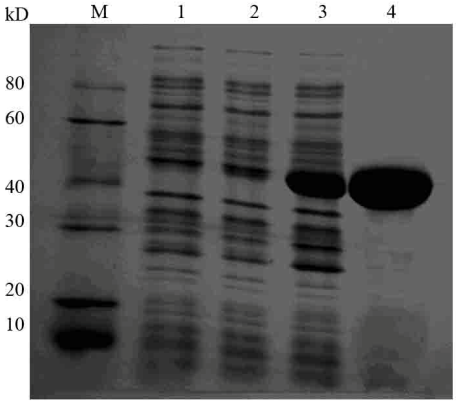

Figure 1. Analysis of TlADH using SDS-PAGE. Lane M, molecular markers; lane 1, BL 21-codonplus; lane 2, BL 21-codonplus pET/TlADH; BL 21-codonplus pET/TlADH after IPTG induction, lane 4 the reconstruction ADH by Ni- NTA affinity chromatography.

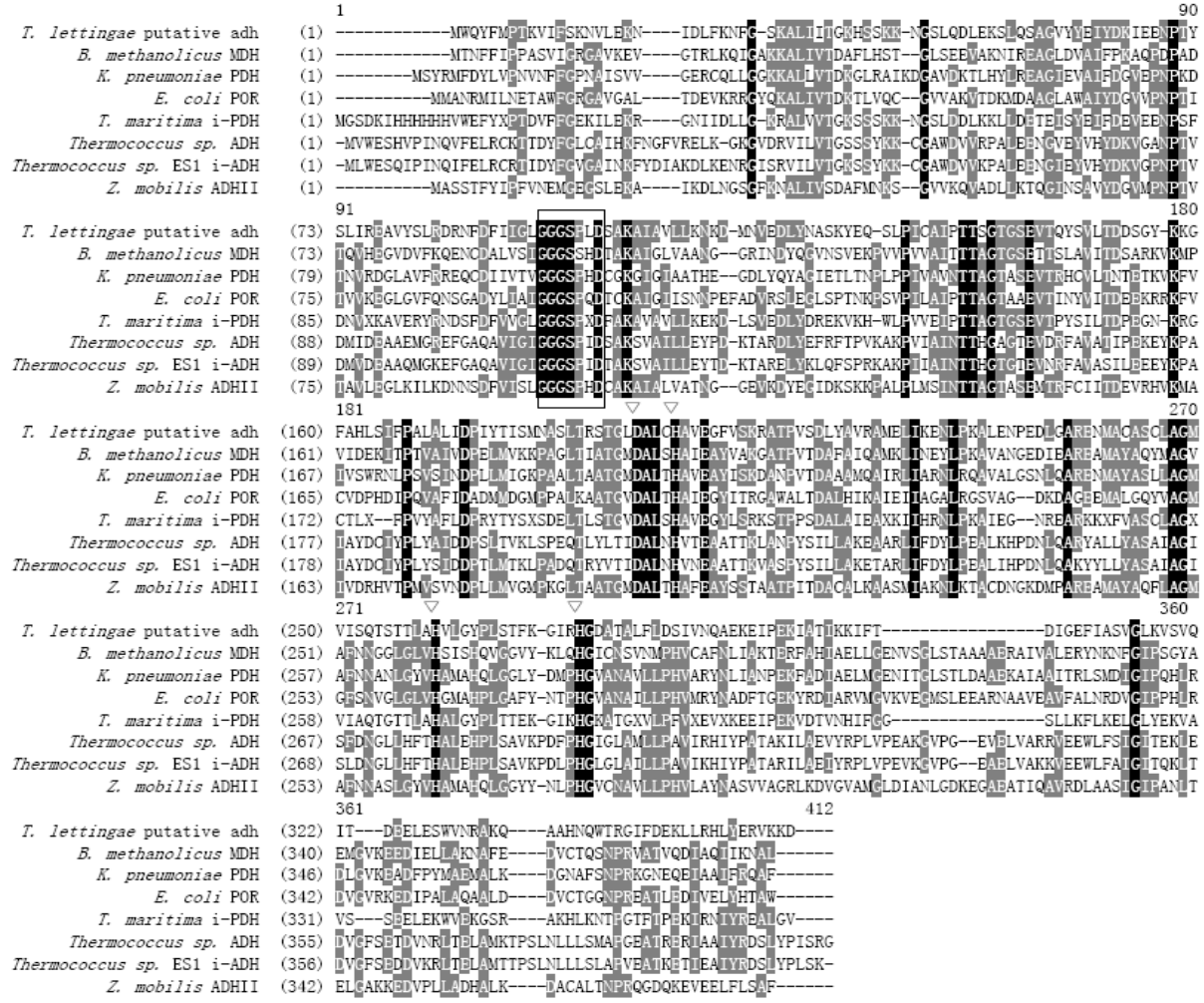

Figure 2. Multi-alignment of amino acid sequences of TlADH and other group 3 bacterial ADHs. The identical amino acids were showed in white with black background and the conserved amino acids were showed in black with gray background. The conserved iron binding sites, His260, His274, Asp191 and His195 were bolded and marked with downtriangles. The typical coenzyme binding sites, GGGSXXD, of iron-containing ADHs were boxed.

