การโคลนและการแสดงออกของยีนอะลานีนดีไฮโดรจีเนสจากไซยาโนแบคทีเรีย
ทนเค็ม *Aphanothece halophytica* ใน *Escherichia coli*

นายสิทธิพล ภู่โกสีย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

CLONING AND EXPRESSION OF ALANINE DEHYDROGENASE GENE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* in *Escherichia coli*

Mr. Sittipol Phogosee

A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Microbiology
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Thesis Title	CLONING AND EXPRESSION OF ALANINE DEHYDROGENASE GENE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* in *Escherichia coli*
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สิทธิพล ภู่โกสีย์ : การโคลนและการแสดงออกของยีนอะลานีนดีไฮโดรจีเนสจากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* ใน *Escherichia coli* (CLONING AND EXPRESSION OF ALANINE DEHYDROGENASE GENE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* in *Escherichia coli*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รุ่งอรุณ วาดิถี สิริศรัทธา, 4 หน้า.

อะลานีนดีไฮโดรจีเนสเร่งปฏิกิริยาผันกลับระหว่างไพรูเวทไปเป็นอะลานีน (PvRAและ ALD) และ ปฏิกิริยาที่ไม่ผันกลับของไกลออกซิเลทไปเป็นไกลซีน (GxRA) จากงานวิจัยที่ผ่านมาพบว่าเอนไซม์ชนิดนี้ทำหน้าที่สำคัญในกระบวนการทางชีวภาพที่หลากหลาย โดยทำหน้าที่เกี่ยวข้องกับการสร้างสปอร์ในแบคทีเรีย ขณะที่เอนไซม์นี้ต้องการสำหรับการย่อยสลายรงควัตถุในสิ่งมีชีวิตที่สามารถสังเคราะห์ด้วยแสง ในการศึกษาครั้งนี้ได้ทำการโคลน และแสดงออกยีนอะลานีนดีไฮโดรจีเนสจากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* (*ApalaDH*) ใน *Escherichia coli ApalaDH* ประสบผลสำเร็จในการแสดงออกเมื่อใช้เวกเตอร์ pColdI และ pColdTF แต่พบว่าไม่สามารถแสดงออกเมื่อใช้เวกเตอร์ pTrcHis2C จากนั้นโปรตีนถูกทำให้บริสุทธิ์ และนำไปศึกษาลักษณะสมบัติเชิงหน้าที่ รีคอมบิแนนท์เอนไซม์ที่ได้สามารถเร่งปฏิกิริยาได้สองปฏิกิริยาด้วยกัน คือ ไพรูเวทไปเป็นอะลานีน และไกลออกซิเลทไปเป็นไกลซีน ซึ่งแตกต่างจากAlaDH ของไซยาโนแบคทีเรียเส้นสาย *Anabaena variabilis* ค่าจลนพลศาสตร์ (Km) ต่อไพรูเวท อะลานีน และไกลออกซิเลทเท่ากับ 0.22 ± 0.02, 0.72 ± 0.04 และ 1.91 ± 0.43 มิลลิโมลาร์ ตามลำดับ ยังได้ทำการศึกษาระดับการแสดงออกของ *ApalaDH* โดยเซมิ-ควอนติเททีฟ อาร์ทีพีซีอาร์ ภายใต้ภาวะความเครียดจากเกลือ *ApalaDH* มีการแสดงออกเพิ่มขึ้นประมาณสองเท่า นอกจากนั้นได้ทำการวิเคราะห์แบบ อิน วิโว ภายใต้ภาวะความเครียดจากเกลือ พบว่าแอกทิวิตี้ของปฏิกิริยา PvRA และ GxRA มีค่าสูงขึ้นประมาณ 1.3 และ 2.7 เท่า ตามลำดับ ผลการทดลองเหล่านี้บ่งชี้เป็นนัยว่า ยีนอะลานีนดีไฮโดรจีเนสมีความสำคัญภายใต้ภาวะความเครียดจากเกลือ จากงานวิจัยในครั้งนี้เป็นครั้งแรกที่ได้มีการศึกษาลักษณะสมบัติเชิงหน้าที่ของเอนไซม์อะลานีนดีไฮโดรจีเนสในไซยาโนแบคทีเรีย

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SITTIPOL PHOGOSEE: CLONING AND EXPRESSION OF ALANINE DEHYDROGENASE GENE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* in *Escherichia coli*. ADVISOR: ASST. PROF. RUNGAROON WADITEE-SIRISATTHA, Ph.D., 4 pp.

Alanine dehydrogenase catalyzes the reversible reaction of pyruvate to alanine (PvRA and ALD) and non-reversible reaction of glyoxylate to glycine (GxRA). It plays a crucial role for sporulation in bacteria while it is required for pigment degradation in photosynthetic organisms. In this study, the putative gene encoding alanine dehydrogenase from halotolerant cyanobacterium *Aphanothece halophytica (ApalaDH)* was cloned and expressed in *Escherichia coli*. *ApalaDH* was successfully expressed into the expression vector pColdI and pColdTF but not in pTrcHis2C. Recombinant ApalaDH was purified homogeneity and then functionally characterized. Recombinant ApalaDH exhibited two catalytic activities of pyruvate to alanine and glycine to glyoxylate, which were different from the AlaDH from filamentous cyanobacterium *Anabaena variabilis*. The kinetic parameter Km of ApalaDH for pyruvate, alanine and glyoxylate were 0.22 ± 0.02, 0.72 ± 0.04 and 1.91 ± 0.43 mM, respectively. These Kms of ApalaDH suggested high affinity for all substrates. The expression level under salt stress was carried out by semi-quantitative RT-PCR. The result showed that *ApalaDH* expression increased approximately two folds under salt stress. Furthermore, *in vivo* analysis was performed. Under salt stress condition, the PvRA and GxRA activities were increased about 1.3- and 2.7-folds, respectively. These results implicated that ApalaDH would important under salt stress condition. To our knowledge, this is the first functional characterization of AlaDH in cyanobacteria.

Department:	Microbiology	Student's Signature	.....
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	.....
Academic Year:	2015		

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CHAPTER I

INTRODUCTION

Glycinebetaine (*N*,*N*,*N*-trimethylglycine) is a high protection efficiency compatible solute that helps cells to survive under stress conditions. It is biosynthesized and accumulated in extremely stress tolerant organisms such as a halotolerant cyanobacterium *Aphanothece halophytica* (Waditee *et al.,* 2003). This cyanobacterium can grow in a wide range of salinity conditions from 0.5 to 2.5 M NaCl. In *A. halophytica*, glycinebetaine is biosynthesized from glycine by a novel pathway of three steps methylation (Waditee *et al.,* 2003). Thus, provision of glycine is very important for glycinebetaine biosynthesis in this extremophile. Thus far, there are four pathways of glycine biosynthesis reporting in living organisms. There are threonine aldolase, glyoxylate aminotransferase, glycine-cleavage system and serine hydroxymethyltransferase (Schlupen *et al.*, 2003). In the glyoxylate pathway, glycine is produced from glyoxylate by aminotransferase. This enzyme also catalyzes the reaction from alanine to pyruvate (Schlupen *et al.*, 2003). This reaction can be catalyzed by alanine dehydrogenase (Giffin *et al.*, 2012).

Alanine dehydrogenase (AlaDH) (L-alanine: NAD^+ oxidoreductase, deaminating, EC 1.4.1.1) catalyzes the reversible deaminating of L-alanine to pyruvate and ammonia in the presence of NAD^+. Accumulating evidence has shown that AlaDH involved in various biological processes. In spore-forming bacterium *Bacillus subtilis,* AlaDH is essential for growth when alanine is the sole carbon or nitrogen source. It is also required for sporulation (Siranosian *et al.*, 1993). In pathogenic bacterium

Mycobacterium tuberculosis, it has shown that AlaDH performs the glyoxylate reductive aminase activity. It catalyzes the reductive amination reaction of glyoxylate to glycine with the oxidation of NADH to NAD^+ but the reversible reaction cannot be detected (Giffin *et al.*, 2012). In cyanobacterium *Synechococcus elongatus* PCC 7942, AlaDH is required for pigment degradation during nitrogen starvation condition. Expression level of *ald* gene encoding AlaDH was up-regulated under nitrogen starvation condition (Lahmi *et al.*, 2006) . The non-reversible reaction between glyoxylate and glycine is one of important steps of photorespiration C2 cycle in photosynthetic organisms. AlaDH catalyzes the reaction of glyoxylate that is generated from 2-phosphoglycolate (2PG) in the first reaction. This pathway facilitates cells to detoxification toxic compound 2PG.

The products of carboxylation with CO_2 are two molecules of 3-phosphoglycerate (3PG) while the oxygenase reaction with O_2 produces one molecule of 3PG and 2PG. The product 3PG can be used in the Calvin-Benson cycle but 2PG is toxic for cells. To solve this problem, 2PG must be converted into 3PG by the photorespiration (Kern *et al.*, 2013). There are a number of enzymes involved in photorespiration. Some mutants lacking enzyme functions in photorespiration pathway have been generated. For instance, the unicellular cyanobacterium *Synechocystis* sp. PCC6803 mutant lacking carboxysome in which RuBisCo is packed cannot grow under air without CO_2 (Hagemann *et al.*, 2013). The similar phenomena were showed in green alga *Chlamydomonas reintardtii* disrupted in *glycolate dehydrogenase* (Nakamura *et al*., 2005) and C4 plant *Zea mays* mutant disrupted in *glycolate oxidase1* (Zelitch *et al.*, 2009). These evidences showed the importance of

photorespiration in detoxification of 2PG. To date, there are a few reports showing the importance of photorespiration in stress response. For example, under salt stress condition, transcription levels of the photorespiration associated genes were induced (Srivastava *et al.*, 2011). Photorespiration also plays a role under oxidative stress condition. The mutant disrupted gene in photorespiration could not eliminate the Relative Oxygen Species (ROS) (Moreno *et al.*, 2005). All these evidences indicated that photorespiration is important to survive under stress condition.

This study aims to clone, express and functionally characterize of putative AlaDH from halotolerant cyanobacterium *A. halophytica* (hereafter ApalaDH). This study would be important to clarify a role of this enzyme that may important to response under stress conditions. This knowledge would be possible to boost glycinebetaine accumulation and its application in agricultural field.

The objectives of this research

1. To clone and express *AlaDH* from halotolerant cyanobacterium *A. halophytica* into *E. coli*.
2. To functionally characterize the recombinant ApalaDH.
3. To analyze the expression level of *ApalaDH* under salt stress condition

CHAPTER II

LITERATURE REVIEW

2.1 Compatible solute glycinebetaine

Glycinebetaine (*N*, *N*, *N*-trimethylglycine) is a high protection efficiency compatible solute that helps cells to survive under abiotic stress conditions. It is biosynthesized and accumulated in some microorganisms, higher plants and animals. Under normal condition, the accumulation of glycinebetaine in cells is generally low. However, its accumulation increases dramatically under stress condition. It has been shown in *Avicennia marina* (commonly known as mangrove) that glycinebetaine accumulation increased to about 2-folds under the treatment of 400 mM NaCl (Hibino *et al.*, 2001). It was also shown in *Spinacia oleracea,* that the increased of glycinebetaine were enhanced during salt stress treatment. In this case, glycinebetaine level strongly increased (approximately 20 folds) after treatment with 170mM NaCl (Martino *et al.,* 2003).

Glycinebetaine is biosynthesized and accumulated at high level in extremely stress tolerant organisms such as a halotolerant cyanobacterium *Aphanothece halophytica* (Waditee *et al.,* 2003), *Avicennia marina* (Hibino *et al.*, 2001) and *Spinacia oleracea* (Martino *et al.,* 2003). In higher plants, protective effects of glycinebetaine under various abiotic stresses have been reported (Hayashi *et al.*, 1997 and Sakamoto *et al.*, 1998). Thus, manipulation of non-glycinebetaine accumulating plants by glycinebetaine biosynthetic gene is of great interest. For example, in non-betaine accumulating plant *Arabidopsis thaliana* transformed with

codA gene that encodes choline oxidase showed the accumulation of glycinebetaine and improved tolerance to salt- and cold stresses (Hayashi *et al.*, 1997). The similar result was observed in *Oryza sativa* expressing *codA* gene (Sakamoto *et al.*, 1998). Moreover, co-expression of glycine ***N***-methyltransferases genes *ApGSMT* and *ApDMT* in a freshwater cyanobacterium *Synechococcus elongatus* PCC7942 and higher plant *Arabiodopsis thaliana* has been reported. It was found that both organisms accumulated high level of glycinebetaine and could survive under salt stress condition (Waditee *et al.*, 2005). These results showed benefit of transfer genes which involved in glycine betaine biosynthesis into non-accumulating organisms, and resulting transformed organisms confer salt tolerance.

To date, the halotolerant cyanobacterium *A. halophytica* is one of microorganisms that can biosynthesize and accumulate glycinebetaine very higher level (Ishitani *et al.,* 1993). This cyanobacterium was originally isolated from the Dead Sea (Waditee *et al.,* 2012). The cells can grow in wide range of salinity conditions from 0.5 to 3.0 M NaCl. Accumulation of glycinebetaine in *A. halophytica* is up to 1 M under salt stress (unpublished data). It has shown that glycinebetaine is biosynthesized from glycine by a novel pathway of three steps methylation in *A. halophytica* (Waditee *et al.,* 2003). The first reaction, glycine is converted to sarcosine and then sarcosine is converted to dimethylglycine. The final reaction, dimethylglycine is methylated to glycinebetaine. Thus, provision of glycine is very important for glycinebetaine biosynthesis in this extremophile. Thus far, there are four known pathways of glycine biosynthesis reporting in living organisms. There are serine hydroxymethyltransferase, threonine aldolase, glycine-cleavage system and

glyoxylate aminotransferase (Figure 1) (Schlupen *et al.*, 2003). For the first pathway, serine hydroxymethyltransferase (SHMT) catalyzes the reversible reaction of glycine to serine. This enzyme has been studied in many organisms. In cyanobacteria, the *SHMT* gene seems to be crucial for survival because the complete gene disruption cannot be generated (Hagemann *et al.*, 2005). The second pathway is threonine aldolase pathway, glycine is produced from threonine by threonine aldolase. Threonine aldolase catalyzes the reversible reaction between threonine and acetaldehyde and glycine. In *E. coli*, this reaction plays the alternative route for glycine biosynthesis while SHMT is the major pathway (Ogawa & Fujioka, 1999). Next pathway is known as the glycine cleavage system (GCS) or glycine decarboxylase complex (GDC). It has been shown that this complex catalyzes glycine cleavage in bacteria. In plants and animals, GDC complex was found to be located on mitochondria. It consists of three enzymes and a carrier protein (Kikuchi *et al.*, 2008). Three enzymes contain P-protein or glycine dehydrogenase (EC 1.4.4.2), T-protein or aminomethyltransferase (EC 2.1.2.10), L-protein or dihydrolipoamide dehydrogenase (EC 1.8.1.4) and the carrier protein namely H-protein (Kikuchi *et al.*, 2008). This complex catalyzes the reversible reaction of glycine to generate CO_2 and NH_4^+. The fourth pathway for generating glycine is glyoxylate aminotransferase. In this pathway, glycine is biosynthesized from glyoxylate by aminotransferase. This enzyme also catalyzes the reaction from alanine to pyruvate (Schlupen *et al.*, 2003). This reaction can be also catalyzed by AlaDH (Giffin *et al.*, 2012). This pathway has been studied in *Saccharomyces cerevisiae* (Schlosser *et al*., 2004). It has shown that *Saccharomyces* AlaDH is important for growth with a non-fermentable carbon source, such as ethanol and acetate.

