การโคลนยีน SHMT จากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* และการแสดงออกใน

Escherichia coli

นางสาวดวงใจ สิทธิพล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CLONING OF SHMT GENE FROM A HALOTOLERANT CYANOBACTERIUM

Aphanothece halophytica AND EXPRESSION IN *Escherichia coli*

Miss Duangjai   Sittipol

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By   Duangjai Sittipol

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Thesis Advisor   Dr. Rungaroon Waditee - Sirisattha

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the

Requirements for the Master's Degree

…………………………………………..Dean of Faculty of Science

(Professor Supot Hannongbua, Ph. D.)

Thesis committee

…………………………………………..Chairman

(Associate Professor Suthep Thaniyavan, Ph. D.)

…………………………………………..Thesis Advisor

(Rungaroon Waditee – Sirisattha, Ph. D.)

…………………………………………..Member

(Associate Professor Pairoh Pinpanichakarn, Ph. D.)

…………………………………………..Member

(Assistant Professor Chulee Yompakdee, Ph. D.)

…………………………………………..Member

(Sophon Sirisattha, Ph. D.)

ดวงใจ สิทธิพล : การโคลนยีน *SHMT* จากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* และการแสดงออกใน *Escherichia coli*. (CLONING OF *SHMT* GENE FROM A HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* AND EXPRESSION IN *Escherichia coli*)

อ. ที่ปรึกษาวิทยานิพนธ์ : อาจารย์.ดร. รุ่งอรุณ วาดิถี สิริศรัทธา, 94 หน้า

   Serine hydroxymethyltransferase (SHMT) เป็นเอ็นไซม์ที่มีความสำคัญในการเร่งปฏิกิริยาผันกลับของกรดอะมิโนเซอรีน-ไกลซีน และเป็นส่วนหนึ่งของกระบวนการเมแทบอลิซึมของคาร์บอนหนึ่งหน่วย (C-1) ซึ่งเป็นกระบวนการที่สำคัญกับสิ่งมีชีวิตทุกชนิด ในการศึกษา SHMT พบอย่างกว้างขวางในสิ่งมีชีวิตตั้งแต่ในแบคทีเรียจนถึงพืชชั้นสูงและสัตว์เลี้ยงลูกด้วยนม แต่ไม่พบว่ามีการศึกษาคุณลักษณะของ SHMT ในไซยาโนแบคทีเรีย ดังนั้นงานวิจัยนี้จึงมุ่งศึกษา SHMT ในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* (ApSHMT) และแสดงออกใน *Escherichia coli* พบว่ารีคอมบิแนนท์เอ็นไซม์ ApSHMT แสดงการเร่งปฏิกิริยาได้ 2 แบบคือ ปฏิกิริยาที่ขึ้นกับเตตระไฮโดรโฟเลต (THF-dependent) และ ปฏิกิริยาที่ไม่ขึ้นกับเตตระไฮโดรโฟเลต (THF-independent) โดยมีกรดอะมิโนเซอรีนเป็นสารตั้งต้นที่สำคัญ ในปฏิกิริยาของ SHMT ถูกยับยั้งโดยการเติมโซเดียมคลอไรด์แต่ SHMT ถูกปกป้องอย่างมีประสิทธิภาพเมื่อเติมบีเทนลงในปฏิกิริยา

   การวิเคราะห์ปริมาณกรดอะมิโนเซอรีนและไกลซีนในเซลล์สำหรับการแสดงออก พบว่ามีปริมาณการสร้างเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับชุดควบคุม และนอกจากนี้เมื่อวิเคราะห์ปริมาณสารเมทาบอไลต์ ได้แก่ บีเทน และ โคลีน พบว่าในเซลล์สำหรับการแสดงออกมีการสะสมบีเทน และโคลีน สูงขึ้นหลายเท่าเมื่อเปรียบเทียบกับชุดควบคุม ดังนั้นเซลล์สำหรับการแสดงออกจึงมีการเจริญเติบโตได้เพิ่มขึ้นภายใต้สภาวะที่มีเกลือ

ภาควิชา จุลชีววิทยา   ลายมือชื่อนิสิต ....
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์……......................
ปีการศึกษา 2554

5272310023 : MAJOR INDUSTRIL OF MICROBIOLOGY

KEYWORDS : *Aphanothece halophytica*/ one-carbon metabolism/ halotolerant cyanobacterium/ serine hydroxymethyltransferase/ salt tolerance

DUANGJAI SITTIPOL: CLONING OF SHMT GENE FROM A HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* AND EXPRESSION IN *Escherichia coli*.

ADVISOR : RUNGAROON WADITEE-SIRISATTHA, Ph.D., 94 pp.

Serine hydroxymethyltransferase (SHMT) is an important enzyme for serine-glycine cycles and plays important role as a central position in one-carbon metabolism. SHMT has been studied in many living organisms from bacteria to higher plants and mammals; however, biochemical and molecular characterization of SHMT has never been investigated in photoautotrophic microorganisms. In this study, we have isolated SHMT gene from a halotolerant cyanobacterium *Aphanothece halophytica* (*ApSHMT*) and expressed in *Escherichia coli*. Recombinant ApSHMT exhibited catalytic reactions for both THF-dependent/independent cleavages. Catalytic reaction for L-serine, a physiological substrate, was strongly inhibited by NaCl but it could be protect efficiently by osmoprotectant betaine. Amino acid analysis revealed the increased levels of amino acids (glycine and serine) in the expressing cells in all condition examined. Furthermore, metabolite analysis revealed alleviated levels of choline as well as betaine. ApSHMT expressing cells could accumulate betaine several folds higher than the control cells, which caused the enhanced growth rate of the expressing cells particularly under salt-stress conditions.

Department Microbiology   Student's Signature

Field of study Industrial of Microbiology   Advisor's Signature

Academic Year 2011

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LIST OF ABBREVIATIONS

A	Absorbance
Bp	Base pair
BSA	Bovine serum albumin
SHMT	Serine hydroxymethyltransferase
cm	Centimeter
°C	Degree celsius
kDa	Kilodalton
g	Gram
hr	Hour
l	Liter
mA	Milliampare
min	Minute
μl	Microliter
ml	Milliliter
mM	Millimolar
M	Molar
nm	Nanometer
PCR	Polymerase chain reaction
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
V	Volume

CHAPTER I

INTRODUCTION

1.1 Background

One carbon (C-1) metabolism comprises a complex set of reactions that are essential to all living organisms. These reactions supply the C-1 unit need to the synthesize nucleic acid, protein and numerous biological compounds Cossin and Chen, 1997; Chen et al., 1997; Hanson et al., 1994). There are many enzymes involved in C-1 metabolism network, for instance, *N5, N10*-methylenetetrahydrofolate reductase (MTHFR), cobalamin-independent methionine synthase (MetSyn), *S*-adenosylmethionine synthetase (AdoMet Syn), *S*-adenosylmethionine decarboxylase (SAMDC) and serine hydroxymethyltransferase (SHMT), and, etc. Among these enzymes, it is believed that SHMT is the first enzyme for interconversion of C-1 compound, which catalyzes the reversible conversion of serine and tetrahydrofolate (THF), to glycine and *N5, N10*-methylenetetrahydrofolate (Schirch et al., 1985).

Accumulating evidence indicates the biological significance of SHMT. In mammals, SHMT has been shown to be involved in *de novo* biosynthesis of thymidylate (Anderson and Stover, 2009). Disruption of SHMT increases the risk of neural tube defects (Anderson and Stover, 2009; Agrawal et al., 2003). In prokaryotes such as *Escherichia coli*, 15% of all carbon atoms assimilated from glucose is estimated to pass through the glycine-serine pathway (Wilson et al., 1993). In plants, SHMT cooperates with the glycine decarboxylase complex (GDC) to mediate photorespiratory glycine-serine interconversion (Bauwe et al., 2010). An Arabidopsis mutant deficient in SHMT exhibited

a lethal phenotype under ambient CO_2 environment (Voll et al., 2006). In photosynthetic organism such as cyanobacteria, the enzyme activity of SHMT from a cyanobacterium *Synechocystis* sp. PCC 6803 has been determined (Eisenhut et al., 2006), however, molecular properties of cyanobacterial SHMT remain largely unknown. In this study, we aim to study SHMT from a halotolerant cyanobacterium, *Aphanothece halophytica* (hereafter called *A. halophytica*). This organism is the unique cyanobacterium which originally isolated from Dead sea. It can produce a large amount of glycine betaine (hereafter called betaine) under stress condition by *de novo* biosynthesis of betaine using three-step methylation of glycine (Waditee et al., 2003). Previous study suggested the provision of substrate glycine/serine enhanced accumulation levels of betaine in *A. halophytica* (Waditee et al., 2007). Thus, the study of the *Aphanothece* SHMT (hereafter called ApSHMT) which involved in glycine-serine interconversion would provide useful information for metabolic engineering to increase betaine level.

1.2 Objectives

1. To produce recombinant ApSHMT and study its biochemical properties.

2. To investigate metabolic flux in the ApSHMT expressing cells (i.e., serine, glycine, choline and betaine).

3. To investigate transcription level of *ApSHMT* under salt up- and down-shock conditions.

1.3 Hypothesis

1. ApSHMT is capable of catalyzing THF- dependent and THF -independent reactions.

2. Overexpression of the *ApSHMT* gene in *E.coli* increases of metabolites levels; namely, glycine, serine, choline and betaine.

3. ApSHMT overexpressor exhibits stress-tolerance phenotype.

4. *ApSHMT* is a salt - inducible gene.