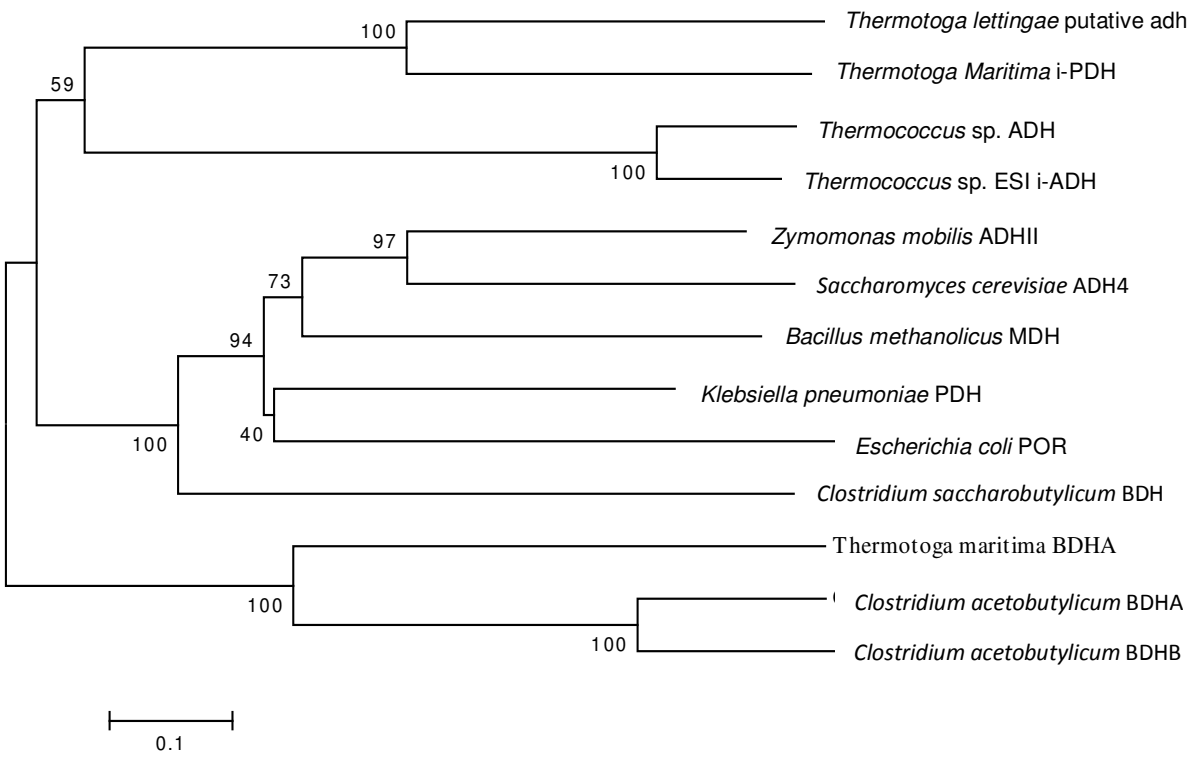

Figure 3. A phylogenetic tree of group 3 alcohol dehydrogenases from various organisms was constructed by the neighbor-joining method on MEGA 3. The numbers on the branches represented bootsrap support for 1000 replicates. TlADH was gathered with some group 3 ADHs from thermophilic or hyperthermophilic bacterias while butanol dehydrogenases were gathered together. The sequences used were listed below with GenBank or PDB accession number: *Thermotoga Maritima* Iron-Containing 1,3-Propanediol Dehydrogenase, 1O2D_A; *Thermococcus* sp. *alcohol dehydrogenase (Adh)*, AAB63011.1; *Thermococcus* sp. *iron alcohol dehydrogenase ES1*, ACK56133; *Zymomonas mobilis*, GGC70367; *Saccharomyces cerevisiae*, CAA64131; *Bacillus methanolicus* Methanol Dehydrogenase, AAA22593*; Klebsiella pneumoniae* 1,3-propanediol dehydrogenase, YP_002236499; *Escherichia coli* L-1,2-propanediol oxidoreductase, YP002330547; *Clostridium saccharobutylicum* NADPH-dependent butanol dehydrogenase, AAA83520; Thermotoga maritime NADH-dependent butanol dehydrogenase A (TM0820), AAD35902; *Clostridium acetobutylicum* butanol dehydrogenase isozyme A, AAA23206; *C. acetobutylicum* butanol dehydrogenase isozyme B, AAA23207.

in the group 3 and long-chain multifunctional ADHs and related enzymes. It served as a consensus signature sequence to probe group 3ADH homology in other organisms (Li and Stevenson, 1997) and can be found in polypeptide sequence