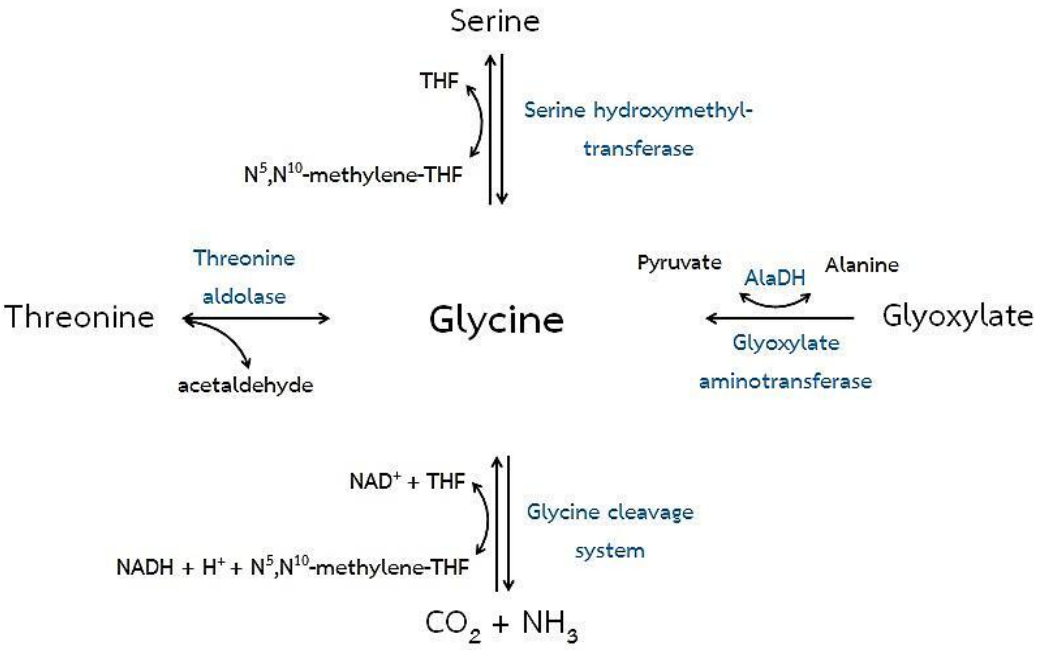


Figure 2.1: Purposed enzymatic reactions of glycine biosynthesis in living organisms. (Modified from; Schlupen et al., 2003)

2.2 Alanine dehydrogenase

Alanine dehydrogenase (AlaDH) (L-alanine: NAD^+ oxidoreductase, deaminating, EC 1. 4. 1. 1) catalyzes the reversible deaminating of L-alanine to pyruvate and ammonia in the presence of NAD^+. It was firstly described in *Bacillus subtilis* (Wiame *et al*., 1955). This enzyme has been examined its function in various organisms. Implication roles in biological processes of AlaDH were described in various microorganisms. In spore-forming bacterium *Bacillus subtilis,* AlaDH is essential for growth when alanine is the sole carbon or nitrogen source. It is also required for sporulation (Siranosian *et al.*, 1993). The defective sporulation was found in the mutant *ald* which lacking *alaDH* gene. AlaDH also provides the energy for sporulation bygenerating pyruvate. The *Bacillus* AlaDH molecular mass about 70 kDa and the

optimal pH is 8.5 (Siranosian *et al.*, 1993). In phototrophic bacterium *Rhodobacter capsulatus,* AlaDH plays an alternative route in ammonia assimilation if glutamate synthetase is not functional. The *Rhodobacter* AlaDH is hexamer subunits with native molecular mass about 240 kDa. The optimal pH of this enzyme is 9.8 (Caballero *et al.*, 1989). In pathogenic bacterium *Mycobacterium tuberculosis,* it has shown that AlaDH performs the glyoxylate reductive aminase activity. It catalyzes the reductive amination reaction of glyoxylate to glycine with the oxidation of NADH to NAD^+ but the reversible reaction cannot be detected (Figure 2) (Giffin *et al.*, 2012). Another pathogenic bacterium *Mycobacterium smegmatis*, there was the report showing that a knockout mutant of *ald* gene did not exhibit AlaDH activity but still performed glycine dehydrogenase activity (Feng *et al.*, 2002). In nitrogen-fixing organisms *Bradyrhizobium japonicum,* this organism lacks glutamate dehydrogenase which is important for nitrogen accumulation. This case, AlaDH is an alternative pathway to store nitrogen. In filamentous cyanobacterium *Anabaena* sp. PCC 7120, AlaDH plays a role in alanine catabolism in the heterocyst that is important for normal diazotrophic (Pernil *et al.*, 2010). In unicellular cyanobacterium *Synechococcus elongatus* PCC 7942, AlaDH is required for pigment degradation during nitrogen starvation condition. Expression level of *ald* gene encoding AlaDH was up-regulated under nitrogen starvation condition (Lahmi *et al.*, 2006). The non-reversible reaction between glyoxylate and glycine is one of important steps of photorespiration C2 cycle in photosynthetic organisms. AlaDH catalyzes the reaction of glyoxylate, that is generated from 2-phosphoglycolate to glycine in the first reaction (Figure 3). This pathway facilitates cells to detoxification toxic compound 2PG.

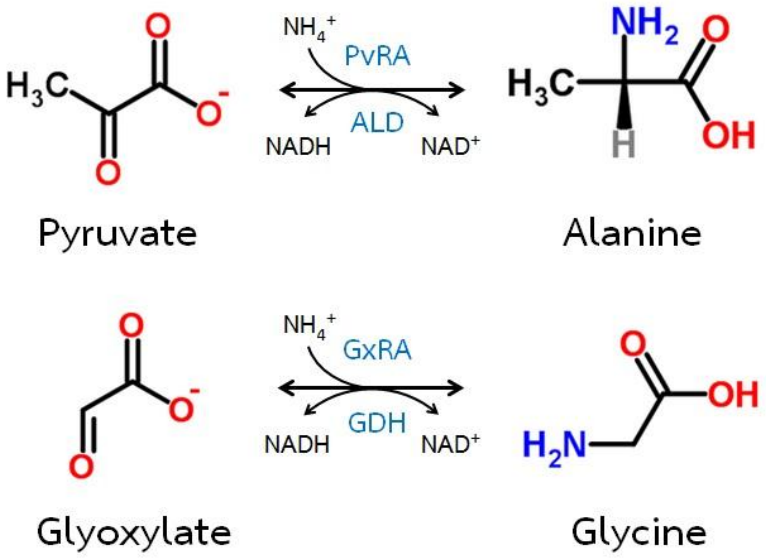


Figure 2.2: Catalytic reactions of alanine dehydrogenase. Pyruvate reductive amination activity (PvRA), Glyoxylate reductive amination activity (GxRA), Alanine oxidative dehydrogenase activity (ALD) and Glycine oxidative dehydrogenase activity (GDH).

2.3 Photorespiration C2 cycle

In photosynthetic organisms, ability to fix CO_2 with ribulose 1,5-bisphosphate carboxylate/ oxygenase (RuBisCo) is well known. However, RuBisCo also reacts with O_2 in a competitive manner. The products of carboxylation with CO_2 are two molecules of 3-phosphoglycerate (3PG) while the oxygenase reaction with O_2 produces one molecule of 3PG and 2PG. The product 3PG can be used in the Calvin-Benson cycle but 2PG is toxic for cells. To solve this problem, 2PG must be converted into 3PG by the photorespiration (Kern *et al.*, 2013). There are a number

of enzymes involved in photorespiration (Figure 3). The first reaction is dephosphorelation of 2PG to glycolate. After that, glycolate is oxidized to glyoxylate and then glyoxylate is transaminated to glycine transamination. Glycine is used to produce serine. Then, serine is converted to hydroxypyruvate by deaminated. Hydroxypyruvate is converted to glycerate. Finally, it is phosphorylated to glycerate-3-phosphate. Glycerate-3-phosphate generating from this pathway will be used in the Calvin cycle. The photorespiration rate will increase when environment is high O_2 and low CO_2. For example, when the stomata are closed to prevent water loss during drought stress. This condition limits the CO_2 while O_2 production in the leaf will further continue. Importance of this pathway in photosynthetic organisms has been studied. Some mutants lacking enzyme functions in photorespiration pathway have been generated. For instance, the unicellular cyanobacterium *Synechocystis* sp. PCC6803 mutant lacking carboxysome in which RuBisCo is packed cannot grow under air without CO_2 (Hagemann *et al.*, 2013). The similar phenomena were showed in green algae *Chlamydomonas reintardtii* disrupted in *glycolate dehydrogenase* (Nakamura *et al*., 2005) and C4 plant *Zea mays* mutant disrupted in *glycolate oxidase1* (Zelitch *et al.*, 2009) . In evolutionary term, it has shown that the photorespiration C2 cycle in higher plants originated from cyanobacteria and conveyed to plants by endosymbiosis (Eisenhut *et al.*, 2008). For 2PG metabolism in cyanobacteria, *in silico* analysis showed the presence of two possible pathways, one similar to the bacterial glycerate pathway in which glyoxylate is converted to glycerate and a second similar to plant photorespiratory metabolism (Bauwe *et al.*, 2010).

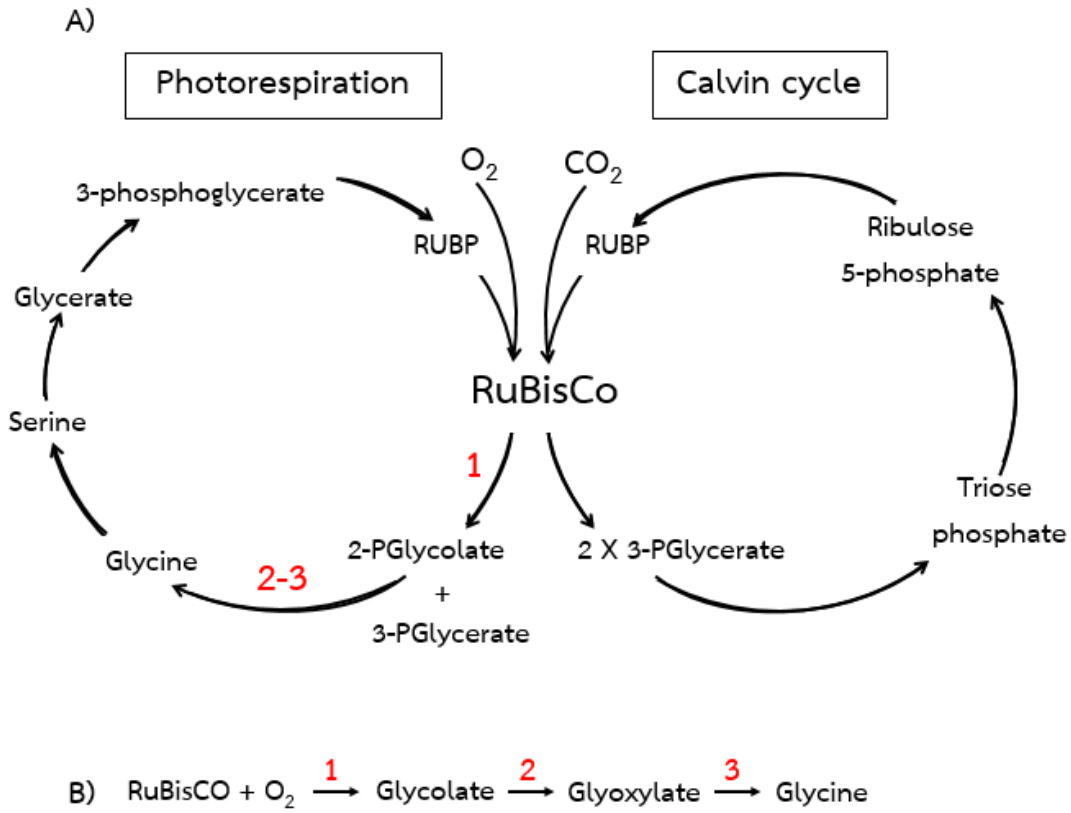


Figure 3: (A). The calvin cycle and photorespiration pathway in photosynthetic organisms. (B). Three reaction in the photorespiration. First reaction, glycolate is generated form RuBisCO and oxygen. Second reaction, glycolate is oxidized to glyoxylate by glycolate dehydrogenase. Third reaction, alanine dehydrogenase catalyzes the reaction of glyoxylate to glycine.

In addition, photorespiration pathway generates some metabolites such as glycine and serine in mitochondria and can be exported to other organelles. These metabolites can be further used as substrates in other metabolic pathways. For

example, glycine is the substrate for synthesis of glutathione that is an antioxidant in plants (Noctor *et al*., 1998). Glycine is substrate for biosynthesis of glycinebetaine which is important to protect cells under stress condition (Klahn *et al*., 2011). The mentioned above showed the relationship between photorespiration products and stress response. Many studies have been report about this association. In *Anabaena* PCC 7120, transcription levels of the photorespiration associated genes (phospho-glycolate-phosphatase, glycolate oxidase, alanine-glyoxylate aminotransferase and serine hydroxymethyltransferase (SHMT) were investigated after treatment with NaCl. All of genes were induced by 150 mM NaCl. The highest induction was found in the gene encoding SHMT (7.5 folds). Amino acids glycine and serine were determined and showed higher levels under salt stress condition (Srivastava *et al.*, 2011). The transgenic rice plants *Oryza sativa* transformed with *glutamine synthetase* showed high photorespiration rate. The transformant also showed more tolerance to NaCl than control plants (Hoshida *et al.*, 2000). The other problem is relative oxygen species (ROS). In higher plants which grow under abiotic stress conditions such as salt and drought stresses, disruption of photosynthesis is occurred. The relative oxygen species is generated and causes some effects to cells (Miller *et al.*, 2010). Photorespiration is one of important way to solve this problem. The growth of *A. thaliana* mutant in a SHMT gene (*shmt1-1*) was lower and increased accumulation of ROS when treated with NaCl. The deficient of *shmt* in mutant caused the high amount of ROS and led plants vulnerable to salt stress (Moreno *et al.*, 2005).

To date, little is known for AlaDH pathway in cyanobacteria. To understand the physiological role and the mechanism of putative AlaDH, this study aims to

clone, express and functionally characterize of putative AlaDH from halotolerant cyanobacterium *A. halophytica*. This study would be important to clarify a role of this enzyme that may important for respond to stress conditions. This knowledge would be possible to improve glycinebetaine accumulation and its application in agricultural field.

The objectives of this research

1. To clone and express *AlaDH* from halotolerant cyanobacterium *A. halophytica* into *E. coli*.
2. To functionally characterize the recombinant ApalaDH.
3. To analyze the expression level of *AlaDH* under salt stress condition.

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

Affinity chromatography column: Hitrap HP column, GE Healthcare Life Sciences, USA

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetteman, Gilson, France

Electrophoresis unit: Model mini protein II cell: Biorad, USA

Gel imaging: Model Gel Doc EZ Imager, Biorad, USA

Incubator: Haraeus, Germany

Laminar flow BVT-124: International scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

Nanodrop 200 UV-Vis Spectrophotomater: Thermo scientific, USA

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Power supply: Pharmacia, England

Refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Spectrophotometer UV-240: Shimadzu, Japan

Vortex: Model K-550-GE: Scientific Industrie, USA

3.2 Chemicals

Acrylamide: Merck, Darmstadt, Germany

Ammonium persulfate: Katayama Chem, Japan

Ammonium sulfate: Merck, USA

An antibody raised against 6-histidine: R&D systems, USA

An antibody raised against mouse: Biolab, England

Ampicillin: Katayama Chem, Japan

Bacto tryptone: Merck, Darmstadt, Germany

Bacto Yeast extract: Merck, Darmstadt, Germany

Calcium chloride: Merck, Darmstadt, Germany

Coomasie brilliant blue R-250: Sigma. USA

Ethanol: Katayama Chem, Japan

Glycerol: Merck, Darmstadt, Germany

Glycine: Sigma, USA

L-serine: Sigma, USA

Hydrochloric acid: Sigma, USA

Isopropyl β-D-1-thiogalactopyranoside: Sigma, USA

Imidazole: Sigma, USA

Magnesium chloride: Merck, Darmstadt, Germany

Magnesium sulfate: Merck, Darmstadt, Germany

Potassium chloride: Merck, Darmstadt, Germany

SYBR safe DNA gel strain: Life Technologies, USA

Sodium acetate: Sigma, USA

Sodium chloride: Sigma, USA

Sodium nitrate: Sigma, USA

Sodium sulfate: Sigma, USA

Streptomycin: Sigma, USA

3.3 Membranes

PVDF: Milipore Cooperation, USA

Nitrocellulose membrane: Biorad, USA

3.4 Kits

Amicon Ultra-2: Sigma, USA

DNeasy plant mini kit: Qiagen, Germany

Gel extraction kit: Invitrogen, USA

HRP conjugate substrate kit: Biorad, USA

RNeasy Plant Mini Kit: Qiagen, Germany

Standard molecular weight: Biorad, USA

3.5 Enzymes and restriction enzymes

*Nco*I: New England Biolabs, USA

*Nde*I: New England Biolabs, USA

*Sal*I: New England Biolabs, USA

3.6 Bacterial strains and plasmids

Table 3.1 Bacterial strains and plasmids used this study

Strains and plasmids	Descriptions	Sources/references
Aphanothece halophytica	Halotolerant cyanobacterium	Waditee *et al.,* 2012
ApalaDH/pColdI	1.1 kb *ApalaDH* fragment cloned into pColdI	This study
ApalaDH/pColdTF	1.1 kb *ApalaDH* fragment cloned into pColdTF	This study
ApalaDH/pTrcHis2C	1.1 kb *ApalaDH* fragment cloned into pColdTrcHis2C	This study
Escherichia coli DH5α	*sup*E44 Δ*lac*U169 (80 *lacZ* ΔM15) *hsd*R17 *recA1 endA1 gyrA96 thi*-l *relA1*	Invitrogen, USA
Escherichia coli BL21	(B F-*dcm omp*T *hsd*S (rB-mB-) *gal* [malB+] K-12 ()	Invitrogen, USA
pMD20	TA-cloning vector	Takara, Japan
pColdI	Expressing vector	Takara, Japan
pColdTF	Expressing vector	Takara, Japan