CHAPTER II

LITERATURE REVIEW

2.1 One-carbon (C-1) metabolism

One-carbon (C-1) metabolism comprises a complex set of reactions that are essential to all living organisms. It involved in the addition or removal of C-1 unit for biosynthetic or regulatory functions, thus, it is crucial for the synthesis of proteins, nucleic acids, and numerous biological compounds (Chen et al., 1997; Cossins and Chen, 1997; Hanson et al., 1994). Current progress in bioinformatics databases suggest a number of enzymes for C-1 metabolism network such as *N5, N10* -methylenetetrahydrofolate reductase (MTHFR), cobalamin-independent methionine synthase (Met Syn), S-adenosylmethionine synthetase (AdoMet Syn), S-adenosylmethionine decarboxylase (SAMDC), serine hydroxymethyltransferase (SHMT), and *N5, N10*-methylenetetrahydrofolate dehydrogenase (MTHFD), Betaine-homocysteine S-methyltransferase (BHMT) and Methionine synthase reductase (MTRR). Schematic pathway for C-1 metabolic network was purposed in mammals (Figure. 2.1).

Among these enzymes, it is believed that SHMT is the first enzyme for interconversion of C-1 compound. It is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and catalyzes the reversible conversion of serine and tetrahydrofolate (THF), to glycine and *N5, N10*-methylenetetrahydrofolate (Schirch et al., 1985). Serine is an essential amino acid. It has important roles as a precursor in metabolism participating in purine and pyrimidine biosynthesis (Beaudin et al., 2011) and also serves for the generation of

activated C‑1 unit via SHMT. These evidences indicate that SHMT is not only a key

enzyme not only in nucleic acids, but also in amino acids including biological compounds.
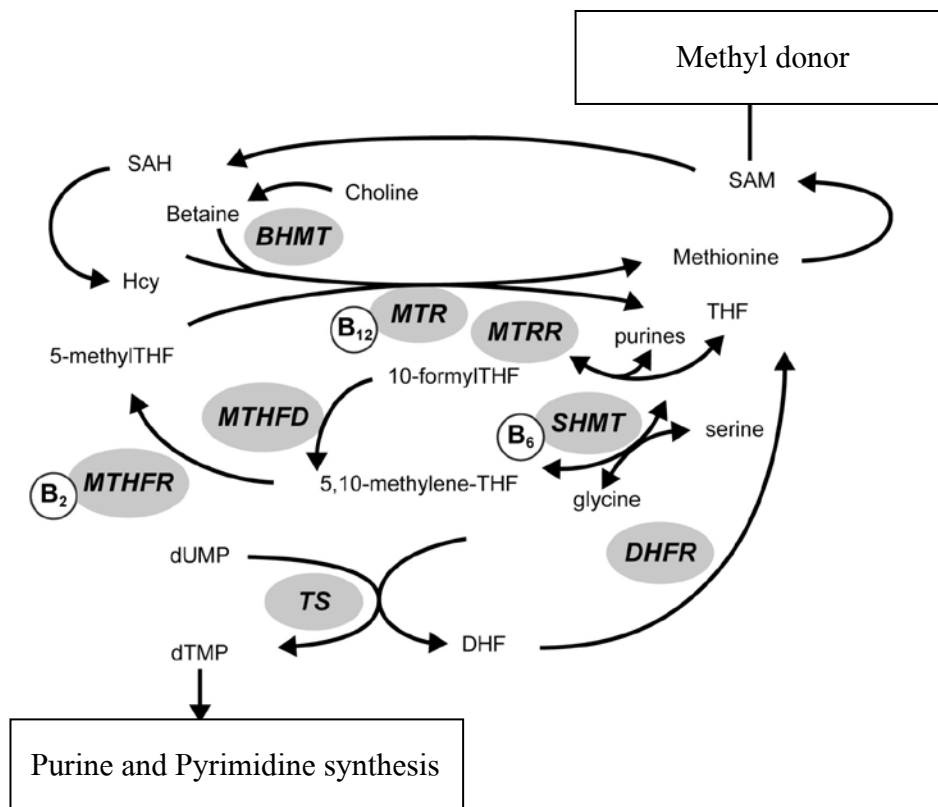
Figure 2.1 Purposed pathway For C‑1 metabolism in mammals (adapted from Shivani et

al., 2010).

BHMT : Betaine‑homocysteine S‑methyltransferase

SHMT: Serine hydroxymethyltransferase

MTRR : Methionine synthase reductase

MTR : Methionine synthase

MTHFD : *N5, N10*-methylenetetrahydrofolate dehydrogenase

MTHFR : Methylenetetrahydrofolate reductase

DHFR : Dihydrofolate reductase

TS : Thymidylate synthase

2.2 Serine hydroxymethyltransferase

2.2.2 Catalytic mechanism of SHMT

SHMT is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and catalyzes the interconversion of serine and THF to glycine and *N5, N10*-methylene-THF (Schirch et al., 1985).

PLP interacts with the active site of SHMT and links to a serine molecule which is also attracted to the active site through interactions with residues. The carbon of serine is transferred to THF during the reaction which yields the products of glycine and *N5, N10*-methylene-THF. The conversion of serine to glycine causes the release of a C-1 unit which is transferred to THF; this is the main mechanism in which C-1 units enter into the THF pool. *N5, N10*-methylene-THF is mainly used in synthesizing thymine nucleotides. The general mechanism for how serine is conversion to glycine is representing (Figure 2.2A). Therefore, the reaction is called THF dependent.

There are also proposed mechanisms for how the serine is converted to glycine. The first of these two mechanisms is a retroaldol cleavage mechanism. In retro aldol cleavage, the SHMT enzyme which catalyzes the cleavage of C_3-OH amino acids produced from benzaldehyde from such as aldolase reaction (Figure 2.2B). Within the active site of SHMT, there is a hydrophobic that interacts with C_3. In serine, there are two different isomer forms of C_3 which played the -erythro and the -threo isomers of either conformations. Therefore, the reaction played THF independent (Metha and Christen, 2000; Rao et al., 2000; Schirch and Gross, 1968; Ulevitch and Kallen, 1977)

A)   **Serine + THF** $\longleftrightarrow$ *N5, N10*-**methylene-THF + Glycine**

B)   **DL-threo-3-phynylserine + serine** $\longleftrightarrow$ **Glycine + Benzaldehyde**

Figure 2.2 The coupling reaction of SHMT played two reactions for the enzyme. A) THF-dependent reaction and B) THF-independent reaction. Both reactions use PLP as a cofactor.

2.2.1 Structure of SHMT

SHMT involved in many important reactions of the generation of DNA and protein as well as in many other cellular processes. Thus, mechanisms of SHMT and their structures were examined. It has been shown that SHMT must form a dimer or tetramer in order to have catalytic activity (Figure 2.3). Four structures of SHMT have been determined that form homo-tetramers, these are hcSHMT (Human Liver SHMT) (Skibola et al., 2002), rcSHMT (Rabbit Liver SHMT) (Lim et al., 2005), mcSHMT (Murine Cytoplasmic SHMT) (Heil et al., 2001), and eSHMT (*E.coli* SHMT) (Chang et al., 2006). All of the structures of the mammalian SHMT show structural homology, but the bacterial eSHMT, though forming tetramers, display different organization of the chains at the quaternary structure.
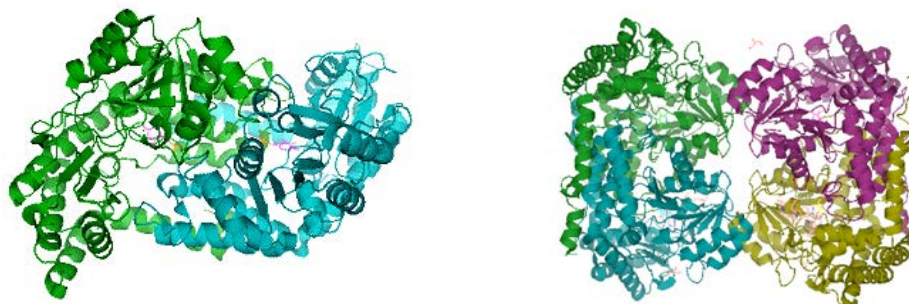
Figure 2.3 Structures of SHMT, in left homo‑dimer (Fu et al., 2003), and in right homo‑tetramer (Lim et al., 2005).

2.2.3 Importance of SHMTs in living organisms

Accumulating evidence has suggested the importance of SHMT in all living organisms. In prokaryotes, SHMT (or another annotation is called GlyA), is a key enzyme in the biosynthesis of purines, thymidine, methionine, choline and lipids. The enzyme also catalyzes several side reactions including hydrolysis of 5,10-methenyl THF to 5‑formyl THF and the reversible cleavage of 3‑hydroxy amino acids (L-threonine, allothreonine, 3-phenylserine) to glycine and an aldehyde (Misano et al., 2005). D-alanine inactivates the enzyme by reacting with the PLP prosthetic group to form pyridoxamine phosphate (Hopkins et al., 1986; Plamann and Stauffer, 1985). A *glyA* mutant is auxotrophic for glycine (Pizer, 1965). In *E.coli glyA* was later shown to be essential for growth on glycerol minimal medium (Joyce et al., 2006). The *glyA* mutants were shown that it can not use glycine as the sole source of nitrogen (Newman et al., 1976). In *Escherichia coli*, 15% of all carbon atoms assimilated from glucose are estimated to pass through the glycine-serine pathway (Wilson et al., 1993).

It has been shown that in mammals, SHMT is important for *de novo* biosynthesis of thymidylate biosynthesis (Anderson and Stover, 2009). Disruption of SHMT, resulting from common genetic variations, nutritional deficiencies and increases the risk of neural tube defects (Anderson and Stover, 2009; Beaudin et al., 2011; Voll et al., 2006). In mouse, it has been shown that cSHMT involved in the conversion of glycine to serine, and also regulates the metabolic partitioning of THF (Narkewicz et al., 1996).

