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Cloning and Expression of Alcohol Dehydrogenase I and II from the Ethanologenic *Zymomonas mobilis* in *Escherichia coli* and Potential for Bioethanol Production

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The *adh I* and *adh II* genes encoding the alcohol dehydrogenase of an ethanologenic *Zymomonas mobilis* TISTR 405 have been cloned and characterized regarding their expression in *Escherichia coli* XL1 Blue. The *adh I* and *adh II* genes contain 1,014 and 1,152 nucleotides encoding 337 and 383 amino acid residues, respectively. The 405-ADH I and 405-ADH II proteins are highly conserved among *Z. mobilis* species but distant from the yeast genera *Saccharomyces* and *Candida*. The molecular weights of the expressed 405-ADH I or II proteins were 34 and 38 kDa, respectively. The ADH activity of the transformants expressing *adh I* and *II* was also detected via native PAGE at the same molecular weights. The comparative models of Zn-dependent 405-ADH I and Fe-dependent 405-ADH II showed 61.95% and 99.74% similarity to the crystal structures of LlAdhA from *Lactococcus lactis* and zmADH2 from *Z. mobilis* ZM4, respectively. Gas chromatography analysis showed that the transformants expressing ADH I and II produced ethanol at 2.5 and 3.9 % (v/v), respectively. Apparently, these two enzymes could function independently for bioethanol production in *E. coli*. The characteristics of ADH I and ADH II enzymes will be further investigated for the potential bioethanol production.

Key words: Alcohol dehydrogenase, ADH I, ADH II, bioethanol, *Zymomonas mobilis*.

Ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the *Saccharomyces* spp. currently used for biofuel alcohol production. With further advances in biotechnology, these bacteria can potentially be employed for more economical production of ethanol[1]. Among the ethanol-producing bacteria, *Zymomonas mobilis* is a well-known organism that has historically been used in tropical areas to produce alcoholic beverages from plant sap[2]. The advantages of *Z. mobilis* are its high growth rate and ability to produce high yield of ethanol from glucose through the Entner-Doudoroff (ED) glycolytic pathway, glyceraldehydes-3-phosphate-to-pyruvate (GP), and pyruvate to ethanol via pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH, EC 1.1.1.1)[3-5]. Two ADH isoenzymes are present in *Z. mobilis* that catalyze the reduction of acetaldehyde derived from pyruvate to ethanol during fermentation, accompanied by the oxidation of NADH to NAD^+. Its high ethanol yield was likely a consequence of an equilibrium reaction toward ethanol synthesis via these two enzymes[6]. ADH I encoded by the *adh I* gene, is a Zn-dependent member of Group I

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while ADH2 encoded by the *adh II* gene, is an Fe-dependent member of Group III[5]. Although, the protein sequence and structure were unrelated, their roles in ethanol cycle were associated with the flexible distribution of reducing equivalents between the two ADHs and growth conditions[7].

The carbohydrates fermented by *Z. mobilis* are limited to glucose, fructose, and sucrose, while other renewable carbon substrates, such as lactose, xylose, starch, and many agricultural cellulosic wastes, cannot be directly converted into ethanol[3,8]. Accordingly, many native ethanologenic strains are not well-suited for efficient ethanol production[9]. Thus, the development of effective strain that produces high yield of ethanol is currently urgent when the alternative biofuel is needed to replace vanishing fossil fuels.

ADH-encoded genes from *Z. mobilis* have been cloned and successfully expressed in *E. coli*[3,10,11]. Recombinant *E. coli* expressing plasmid-borne *pdc* and *adh II* genes efficiently converted both hexose and pentose to ethanol [8]. Despite a high level of ethanol produced, the plasmid-based recombinants were less stable and not well-suited for a long term and upscale fermentation[11]. The genes encoding for PDC and ADH II from *Z. mobilis* have been chromosomally integrated into *E. coli* KO11 and intensively studied for the capacity in ethanol production[9, 12-14]. This strain produced ethanol (92 % of theoretical yield) from dilutic acid and toxic hydrolysate of rice hulls[15]. Fermentation performance for cellulosic ethanol production between three metabolically engineered ethanologenic strains including *E. coli* KO11, *S. cerevisise* 424A(LNH-ST) and *Z. mobilis* AX101 has been reported[9]. Both KO11 and AX101 strains were more effective in consuming sugar and converting to ethanol. In contrast, 424A(LNH-ST) strain showed the highest xylose consumption extent and rate.