of TlADH. The analyzed alcohol dehydrogenases share a common motif involved in iron coordination, composed by four amino acids: His260, His274, Asp191 and His195. These histidine residues were thought to serve as metal binding ligands and be a portion of the active center (Cabiscol et al., 1994). The sequence GGGSXXD considered to be typical coenzyme binding sites was found in all alignment group 3 alcohol dehydrogenases (Figure 2).

Using MEGA version 3.0 based on CLUSTAL W alignments, a phylogenetic tree of group 3 ADH family was constructed that had been characterized. The phylogenetic analysis revealed that group 3ADH family was divided into two groups: one is BDH from *T. maritime* and *C. acetobutylicum*; and the other is other analyzed dehydrogenases of which thermophilic ADHs were gathered together (Figure 3). TlADH belonged to the latter.

Catalytic properties

Optimum pH, temperature and thermal stability

The optimal pH value for butanol oxidation was 11.9 and that for butylaldehyde reduction was 6.0. When the pH values were lower than 9.5, only low activities for butanol oxidation can be detected. Meanwhile, the activities of butylaldehyde reduction were decreased to lower than 50% when the pH values were higher than 7.0 and it almost loss activities of butylaldehyde reduction above pH 10 (Figure 4A). The property of optimal pH for TlADH is similar to that for other thermophilic ADHs. For example, Optimal pH for ethanol oxidation and