In plants, serine can be formed by several pathways, the principal one being associated with photorespiratory glycine metabolism through the THF-mediated conversion of glycine to serine by glycine decarboxylase complex (GDC) and SHMT. The THF-mediated pathway via the C1-THF synthase and SHMT using formate as the C-1 source (Prabhu et al., 1996; King, 2002). Serine and glycine are both potential sources of C-1 units in plants. They are interconversion by SHMT. GDC oxidizes glycine to CO_2, NH_3 and *N5, N10*-methylene-THF. The *N5, N10*-methylene-THF reactions with a second molecule of glycine, catalyzed by SHMT, to produce serine (Leegood et al., 1995 ; Prabhu et al., 1996). Therefore, SHMT cooperates with the glycine decarboxylase complex (GDC) to mediate photorespiratory glycine-serine interconversion. An Arabidopsis mutant deficient in SHMT exhibited a lethal phenotype under ambient CO_2 environment (Eisenhut, 2006). In cyanobacteria, the SHMT gene was suggested to be essential for cell survival since the complete segregation of SHMT gene could not be generated (Hagemann et al., 2005). This result indicates the importance of cyanobacterial SHMT.

2.3 Metabolic engineering of betaine via provision of substrate (s)

Betaine is known as the most potent osmoprotectant found in nature. It

could protect organisms thriving under very high salinity (Kempf and Bremer, 1998; Rhodes and Hanson, 1993; Takabe et al., 1997). Betaine is synthesized by a choline oxidation route and by glycine methylation. The well known biosynthetic pathways of betaine include a classical two-step oxidation of choline. The first step is catalyzed by choline monooxygenase in plants, choline dehydrogenase in animals and bacteria, and choline oxidase in some bacteria. The second step is catalyzed by betaine aldehyde dehydrogenase in all organisms (Boch et al., 1996; Hayashi et al., 1997; Lamark et al., 1991; Rathinasabapathi et al., 1997; Weretilnyk and Hanson, 1990; Yamada et al., 1979). However, the halotolerant cyanobacterium *A. halophytica* possesses a novel biosynthetic pathway for betaine. Waditee et al. have successfully identified a novel biosynthetic pathway for betaine which is different from other living organisms via a three-step methylation of glycine catalyzed by two new methyltransferases. The first *N*-methyltransferase, glycine/sarcosine-*N*-methyltransferase, catalyzes the methylation reactions of glycine and sarcosine with *S*-adenosylmethionine acting as the methyl donor. The other, dimethylglycine-*N*-methyltransferase, specifically catalyzes the methylation of dimethylglycine to betaine (Waditee et al., 2003).

Betaine accumulates as an osmoprotectant in stressed cells. So far, there are only a few different pathways (as described above) for the biosynthesis of betaine in living cells: (i) by a choline oxidation, and (ii) by glycine methylation. Choline is synthesized from ethanolamine, which is derived from serine. Serine and glycine are interconvertible through the activity of SHMT. Therefore, two routes for the biosynthesis of betaine can utilize serine as an upstream precursor. Metabolic engineering of betaine was previously reported by the achievement of the introduction of *ApPGDH* gene which catalyzes the first

step of the phosphorylated pathway of serine biosynthesis (Waditee et al., 2007). Expression of the *ApPGDH* in *E.coli* increases betaine accumulation. These data suggest the importance of the *ApPGDH* gene in serine or glycine engineering for the enhancement of betaine accumulation through the choline oxidation or the glycine methylation pathway in microorganism.

SHMT is an enzyme for not only glycine/serine interconversion, but it is also an upstream enzyme for generating *S*-adenosylmethionine (SAM) (See Figure. 2.1). Thus, overexpression of SHMT might be possible to increase both glycine/serine and SAM in the overexpressor. This enzyme would be one of interesting target for metabolic engineering of betaine in living cells.

In this study, we aim (i) to express the ApSHMT in *E. coli* in order to produce recombinant protein for biochemical characterization, (ii) to analyse metabolites in the overexpressor, (iii) to investigate the increase level of betaine in the overexpressor, and (v) to analyse transcript level of *ApSHMT* under salt-stress condition.

CHAPTER III

MATERIALS AND METHODS

2.1 Instrument

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetteman, Gilson, France

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

Digital Lux meter FT710: Taiwan

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

High performance liquid chromatography: Model Hewlette Packard series 1050, Japan

Illuminated/Refrigerated orbital: Sanyo, England

Incubator: Haraeus, Germany

Incubator shaker: Psyco-therm, New Bruncwick Scientific Supply, Thailand

Laminar flow BVT-124: International Scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

Microscope: Olympus, USA

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

Power supply: Pharmacia, England

Spectrophotometer UV-240: Shimadzu, Japan and Du series 650: Beckman, USA

Time of flight Mass spectrophotometer : Shimadzu, Japan

Vacuum dry: Taitech, Japan

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

Water bath: Charles Hearson, England

2.2 Chemicals

Acrylamide: Merck, USA

Amino acid and derivatives: Sigma, USA

Ammonia: Sigma, USA

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

An antibody raised against mouse: Biolab, England

Ampicillin: Katayama, Japan

Bacto tryptone: Merck Ag Darmstadt, Germany

Bacto Yeast extract: Merck Ag Darmstadt, Germany

Beta-mercaptoethanol: Katayama Chem, Japan

Calcium chloride: Merck Ag Darmstadt, Germany

Calcium chloride dihydrate: Merck Ag Darmstadt, Germany

Chloramphenicol: Sigma, USA

Coomasie brilliant blue G-250: Sigma, USA

Coomasie brilliant blue R-250: Sigma, USA

Dialysis tube: Sigma, USA

D (+)glucose: Sigma, USA

2, 4 dinitrophenol, Sigma, USA

DTT (Dithiothreitol): Sigma, USA

D9-choline : Sigma Aldrich, USA

D11-betaine : Sigma Aldrich, USA

EDTA (Ethylenediaminetetraacetic acid): Sigma, USA

Ethanol: Katayama Chem, Japan

Ferric sulfate: Mallinckrodt Chemical, USA

Glycerol: Merck Ag Dramstadt, Germany

Glycine: Sigma, USA

Hydrochloride: Sigma, USA

HEPES: Sigma, USA

Imidazole: Katayama Chem, Japan

Lithium chloride: Katayama Chem, Japan

Magnesium chloride: Merck Ag Dramstadt, Germany

Magnesium sulfate: Merck Ag Dramstadt, Germany

Morpholinopropanesulphonic acid (MOPS): Katayama Chem, Japan

N,N'-methylene-bis-acrylamide: Sigma, USA

Nitrobluetetrazolium (NTB): Katayama Chem, Japan

Potassium chloride: Merck Ag Dramstadt, Germany

Sodium acetate: Sigma, USA

Sodium chloride: Sigma, USA

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: Sigma, USA

Sodium nitrate: Sigma, USA

Sodium phosphate: Sigma, USA

Tris-hydrochloride: Katayama Chem, Japan

2.3 Supplied

Nitrocellulose membrane: Millipore Cooperation, USA

Whatman 3MM paper: Whatman International, England

2.4 Kit

Ligation kit version 1: Takara, Japan

PCR amplification kit: Applied Biosystems, USA

Plasmid extraction kit: Invitrogen, USA

Standard molecular weight: Sigma, USA

RNA extraction kit: QIAGEN, USA

2.5 Enzyme and restriction enzyme

*Bam*HI: Biolabs, USA

*Nde*I: Biolabs, USA

RNase: Katayama Chem, Japan

3.6 Bacterial strains and plasmids

Table 1 Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Description	Source/reference
A.halophytica	Halotolerant cyanobacterium	Ishitani, 1993
E. coli DH5α	(φ80d*lacZ*ΔM15 *recA1endA1gyrA96 thi*-1 *hsdR*17($r_k^- m_k^+$) *sup*E44 *relA*1 *deoR* Δ(*lacZYA-argF*)U169)	Meijo University, Japan
E. coli BL21	(B F- dcm ompT hsdS(rB- mB-) gal [malB+]K-12(λS)	Meijo University, Japan
E. coli BL21(DE3)	(F–ompT gal dcm lon hsdSB (rB- mB-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Meijo University, Japan
pCR2.1	Cloning vector	Invitrogen, USA (Appendix 1)
pColdI	Expressing vector	Takara , Japan (Appendix 2)
pSHMT_C	1.2 kb ApSHMT pCR fragment cloned into pCR 2.1 vector	Waditee-Sirisattha (unpublished)
pSHMT_E	1.2 kb *Nde*I-*BamH*I from pSHMT_C cloned into pColdI vector	This study

Table 2 Primers for isolation and expression of *ApSHMT* gene and for detection of their mRNA levels

Primers	5'-----3'	Base pairs (mer)
ApSHMT-Bam	CAACATATGGTGACGCAAACAAAC	24
ApSHMT-Nde	AGGGATCCTTAT GCCATTGCGGG	23
ApSHMT-RT_F	CAAGGGTCTGTTCTCACC	18
ApSHMT-RT_R	GTTTCTTGGCTTACGCCG	18
AprnpB_ F	TGAGGAAAGTCCGGGCTTCC	20
AprnpB_ R	GGACATAAGCCGGGTTCTGT	20

3.7 Bioinformatic sequence analysis

A sequence of ApSHMT (nucleotide sequence data for ApSHMT is available in the DDBJ databases under the accession number AB695121) was compared with various SHMTs and analysed phylogenetic tree. SHMT sequences from various organisms (from bacteria to higher plants) were obtained from GENBANK and Cyanobase databases. These are *Anabaena variabilis* ATCC29413 (AC225679), *Arabidopsis thaliana* SHMT1 (AJ271726.1)*, Arabidopsis thaliana* SHMT2 (AL034567.1), *Chlamydomonas reinhardtii* (DS496176.1), *Escherichia coli* K12 (AP009048.1), *Gloeobacter violaceus* PCC7421 (BA000045.2), *Micromonas* sp. RCC299 (CP001325.1), *Nodularia spumigena* CCY9414 NZ (AAVW01000129.1), *Nostoc* sp. PCC7120 (BA000019.2), *Oryza sativa* (DP000011.2), *Ostreococcus tauri* XM (003084227.1), *Phaeodactylum tricornutum* (CP001142.1), *Populus trichocarpa* (CM000337.1), *Prochlorococcus marinus*. MIT 9313

(BX548175.1), *Rhodopseudomonas palustris* CGA009 (NC_005296.1), *Synechococcus elongatus* PCC 6301 (AP008231.1), *Synechococcus* sp. CC9902 (CP000097.1), *Synechococcus* sp. PCC7002 (NC_010475.1), *Synechococcus sp.* WH5701NZ (AANO01000004.1), *Synechococcus sp*. WH 8102 (BX569689), *Synechocystis* sp. PCC6803 (BA000022.2), *Thalassiosira pseudonana* CCMP1335 (XM_002293957.1) and *Zea mays* (NM_001158393.1)

The phylogenetic tree for SHMTs was constructed by using the software package ARB (http://www.mikro.biologie.tu-muenchen.de.). The physicochemical properties for SHMTs (i.e., molecular mass and pI) were analyzed by ARB software via EXPAXY server.