Table 3. 2 Primers used in this study

Primers	5’ ----▶ 3’	Base pairs
ApalaDH-NcoI_F	CCATGGAAATCGGCGTTCCC	20
ApalaDH-SalI_R	GTCGACTAAATCAGGAAAAACTTCTTTAA	29
ApalaDH-NdeI_F_pColdI	CATATGGAAATCGGCGTTCCCAAAGAAA	28
ApalaDH-SalI_R_pColdI	GTCGACCTATAAATCAGGAAAAACTTCT	28
ApalaDH-RT_F	AACCCAATGAGTATTATTGCGG	22
ApalaDH-RT_R	ACTGCGCCAATCAGTAAATC	20
AprnpB_F	TGAGGAAAGTCCGGGCTTCC	20
AprnpB_R	GGACATAAGCCGGGTTCTGT	20
ApSHMT_F	CAAGGGTCTGTTCTCACC	18
ApSHMT_R	TGAGGAAAGTCCGGGCTTCC	20

3.7 Bioinformatics analysis

Amino acid sequence of *Aphanothece* alanine dehydrogenase (ApalaDH) was obtained from shot-gun genome sequence of *A. halophytica* (Meijo University, Japan). It was compared with alanine dehydrogenase from other organisms and analyzed phylogenetic tree. Twenty-three alanine dehydrogenases from various organisms were obtained from GENBANK database. There are

Synechococcus elongatus PCC 7942 (accession number: ABB57790.1, *Staphylococcus aureus* (accession number: KII21197.1), *Mycobacterium tuberculosis* (accession number: AIH99526.1), *Bacillus subtilis* (accession number: KFK77428.1), *Anabaena variabilis* (accession number: WP_011317065.1), *Arthrospira platensis* (accession number: WP_006625337.1), *Rhodobacter capsulatus* (accession number: WP_023922707.1), *Nostoc* sp. PCC 7120 (accession number: WP_010996511.1), *Rhizobium* sp. (accession number: CCF19270.1), *Enterobacter aerogenes* (accession number: BAA77513.1), *Pseudomonas* sp. LFM046 (accession number: WP_044872962.1), *Halobacterium salinarum* R1 (accession number: CAP14650.1), *Listeria monocytogenes* (accession number: KKF73774.1), *Deinococcus maricopensis* DSM 21211 (accession number: ADV67730.1), *Halothece* sp. PCC 7418 (accession number: WP_015225136.1), *Dactylococcopsis salina* (accession number: WP_015228889.1), *Planktothrix prolifica* (accession number: WP_026796513.1), *Microcystis aeruginosa* (accession number: WP_002801833.1), *Oscillatoria acuminate* (accession number: WP_015147963.1), *Bacillus cereus* E33L (accession number: AJI27581.1), *Helicobacter pylori* B8 (accession number: CBI67257.1), *Trichoderma reesei* QM6a (accession number: EGR52368.1) and *Vibrio cholera* (accession number: AKB07284.1). The phylogenetic tree was constructed and analyzed using MEGA6 software (http://www.megasoftware.net/).

The physicochemical property of alanine dehydrogenase (for example theoretical isoelectric point (pI) and molecular mass) was analyzed using Prot Param software (http://web.ezxpasy.org/cgi-bin/protparam/protparam).

3.8 Cloning and expression of alanine dehydrogenase gene from *A. halophytica* into *E. coli*

3.8.1 Strains and culture conditions

3.8.1.1 *A. halophytica* culture condition

The halotolerant cyanobacterium *A. halophytica* was cultured photoautotrophically (70 μE m^{-2} s^{-1}) in BG11 medium containing 18 mM $NaNO_3$ and Turk Island salt solution with shaking at 30 °C , as previously described (Appendix 1) (Waditee *et al*., 2012). The concentration of NaCl in the medium was typically used from 0.5 M - 2.5 M. The growth of *A. halophytica* cells was monitored by measuring absorbance at 730 nm by spectrophotometer (Shimadzu, Japan).

3.8.1.2 *Escherichia coli* culture condition

E. coli strain DH5α and BL21 were grown in the Luria-Bertani (LB) medium (Appendix 2) at 37 °C. *E. coli* transformed cells were grown under the same condition as wild type and supplemented with 50 μg/ml ampicillin. The growth of *E. coli* was monitored by measuring absorbance at 620 nm by spectrophotometer (Shimadzu, Japan).

3.8.2 Genomic DNA preparation

Genomic DNA of *A. halophytica* was extracted from the cells in the mid-log phase (approx. 14 days). Cells were harvested by centrifugation at 8,000 rpm for 15 minutes at 4 °C. The cell pellet was used for genomic DNA extraction using DNeasy

Plant mini kit (Qiagen, USA) according to the manufacturer's instructions. The concentration and purity of DNA was measured using Nanodrop and confirmed the intact genomic DNA by electrophoresis on 1% (w/v) agarose gel.

3.8.3 Construction of expression plasmids harboring *ApalaDH*

3.8.3.1 Construction of *ApalaDH* into pTrcHis2C vector

To clone *ApalaDH* from *A. halophytica* (hereafter *ApalaDH*), the specific gene primer pairs were designed using sequence information of genomic DNA from shot-gun genome sequencing database. The coding region of *ApalaDH* was amplified from genomic DNA of by Polymerase Chain Reaction (PCR) using specific primers ApalaDH-NcoI_F and ApalaDH-SalI_R (Table 3.6.2). Tag gold polymerase was used in the PCR reaction and the condition of PCR was 95 ºC for 3 minutes followed by 95 ºC for 30 seconds, 60 ºC for 30 seconds, 72 ºC for 90 seconds (30 cycles) and 72 ºC for 10 minutes. The PCR products (approximately 1.1 kbs) were cloned into pMD20 cloning vector (Appendix 3) (Takara, Japan). The resulting plasmid *ApalaDH*/pMD20 was sequenced to determine nucleotide sequence. The *ApalaDH*/pMD20 double digested with restriction enzymes *Nco*I and *Sal*I. The fragment harboring *ApalaDH* was ligated into corresponding sites of the expression vector pTrcHis2C (Appendix 4) (Takara, Japan). The resulting plasmid *ApalaDH*/pTrcHis2C was transformed into *E. coli* DH5α (Appendix 5) for propagation according to standard protocol (Sambrook *et al*., 1989). For transformation, one hundred microliters of *E. coli* DH5α competent cells were mixed with 100 ng of *ApalaDH*/pTrcHis2C. The transformation mixture was flicked 2-3 times and stood on ice for 10 minutes. The mixture was heated at 42°C

for 90 seconds and stood on ice for 5 minutes. Subsequently, the mixture was added with 900 µl LB medium and shaked at 37°C for an hour. Cell suspension was spread and selected on LB agar supplemented with 50 µg/ml ampicillin. After incubation at 37°C for 16 hours, several candidates of transformants were picked up and re-streaked on LB agar supplemented with 50 µg/ml ampicillin. Each colony was checked by colony PCR using specific primers ApalaDH-NcoI_F and ApalaDH-SalI_R (Table 3.6.2) and then positive clones were used for plasmid preparation using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, USA) according to the manufacturer's instructions. The expression vector *ApalaDH*/pTrcHis2C was confirmed by restriction enzyme analysis. For expression of the ApalaDH recombinant protein, the expression vector *ApalaDH*/pTrcHis2C was transformed into *E. coli* BL21 and selected with the same protocol described above.

8.3.3.2 Construction of an expression plasmid containing *ApalaDH* in pColdI and pColdTF vectors

ApalaDH was amplified from *ApalaDH*/pMD20 by PCR using specific primers ApalaDH-NdeI_F_pColdI and ApalaDH-SalI_R_pcoldI (Table3.6.2). The PCR fragment (approximately 1.1 kbs) was ligated into cloning vector pMD20 and prepared by double digestion with restriction enzymes *Nde*I and *Sal*I. Then, the fragments were ligated into corresponding sites of pColdI (Appendix 6) (Takara, Japan) and pColdTF vectors (Appendix 7) (Takara, Japan), respectively (hereafter *ApalaDH*/pColdI and *ApalaDH*/pColdTF). The expression vectors *ApalaDH*/pColdI and *ApalaDH*/pColdTF were transformed into *E. coli* DH5α for propagation and selected

the positive clones with the same protocol for transformation that described in 3.8.3.1. The plasmids were extracted from positive clones using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, USA) according to the manufacturer's instructions and transformed into *E. coli* BL21 for expression using the same protocol of transformation in 3.8.3.1.

3.8.4 Expression of recombinant ApalaDH into *E. coli* BL21

E. coli BL21 cells harboring expression vectors *ApalaDH*/pTrcHis2C, *ApalaDH*/pColdI and *ApalaDH*/pColdTF were cultured in LB medium containing 50 µg/ml ampicillin with shaking (200 rpm) at 37 °C until OD_{620} = 0.5-0.6 (approximate 3 – 4 hours). Cells were induced the expression of recombinant protein by adding 0.5 mM IPTG and incubated for 0, 3, and 24 hours at two temperatures (16 °C and 37 °C). *E. coli* cells harboring *ApalaDH*/pTrcHis2C were incubated at 37 °C while *ApalaDH*/pColdI and *ApalaDH*/pColdTF were incubated at 16 °C. After induction, cells were harvested by centrifugation at 10,000 rpm for 2 minutes at 4 °C. Pellets were resuspended in 100 mM sodium phosphate buffer pH 7.0. Then, cells were broken by sonication and collected as cell lysate. Then, cell lysate was centrifuged at 10,000 rpm for 2 minutes at 4 °C. The soluble fraction was collected as supernatant. Cell lysate and soluble fraction (supernatant) were analyzed the protein expression by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis.

3.8.5 SDS-PAGE and Western Blot analysis

Both cell lysate and supernatant were analyzed by SDS-PAGE (Appendix 8) and Western Blot analysis, respectively. Protein concentration was determined by Bradford method (Bradford, 1976) (Appendix 9). SDS-PAGE was analyzed according to standard protocol (Laemmli, 1970).The molecular mass of recombinant protein was determined by comparing standard protein marker (Biorad, USA). The SDS-PAGE gel was stained with coomassie brillian blue. Western blot analysis was carried out to confirm that the recombinant ApalaDH was fused in-frame to six-histidine. For Western Blot analysis, 30 μg of the recombinant protein ApalaDH was separated by 10% SDS-PAGE and protein band was transferred to PVDF membrane using transfer buffer (Appendix 10). Blotting was done at 100 mA/inch^2 for 2 hours and then blocking with blocking solution (Appendix 5) for an hour. The membrane was incubated with primary antibody (antibody raised against His-tagged) for an hour. The membrane was washed with PBS solution (Appendix 5) for 15 minutes by gently shaking for three times. After washing, the membrane was incubated with secondary antibody (antibody against mouse conjugated with alkaline phosphatase or horseradish peroxidase) (Biolab, England) for an hour, followed by washing with PBS solution for 15 minutes by gently shaking for three times. To detect the signal, membrane was developed with substrate for alkaline phosphatase (150 mM Barbital pH 9.6, 0.1% NTB (Nitro Blue Tetrazolium), 1 M $MgCl_2$, 0.5% 5-bromo-4-chloro-3-indolyl phosphate)) or detection reagent for HRP (Biorad, USA).

3.8.6 Purification of recombinant ApalaDH

In this study, the recombinant ApalaDH protein was fused with six-histidine at N terminus. Thereby, it can be purified by Ni^{2+}-NTA affinity column chromatography. The supernatant fraction from 3.8.4 was purified by using Hi-Trap HP column (GE Healthcare, USA). After induction the expression for 24 hours, cells were harvested by centrifugation at 12,000 rpm for two minutes. After that, cell pellets were resuspended in 100 mM sodium phosphate buffer pH 7.0. Cells were interrupted by sonication. Then, the suspension was centrifuged at 12,000 rpm for two minutes. The protein in supernatant was collected and mixed with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole). The Hi-Trap HP column (1 ml) was equilibrated with binding buffer before using. The supernatant (5 mg soluble protein fraction) was loaded into the 1 ml Hi-Trap HP column. After loading, the column was washed with 5 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl and 40 mM imidazole). Then, 5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole) was applied into column. The purified recombinant protein was measured the protein concentration using Bradford method and analyzed by SDS-PAGE and Western Blotting as described above.

3.9 Biochemical characterization of recombinant ApalaDH protein

3.9.1 Pyruvate reductive amination activity (PvRA)

Reductive amination of pyruvate to alanine was assayed by measuring the oxidation rate of NADH during the reaction. Firstly, 80 μM NADH was added into 100 mM sodium phosphate buffer or Tris buffer containing 400 mM ammonium sulfate. The A_{340} was increased to 0.5, then 10 mM of sodium pyruvate was added. The reaction was started by addition of 0.2 μg of purified recombinant ApalaDH. The reaction was monitored the rate of decrease of the A_{340} within two minutes using spectrophotometer (Giffin *et al.*, 2012).

3.9.2 Glyoxylate reductive amination activity (GxRA)

Reductive amination of glyoxylate to glycine was assayed in the same protocol of pyruvate reductive amination activity but sodium pyruvate was replaced by 10 mM of glyoxylate (Giffin *et al.*, 2012).

3.9.3 Alanine oxidative dehydrogenase activity (ALD)

For oxidative dehydrogenase reaction which pyruvate was generated from alanine, the activity was assayed by measuring the reduction of NAD^+. The assay begins with the addition of 1 mM NAD^+ into 100 mM sodium phosphate buffer or Tris buffer and 10 mM alanine was added. To start the reaction, 1 μg of purified recombinant enzyme was added. The reaction was monitored the rate of increase of the A_{340} in two minutes using spectrophotometer (Giffin *et al.*, 2012).

3.9.4 Glycine oxidative dehydrogenase activity (GDH)

For oxidative dehydrogenase reaction that converted glycine to glyoxylate, the same experiments in step 3.9.3 were used except alanine was replaced by 10 mM glycine.

For steps 3.9.1 – 3.9.4, the reaction without purified recombinant ApalaDH was used as control to measure its background. All of reactions were calculated activities from the linear slope of curves. An extinction coefficient 6220 M^{-1} cm^{-1} was used for calculation. Specific activity was shown as nanomole of NADH oxidized per minutes per mg of protein. All reactions were performed in three independent replications at room temperature (25 ºC ± 1) (Giffin *et al.*, 2012).

3.9.5 Effect of pH on recombinant ApalaDH activity

To determine the effect of pH, 100 mM phosphate buffer (pH 4.0 to 9.0) and 100 mM Tris buffer (pH 6.0 to 11.0) were used. Other constituents were used as listed in steps 3.9.1-3.9.4. The activity of enzyme was measured at different pHs with the same protocols that described above. All assays were determined in three independent replications and the results were showed in term of relative activity (%).

3.9.6 Kinetic parameters of ApalaDH

To determine the K_m and V_{max} values, the concentration of substrates pyruvate, alanine and glyoxylate were varied. The K_m and V_{max} values were analyzed from Michaeis-Menten kinetics plot using GraphPad Prism 6.0 software

(www.graphpad.com/guides/prism/6). All assays were performed in three independent replications.

3.9.7 Product Inhibition assay

To analyze the inhibition assay on alanine dehydrogenase activity, end products of reactions and analogs were used. We hypothesized that it might affect the enzyme activity. The assay was performed by adding the products, namely alanine, glycine and pyruvate into the reaction mixtures. The five-fold concentrations calculating from K_m values of each compound were added into the reactions. The enzyme activities were measured according to the protocol described in step 3.9.1-3.9.4. The reaction mixtures without alanine, glycine and/or analogs were used as positive controls. All assays were determined in three independent replications and the results were showed in term of relative activity (%).

3.9.8 Effect of NaCl on purified recombinant ApalaDH activity

The effect of NaCl on ApalaDH activity was determined by adding various concentration of NaCl from 0-2.5 M in all assay reactions. The enzyme activity was measured with the same protocol in step 3.9.1-3.9.4. All assays were determined in three independent replications. The results were expressed as relative activity (%). The ApalaDH activity measured in the absence of NaCl was taken as 100%.

3.9.9 Effect of KCl on purified recombinant ApalaDH activity

The effect of KCl on ApalaDH activity was determined by adding various concentration of NaCl from 0-1.5 M in all assay reactions. The enzyme activity was measured with the same protocol in step 3.9.1-3.9.4. All assays were determined in three independent replications. The results were expressed as relative activity (%). The ApalaDH activity measured in the absence of NaCl was taken as 100%.