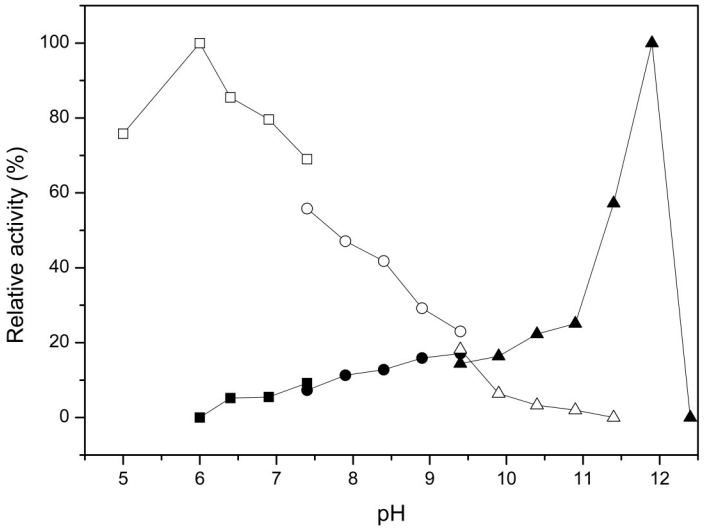

Figure 4A. Biochemical characterizations of the recombinant TlADH. pH dependence of butanol oxidation and butyaldehyde reduction activities catalyzed by the recombinant ADH. The optimal pH value for butanol oxidation was 11.9 and that for butylaldehyde reduction was 6.0. Filled symbols represent the oxidation of 1-butanol; open symbols represent the reduction of butyraldehyde. Squares, pH 6.0 - 7.4 (phosphate buffer); circles, pH 7.4 - 9.4 (Tris-HCl buffer); triangles, pH 9.4 - 12.4 (glycine-NaOH buffer).

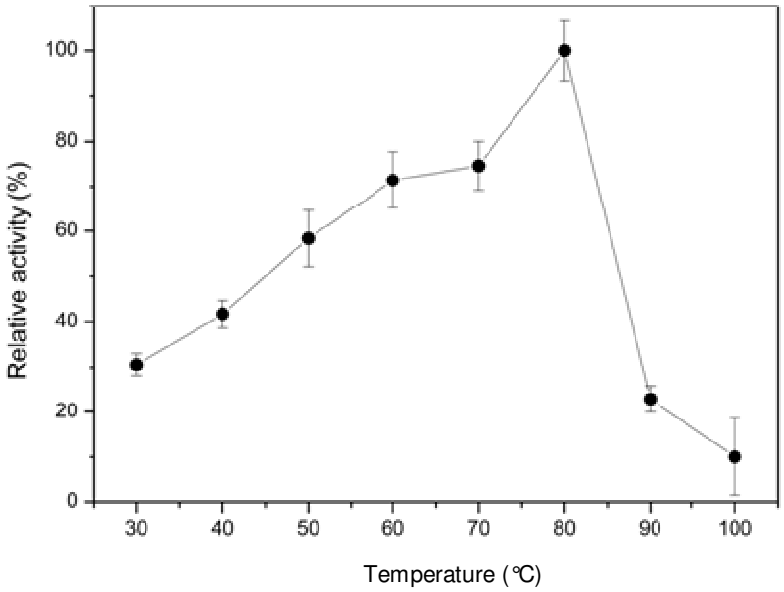

Figure 4B. Biochemical characterizations of the recombinant TlADH. Optimal temperature measured by butanol-dependent oxidative reaction and was highest at 80℃.

acetaldehyde reduction of *Thermococcus* strain ES1 i-ADH were at 10.4 and 7.0 and those of *T. hypogea* i-ADH were at 11.0 and 8.0 (Ying et al., 2009; Ying et al., 2007).

Incubation in the temperature range from 30 to 100℃, the activity of TlADH was highest at 80℃ and lost 80% at 90℃ (Figure 4B). The thermal stabilities of TlADH were further determined after incubation at 85 and 95℃. After incubation for 1 h at 85 and 95℃, the residual activities of butylaldehyde reduction were retained for 50 and 20%, respectively. No activities were detectable after 6 h at