3.8 Culture conditions

A. halophytica cells were grown photoautotrophically in modified BG11 medium, BG11 plus 18 mM $NaNO_3$ and Turk Island salt solution, as previously described (Appendix 3) (Ishitani et al., 1993; Takabe et al., 1988) except that NaCl concentration of the culture medium was adjusted to a range from 500 to 2500 mM as desired. Cotton plugged 500 ml conical flasks containing 100-200 ml of medium each were used and shaken on reciprocal shaker without supplementation of CO_2. The culture flasks were incubated at $30^{\circ}C$ under continuous supplying of fluorescent white light ($70\mu Em^{-2}S^{-1}$). The growth of cyanobacterial cells were monitored by measuring absorbance at 730 nm. Chlorophyll content was determined according to Giusy (Appendix 4).

3.9 Construction of expression plasmid (ApSHMT)

The coding region of *ApSHMT* was amplified from genomic DNA of *A.halophytica* by the PCR reaction and cloned into pCR2.1 generating, pSHMT_C

(Waditee-Sirisattha, Unpublished). Nucleotide sequence of *ApSHMT* was determined to exclude PCR error. The full-length *ApSHMT* frangment in pSHMT_C was prepared by double digestion with *Nde*I and *Bam*HI ligated into corresponding sites of the digested pColdI expression vector. The resulting plasmid, pSHMT_E, encoding the ApSHMT fused in the frame to six histidines at N-terminal, was transformed first into *E.coli* DH5α, then into BL21 and BL21 (DE3) cells (Appendix 5). The transformants were selected on LB agar containing ampicillin at concentration 50 µg/ml. After incubation at 37 $^{\circ}$C for 12-16 h, single colony of several transformants were picked up and inoculated into LB medium, allowed to grow overnight and used for plasmid DNA extraction. The expression plasmids harbouring ApSHMT were confirmed by restriction enzyme analysis (Appendix 6).

3.10 Expression of ApSHMT and purification

To express recombinant ApSHMT, *E.coli* expressing cell were grown in LB medium and induced with 0, 0.05, 0.1, 0.5 and 1.0 mM isopropyl-D-thiogalactopyranoside at 16°C, according to manufacturer's instruction with a slight modification. After 16 h, cells were harvested by centrifugation at 5,000 rpm for 15 min. The bacterial pellets were resuspended in buffer A (100 mM Tris, pH 8.0), sonicated and centrifuged. Clear supernatant was used to determine expression level of the ApSHMT product by SDS-PAGE (Laemmli, 1970) (Appendix 7). Protein content was determined by the method of Bradford (Bradford, 1976) (Appendix 8). The molecular mass of ApSHMT in the linear polypeptide state was determined by comparing with protein molecular weight marker. The gel was stained with coomassie brillian blue (CBB).

To purify recombinant enzyme, the expressing cells cultured in 300 ml scale were prepared. Cells were harvested by centrifugation and cell pellets were resuspended in 100 mM Tris pH 8.0 and then subjected to sonicate. The suspension was then centrifuged at 14,000 rpm for 15 min at 4 $^{\circ}$C to collect crude enzyme in the supernatant. Crude enzyme was purified by Affinity chromatography (Ni^{2+}NTA) (GE Healthcare). The purification procedure was carried out according to manufacturer's procedure with a slight modification. It can be divided into three stages (i) preparation of the cell lysate and binding of the 6xHis-tagged protein to Ni-NTA silica, (ii) washing, and (iii) elution of the 6xHis-tagged protein (Appendix 9). Finally, purified recombinant ApSHMT was desalted by spin column.

Western blot analysis was carried out to confirm that the recombinant ApSHMT was fused in-frame to six histidine tag. For this, twenty-microgram of purified recombinant ApSHMT was separated by 10 % SDS-PAGE and transferred to nitrocellulose membrane using blotting transfer buffer (Appendix 10). Blotting was done at 150 mA/inch^{2} for 1 hr followed by blocking in blocking solution (Appendix 10) for 1 h. The nitrocellulose membrane was incubated with primary antibody (an antibody raised against 6-histidine, 6X-His tag) for 1 h and washed with 100 ml of PBS plus 5% skim milk solution for 15 min, 3 times. After washing the membrane with PBS buffer plus 5% skim milk, the membrane was immediately incubated with secondary antibody (an antibody raised against mouse) for 1 h and washed 3 times with 100 ml of PBS plus 5% skim milk buffer for 15 min. The nitrocellulose membrane was visualized after incubation with the detection reagent for 30 min (Appendix 10).

3.11 Biochemical characterization of ApSHMT

3.11.1 Activity assay

3.11.1.1 THF-dependent activity

ApSHMT assay for THF-dependent direction was carried out according to the protocol of Simic with a slight modification (Simic et al., 2002). The ApSHMT was assayed with 300 µl of Tris-Cl (200 mM, pH 9.0), 200 µl of purified ApSHMT 100 µl of PLP (2 mM), 50 µl of THF (18 mM in 0.1% [wt/vol] dithiothreitol), 200 µl substrate L-serine and 150 µl of water. Reaction mixtures were incubated for up to 15 min, and 500 µl samples were mixed with 125 µl of 25% (wt/vol) trichloroacetic acid, placed on ice, and centrifuged in the cold. Then, 480 µl of the resulting supernatant was neutralized with buffer and glycine was quantified by Amino Acid Analysis System (Shimadzu, Japan) (Appendix 11).

3.11.1.2 THF-independent activity

ApSHMT assay for THF-independent direction was carried out according to the protocol of Misano with a slight modification (Misano et al., 2005). The standard reaction mixture contained 10 µmol of DL-threo-3-phenylserine, 10 nmol of PLP, 100 µmol of Tris-Cl buffer (pH 9.0), and enzyme in a final volume of 0.5 ml. In a blank, the enzyme was replaced with water. Incubation was performed at 30°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 100 mM HCl. The benzaldehyde formed was determined by the 2,4-dinitrophenylhydrazine method as follows. To the reaction mixture (0.55 ml), 0.15 ml of 0.1% 2, 4-dinitrophenylhydrazine solution in 2000 mM HCl was added, and the mixture was incubated at 30°C for 20 min. Three milliliters of 99% ethanol and 0.85 ml of 3 M NaOH were then added to the mixture, and the color

intensity of 2,4-dinitrophenylhydrazone of benzaldehyde was measured at 475 nm with a spectrophotometer. (Appendix 12). Specfic activity was expressed as units per milligram of protein. Protein was measured by the method of Bradford method.

3.11.1.3 The kinetic of ApSHMT

Kinetics parameter of the ApSHMT was carried out using purified recombinant ApSHMT. The reaction was started by the addition of varying concentrations of either L-serine and THF for THF dependent and DL-threo-3-phenylserine for THF independent. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) were calculated.

3.11.1.4 Effect of NaCl on THF-dependent activity

Purified recombinant ApSHMT was assayed in the reaction mixture as described in 3.11.1.1. Various NaCl concentrations (0-2500 mM) were added to the reaction mixture. ApSHMT activity in THF-dependent direction was expressed as the percentage of remaining activity of the enzyme without addition of NaCl.

3.11.1.5 Effect of betaine for ApSHMT activity at high salinity

Purified recombinant ApSHMT was assayed in the reaction mixture as described in 3.11.1.4, with the addition of betaine (0-2500 mM). ApSHMT activity in THF-dependent direction was expressed as the percentage of remaining activity of the enzyme without addition of NaCl.

3.12 Metabolite analysis

3.12.1 Amino acid analysis

The expressing cells of ApSHMT were grown in minimal M9 medium (Appendix 13) and LB medium (Appendix 14) with the supplementation of

exogenous NaCl (0-500 mM) at 37 $^{\circ}$C for overnight. Then, cells were harvested from 3 ml culture medium. Cells pellets were extracted by homogenizing with methanol (90% vol/vol) at room temperature. Cell suspensions were centrifuged at 14,000 rpm for 10 min. Total supernatant was pooled and dried by evaporate at 45 $^{\circ}$C. The cell dry was dissolved with mobile phase and determining amino acid by using amino acid analysis System (Shimadzu, Japan) (Appendix 11).

3.12.2 Choline analysis

The expressing cells of ApSHMT were grown in the same condition as described in 3.12.1. Cells pellets were resuspended with 1N H_2SO_4, and incubated at 25 $^{\circ}$C for overnight. Cell-lysed suspension was centrifuged 14,000 rpm 10 min, collected supernatant, and the supernatant was mixed with 80 ul KI-I_2 solution and precipitated at 4 $^{\circ}$C for 3 h. Suspension was centrifuged at 14,000 rpm for 1 h at 4 $^{\circ}$C to collect the pellets. Final pellets were dissolved with 2000 mM NH_3, Choline was analyzed by TOF-MS using d9-choline as an internal standard (Appendix 15).