It should be noted that despite the wealth of publications on *Z. mobilis* genes for the ethanol pathway, variations in experimental conditions make a troublesome comparison between a variety of genotypic and phenotypic ethanologenic strains. To better understand the nature of ethanol-production enzymes, observation of more *Z. mobilis* strains could definitely provide insight into the greatest value obtained from these bacteria and how their ethanol production were further achieved.

In this study, we cloned the *adh I* and *adh II* structural genes encoding alcohol dehydrogenase from *Z. mobilis* TISTR 405 and characterized their expression in the *E. coli* XL1 Blue. Understanding the physico-chemical and functional properties of these genes could lead to the development of an effective and well-defined system for microbial bioethanol production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Z. mobilis TISTR 405 was grown in *Zymomonas* medium at 30 °C without agitation. *E. coli* XL1 Blue used as a bacterial host for DNA transformation, was grown at 37°C in Luria-Bertani (LB) broth. The pBluescript II KS+ (pBKSII) plasmid was used as the vector for cloning. Positive clones were selected on LB agar plates containing 100 µg/ml ampicillin, 5 mM IPTG, and 20 µg/ml X-gal.

Cloning and expression of *adh I* and *adh II*

The chromosomal DNA of *Z. mobilis* TISTR 405 was extracted according to Sambrook and Russell (2001), with some modifications. For plasmid isolation, the QIAprep Spin Miniprep Kit (Qiagen) was used. Two pairs of specific primers, ZadhIF (5' CCCTCGAGGTAATCGGCT GGCAA TCGTTTTCC3') and ZadhIR (5' GTTCTA GAGA TAGCGGCTTATAGCAACGAGTG3') for the *adh I* gene and ZadhIIF (5' CTCTCGAGAAAGGCAA AATCGGTAACCACATC3') and ZadhIIR (5' GCTCTAGAACAAATGCCTCCGATTAGAAATCG3') for the *adh II* gene, were used for PCR amplification. The engineered restriction sites for *Xho*I in the *adh I* primers and *Xba*I in the *adh II* primers are underlined. The PCR amplification reactions contained 2.5 mM dNTPs, 20 µmol of each primer, 5 µg of genomic DNA template, and 2.5 U *Taq* polymerase . The amplification program consisted of 40 cycles of the following conditions: 30 sec at 94 °C, 30 sec at 58 °C for *adhI* or 64 °C for *adh II*, and 2 min at 72 °C, with a final extension for 3 min at 72 °C. The PCR fragments corresponding to the *adh I* or *adh II* gene cloned into the pGEM®-T Easy vector were sub-cloned into pBKSII and transformed into *E. coli* XL1 Blue via the heat shock method. Restriction, ligation, and cloning essentially followed the standard procedures

described by Sambrook and Russell (2001)[16]. Total protein composition of the transformant cells was analyzed through 12.5% SDS-PAGE.

Sequence analysis and structure comparative modelling

The PCR products containing the *adh I* and *adh II* geneswere cloned into the pGEM®-T Easy vector (Promega) and sequenced. The *adh I* (GenBank accession JX962697) and *adh II* (GenBank accession JX962698) nucleotide sequences were analyzed using the standard BLAST system. The amino acid translation and alignment were analyzed with BioEdit5 and ClastalX version 2.0.9. Phylogenetic tree analysis of the ADH I and II proteins was performed using a maximum likelihood program of the MeGa 5.05. The secondary structure and comparative modeling of three-dimensional (3D) protein structures was analyzed using Modelling-Automated Mode of SWISS-MODEL server.

Alcohol dehydrogenase activity assay

The activity of the ADH I and ADH II proteins was analyzed and visualized using nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) via native PAGE as described by Dewey and Conklin (1960)[17]. Briefly, wild-type *E. coli* and *E. coli* transformants were grown in 10 mL of LB broth until the optical density at 600 nm reached 0.6. Cells were harvested by centrifugation for 5 min at 13,000 rpm. The cell pellet was washed with 700 µl of ADH lysis buffer (85 mM KCl, 30 mM Tris-HCl (pH 7.5), 3 mM Mg acetate, 25 % glycerol). The cell pellet was frozen on dry ice for 30 min and incubated overnight at -70 °C. The cell pellet was thawed on ice, and 0.2 g glass beads and 200 ml of ADH lysis buffer containing 0.07 % α-mercaptoethanol were added. The cells were lysed by vortexing at 4 °C. The cell lysate was centrifuged at 13,000 rpm for 10 min. The protein concentration of the supernatant was determined using the Bio-Rad Protein assay. Protein samples (100 µg per lane) were mixed with loading buffer and analyzed by native-PAGE. After electrophoresis, the gel was soaked in 100 mM Tris-HCl, pH 8.8, for 5 min. ADH enzyme activity was visualized by staining the gel with 50 ml of ADH activity buffer (50 mg NAD^+, 10 mg NBT, 4 mg (PMS), 1.5 M Tris-HCl (pH 8.0), 50 µL ethanol), followed by incubation at 37 °C until the signal could be detected.