3.9.10 *In vivo* analysis

A. halophytica cells were cultured in BG11 medium with different concentration of NaCl (0.5 and 2.0 M) for 10-14 days. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes. Then, pellets were resuspened in 100 mM phosphate buffer pH 7.0. Cells were disrupted by sonication and collected as crude extraction. The protein concentration of crude extract was determined by Bradford method. To examine the AlaDH activity, 50 µg of crude extract were used. All of AlaDH activities (PvRA, GxRA, ALD and GDH) were performed in the same protocol in step 3.9.1 – 3.9.4. All assays were determined in three independent replications. The results were expressed as relative activity (%).The activity of crude extracts from cells growing under 0.5 M NaCl was taken as 100%

3.10 Semi-quantitative RT-PCR analysis of *ApalaDH*

A. halophytica cells were cultured in BG11 medium photoautotrophically at mid-log phase (approx. 14 days) before changing to stress conditions. For salt up shock condition, the concentration of NaCl was changed from 0.5 M to 2.0 M. After treatment with salts, cells were harvested at 0, 3, 6, 9 and 24 hours by centrifugation at 8,000 rpm for 15 minutes at 4 ° C. Total RNA was extracted from *A. halophytica* cells using Purelink RNA mini kit (Ambion, USA) according to the manufacturer's instructions. One microgram of total RNA was converted to cDNA using the Superscript III RT kit (Invitrogen, USA) according to the manufacturer's instructions. The cDNA products were used as templates in the PCR amplification with specific primers ApalaDH-RT_F and ApalaDH-RT_R (Table 3.6.2). The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis. The relative intensity were determined and analyzed by Imagelab software. All RT-PCR experiments were examined three independent replications.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Phylogenetic analysis

Amino acids sequence of *Aphanothece* alanine dehydrogenase (ApalaDH) and other living organisms were searched and obtained from databases as described in Materials and Methods. The information from NCBI database showed that *alanine dehydrogenase* could not be found in higher plants and animals. Thirteen amino acid sequences from bacteria, nine amino acid sequences from cyanobacteria and one amino acid sequence of fungi were found on databases. These sequences were analyzed phylogenetic tree using MEGA6 software. Twenty-four amino acid sequences could be separated into three groups. First group consisted of AlaDH in *Aphanothece halophytica*, *Halothece sp. PCC 7418*, *Dactylococcopsis salina*, *Arthrospira platensis, Anabaena variabilis, Nostoc sp. PCC 7120,* and *Synechococcus elongatus PCC 7942*. All of members in this group were cyanobacteria which consisted of both unicellular and filamentous cyanobacteria. The second group was bacterial group. It consisted of *Mycobacterium tuberculosis, Pseudomonas sp. LFM046*, *Rhodobacter capsulatus*, *Rhizobium sp*, *Deinococcus maricopensis DSM 21211*, *Listeria monocytogenes*, *Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus, Helicobacter pylori* and *Halobacterium salinarum R1.* The final group was the most variable group. The members were bacteria, cyanobacteria and fungi. It consisted of *Vibriocholera*, *Bacillus cereu E33L, Oscillatoria acuminate*, *Planktothrix prolifica*, *Microcystis aeruginosa*and *Trichoderma reesei QM6a*.

Physiochemical property, namely theoretical namely isoelectric point (pI) and molecular mass, for *Aphanothece* alanine dehydrogenase (ApalaDH) and alanine dehydrogenase in other organisms was analyzed from amino acid sequence using ProtParam software. All AlaDHs composed of 320-370 amino acid residues. For ApalaDH, it consisted of 360 amino acid residues. Theoretical pI and molecular mass were 5.63 and 38.6 kDa, respectively. The pI and molecular mass were similar to AlaDH from other organisms. Other organisms had theoretical pI ranging from 5-7 with molecular mass about 38 – 40 kDa. The physiochemical property from other living organisms were compared and shown in Table 4.1. Some AlaDHs were functionally characterized. For example, the *Rhodobacter* AlaDH is hexamer subunits with native molecular mass about 240 kDa. The optimal pH of this enzyme is around 9.8 (Caballero *et al.*, 1989).

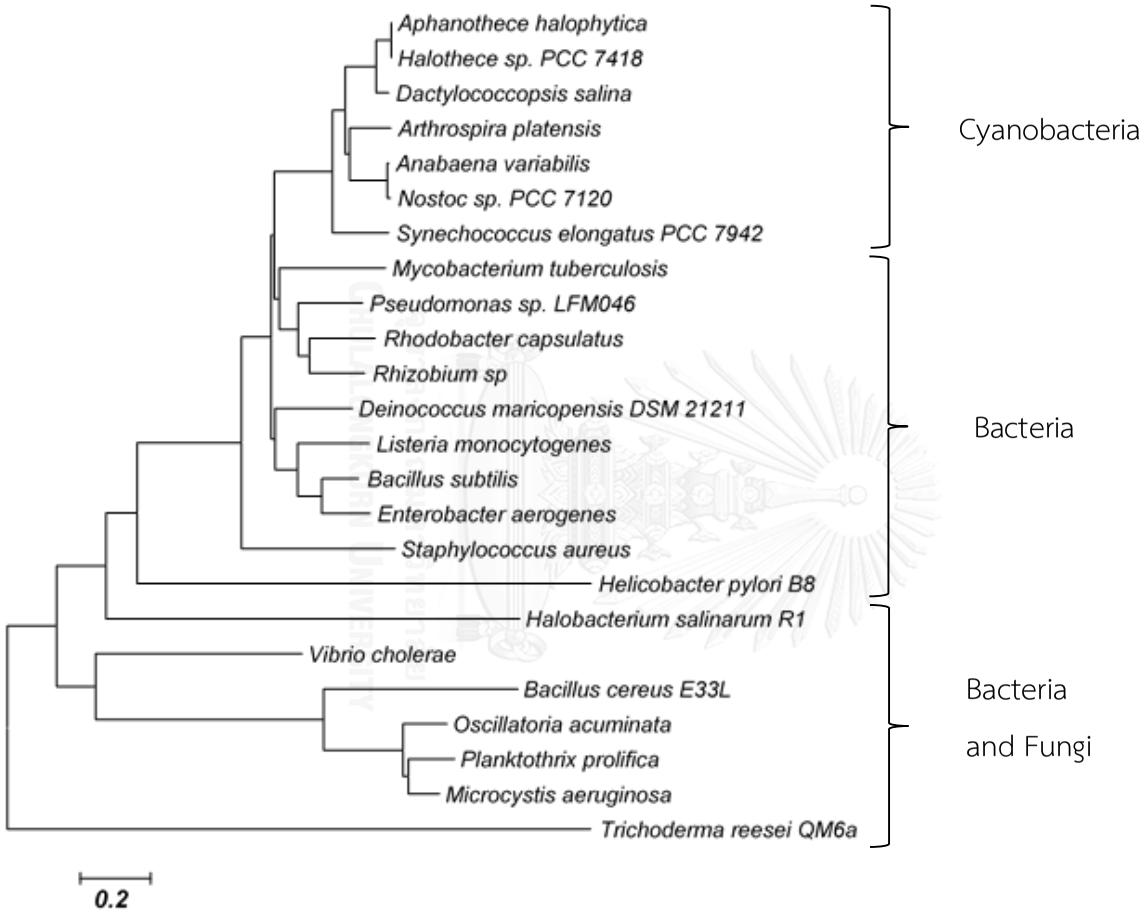


Figure 4.1: Phylogenetic analysis of AlaDH in bacteria, photosynthetic organisms and fungi. Aphanothece alanine dehydrogenase (ApalaDH) was obtained from shot-gun genome sequence of A. halophytica. Twenty three alanine dehydrogenase from various organisms were obtained from GENBANK database. Accession number of each AlaDH was described in Materials and Methods. The phylogenetic tree was constructed using MEGA6 software with Neighbor joining system (bootstrap value from 1,000

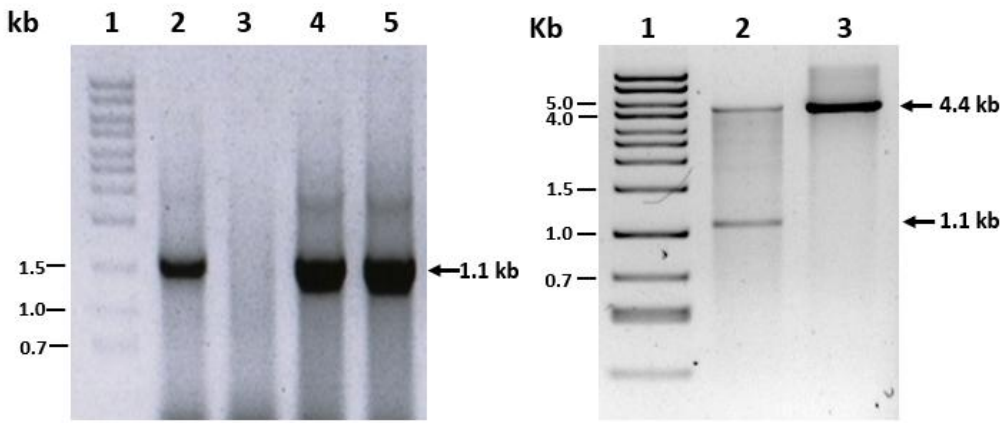
Table 4.1 Physiochemical property of alanine dehydrogenase

Organisms	Accession number	Amino acid residue	Theo-retical pI	Molecular-mass (kDa)
1. Bacteria				
Bacillus subtilis	KFK77428.1	378	5.36	39.6
Bacillus cereus E33L	KFK77428.1	378	5.36	39.6
Mycobacterium tuberculosis	AIH99526.1	371	5.81	38.7
Staphylococcus aureus	KII21197.1	372	5.19	40.2
Rhodobacter capsulatus	WP_023922707.1	372	5.91	38.2
Enterobacter aerogenes	BAA77513.1	377	5.56	39.8
Pseudomonas sp. LFM046	WP_044872962.1	373	6.36	39.2
Rhizobium sp.	CCF19270.1	372	5.79	39.0
Halobacterium salinarum R1	CAP14650.1	328	3.97	34.9
Listeria monocytogenes	KKF73774.1	370	5.24	39.6
Deinococcus maricopensis DSM 21211	ADV67730.1	368	5.72	38.2
Helicobacter pylori B8	CBI67257.1	380	6.27	40.9
Vibrio cholerae	AKB07284.1	374	5.88	39.0
2. Cyanobacteria				
Aphanothece halophytica	**-**	**360**	**5.63**	**38.6**
Halothece sp. PCC 7418	WP_015225136.1	360	5.63	38.6
Synechococcus elongatus PCC 7942	ABB57790.1	363	5.45	38.3
Anabaena variabilis	WP_011317065.1	362	5.95	38.7
Nostoc sp. PCC 7120	WP_010996511.1	363	6.01	38.8
Arthrospira platensis	WP_006625337.1	361	5.64	38.72
Microcystis aeruginosa	WP_002801833.1	361	6.12	38.5
Oscillatoria acuminate	WP_015147963.1	362	5.78	38.9
Dactylococcopsis salina	WP_015228889.1	359	5.36	38.6
Planktothrix prolifica	WP_026796513.1	361	5.68	38.8
3. Fungi				
Trichoderma reesei QM6a	EGR52368.1	368	5.33	40.6

4.2 Construction of expression plasmids harboring *ApalaDH*

4.2.1 Construction of expression plasmid *ApalaDH* into pTrcHis2C vector

In this study, putative *ApalaDH* was first constructed in pTrcHis2C. This gene was amplified from genomic DNA using specific primers as described in Materials and Methods. The gene fragments approximately 1.1 kb were ligated into pMD20 cloning vector. The resulting plasmid *ApalaDH*/pMD20 was sequenced to determine nucleotide sequence (data not shown). The recombinant plasmid was confirmed by double digestion with restriction enzymes *Nco*I and *Sal*I. To construct *ApalaDH* in expression vector pTrcHis2C, the fragments inserted into pMD20 were prepared by double digestion with *Nco*I and *Sal*I. Then, the digested fragments were ligated into corresponding sites of pTrcHis2C (hereafter *ApalaDH/*pTrcHis2C) and transformed into *E. coli* DH5α for propagation. The correct recombinant plasmids were selected and transformed into *E. coli* BL21 for expression as described in Materials and Methods. To screen the recombinant clones, several colonies were picked and used as templates for colony PCR. PCR products were examined on 1% (w/v) agarose gel electrophoresis (Figure 4.2A). Positive clones from colony PCR screening were further confirmed gene insertion. As shown in Figure 4.2B, the recombinant plasmids harboring *ApalaDH* had 1.1 kb fragments when digestion with *Nco*I and *Sal*I. These results indicated that *ApalaDH* was correctly inserted in pTrcHis2C. The correct plasmids were further transformed into *E.coli* strain BL21 for expression. *E. coli* BL21 cells harboring *ApalaDH/*pTrcHis2C were cultured in LB medium containing 50 µg/ml ampicillin for expression recombinant protein.



(A) (B)

Figure 4.2: (A) Colony PCR analysis of ApalaDH in expression vector, pTrcHis2C. The PCR products were analyzed by 1% (w/v) agarose gel precasted with with SYBR safe DNA (diluted the concentration 10,000X to 1X). Lane 1: DNA ladder, lane 2 to 5: candidate transformants of ApalaDH/pTrcHis2C. (B) Restriction enzyme analysis of ApalaDH. ApalaDH/pTrcHis2C plasmids were extracted from E. coliDH5α and double digestion of *Nco*I and *Sal*I, and then were separated on 1% (w/v) agarose gel precasted with SYBR safe DNA. Lane 1: DNA ladder, lane 2: double digestion of ApalaDH/pTrcHis2C with *Nco*I and *Sal*I, lane 3: double digestion of empty vector pTrcHis2C with *Nco*I and *Sal*I.

4.2.2 Construction of expression plasmid *ApalaDH* in pColdI and pColdTF vectors

The *ApalaDH* was amplified from *ApalaDH*/pMD20 using specific primers as described in Materials and Methods. The PCR products approximately 1.1 kb were ligated into pMD20 cloning vector. The recombinant plasmid *ApalaDH*/pMD20 was confirmed by double digestion with *Nde*I and *Sal*I. To construct *ApalaDH* in expression vector pColdI and pColdTF, the cloning vector *ApalaDH*/pMD20 was prepared by double digestion with *Nde*I and *Sal*I. Then, the digested fragments were ligated into corresponding sites of digested pColdI and pColdTF, respectively (hereafter *ApalaDH*/pColdI and *ApalaDH*/pColdTF), and transformed firstly into *E. coli* DH5α for propagation. The recombinant plasmids were selected and further transformed into *E. coli*BL21 for expression as described in Materials and Methods. Colony PCR was conducted for screening the correct plasmids (Figure 4.3A and 4.4A). To confirm the insertion, restriction enzymes analysis with *Nde*I and *Sal*I were performed. The electrophoresis result showed the inserted of *ApalaDH* size approximately 1.1 kb in both pColdI and pColdTF (Figure 4.3B and 4.4B). These results confirmed that *ApalaDH* was correctly inserted in pColdI and pColdTF. *E. coli* BL21 cells harboring *ApalaDH*/pColdI and *ApalaDH*/pColdTF were cultured in LB medium containing 50 μg/ml ampicillin for expression.

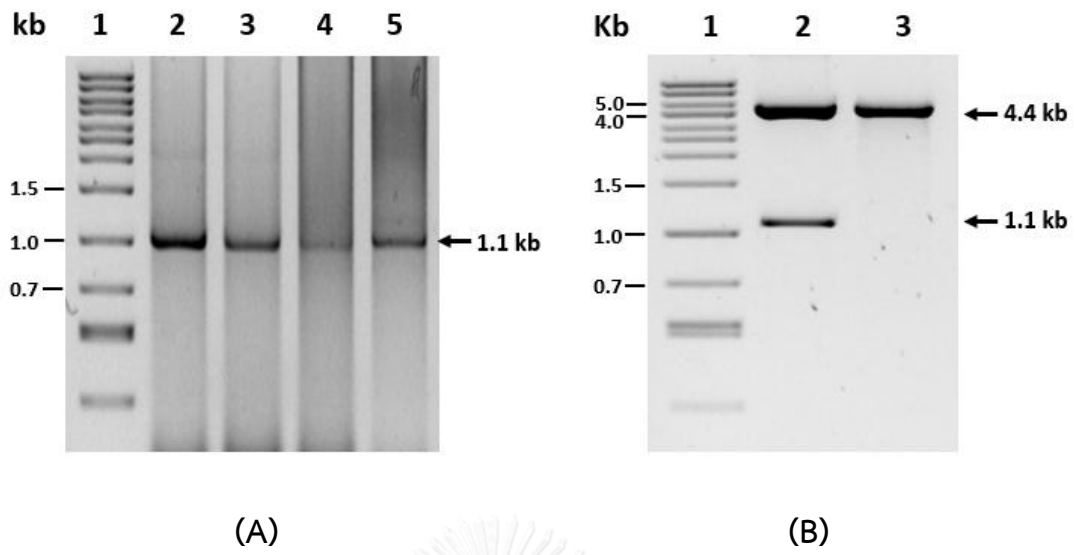
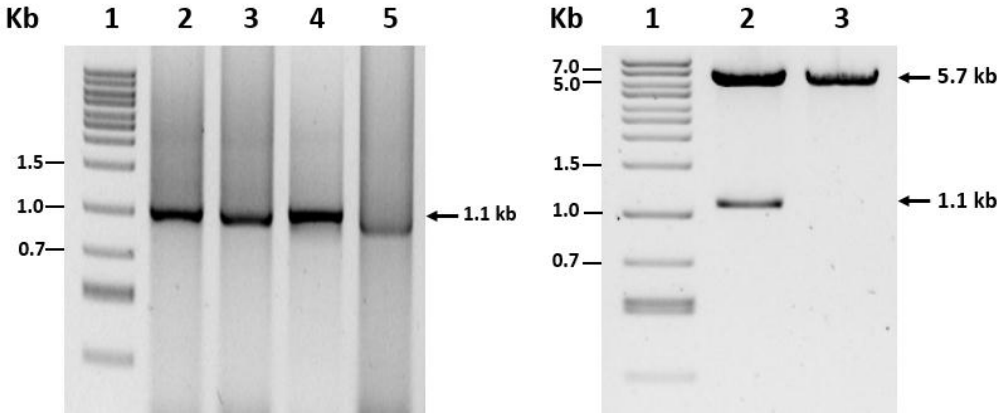


Figure 4.3: (A) Colony PCR analysis of *ApalaDH* in expression vector, pColdI. The PCR products were analyzed by 1% (w/v) agarose gel precasted with SYBR safe DNA (diluted the concentration 10,000X to 1X). Lane 1: DNA ladder, lane 2 to 5: candidate transformants of *ApalaDH*/pColdI. (B) Restriction enzyme analysis of *ApalaDH*. *ApalaDH*/pColdI plasmids were extracted from *E. coli*DH5α and double digestion of *Nde*I and *Sal*I, and then were separated on 1% (w/v) agarose gel precasted with YBR safe DNA. Lane 1: DNA ladder, lane 2: double digestion of *ApalaDH*/pColdI with *Nde*I and *Sal*I, lane 3: double digestion of empty vector pColdI with *Nde*I and *Sal*I.