3.12.3 Betaine analysis

The expressing cells of ApSHMT were grown in the same condition as described in 3.12.1. Cells pellets were resuspended with 1N H_2SO_4, and incubated at 25 $^{\circ}$C for overnight. Cell-lysed suspension was centrifuged 14,000 rpm 10 min, collected supernatant, and the supernatant was mixed with 80 ul KI-I_2 solution and precipitated at 4 $^{\circ}$C 3 h. Suspension was centrifuged 14,000 rpm for 1 h at 4 $^{\circ}$C to collect the pellet. Final pellets were dissolved with distill water. Betaine was analysed by TOF-MS using d11-betaine as an internal (Appendix 15).

3.13 Stress tolerance of expressing cells

Control and ApSHMT expressing cells were grown under (i) salt-stress (ii) oxidative stress and (iii) pH stress condition using minimal M9 medium or LB medium. For salt stress, cells were grown in LB medium supplemented with 0, 300 and 500 mM NaCl. For oxidative stress, cells were grown in LB medium supplemented 0.02% and 0.2 % rose bengal. For pH stress, cells were grown in LB medium at various pH (pH 4-10).

3.14 Quantitation of ApSHMT mRNA expression level in *A.halophytica* upon the salinity change by semiquantitative RT-PCR

A. halophytica cells were grown in the BG11 growth medium photoautotropically for 14 days prior to the up- and down-shock experiments. For up-shock experiment, the concentration of NaCl in growth medium was changed from 500-2500 mM. For the down-shock experiment, the concentration of NaCl was changed from 500-2500 mM. Total RNA was extracted from *A. halophytica* cells using the RNeasy kit (QIAGEN, Germany). Five microgram of the total RNA was reverse transcribed using the Superscript II RT kit (Invitrogen, USA) according to the manufacturer's instructions. The PCR amplification was performed with oligonucleotides specific for targeted genes ApSHMT-RT_F and ApSHMT-RT_R (Table 1) and AprnpB_F and AprnpB-R (Table 1). The PCR-amplified samples were electrophoresed on 1.2% (w/v) into agarose gels. All RT-PCR experiments were repeated at least three times.

CHAPTER IV

RESULTS

4.1 Bioinformatics sequence analysis

Protein sequences of representative bacteria, cyanobacteria, microalgae and higher plants were refined from databases as described in Materials and Methods. The 23 strains of SHMT protein sequences clustered into three main groups on the phylogenetic tree (Figure 4.1).

The first group (group I: higher plant and microalgae) consisted of *Arabidopsis thaliana* SHMT1*, Arabidopsis thaliana* SHMT2, *Oryza sativa*, *Zea mays*, *Nodularia spumigena* CCY9414. *Chlamydomonas reinhardtii*, *Gloeobacter violaceus* PCC7421, *Micromonas* sp. RCC299, *Ostreococcus tauri*, *Thalassiosira pseudonana*. The second group (group II: bacteria) was *Escherichia.coli* K12 and the last group cyanobacteria (group III) consisted of *Anabaena variabilis* ATCC 29413, *Aphanothece halophytica*, *Nostoc* sp. PCC 7120, *Phaeodactylum tricornutum*, *Populus trichocarpa*, *Prochlorococcus marinus*. MIT 9313, *Rhodopseudomonas palustris*, *Synechococcus* sp. CC9902, *Synechococcus* sp. PCC 7002, *Synechococcus sp.* WH 5701, *Synechococcus sp.* WH 8102, and *Synechocystis* sp. PCC 6803.

In addition, protein sequences of all SHMTs were analyzed for their physicochemical properties (i.e., theoretical pI and molecular mass) as shown in Table 3. The predict *ApSHMT* gene product consists of 427 amino acids with isoelectric point 6.32 and molecular mass of 44.453 kDa (Table 3).
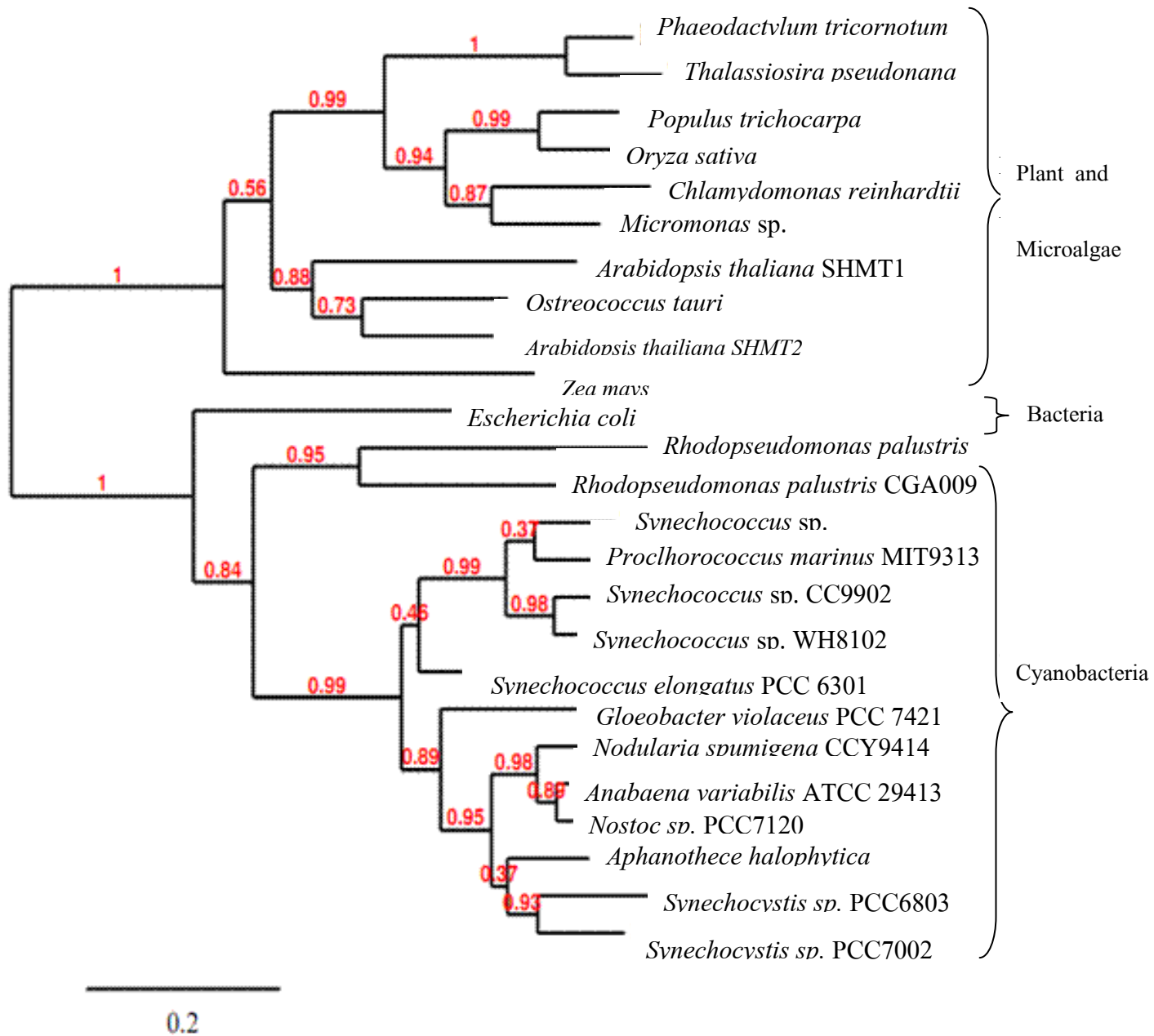
Figure 4.1 Phylogenetic tree of SHMTs from representative bacteria, cyanobacteria, micro algae and higher plant species.

Table 3 Physicochemical properties of SHMT proteins.

Organisms	Amino acid residue	pI	Molecular mass (kDa)
Anabaena variabilis ATCC 29413	473	5.77	53.343
Aphanothece halophytica	427	6.32	44.453
Arabidopsis thaliana SHMT1	542	9.43	59.505
Arabidopsis thaliana SHMT2	517	8.13	57.4
Chlamydomonas reinhardtii	462	8.13	50.735
Escherichia coli strain K12	471	7.15	51.911
Gloeobacter violaceus PCC 7421	528	6.48	57.907
Gloeobacter violaceus PCC7421	466	8.45	51.214
Micromonas sp. RCC299	422	6.87	46.322
Nodularia spumigena CCY9414	427	6.25	51.862
Nostoc sp. PCC 7120	476	6.38	52.998
Oryza sativa	427	6.16	46.271
Ostreococcus tauri	427	6.23	46.479
Phaeodactylum tricornutum CCAP 1055/1	427	6.12	46.308

Organisms	Amino acid residue	pI	Molecular mass (kDa)
Prochlorococcus marinus str. MIT 9313	427	5.97	46.356
Rhodopseudomonas palustris CGA009	426	6.09	45.703
Synechococcus elongatus PCC 6301	384	6.40	41.492
Synechococcus sp. CC9902	428	6.16	45.983
Synechococcus sp. PCC 7002	429	6.09	46.180
Synechococcus sp. WH 5701	429	5.92	45.756
Synechococcus sp. WH 8102	429	5.78	46.383
Synechocystis sp. PCC 6803	433	7.76	46.133
Thalassiosira pseudonana CCMP1335	434	6.27	45.360
Zea mays	417	6.03	45.316