Ethanol production analysis

The ethanol concentration in supernatants produced by the transformants expressing the *adh I* and *adh II* genes was detected using gas chromatography (GC), as described by Beall *et al*. (1991)[24]. The gas chromatographer equipped with a HP-INNOWAX capillary column and flame ionization detector (FID), using nitrogen as the carrier gas. All tests were run in triplicate.

RESULTS

Cloning of *adh I* and *adh II*

Ethanologenic *Z. mobilis* TISTR 405 was thermotolerant[4] and able to produce approximately 2% ethanol in the culture broth and tolerate up to 5% ethanol (Fig. 1). The *adh I* and *adh II* DNA fragments were amplified from the genomic DNA and the PCR products were visualized through 0.8% agarose gel electrophoresis. The products containing *adh I* and *adh II* were approximately 1.2

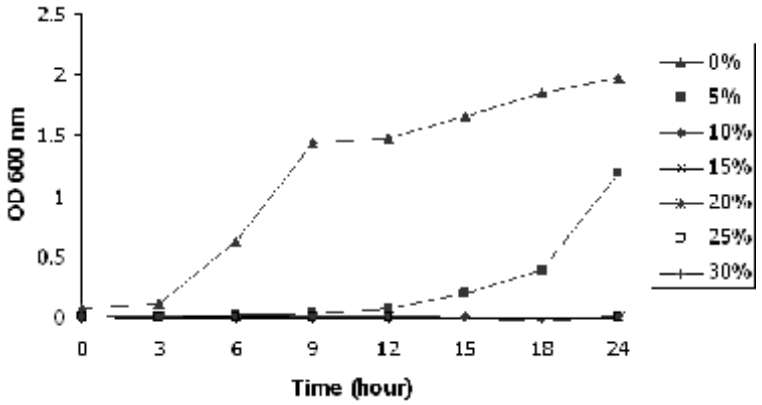


Fig. 1. Effect of 5-30% ethanol concentration on the growth of Z. *mobilis* TISTR405

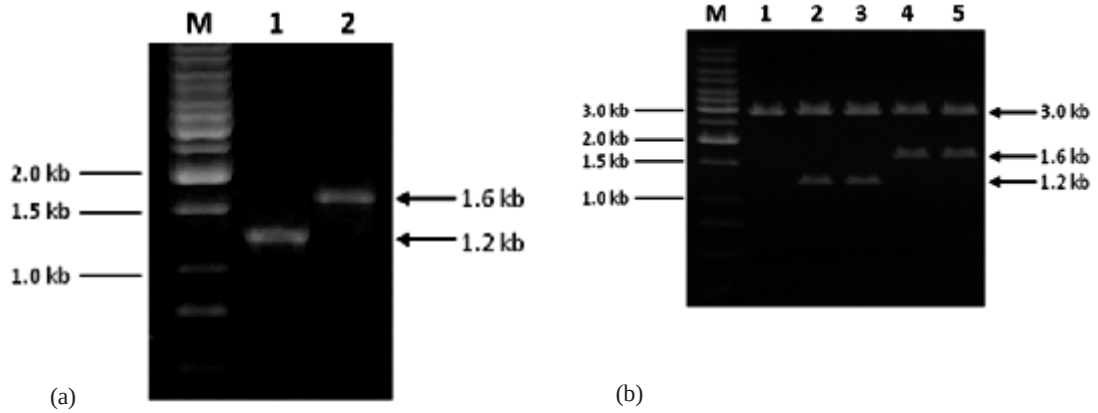


Fig. 2. (a) PCR products of *adh I* and *II* M: DNA marker; 1: *adh I* ; 2: *adh II* (b) Analysis of positive plasmid clones digested with *Xba*I and *Xho*I M: DNA marker; 1: pBKSII; 2-3: pBKS*adh I*; 4-5: pBKS*adh II*

and 1.6 kb in length, respectively (Fig. 2a). The PCR fragments containing each gene were sub-cloned into the pBKSII vector and transformed into *E. coli* XL1 Blue. Positive clones were selected on LB medium containing ampicillin, IPTG, and X-gal. Based on *Xba*I and *Xho*I digestion, potential recombinant plasmids containing the *adh I* or *adh II* gene should be cleaved into two fragments: a 3.0 kb vector fragment and an insert fragment of 1.2 kb or 1.6 kb, respectively (Fig. 2b). The pBKSII vectors with *adh I* and *adh II* inserts were designated as pBKS*adhI* and pBKS*adhII*, respectively.