(A) (B)

Figure 4.4: (A) Colony PCR analysis of *ApalaDH* in expression vector, pColdTF. The PCR products were analyzed by 1% (w/v) agarose gel precasted with with SYBR safe DNA (diluted the concentration 10,000X to 1X). Lane 1: DNA ladder, lane 2 to 5: candidate transformants of *ApalaDH*/pColdTF. (B) Restriction enzyme analysis of *ApalaDH*. *ApalaDH*/pColdTF plasmids were extracted from *E. coli*DH5α and double digestion of *Nde*I and *Sal*I, and then were separated on 1% (w/v) agarose gel precasted with with SYBR safe DNA. Lane 1: DNA ladder, lane 2: double digestion of *ApalaDH*/pColdTF with *Nde*I and *Sal*I, lane 3: double digestion of pColdTF with *Nde*I and *Sal*I.

4.2.3 Expression of recombinant ApalaDH

The vector pTrcHis2C was firstly used to express ApalaDH. This vector contains *trc* promoter (the hybrid of *trp* and *lac* promoters). This vector enhances translation efficiency in *E. coli* system. Example of a successful expression was shown in *E.coli*, using *CRP* gene from *Candida albican* (Van Bogaert *et al.*, 2007). In this study, *E. coli* BL21 expressing cells harboring *ApalaDH*/pTrcHis2C were grown until mid-logarithm phase (OD_{620} = 0.5-0.6) and induced the expression by addition of 0.5mM IPTG. After induction for 0, 3, 24 hours at 37 ºC, cells were harvested, resuspended in appropriate buffer and further disrupted suspension by sonication. Cell lysate and supernatant fraction were analyzed by 10% SDS-PAGE and stained with CBB. The results showed that control and expressing cells had the same protein patterns under SDS-PAGE (Figure 4.5). This result likely showed that ApalaDH could not be expressed under the driven of *trc* promoter. To further confirm this result, Western Blotting was performed; however, we could not detect any cross reacting band (data not shown). These results indicated that ApalaDH could not express in pTrcHis2C. This may be due to the promoter of this vector is not appropriate for *ApalaDH*. The other reason was the different of codon usage in *E. coli* and *A. halophytica*. Thus, the codon in *ApalaDH* sequence could not encode the ApalaDH protein in *E. coli* expression system

Next, *ApalaDH* was expressed in cold shock expression vector pColdI. This vector contains a *cspA* promoter which could be used to express a protein under low temperatures together with induction with IPTG. *E.coli* BL21 expressing cells harboring *ApalaDH*/pColdI were cultured and inducted by 0.5 mM IPTG at 16 ºC for

0, 3, and 24 hours. After induction with IPTG, cells were collected, resuspended in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole) and sonicated. Cell lysate and supernatant were analyzed by 10% SDS-PAGE and stained with CBB. SDS-PAGE showed the expression of recombinant ApalaDH was mostly in cell lysate with a molecular mass 46 kDa (theoretical molecular mass is 38.6 kDa) (Figure 4.6 C) while the expected ApalaDH almost could not be detected in supernatant (Figure 4.6B). However, the expression of ApalaDH could be detected by Western blot analysis. As shown in Figure 4.6D, the supernatant showed a cross reacting band corresponding to 46 kDa.

The other expression was performed using pColdTF vector. Cells were cultured with the same condition as doing in the expression using pColdI. Both cell lysate and supernatant had strongly induced bands of ApalaDH. The fusion protein of *ApalaDH* had molecular mass approximately 90 kDa (52 kDa of Trigger Factor (TF) and 38 kDa of ApalaDH) revealed on SDS-PAGE (Figure 4.7B). These results showed that the very high expression level was detected using pColdTF vector. To confirm ApalaDH was fused with 6xHis-tag, Cell lysate and supernatant fractions were analyzed by Western Blotting. Western Blotting analyze was performed using antibody raised against 6x-His tag and developed as described in Materials and Methods. Both cell lysate and supernatant fractions of *ApalaDH*/pColdTF shown a single cross reaction band at 90 kDa (Figure 4.7D). These results confirmed that ApalaDH could be express in pColdI and pColdTF vector and the recombinant protein was fused with 6x His-tag correctly.

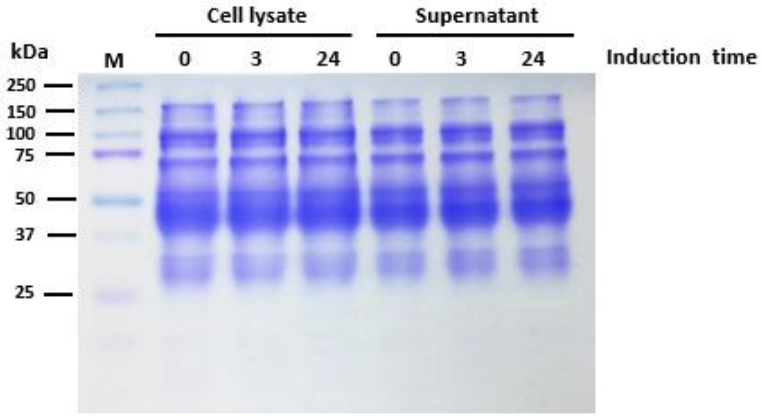


Figure 4.5: Expression of *ApalaDH*/pTrcHis2C was analyzed by 10% SDS-PAGE. Equal amount of protein (30 µg) was applied per lane. SDS-PAGE was visualized by CBB.

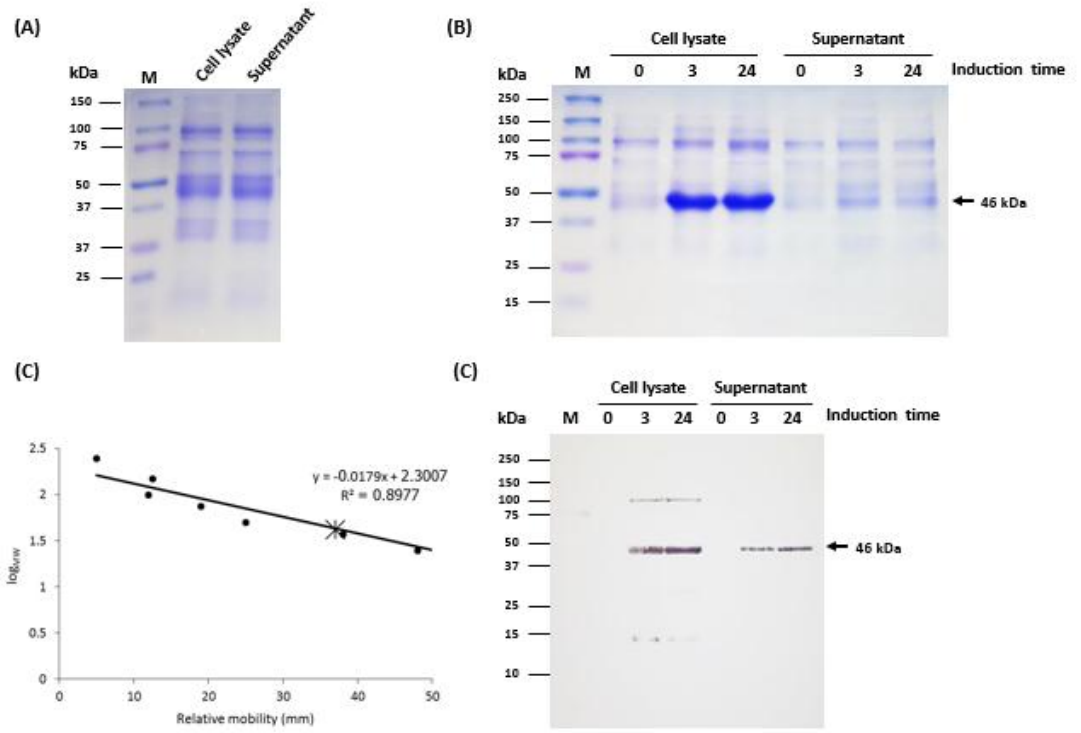


Figure 4.6: Expression of pColdI (A) and *ApalaDH*/pColdI (B) were analyzed by 10% SDS-PAGE. Equal amount of protein (30 µg) was applied per lane. SDS-PAGE was visualized by CBB. (C) Standard curve between $\log_{MW}$ and relative mobility (mm) of standard protein. (D) Western Blotting of cell lysate and supernatant fractions after induction was done on PVDF membrane. Antibody raised against 6x His-tag and antibody raised against mouse HRP conjugated were used as primary antibody and secondary antibody, respectively.

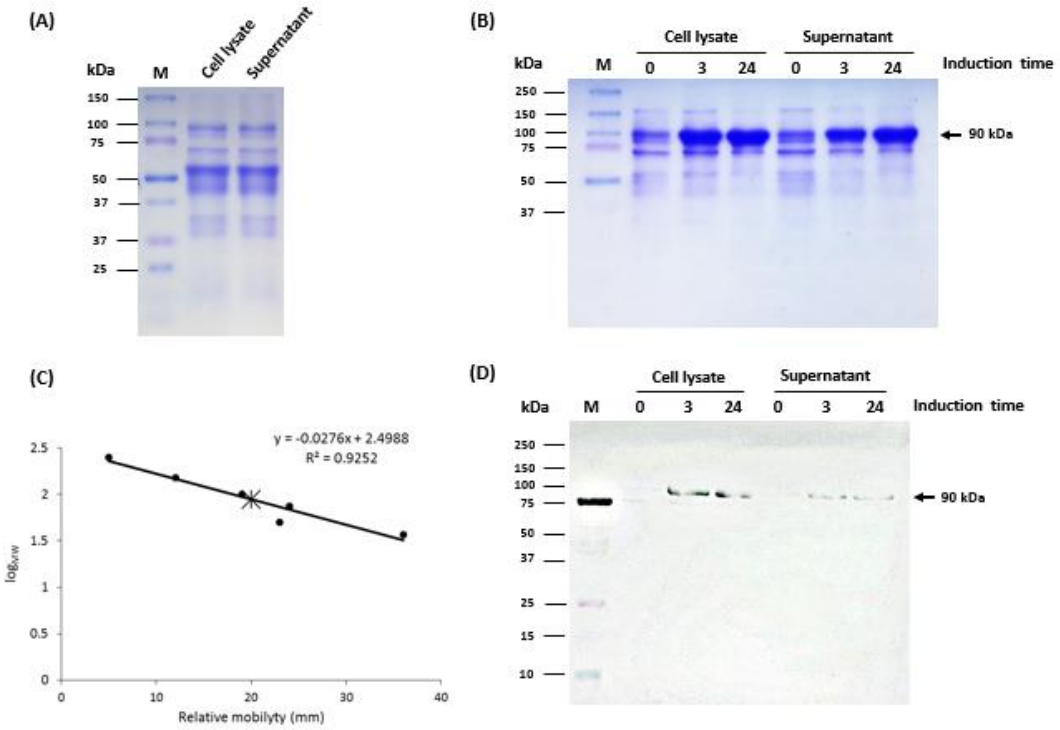


Figure 4.7: Expression of pColdTF (A) and *ApalaDH*/pColdTF (B) were analyzed by 10% SDS-PAGE. Equal amount of protein (30 µg) was applied per lane. SDS-PAGE was visualized by CBB. (C) Standard curve between $\log_{MW}$ and relative mobility (mm) of standard protein. (D) Western Blotting of cell lysate and supernatant fractions after induction was done on PVDF membrane. Antibody raised against 6x His-tag and antibody raised against mouse HRP conjugated were used as primary antibody and secondary antibody, respectively.

4.2.3 Purification of recombinant protein

Recombinant ApalaDH from *ApalaDH*/pColdI and *ApalaDH*/pColdTF were purified by using 1 ml Hi-trap HP column as per manufacturer's instructions. Firstly, 5 mg of crude extract were gently mixed with 100 mM sodium phosphate buffer pH 7.0 and filtrated through 0.45 µm filter. Then, the suspension was loaded into the column that was pre-equilibrated with binding buffer. After loading, non-binding proteins were washed with washing buffer (10x column volume). Finally, binding recombinant proteins were eluted with elution buffer. The elution fractions were analyzed by 10% SDS-PAGE. After purified with affinity column, ApalaDH exhibited the single band at 46 kDa as shown in Figure 4.8. Purified ApalaDH from both plasmids were preliminary tested the activity with pyruvate, alanine, glyoxylate and glycine. We found only ApalaDH from *ApalaDH*/pColdI could detect the AlaDH activity. This phenomenon may cause from many reasons. For example, the size of 90 kDa fusion protein (52 kDa of TF protein and 38 kDa of ApalaDH) might obstruct the reaction between substrate and enzyme. In addition, the folding of recombinant ApalaDH (fusion with TF) might not appropriate. To test this hypothesis, the TF protein should be digested to remove TF but the recombinant ApalaDH would be similar to recombinant protein from pColdI. So, we decided to use recombinant protein obtaining from the construct of pColdI.

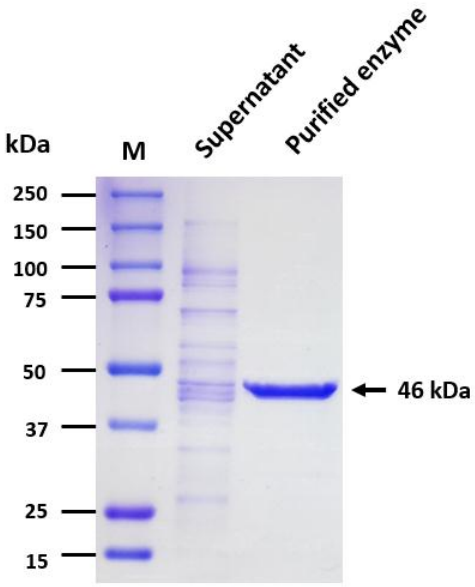


Figure 4.8: Purification of recombinant ApalaDH from *ApalaDH*/pColdI by Hi-trap column. Fifteen milligrams of total protein was resuspended in 100 mM sodium phosphate buffer pH 7.0, filtrated and loaded in to 1 ml Hi-trap column which was pre-equilibrated with binding buffer. Then, the column was washed with washing buffer and eluted recombinant ApalaDH. The purified ApalaDH was examined by 10% SDS-PAGE. Equal amount (15 µg) of non-purified or purified ApalaDH proteins was applied per lane. SDS-PAGE was visualized by CBB.

4.3 Biochemical characterization of recombinant ApalaDH

4.3.1 Effect of pH

The purified recombinant ApalaDH from *ApalaDH*/pColdI and *ApalaDH*/pColdTF were used for biochemical characterization as mentioned in section 4.2.3. After preliminary test, only recombinant ApalaDH from pColdI exhibited AlaDH activity. Therefore, only purified ApalaDH from pColdI was assayed in four reactions, namely PvRa, ALD, GxRa and GDH. Each enzymatic activity was determined the pH dependence at various pH values (phosphate (pH 4.0 – 9.0) and Tris buffer (pH 6.0 – 11.0)). It should be noted that the purified enzyme from *ApalaDH*/pColdTF could not detect any activities in all condition tested. This result might be due to the 50 kDa TF fusion protein from pColdTF vector. This TF fusion protein might affect the protein folding of this enzyme. We therefore used only purified enzyme from *ApalaDH*/pColdI for biochemical characterization. Purified enzyme obtaining from *ApalaDH*/pColdI was assayed for PvRA, ALD, GxRA and GDH activities. For the PvRa reaction, the highest activity was observed when phosphate buffer pH 9.0 and Tris buffer pH 9.5 were used (Figure 4.9A). For the ALD reaction, the optimal pH was 9.0 (phosphate buffer) and 8.0 (Tris buffer) (Figure 4.9B). These optimal pHs for PvRA and ALD were similar to AlaDH reporting from other organisms. Previous studies showed the optimal pH of AlaDH was an alkaline pH. In cyanobacteria, there was no reported in the optimum pH of this enzyme while in some organisms has been studied. The previous study showed the optimal pH of AlaDH from *Mycobacterium tuberculosis* was 8.5 – 9.0 and 9.0 – 11.0 for PvRa and ALD reactions, respectively (Hutter *et al*., 1999, Giffin *et al.*, 2012). Another study in *Bacillus subtililis* showed the optimum of

AlaDH for PvRa reaction was8.8 – 9.0 and 10 -10.5 for ALD reaction, respectively (Yoshida *et al*., 1965). These results indicated that the optimal pH of ApalaDH preferred at the alkaline pH which is very similar to *Mycobacterium* and *Bacillus* AlaDHs.