4.2 Growth profile of *A.halophytica*

      A.halophytica cells were grown in modified BG11medium supplemented with 500mM (low salinity) or 2500 mM (high salinity) NaCl, respectively. The shape of *A.halophytica* under microscope represents unicellular cells with cocci shape of younger cells and rod shape of older cells (data not shown). Growth profile was shown in Figure 4.1A. Under low salinity condition (500 mM NaCl), *A. halophytica* grew faster than at 2500 mM NaCl. Chlorophyll content was determined from cell grown at 7, 10 and 14 days, respectively. As shown in Figure 4.1B, chlorophyll content in cell grown under 2500 mM NaCl was much higher than those of 500 mM NaCl.
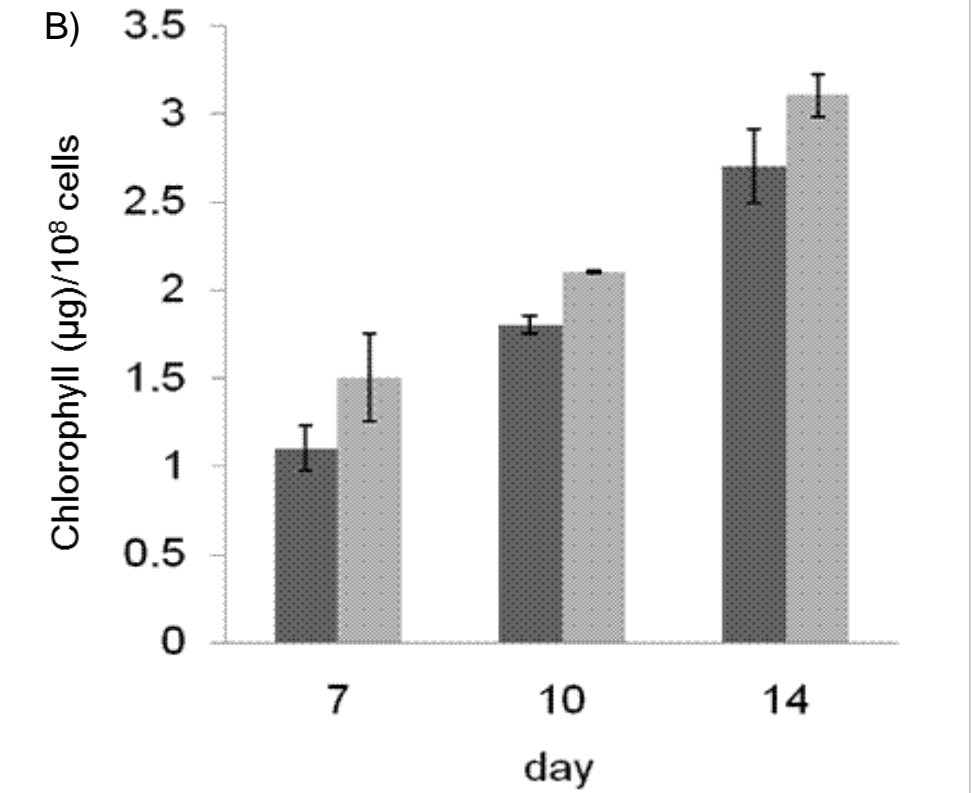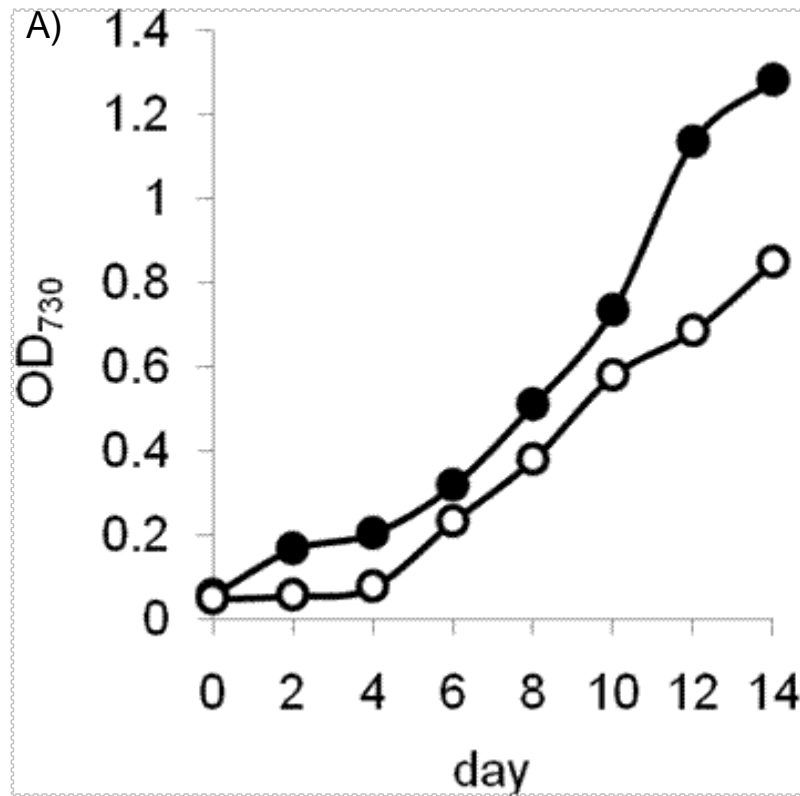
Figure 4.1 A) Growth profile of *A.halophytica* at various NaCl concentrations (500 mM, closed circle and 2500 mM, open circle).

B) Chlorophyll contents of *A.halophytica* cells at various NaCl concentrations (500 mM, black square and 2500 mM, gray square).

4.3 Construction of expression plasmid

The full length of *ApSHMT* gene was amplified by the PCR reaction and cloned into pCR2.1 as described in material and methods (Waditee‑Sirisattha, Unpublished). Nucleotide sequence of *ApSHMT* in pCR 2.1 was confirmed. To express recombinant *ApSHMT*, pColdI was used as expression vector. The full‑length *ApSHMT* fragment was prepared by double digestion pSHMT_C with *Nde*I and *Bam*HI and ligated into the corresponding sites of pColdI vector, resulting pSHMT_E. Figure 4.2 showed restriction enzyme analysis of recombinant plasmid harbouring *ApSHMT*. In lane 2, recombinant plasmid yielded 2 bands, corresponding to 4.4 and 1.2 kb, respectively. This result confirmed recombinant plasmid carrying *ApSHMT* gene.
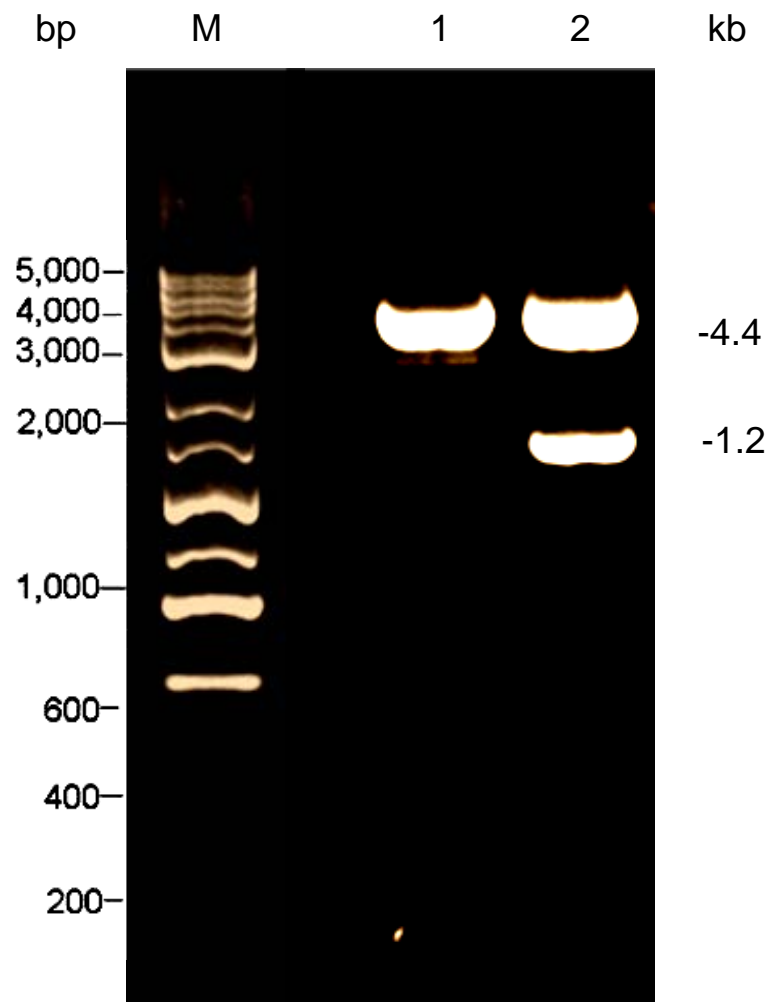
Figure 4.2 Analysis of expression plasmids harbouring *ApSHMT* on 1% agarose gel.