ADH I and ADH II expression in *E. coli* XL1 Blue

The total protein samples (15 µg per lane) of *E. coli* XL1 Blue and transformants were loaded into a 12.5 % SDS-PAGE. The expression levels of *Z. mobilis* 405 ADH I (405-ADH I) and ADH II (405-ADH II) were similar in the absence and presence of IPTG. The corresponding molecular weights of 405-ADH I and 405-ADH II were 34 kDa (lanes 5-6) and 38 kDa (lanes 7-8), respectively (Fig. 3a).

ADH activity assay

The ADH activity assay is based on the ability of the ADH to reduce NBT when PMS is used as an intermediate electron carrier to form a blue formazan. On native gel, the 405-ADH I and II bands migrated to positions corresponding to sizes of 34 and 38 kDa, respectively (Fig. 3b, lanes 3-4) which was in according to the expressed proteins observed in SDS-PAGE (Fig. 3a). No 405-ADH activity bands were observed in the control extracts from *E. coli* XL1 Blue with or without the intact pBKSII (Fig. 3b, lanes 1-2).

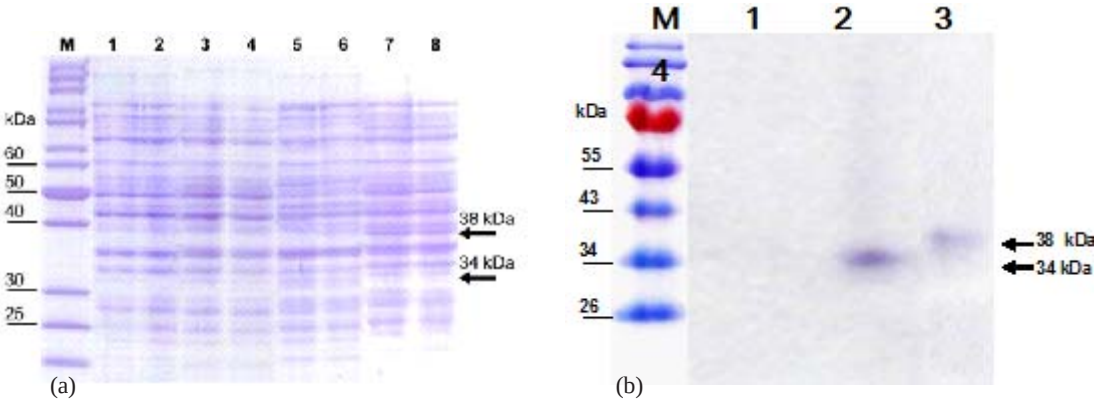


Fig. 3 (a) Total protein analysis via 12.5% SDS-PAGE of *E. coli* XL1 Blue and transformants. M: protein marker, 1: *E. coli*, 2: *E. coli*+ IPTG, 3: pBKSII 4: pBKSII + IPTG, 5: pBKS*adh I*, 6: pBKS*adh I* + IPTG, 7: pBKS*adh II*, 8: pBKS*adh II* + IPTG (b) Analysis of 405-ADH I and II activity via native PAGE. M: protein marker, 1: *E. coli*, 2: *E. coli*/pBKSII, 3: Transformant/pBKS*adh I*, 4: Transformant/pBKS*adh II*

Nucleotide sequence and amino acid analysis

The *adh I* and *adh II* nucleotide sequences were 1,014 and 1,152 bp in length and subjected to homology searches using BLAST, and showed 99% identity with *adh I* and *adh II* from *Z. mobilis* ZM4, the ethanol-producing type strain[5]. The amino acid sequences of the 405-ADH I and II were 337 and 383 amino acids in length, respectively. From BLAST analysis, the amino acid sequences showed 98 and 99 % identity with zmADH1 and zmADH2 from *Z. mobilis* ZM4, respectively. The ADH I and II proteins were highly