Next, the purified protein was determined activity for GxRa and GDH reactions. For the GxRA reaction, the highest activity was detected in phosphate buffer (pH 8.0 - 9.0) and Tris buffer (pH 8.0). It had shown in *Mycobacterium* AlaDH, optimal pH for GxRA is 8.5 (Figure 4.9C). The activity of GDH was not detected activities in all condition tested (Figure 4.9D).

These results indicated that AlaDH from *A. halophytica* is capable of catalyzing two reactions. The first reaction is the reversible PvRa reaction that uses pyruvate to produces alanine. The second reaction is the non-reversible GxRa reaction that produces glycine from glyoxylate. AlaDHs from many organisms have been functionally characterized. To date, only three AlaDHs have ability to catalyze the GxRa reaction to generate glycine from glyoxylate (Yoshida & Freese, 1965, Hagins *et al.*, 2009, Giffin *et al*., 2012). Previous reports showed that AlaDH from *Streptomyces fradiae* (Vancura *et al.*, 1989) and *Anabaena cylindrical* (Rowell & Stewart, 1975) could not use glyoxylate as substrate. However, AlaDHs from *Mycobacterium tuberculosis* (Giffin *et al.*, 2012), *Bacillus subtililis* (Yoshida & Freese, 1965) and *Pseudomonas aeruginosa* (Hagins *et al.*, 2009) have the capability to convert glyoxylate to glycine. Substrate specificity and biocatalyst is of AlaDH from all mentioned above implicated that AlaDH may have various roles and involved in various biological processes.

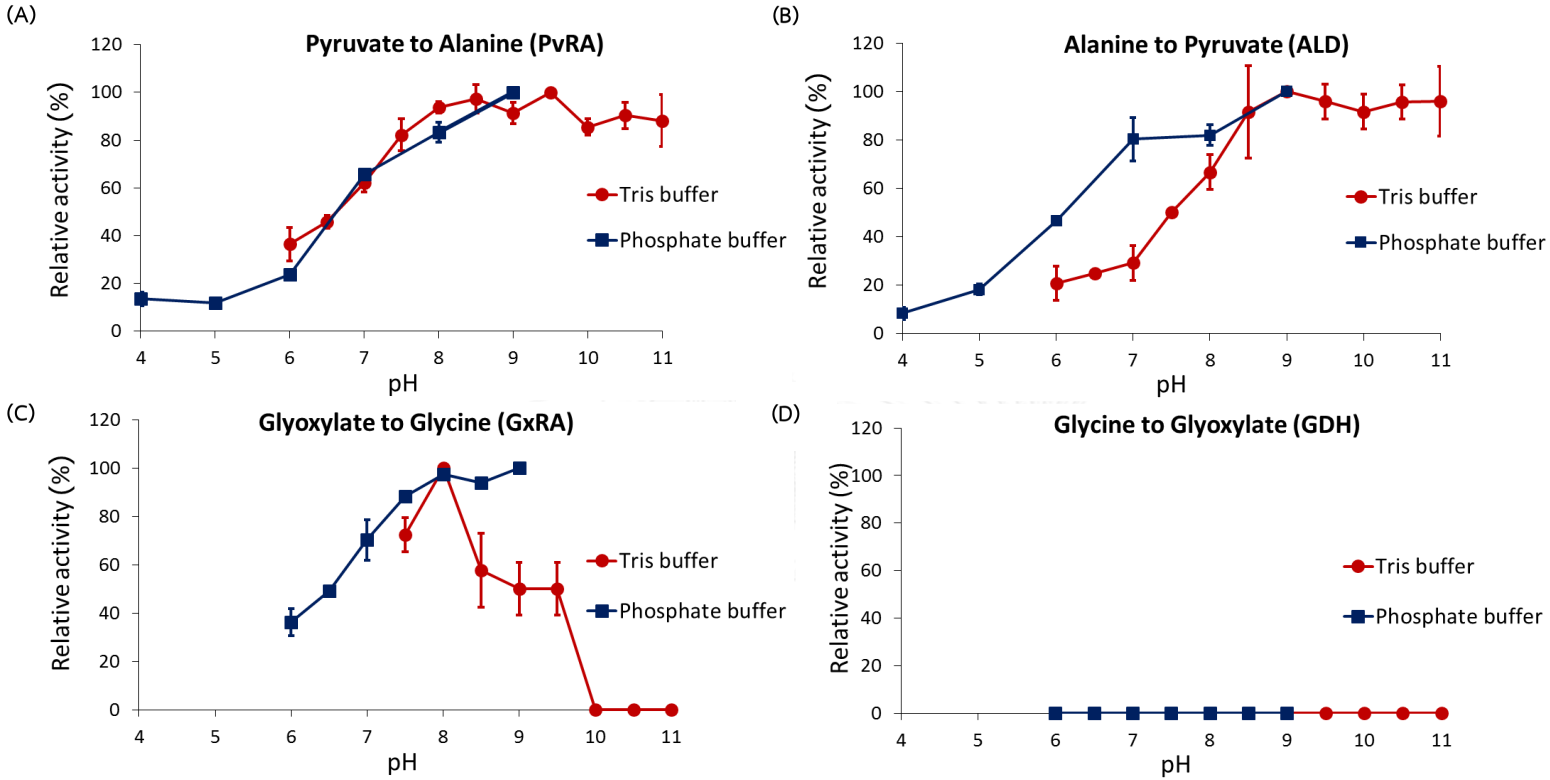


Figure 4.9: Effects of pH for ApalaDH activity. (A) Pyruvate reductive amination activity (PvRA), (B) Glyoxylate reductive amination activity (GxRA) ,(C) alanine oxidative dehydrogenase activity (ALD) and (D) Glycine dehydrogenase (GDH). Data shown as mean ± standard deviation of three independent replications.

4.3.2 Kinetic parameters (K_m and V_{max}) of ApalaDH

Results from 4.3.1 showed that Tris buffer pH 9.0 yielded the highest activities of ApalaDH for PvRA and ALD reactions while pH optimal for GxRA was 8.0. Thus, these conditions were further used to determine kinetic parameters of ApalaDH. The purified protein was determined the K_m and V_{max} values with different concentration of substrates. Here, Tris buffer pH 9.0 was used for PvRa and ALD reactions and Tris buffer pH 8.0 was used for GxRa reaction. The concentration of each substrate was varied and enzyme kinetics were analyzed using analyzed from Michaeis-Menten kinetics plot using GraphPad Prism 6.0 software. When pyruvate was used as substrate, the apparent K_m and V_{max} values of ApalaDH were 0.22 mM and 105.9 nmol NADH oxidized/min/µg protein, respectively (Figure 4.10). This K_m value is similar to K_m value of alanine dehydrogenase from *Enterobacter aerogenase* (Chowdhury *et al.*, 1998) and lower when compared with other organisms such as *Bacillus subtililis* (0.54 mM) (Yoshida & Freese, 1965) and *Mycobacterium tuberculosis* (2.8 mM) (Giffin *et al.*, 2012). The apparent K_m value of ApalaDH for alanine was 0.72 mM with V_{max} 45.6 nmol NADH oxidized/min/µg protein (Figure 4.10). This K_m value was closed to the report from *Enterobacter aerogenase* (Chowdhury *et al.*, 1998). For glyoxylate, the apparent K_m was 1.91 mM and V_{max} value was 37.63 nmol NADH oxidized/min/µg protein, respectively (Figure 4.10) which was lower than the previous reported in *Bacillus subtililis* (16 mM) (Yoshida & Freese, 1965) and *Mycobacterium tuberculosis* (5.5 mM) (Giffin *et al.*, 2012). The comparison of K_m values from other organisms that had been studied and reported were summarized in Table 4.2. These

results indicate that ApalaDH has a greater affinity for pyruvate than alanine and has low affinity for glyoxylate

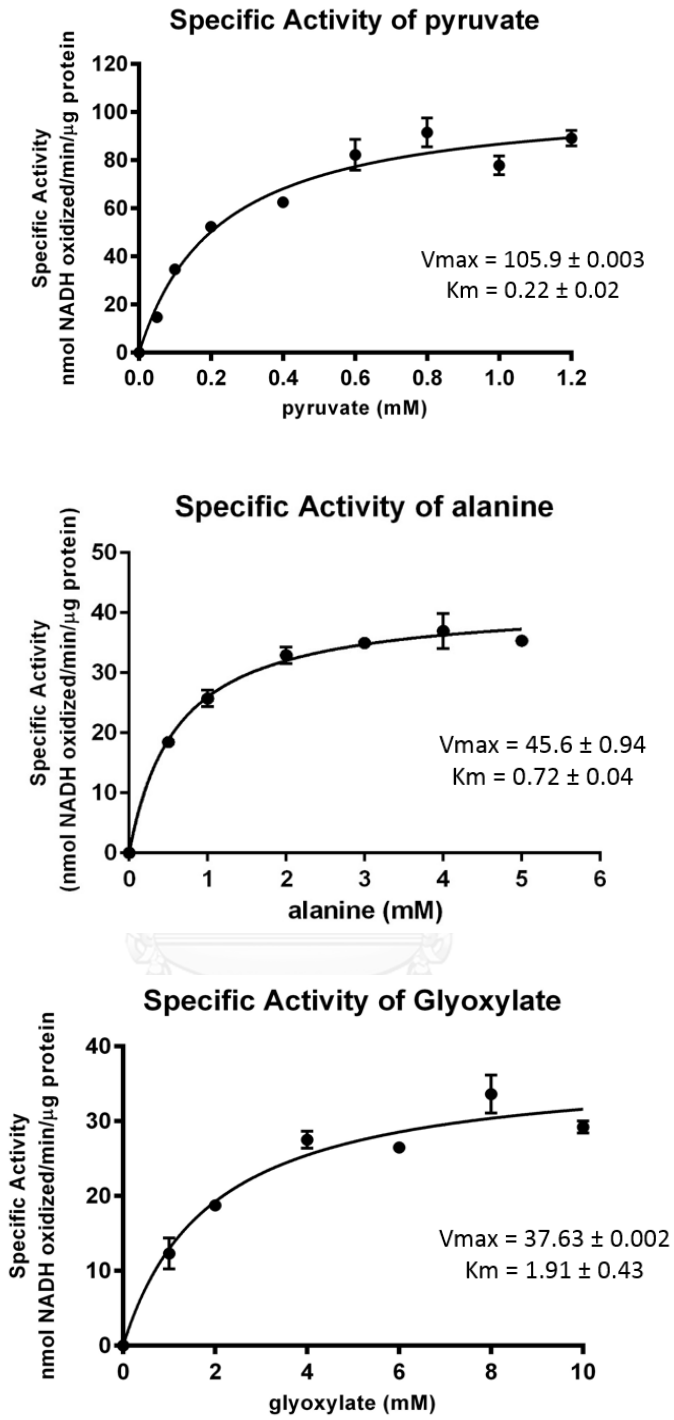


Figure 4.10: Kinetic parameters of recombinant ApalaDH for (A) pyruvate, (B) alanine and (C) glyoxylate. The kinetic parameters analyzed from Michaeis-Menten kinetics plot using GraphPadPrism 6.0 software (www.graphpad.com/guides/prism/6). All assays were performed in three independent replications.

Table 4.2 Comparison of AlaDH K_m for pyruvate, alanine and glyoxylate in eight species of bacteria and photosynthetic organisms.

Organisms	K_m (mM)			Reference
	Pyruvate	Alanine	Glyoxylate	
Mycobacterium tuberculosis	2.8	4.3	5.5	Giffin *et al*., 2012
Rhizobium sp.	0.43	0.37	-	
Enterobacter aerogenase	0.22	0.47	-	Chowdhury *et al*., 1998
Bacillus subtililis	0.54	1.73	16	Yoshida & Freese, 1964
Bacillus stearothermophilus	5.0	13.3	-	Sakamoto *et al*., 1990
Pseudomonas sp.	4.3	-	-	Brunhuber & Blanchard, 1994
Halobacterium salinarum	0.95	7.0	-	Brunhuber & Blanchard, 1994
Thermus thermophilus	0.75	0.18	-	Vali *et al*., 1980
Aphanothece halophytica	**0.22**	**0.72**	**1.9**	**This study**

4.3.3 Inhibition assay

As mentioned in section 4.3.2, pyruvate, alanine and glycine were generated from ApalaDH activity. The K_m of each substrate and product was determined. Therefore, effects of end products and analogs for each reaction are interesting to be tested. To analyze the effect of end product in the reaction, the product of each reaction was added in to the reaction and determined the enzyme activity. In PvRA reaction which generated alanine from pyruvate, 1 mM and 2 mM alanine (5 and 10 folds, calculating from K_m for pyruvate) were added and the activity of enzyme was

decreased 40% and 70%, respectively (Figure 4.11A). For the reverse reaction, ALD that used alanine as substrate, 3.5 mM and 7 mM pyruvate (5 and 10 folds, calculating from of K_m for alanine) were used. The results showed that 3.5 mM 7 mM pyruvate reduced the activity of enzyme to 70% and 55%, respectively (Figure 4.11B). The second reaction of alanine dehydrogenase GxRA was determined the product inhibition. Glycine was added into the reaction at the final concentration 10 mM and 20 mM (5 and 10 folds, calculating from of K_m for glyoxylate), respectively. The product glycine could inhibit the reaction. Addition of 10 and 20 mM glycine inhibited the reaction of this enzyme about 40% and 60%, respectively (Figure 4.11C).

Alanine dehydrogenase catalyzes two reactions so in this experiment the analogs of end product were examined. Effect of glycine was analyzed in the PvRA reaction. Addition of 1 mM and 2 mM glycine (5 and 10 folds, calculating from of K_m for pyruvate) showed slightly decreased in PvRA reaction (Figure 4.11A). Next, ALD reaction was determined by adding 3.5 and 7 mM glyoxylate (5 and 10 folds, calculating from of K_m for alanine). The results of both concentration could inhibit ALD reaction for 30% when compare with control (no addition) (Figure 4.11B). The final reaction GxRA, there was no activity of alanine dehydrogenase when adding 10 and 20 mM alanine (5 and 10 folds, calculating from of K_m for glyoxylate) in the reaction (Figure 4.11C). These results suggested that alanine completely inhibited the GxRA reaction at the final concentration 10 mM and 20 mM.