M : 1 kb ladder

Lane 1 : pColdI expression vector, double digestion with *Nde*I and *BamH*I

Lane 2 : pSHMT_E double digestion with *Nde*I and *BamH*I
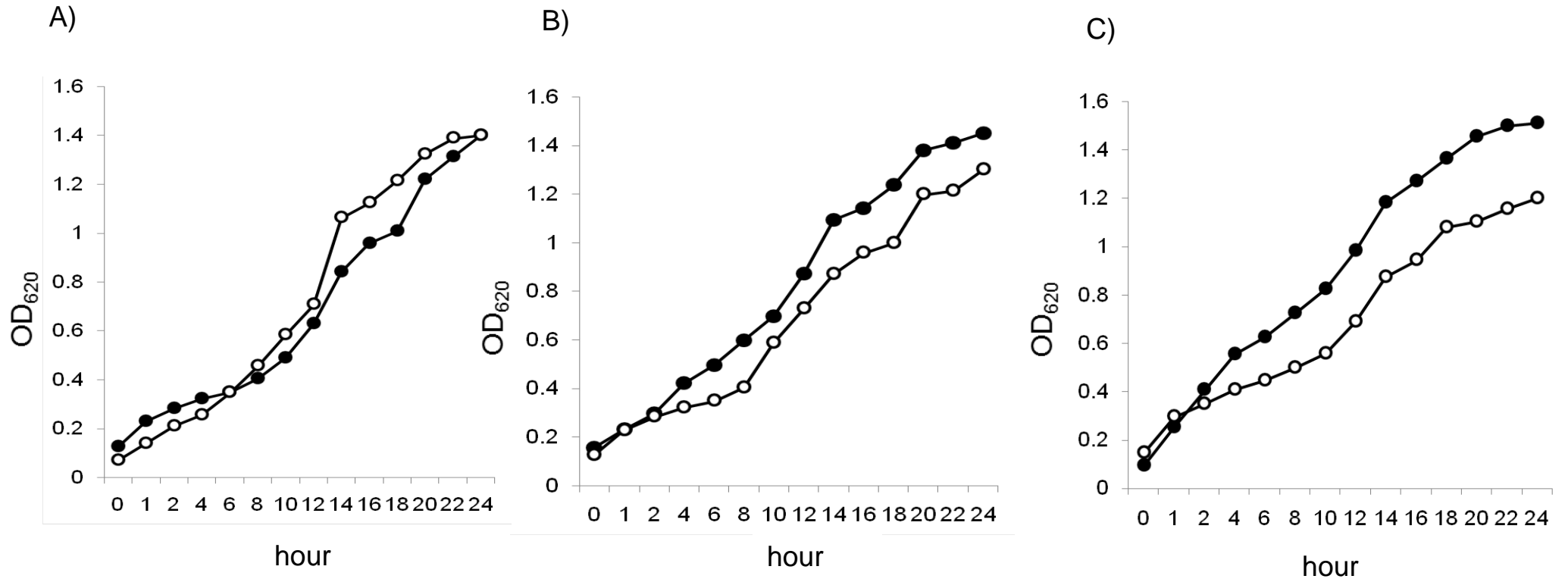
Figure 4.3 Growth profile of three kinds of *E.coli* expressing cells. The control empty vector (open circle) and the expressing cells (closed circle) were grown in LB medium pH 7.0 at 37 °C. A) *E.coli* DH5α, B) *E.coli* BL21(DE3) and C) *E.coli* BL21.

4.4 Expression of ApSHMT

Expression of recombinant ApSHMT protein were investigated in three strains of *E.coli* (*E.coli* DH5α, BL21(DE3) and BL21). Growth profiles of the control and expressing cells were performed. When ApSHMT was expressed in *E.coli* BL21 and BL21(DE3), their growth rates were slightly higher than those of control cells. The expression ApSHMT was analyzed by induction with 0.1 mM isopropyl β-D-thiogalactopyranoside at stationary phase (OD_{620} = 1.0). After 16 h of induction, cells were harvested by centrifugation. Expression level in each strain in both soluble and insoluble fractions were analyzed by SDS-PAGE. The molecular mass of ApSHMT in the linear polypeptide shown approximately 44 kDa as observing in SDS-PAGE. The expressing cells *E.coli* BL21 showed the highest level SDS-PAGE (Figure 4.4A). Western blot analysis was carried out to analyze fusion protein (His6-ApSHMT). Comparing the expressing level in all expressing strains, western blot analysis indicated the expressing cells *E.coli* BL21 showed the highest level (Figure 4.4B). Crude enzyme preparing from *E.coli* BL21 was further prepared and purified by Affinity chromatography (Ni^{2+}-NTA) (Figure 4.4C). The purified recombinant enzyme showed high purity because of very clear and no disturbed contamination of other proteins and the quantity of protein was enough for biochemical activity testing (Figure 4.4C).
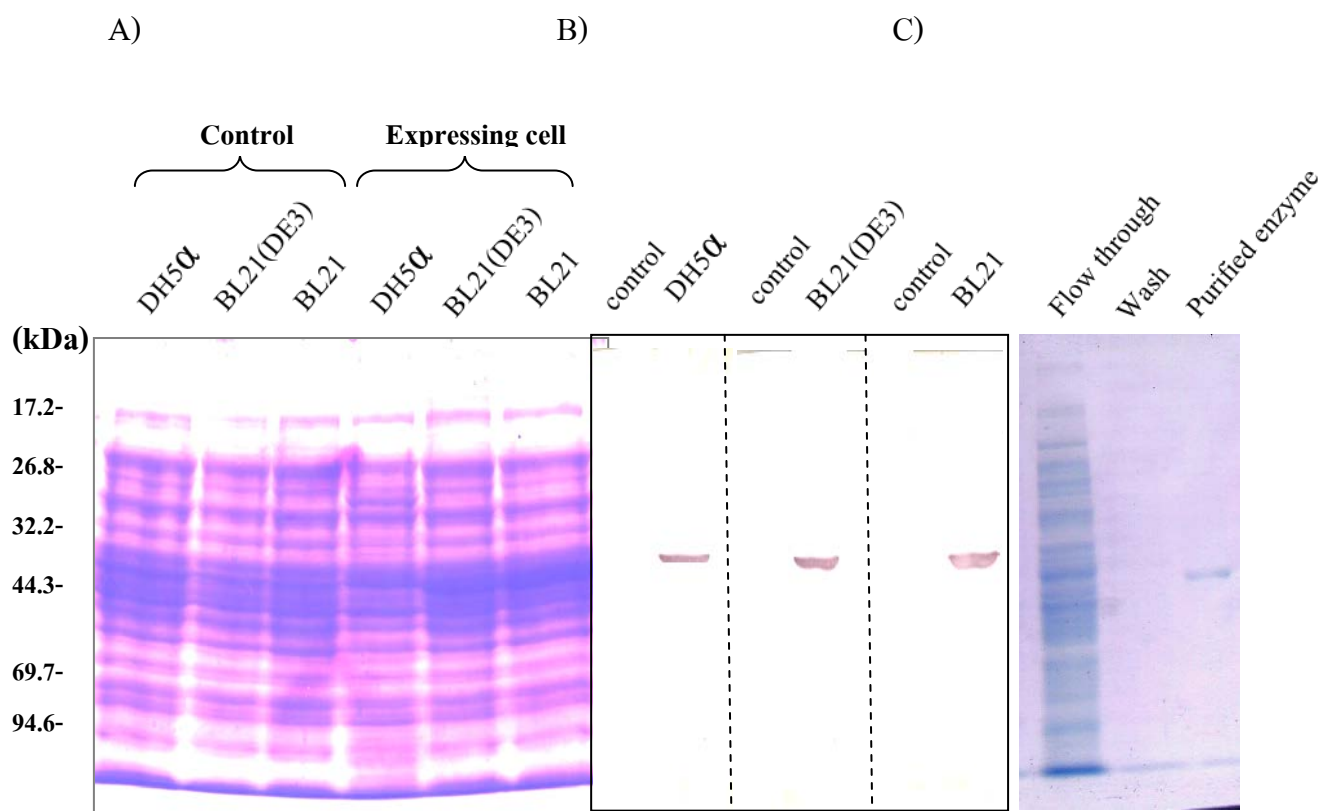
Figure 4.4 SDS-PAGE and western blot analysis**.** A) SDS-PAGE of crude enzyme from control and expressing cells. B) Western blot analysis. C) Purified recombinant ApSHMT protein. Protein content for each lane equals 20 microgram. Protein content was analyzed by Bradford method.

4.5 Biochemical property of ApSHMT

Purified recombinant ApSHMT obtaining from 4.4 was further used for biochemical characterization. ApSHMT activity was assayed in THF-dependent and THF-independent reactions. The reactions in both direction were observed their suitable conditions by using various pHs (using MES buffer (5.8-6.1), Potassium buffer (7-7.5) and Tris-Cl (7.5-9.5)), respectively. The highest activity for both reactions could be observed when Tris-Cl pH 9.0 was used as an assay buffer (Figure 4.5). The THF-dependent reaction was observed the stability of substrate THF by varying times of incubation, and reaction product (glycine) was analyzed by Amino Acid Analysis System. The time course of glycine producing increased in linear line regression. This result suggested the substrate, THF, is stable for the assay in THF-dependent reaction (Figure 4.6).

For THF-dependent reaction, ApSHMT displayed the Michaelis-Menten kinetics when both L-serine and THF were used as substrates. The apparent Km values for L-serine and THF was 0.10 ± 0.007 and 0.25 ± 0.008 mM, respectively (Figure.4.7), and the Vmax values were 750 and 800 nmol/min/mgprotein, respectively (Figure 4.7).