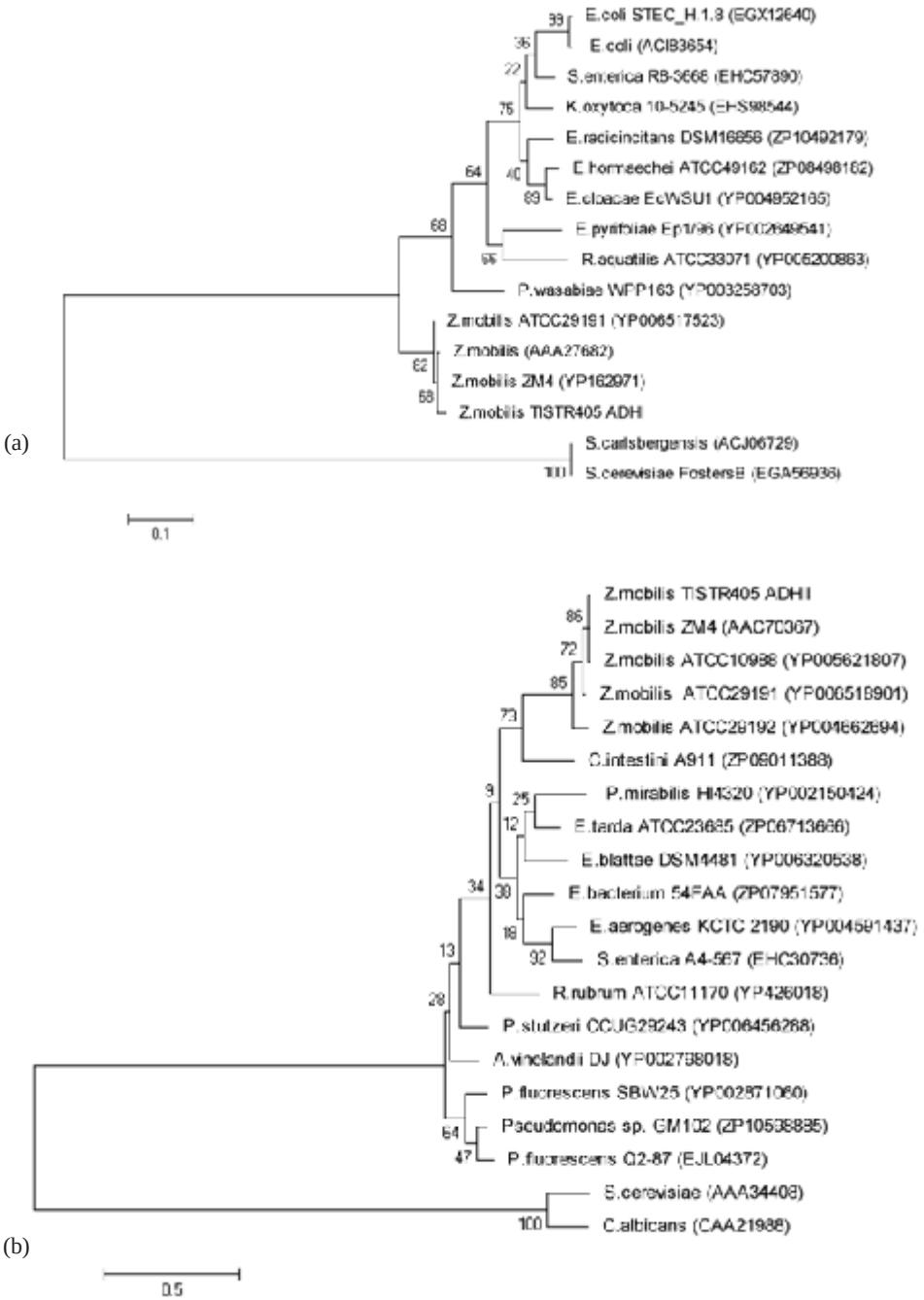


Fig. 4. Phylogenetic analysis of amino acid of (a) 405-ADH I and (b) 405-ADH II

conserved among *Z. mobilis* species, relatively conserved among other gram-negative bacteria, but distant from *Saccharomyces* yeast species (Fig. 4).

Comparative model of 3D protein structures

The 405-ADH I subunit consists of two domains, the zinc-binding domain and the GroES-like domain (Fig. 5a). The 3D comparative 405-ADH I model exhibits 61.95% identity to the crystallized NADH-dependent LlAdhA from *Lactococcus lactis*[18] (Fig. 5b). The catalytic Zn- and NAD(P)-binding Rossmann-fold domains were presented in both L1AdhA and 405-ADH I. The 405-ADH II subunit consists of an Fe-binding domain (Fig. 6a). Both 405-ADH I and II consisted of 22 randomly unstable coil structure and most were presented at the N-terminus. The comparative 3D model of 405-ADH II exhibits 99.74% identity to the crystallized zmADH2 from *Z. mobilis* ZM4 [6] (Fig. 6b). The catalytic function of zmADH2 was likely dependent on cooperative binding of Fe ion and NAD^+ at the active site[5].