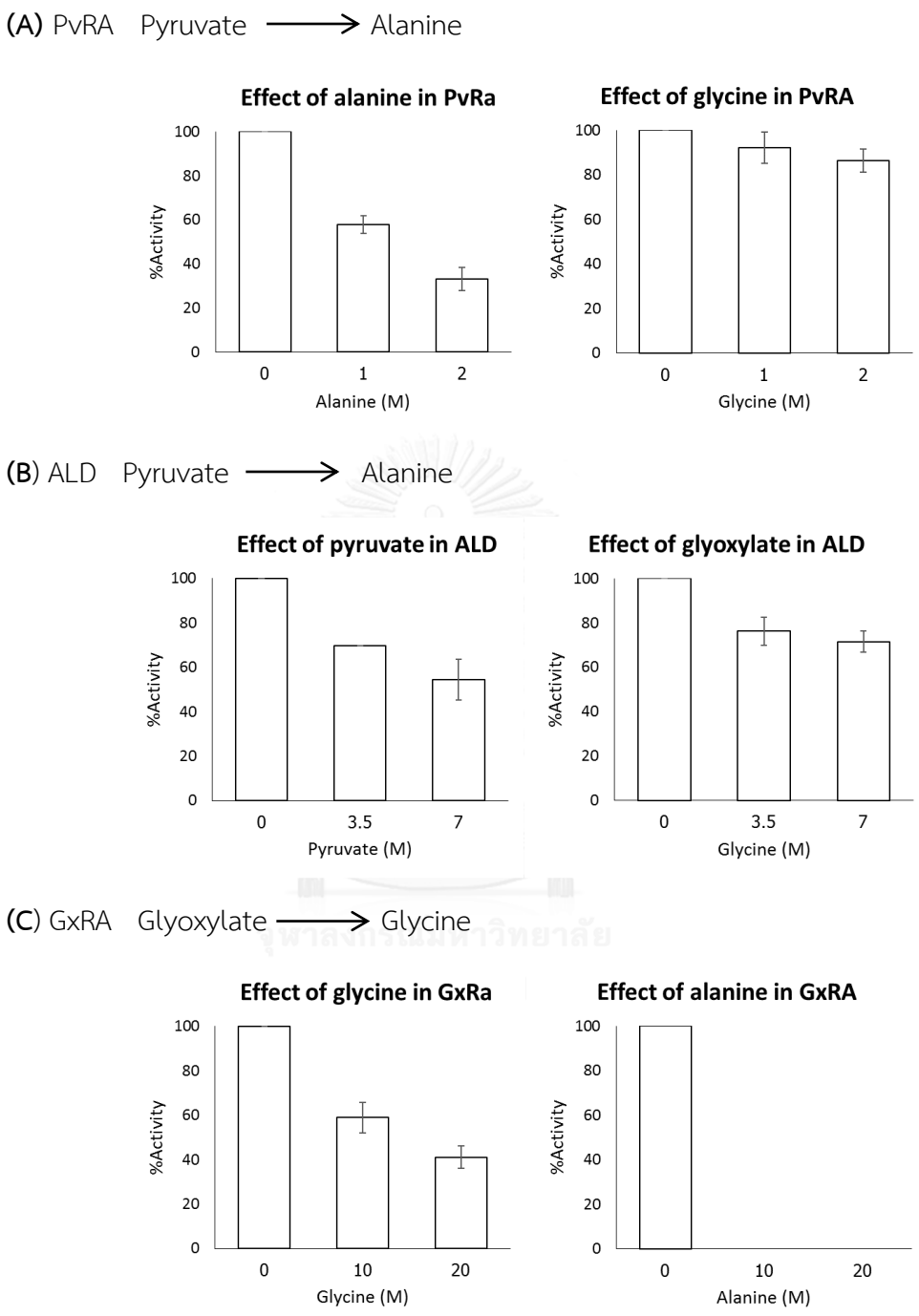


Figure 4.11: Effect of end products and analogs for ApalaDH activity. Three reactions PvRA (A), ALD (B) and GxRA (C) were analyzed. Five folds of Km for substrate were added into the reaction. The ApalaDH activity measured in the absence of end products or analogs were taken as 100%. Data shown as mean ± standard deviation of three independent replications.

4.3.4 Effect of NaCl

NaCl is a salt that can be found in environment. It causes both salt stress and osmotic stress and further generates oxidative stress. These stresses have negatively effect to cell metabolisms. For example, *Eucalyptus citridora* treated with NaCl shows the induction of NADP-malic enzymes activity (Parida *et al*., 2005). *A. halophytica* is a halotolerant cyanobacterium which can grow under high salinity condition so the effect of salt to enzymatic reaction is interesting. In this experiment, NaCl was used to test its activity to purified recombinant ApalaDH. ApalaDH activity was analyzed in the presence of NaCl at different concentration (0 - 2.5 M). For PvRa reaction, enzyme activity was not changed in the presence of 0 – 1 M NaCl but it was decrease approximately 35% with 2.5 M of NaCl (Figure 4.12). The ALD reaction, reversible reaction of PvRa, showed the different results from PvRa reaction. In the presence of 0.1 M and 0.25 M of NaCl, the activity was increased approximately 25% and 40%, respectively but the activity was decreased about 20% when the concentration of NaCl in the reaction was 0.5 – 2.5 M (Figure 4.15). The GxRa reaction that used glyoxylate to produced glycine was showed the positive effect of NaCl. The enzyme activity was increased approximately 80% and 65% when adding 0.1 and 0.25 M, respectively followed by the progressive declined with increasing concentration of NaCl to 2.5 M. In the presence of 2.5 M NaCl, the activity was decreased approximately 60% when compared with no addition of NaCl (Figure 4.12).

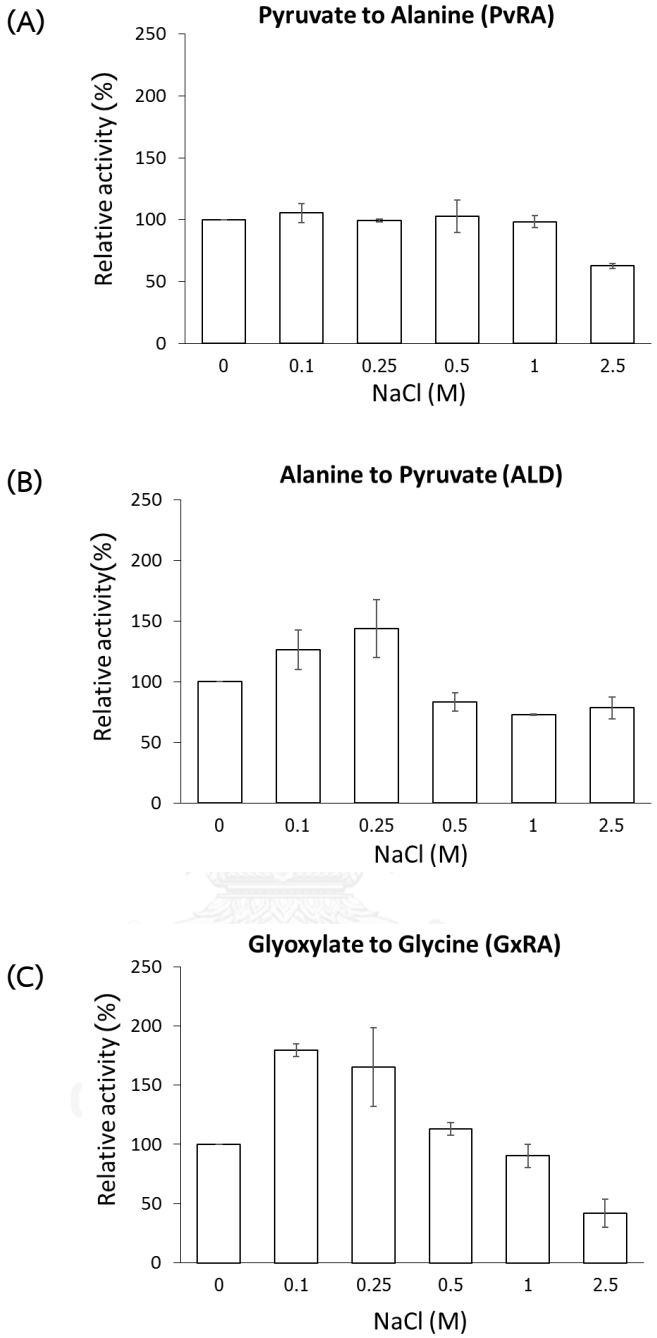


Figure 4.12: Effect of NaCl for ApalaDH activity. The (A) pyruvate reductive amination activity (PvRA), (B) glyoxylate reductive amination activity (GxRA) and (C) alanine oxidative dehydrogenase activity reactions (ALD) were analyzed. The ApalaDH activity measured in the absence of NaCl was taken as 100%. Data shown as mean ± standard deviation of three independent replications.

4.3.5 Effect of KCl

Not only NaCl but KCl was analyzed the effect to AlaDH activity. Balance of KCl is important for cells because it involves in the balance of pH and osmotic pressure. Consequently, the effect of KCl to metabolism of cells was interesting. To analyze the effect of KCl, the various concentrations (0 - 1.5 M) of KCl were added into the reaction mixture. The activity of PvRa reaction was increased when KCl was in the reaction. The activity was increased approximately 20% and 30% when 0.1 M and 1 M NaCl were added into the reaction (Figure 4.16). For ALD reaction, the activity was increased approximately 55% with addition of 0.1 M NaCl followed by the progressive declined with increasing concentration of KCl (Figure 4.13). In the presence of 1 M and 1.5 M KCl, the activity of ApalaDH was decreased about 25%. In the GxRa reaction, the ApalaDH activity was decreased when increasing the KCl concentration. In the presence of 0.1 M KCl, the activity was decreased approximately 15% and lower activity was detected when adding 1.5 M KCl. The activity was decreased approximately 15% (Figure 4.13).

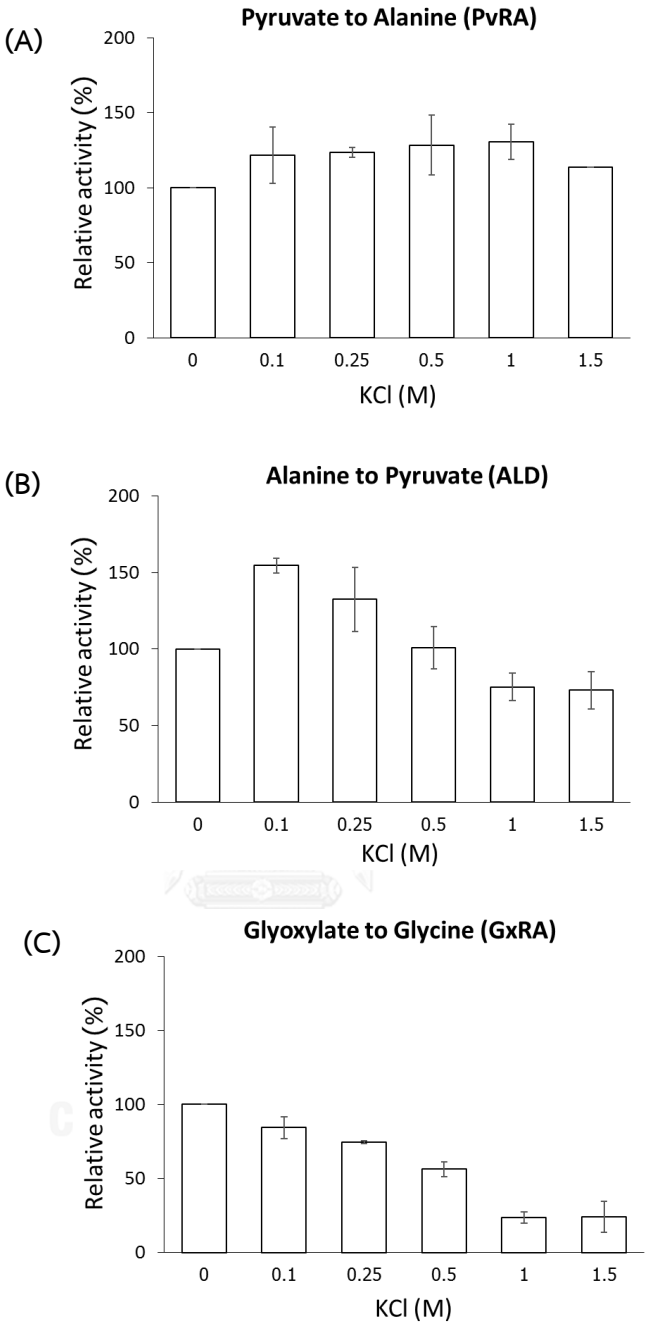


Figure 4.13: Effect of KCl for ApalaDH activity. The (A) pyruvate reductive amination activity (PvRA), (B) glyoxylate reductive amination activity (GxRA) and (C) alanine oxidative dehydrogenase activity reactions (ALD) were analyzed. The ApalaDH activity measured in the absence of NaCl was taken as 100%. Data shown as mean ± standard deviation of three independent replications.

4.3.6 *In vivo* analysis

A. halophytic cells were grown under different concentration of NaCl (0.5 M or 2.0 M). Cells were harvested, resuspended in appropriate buffer and disrupted by sonication. After removal of cell debris, supernatant was used to determine the activity of alanine dehydrogenase. Four reactions PvRa, ALA, GxRA and GDH were analyzed as described in Materials and Methods. The results were showed in term of relative activity (%). For PvRa reaction, the relative activity from cells which grown under 2.0 M NaCl was higher than 0.5 M NaCl approximately 28% (Figure 4.14). The similar result was found in GxRA reaction. The activity from crude extract of cells that grown under 2.0 M NaCl was higher than 0.5 M NaCl approximately 2.7 folds (Figure 4.14). This experiment could not determine the ALD reaction from crude extract. It might be the effect from some metabolites or inhibitors presenting in crude extract. The final reaction GDH was not detected as same as the results from *In vitro* analysis of alanine dehydrogenase. The increasing of GxRA reaction under salt stress condition was interesting. This reaction uses glyoxylate to generated glycine which is a substrate for glycinebetaine biosynthesis (Waditee *et al.*, 2003). These data confirm that alanine dehydrogenase from *A. halophytica* catalyzes two possible reactions *in vivo*. One is the reversible reaction between pyruvate and alanine. The second reaction is non-reversible reaction that produced glycine from glyoxylate.

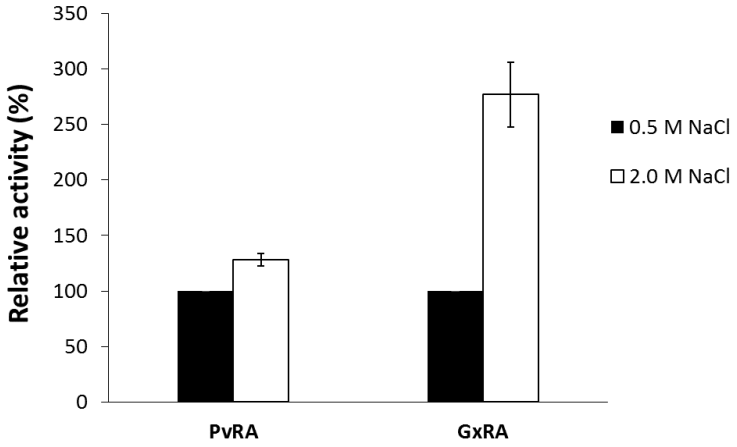


Figure 4.14: *In vivo* analysis of ApalaDH under normal and salt stress conditions. The activity from cells which grown under 0.5 M NaCl was taken as 100%. Data shown as mean ± standard deviation of three independent replications.

4.3.7 Semi-quantitative RT-PCR analysis

A. halophytica cells were grown under normal (0.5 M NaCl) and then subjected to salt stress condition (2.0 M NaCl) for 0, 3, 6, 9 and 24 hours. Cells were collected and extracted RNA. Total RNA was converted to be cDNA and used templates for PCR amplification. The PCR reactions were performed using specific primers for *ApalaDH. Aprnpb* was used as an internal control and *Apshmt* was used as positive control (up-regulated gene). The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis. Electrophoresis results showed the intensity of *AprnpB* was similar in all condition tested (Figure 4.15A). For the *ApalaDH*, band intensity slightly increased. After treating with 2.0 M NaCl for 6 hours, the band intensity of band was stronger than control (0 hour). For the positive control *ApSHMT*, the intensity of band after treated with 2.0 M NaCl was stronger than the initial time (0 hour). Then, all

band intensities were analyzed by Image lab software. The internal control showed equal relative value in all conditions that was similar to electrophoresis results. Relative value of *ApalaDH* was up regulated under salt stress condition when compared with *AprnpB*. The relative value was increased approximately 2.2 folds after treated with 2.0 M NaCl (Figure 4.15B). For the positive control *ApSHMT*, the relative values were increased approximately 2.5 and 5 folds under salt stress condition for 6 and 9 hours, respectively.

The expression levels of *ApalaDH* under salt stress conditions have not been reported so far. In cyanobacteria AlaDH from *S. elongatus* PCC 7942 had been showed the expression under nitrogen starvation condition. The result showed 20 folds increased after 24 hours treatment with nitrogen starvation condition (Lahmi *et al.*, 2006).The positive control *SHMT* has been studied in wide range of organisms. In cyanobacterium *A. halophytica,* the expression level is up-regulated after upshocking with 2.5 M NaCl (Waditee-Sirisattha *et al.*, 2012). Crucial role of SHMT was reported in various organisms (Turner *et al.*, 1993, Hagemann *et al.*, 2005). It was also reported that this gene was crucial to survive under salt stress (Waditee-Sirisattha *et al.,* 2012).