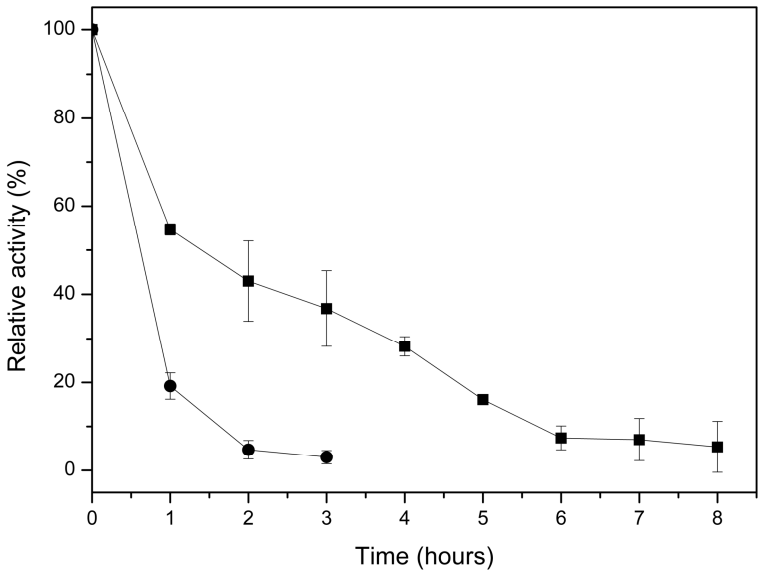

Figure 4C. Biochemical characterizations of the recombinant TlADH. Thermostabiltiy of TlADH: olid squares, 85℃; solid circles, 95℃.

85℃ and 3 h at 95℃ (Figure 4C). The optimal temperatures of those thermophilic group 3 ADHs were similar, all above 80℃, but their thermostabilities were quite different. The most stable ADH was from *P. furious* which $t_{1/2}$ was 160 h at 85℃ and 7 h at 95℃ (Ma and Adams, 1999), whereas, $t_{1/2}$ was 0.25 h at 80℃ for *T. hydrothermalis* ADH (Ying et al., 2007). Previous studies showed that thermostabilities of the ADHs were relative to pH. The enzyme may form polymerized state at alkaline pH that partially overcomes the thermal destabilization of the enzyme molecule (Ying et al., 2007).

Substrate specificity and coenzyme affinity

Various alcohols and aldehydes were used to investigate the substrate specificity coupled with $NADP^+$ reduction or NADPH oxidation. The TlADH was able to oxidize 1, 3-propanediol at the highest specific activity of 56.9 U mg^{-1}. The other alcohols, 1-butanol, 3-methyl-1-butanol, 1-octanol, ethanol and 1, 2-propanediol gave the activities of 21.1, 14.3, 14.2, 10.1 and 4.32 U mg^{-1}, respectively. No activities with 2, 3-butanediol and hexalin were detected. It is indicated that TlADH was active toward straight chain alcohols but was inactive towards branched-chain alcohols and cyclitol. The butylaldehyde, aldehyde, benzaldehyde, formaldehyde gave the activities of 131.5, 67.1, 21.7, 11.0 U mg^{-1} respectively. The higher aldehyde reduction activity was detected to use aldehyde as substrate that may be relative to reactive aldehyde detoxification in cell metabolism. Aldehydes are known to inactivate of key metabolic enzymes and form DNA adducts, which increases mutation rates and inhibits DNA replication (Schweiger and Deppenmeier, 2010). The apparent Km value for 1-butanol oxidation was 0.53 mM and the Vmax was 17.3 U mg^{-1}. The apparent Km value for 1, 3-propanediol oxidation was 0.50 mM and the Vmax was 92.1 U mg^{-1}. It was meant that TlADH had the similar affinity but different catalytic efficiency for them. For alcohol oxidation, the enzyme preferred $NADP^+$ as the cofactor with a Km value of 0.052 mM, while the Km for NAD^+ was 0.519 mM. Some group 3 ADHs were strictly specific for $NADP^+$ as an electron carrier and show no activity with NAD^+ such as *T. litoralis* ADH (Montella et al., 2005) and *H. hydrothermalis* ADH (Ying et al., 2007), some group 3 ADHs are active toward both NAD^+ and $NADP^+$, but they have a relative high affinity for $NADP^+$ (Ma and Adams, 1999).