Determination of ethanol concentration

When grown in LB medium with 8 % glucose and incubated without agitation for 18 h, the transformants expressing *adh I* and *II* produced detectable levels of ethanol at 2.5 and 3.9 % (v/v) of ethanol (volume ratio of culture to supernatant), respectively (Fig. 7), whereas neither *E. coli* XL1 Blue nor *E. coli* XL1 Blue harboring intact pBKSII produced ethanol.

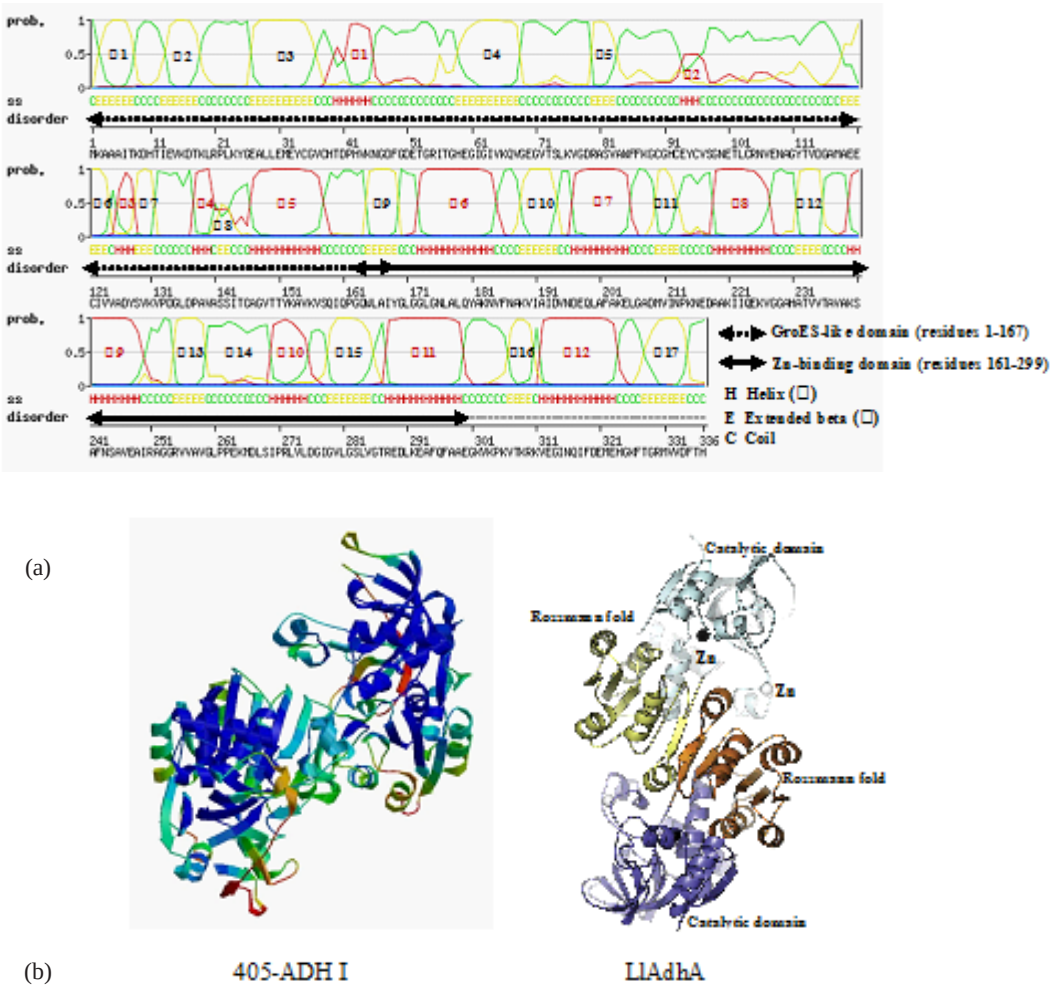
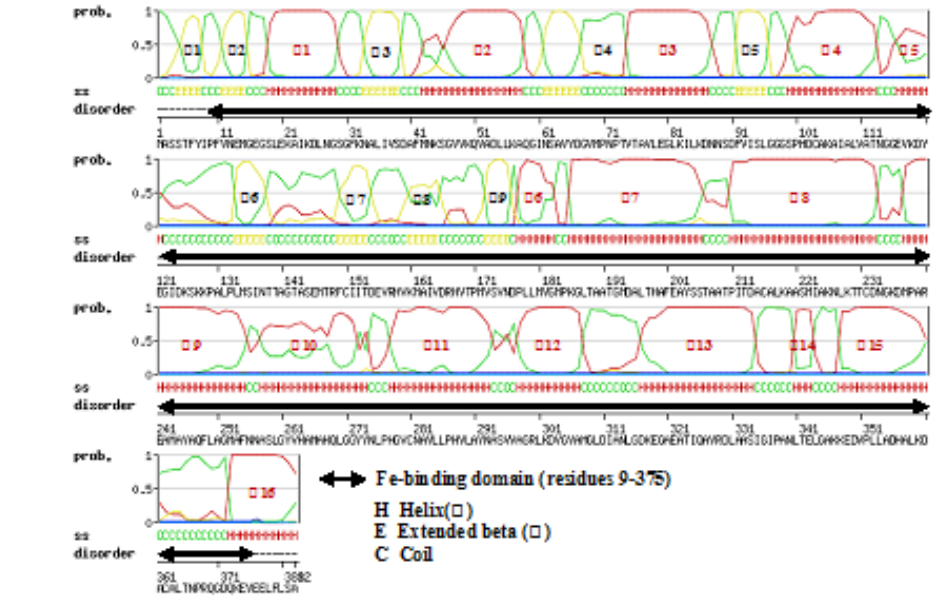