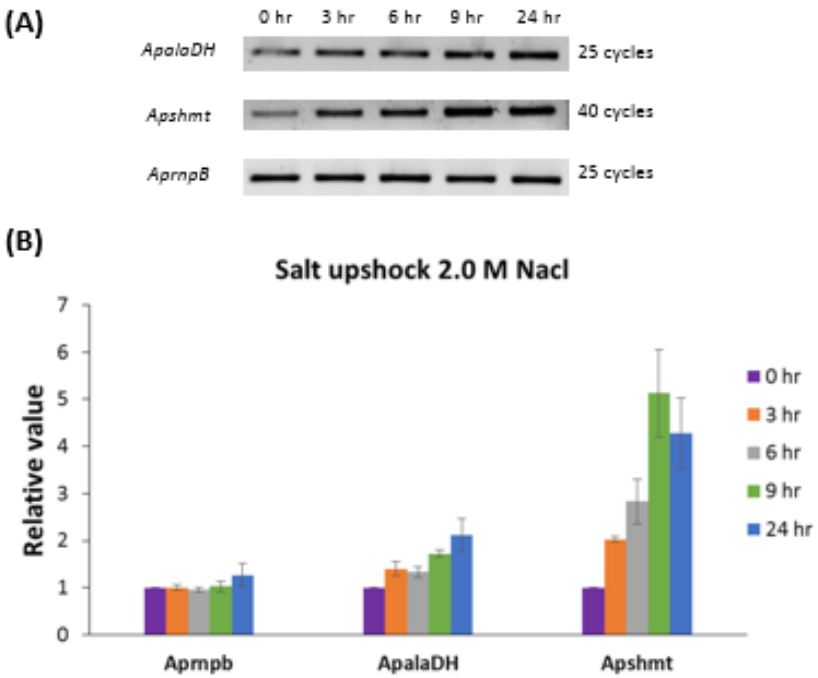


Figure 4.15: (A) Semi quantitative RT-PCR analysis of *ApalaDH* under salt stress condition. Cells were grown under 2.0 M NaCl for 0, 3, 6, 9 and 24 hours, respectively. Total RNA was extracted and converted to cDNA. PCR amplification was performed using cDNA as a template. *AprnpB* and *Apshmt* were used as internal control and positive control, respectively. The PCR products were analyzed by 1% agarose gel electrophoresis. The intensity of bands was analyzed by Image lab software. (B) Relative value compared the expression levels of *AprnpB*, *ApalaDH* and *Apshmt* Data are mean ± standard deviation of three independent replications.

CHAPTER IV

CONCLUSIONS

1. Putative *ApalaDH* was successfully expressed in *E. coli* strain BL21 using pColdI and pColdTF vectors.
2. Purified recombinant ApalaDH catalyzed two reactions. The first reaction was a reversible reaction from pyruvate to alanine (PvRA and ALD) and the second was a non-reversible reaction that produces glycine from glyoxylate (GxRA).
3. The optimal pH of ApalaDH for PvRA, ALD and GxRA were 9.0, 9.5 and 8.0 respectively.
4. The *Km* of recombinant ApalaDH for pyruvate alanine and glyoxylate were 0.02 ± 0.02, 0.72 ± 0.04 and 1.9 ± 0.43 mM, respectively.
5. *In vitro* analysis showed that NaCl enhanced ALD and GxRA activities.
6. *In vitro* analysis showed that KCl enhanced PvRA and ALD activities but strongly inhibited GxRA activity.
7. *In vivo* analysis showed that GxRA activity was increased 2.7-folds under salt stress condition.
8. The expression level of *ApalaDH* was up-regulated under salt stress condition.

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APPENDICES

Appendix 1

BG11 medium (Waditee *et al*., 2012)

Trace element solution

H_3BO_3	2.8	g
$MnCl_2 \cdot 4H_2O$	1.81	g
$ZnSO_4 \cdot 7H_2O$	0.22	g
$CuSO_4 \cdot 5H_2O$	0.079	g
$Co(NO_3)_2 \cdot 6H_2O$	0.049	g

Dissolved all compositions with distilled water to 1 liter.

BG11 solution

$NaNO_3$	1.5	g
K_2PO_4	40	mg
$MgSO_4 \cdot 7H_2O$	75	mg
$CaCl_2 \cdot H_2O$	36	mg
Na_2CO_3	20	mg
EDTA*2Na	1	mg
Citric acid	6	mg
Ferric ammonium nitrate	6	mg
Trace element	1	ml

Dissolved all compositions with distilled water to 1 liter.

BG11 Tark solution

NaCl	28.17	g
KCl	0.67	g
$MgSO_4 \cdot 7H_2O$	6.92	g
$MgCl_2 \cdot 6H_2O$	5.50	g
$CaCl_2 \cdot 2H_2O$	1.47	g

Dissolved all compositions with distilled water to 1 liter.

Appendix 2

LB medium

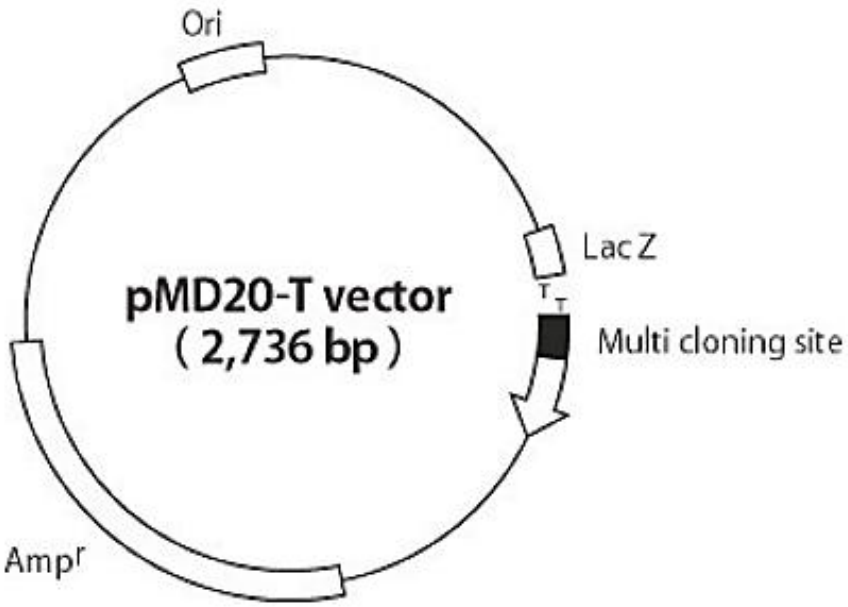
Composition per 1 liter

10	g	Bacto tryptone
5	g	Yeast extract
10	g	NaCl

Dissolve all compositions with 800 ml deionized water, adjust the pH to 7.0 with 6 M NaOH. Adjust volume of solution to 1 liter with deionized water. Autoclave at 121 ºC, 15 lb/in^2 for 15 min. For media containing agar add bactoagar 15 g per liter.

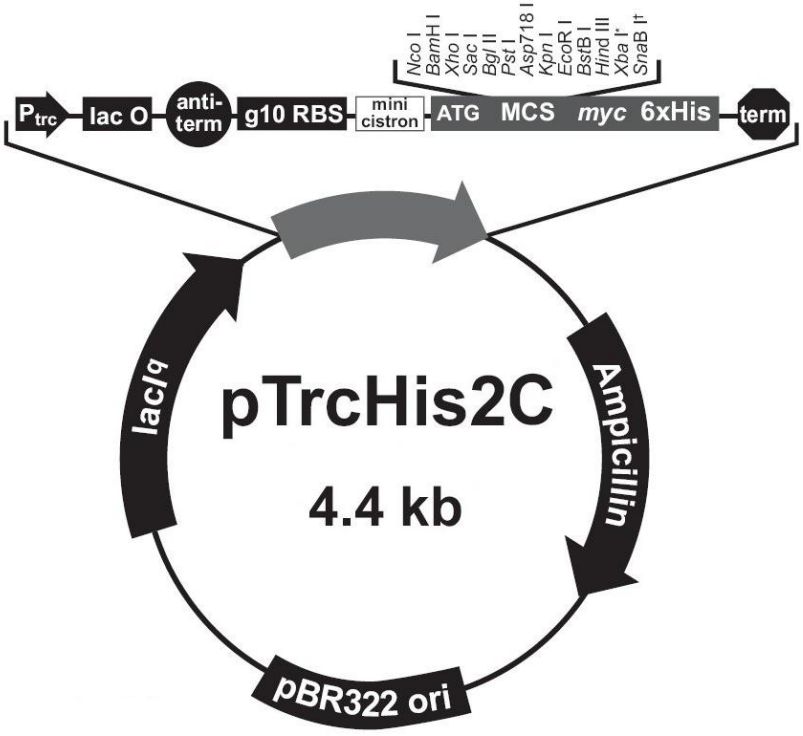
Appendix 3

pMD20 vector (Takara, Japan)



Appendix 4

pTrcHIS2C vector (Takara, Japan)



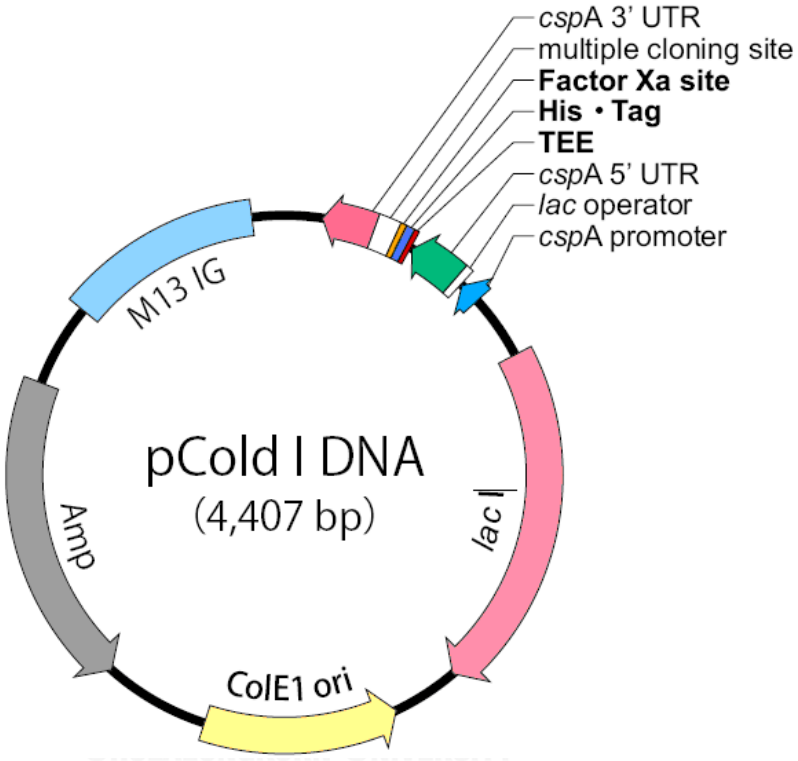
Appendix 5

Preparation of competent *E. coli* cells

A single colony of *E. coil* DH5α was incubated to 2 ml of LB medium and incubation at 37 ºC overnight with vigorous shaking. This culture was reinoculated to fresh LB medium and incubated at 37 ºC with vigorous shaking for 3-4 hours until the OD_{620} reach 0.4-0.6. The culture was stood on ice for 10 minutes and centrifuged at 4000 rpm for 10 minutes at 4 ºC. Cell pellet was resuspended in 0.05 volume of TSB-DMSO free medium and stood on ice for 10 minutes. This cell suspension was dispensed in 100 µl aliquots into 1.5 ml microcentrifuge and stored at -80 ºC

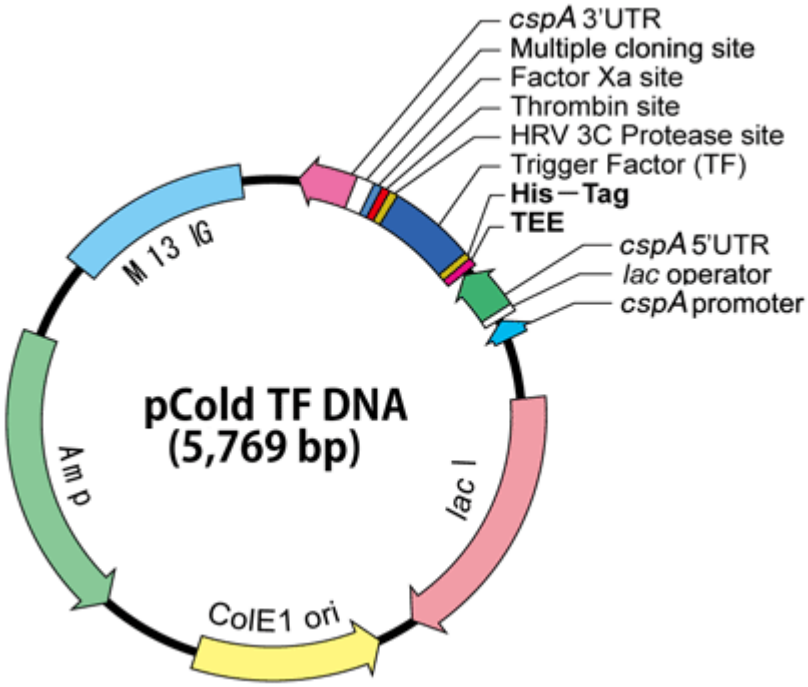
Appendix 6

pColdI vector (Takara, Japan)



Appendix 7

pColdTF vector (Takara, Japan)



Appendix 8

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

30% Acrylamide, 0.8% bis acrylamide, 100 ml

Acrylamide	29.2	g
N, N methylene bis acrylamide	0.8	g

Adjust volume to 100 ml with distill water

1.5 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	18.17	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water

2 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	24.2	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water

0.5 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	6.06	g

Adjust pH to 6.8 and adjust volume to 100 ml with distill water

1 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	12.1	g

Adjust pH to 6.8 and adjust volume to 100 ml with distill water

Solution B (SDS-PAGE)

2 M Tris Cl pH 8.8	75	ml
10% SDS	4	ml
Distill water	21	ml

Solution C (SDS-PAGE)

2 M Tris Cl pH 8.8	50	ml
10% SDS	4	ml
Distill water	46	ml

2. SDS-PAGE

10% separating gel

30% acrylamide solution	3.33	ml
Solution B	2.5	ml
Distill water	5.0	ml
10% Ammonium sulfate	50	µl
TEMED	10	µl

5% stacking gel

30% acrylamide solution	0.67	ml
Solution B	1.0	ml
Distill water	2.3	ml
10% Ammonium sulfate	30	µl
TEMED	5.0	µl

Sample buffer

1 M Tris Cl pH 6.8	0.6	ml
50% glycerol	5.0	ml
10% SDS	2.0	ml
2-mercaptoethanol	0.5	ml
1% bromphenol blue	1.0	ml
Distilled water	0.9	ml

4x of sample buffer is mixture sample to 1x. The mixture heated 5 min in boiling water before loading to the gel.

Electrophoresis buffer for 1 liter

Tris (hydroxymethyl) aminomethane	3.0	g
Glycine	14.4	g
SDS	1.0	g

Adjust volume to 1 liter with distilled water (pH 8.3).

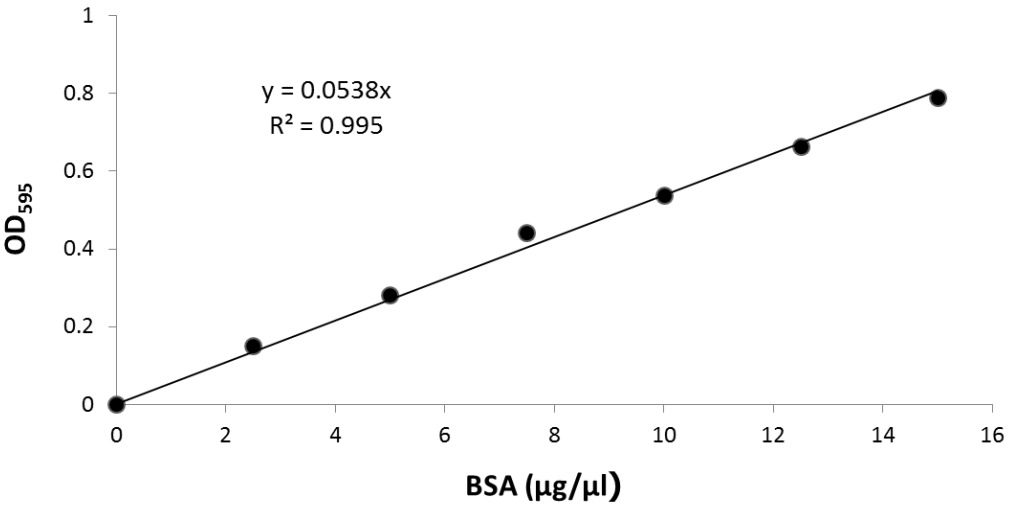
Appendix 9

Protein Assay (Bradford, 1976)

1. Standard procedure (Biorad, USA)

The reagent was prepared by diluting 1 part of reagent with 4 parts of deionized water. Then, the bovine serum albumin was diluted in six dilutions to use as standard protein. One microliter of each dilution was mixed with 900 µl of reagent. The mixtures were incubated at room temperature for 5 minutes and measured the absorbance at 595 nm.

2. Standard curve for protein Assay



Appendix 10

Buffer for western blotting

PBS buffer (Phosphate-buffer-saline)

Final concentration per 1 liter

10 mM sodium phosphate pH 7.4

150 mM NaCl

Blocking buffer

5% (w/v) skim milk and 0.01% Tween20 in 1xPBS buffer

Blotting transfer buffer

Final concentration per 1 liter

39 mM glycine

48 mM Tris base

0.037% SDS

20% methanol

VITA

Mr. Sittipol Phogosee was born on November, 1990 in Bangkok, Thailand. He graduated from Department of Microbiology, Falculty of Science, Chulalongkorn University in 2013 with a Bachelor degree of Science (Microbiology). Recently, he has pursued his Master Degree of Microbiology, Faculty of Science, Chulalongkorn University. Some parts of this work were published in proceeding of The 7th National Conference on Algae and plankton at Narai Hotel, Bangkok. The topic is Expression analysis of ApSHMT gene under stress condition.