Ions, chelator and reducing agents effects

The pre-incubated with TlADH, EDTA, $ZnCl_2$ and $CuCl_2$ inhibited the activity. Conversely, TlADH was activated by $MnCl_2$, $MgCl_2$, $CoCl_2$, DTT and mercaptoethanol, while $CaCl_2$ had little effect on the activity. Interesting, the TlADH incubated with 1mM $MnCl_2$, the activity was largely enhanced by 523% (Figure 4D). The enzymatic activity of 1, 3-propanediol dehydrogenase from *K. pneumoniae* was kept by adding 1 mM $MnCl_2$ (Marçal et al., 2009). However, the sequence alignment showed that TlADH had significant similarity with 1, 3- propanediol dehydrogenase from *K. pneumoniae*, reached at 46.9% identities and 59.7% positives. Although there was no detail to explain the effects of $MnCl_2$, this suggests that

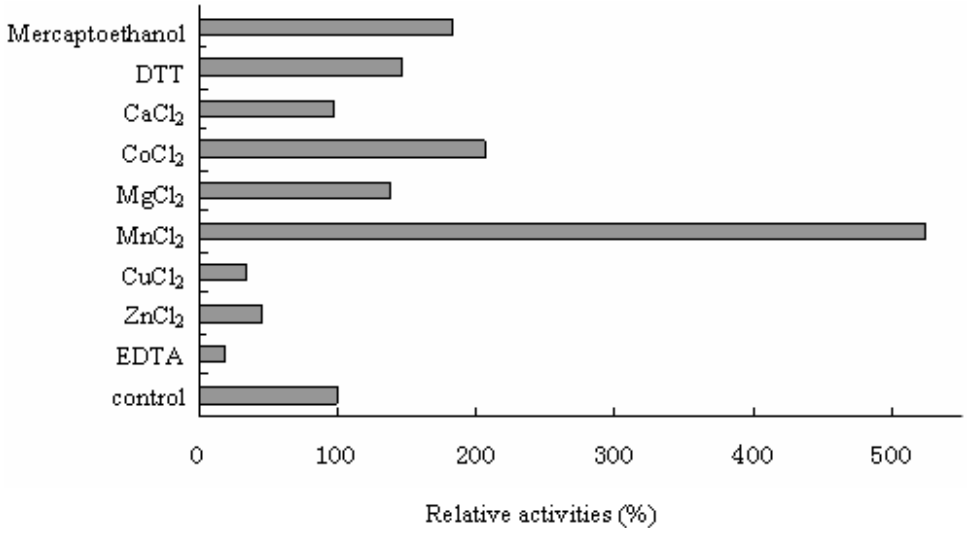

Figure 4D. Biochemical characterizations of the recombinant TlADH. Additive effects of various compounds on TlADH. The pre-incubated with TlADH, EDTA, $ZnCl_2$ and $CuCl_2$ inhibited the activity. $MnCl_2$, $MgCl_2$, $CoCl_2$, DTT and mercaptoethanol activated the activity and $CaCl_2$ had little effect on the activity.

the structure of TlADH active sites were similar with that of 1, 3-popanediol dehydrogenase from *K. pneumoniae* (Schwarzenbacher et al., 2004). The alcohol dehydrogenase family appears to require a divalent metal ion for catalysis. For example, *E. coli* propanediol dehydrogenase was in dependence on Fe^{2+} for activity (Montella et al., 2005), while *Bacillus stearothermophilus* glycerol dehydrogenase showed Zn^{2+} dependence (Ruzheinikov et al., 2001). Whereas methanol dehydrogenase from a thermotolerant *Bacillus* sp. was involved in Zn^{2+} and Mg^{2+} in the active site (Arfman et al., 1997).

In conclusion, we reported the cloning, over-expression, and characterization of ADH from *T. lettingae* TMO. Although in vitro TlADH activities were included, in vivo the role of the enzyme also need to be make sure. *T. lettingae* TMO was isolated in 2002 from a sulfate-reducing bioreactor operated at 65°C with methanol as the sole carbon. The unique ability of *T. lettingae* to utilize methanol made it a very attractive object for biotechnology. However, its molecular mechanism is still unknown. Fernandez et al. emphasized that group 3ADHs were involved in aldehyde detoxication in microorganisms rather than in alcohol turnover (Fernandez et al., 1995). Therefore, analyzed the native ADH in *T. lettingae* TMO should be included in the future.

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