Fig. 5. Features of 405-ADH I (a) Secondary structure (b) 3D model of 405-ADH I compared to the crystal structure of *L. lactis* LIAdhA[25]

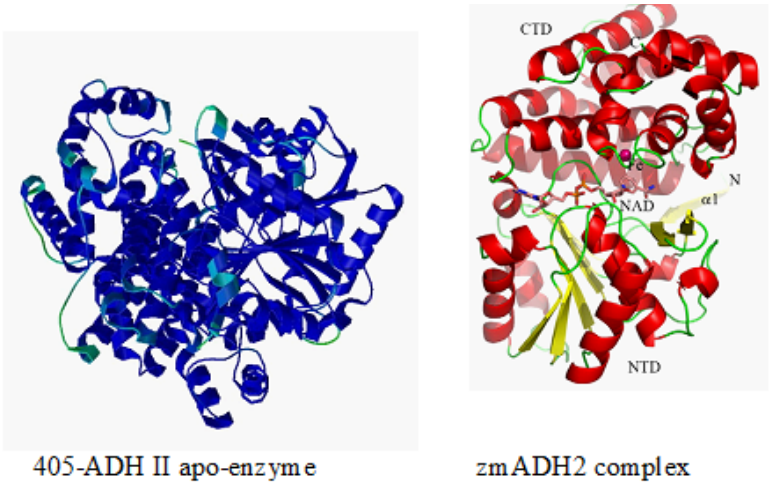
DISCUSSION

Ethanologenic *Z. mobilis* TISTR 405 was a thermotolerant strain. It was able to produce approximately 2% ethanol in the culture broth and tolerate up to 5% ethanol. In addition, it could produced more ethanol than the ethanol-producing *Z. mobilis* ZM4 type strain at 30 and 37 °C [4]. The expression and detectable activity of 405-ADH I and II observed on the SDS-PAGE and native PAGE indicated the functional and active enzymes in *E. coli* which is in accordance to the previous reports describing the high expression level of genes from *Z. mobilis* in *E. coli* [1,3]. The *pdc* and *adhB* genes from *Z. mobilis* were also expressed and active in Gram positive bacteria, *Bacillus subtilis* and *Bacillus polymixa* demonstrating that there is no inherent barrier between unrelated bacteria[19]. This observation could be advantageous for the development of effective ethanologenic strains in a wide variety of bacterial hosts.

The expression of the *adh I* and *II* genes



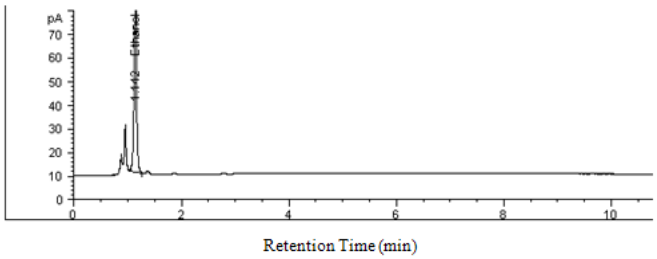
(a)



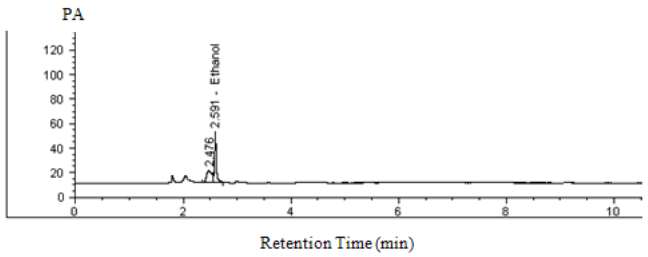
(b)

Fig. 6. Features of 405-ADH II (a) Secondary structure (b) 3D model of 405-ADH II apo-enzyme compared to the crystal structure of zmADH2 in complex with Fe ion and NAD^+ cofactor of *Z. mobilis* ZM4 [6]. NTD, CTD: N-, C- Terminal Domains (a)

(a)



RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ml/100ml]	Grp	Name
2.591	VB	94.31014	2.70314e-4	2.54933e-2		Ethanol



(b)

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ml/100ml]	Grp	Name
1.142	BB	200.88292	3.88114e-4	7.79656e-2		Ethanol

Fig. 7. GC analysis of the ethanol yield produced by the transformants expressing (a) *adh I* and (b) *adh II*

located within the pBKS*adhI* and pBKS*adh* in the absence of IPTG suggested that the additional sequences upstream of the structural genes of *adh I* (~ 200 bp) and *II* (~ 450 bp) within the insert fragments might be the promoter or upstream-regulation sequences *Z. mobilis* which were responsible for gene expression in *E. coli*.

The GroES-like domains found in 405-ADH I were consistent to their function related to aldehyde and alcohol metabolisms[20]. The relative similarity of 405-ADH I and LlAdhA suggested that their enzymatic mechanism might be related to one another. Beside ethanol, both enzymes were able to produce butanol[4,18]. The amino acid engineered L1AdhA variant has shown the improvement in isobutanol synthesis[18]. Hence, the properties of 405-ADH I could be further investigated and may be a promising candidate for effective production of butanol, the next-generation biofuel.

The crystal structure of zmADH2 from *Z. mobilis* ZM4 has been reported[5]. The author stated that the structure of zmADH2-Fe-NAD^+ complex was relatively crucial for the behavior of *Z. mobilis* ZM4 under anaerobic and aerobic conditions. Under anaerobic condition, both zmADH1 and zmADH2 concomitantly catalyzed the reduction of acetaldehyde to ethanol and the oxidation of NADH to NAD^+. However, if oxygen is present, the acetaldehyde reductase activity of zmADH2 to produce ethanol was inhibited. In a reverse reaction, zmADH2 could also reduce ethanol back into acetaldehyde and oxidize NAD^+ into NADH. The ADH II from *Z. mobilis* ATCC 29191 also exhibited those mechanisms similar to zmADH2 [6]. The amino acid sequence similarity between 405-ADH II and zmADH2 make us speculate that their enzymatic mechanisms are possibly much alike.

The conserved properties in amino acid sequence, protein structure and activity of ADH enzymes among ethanologenic *Z. mobilis* strains indicate their closed relationship and well-adapted through the evolution. Based on the bi-functional activities of zmADH2, *Z. mobilis* may favor the ethanol fermentation to maintain a low ATP usage. When ethanol was too high, it may cause deleterious effect to the cell. To reduce ethanol toxicity, the ADH II subsequently oxidizes the

ethanol to acetaldehyde. Furthermore, the coupling reaction causes a release of NADH from the oxidation of NAD^+. The NADH molecule was then utilized in the respiratory chain to produce an energy for a proper survival under ethanol stress. The production of ethanol yield at 2.5 and 3.9 % of *E. coli* transformants expressing 405-ADH I and 405-ADH II under static condition indicated that these enzymes could function independently. It has been reported that the ADH I activity was high at the exponential stage, but ADH II activity was high at the stationary stage[3,5]. From our observation, the ethanol yield was measured after 18 h, at a stationary stage of fast-growing *E. coli* XL1 Blue. Therefore, the 405-ADH II was assumed to behave as a functionally dominant enzyme and lead to more ethanol produced. Some Fe ion trace found in yeast extract in LB medium may be another factor that might facilitate the activity of 405-ADH II but not 405-ADH 1, a zinc-dependent enzyme. To better understand the role of ADHs in the ethanol synthesis pathway of *Z. mobilis*, the co-expression of *adh I* and *adh II* genes as well as effects of some essential factors such as metal ions, cofactors, oxygen on ethanol production are of importance. Further investigation was needed for the development of an effective approach using a well-defined system for microbial bioethanol production.

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