For THF-independent reaction, DL-threo-3-phenylserine was used as a substrate. The enzyme reaction also followed the Michaelis-Menten kinetic. Km and Vmax were calculated by fitting the data into a nonlinear regression, and were found to be 0.2 ± 0.005 mM and 3500 ± 120 nmol/min/mg protein, respectively (Figure 4.8).
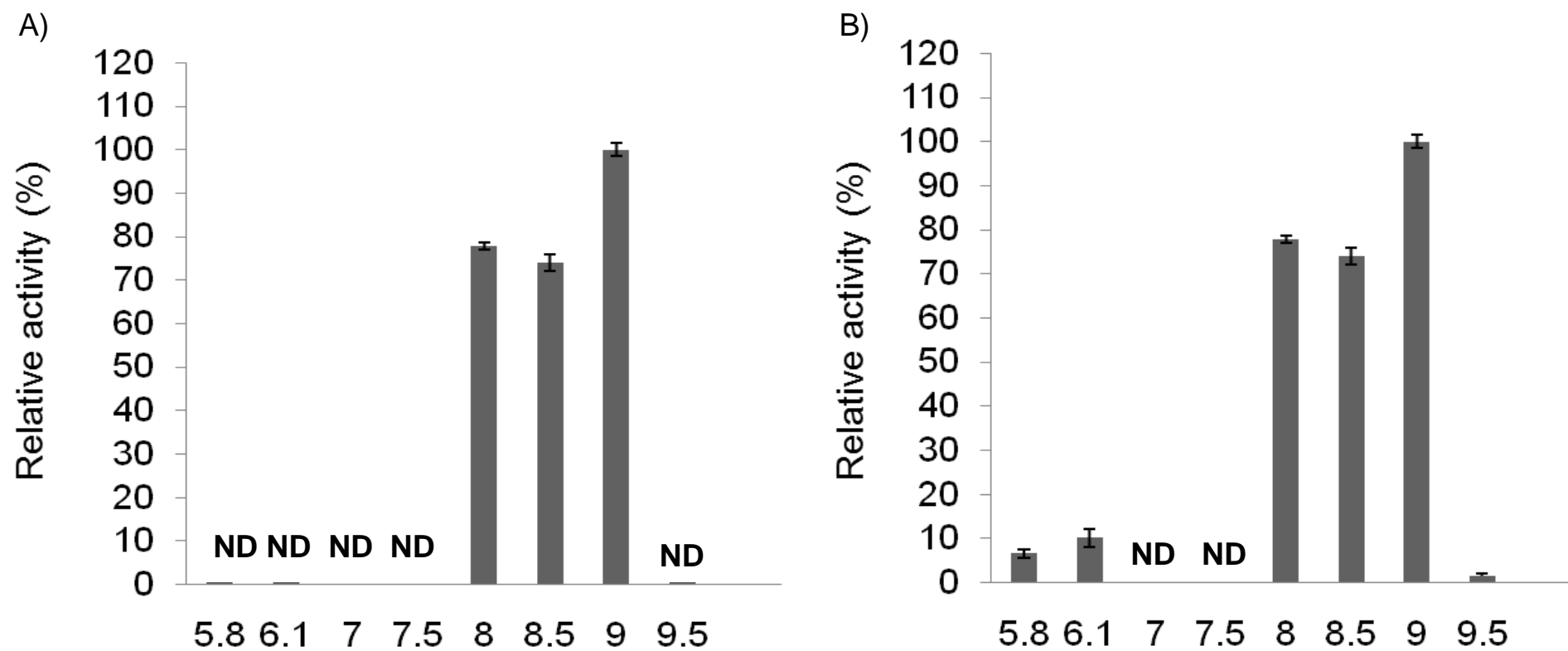
Figure 4.5 The relative activity for ApSHMT at various pHs. A) THF-dependent reaction, B) THF- independent reaction.

ND ; not determined.
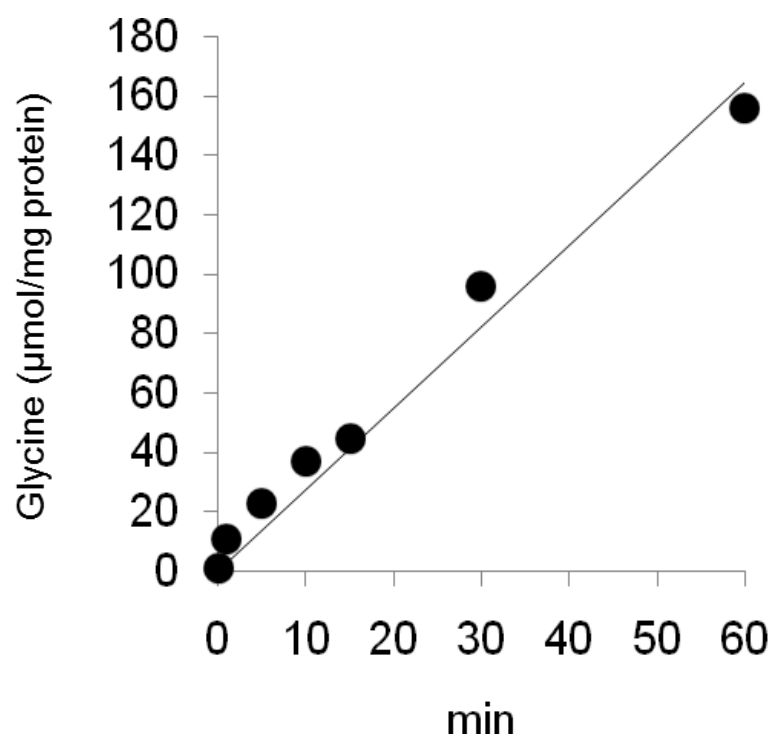
Figure 4.6 The time course of ApSHMT activity in THF-dependent reactio
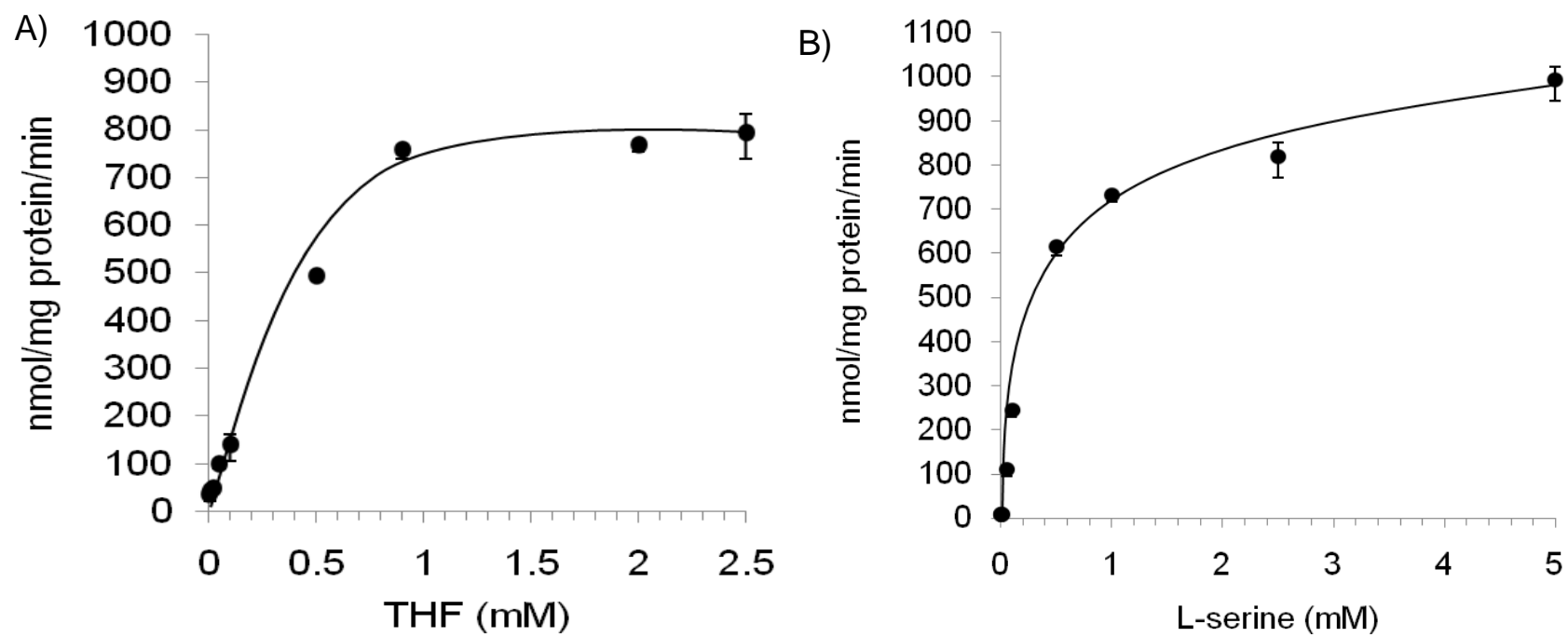
Figure 4.7 The kinetic value (Km and Vmax) of ApSHMT activity for THF- dependent reaction of A) THF , B) L-serine
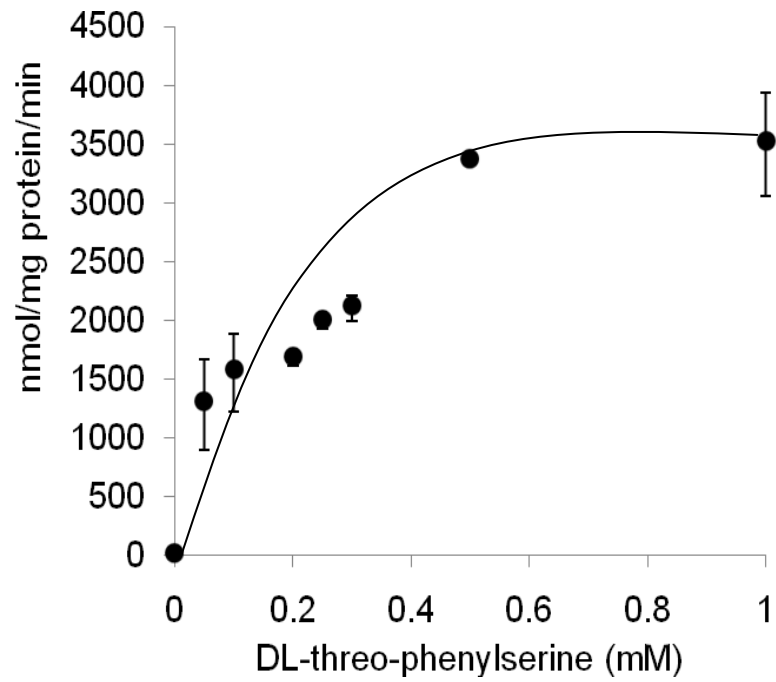
Figure 4.8 The kinetic value (Km and Vmax) of ApSHMT activity for THF- independent

reaction.

4.6 Effect of betaine on ApSHMT activity at high salinity

ApSHMT activity was carried out in the presence of NaCl (0-2,500 mM NaCl). As serine is a physiological substrate for SHMT, so we emphasized to study ApSHMT activity in THF-dependent reaction. As shown in Figure 4.9, ApSHMT activity was inhibited by NaCl.

In presence of 100 mM NaCl, ApSHMT activity was decreased by approximately 60% (Figure 4.9), followed by the progressive declined in its activity with increasing NaCl concentrations. As betaine is an osmoprotectant in *A. halophytica*, therefore, we investigated effect of betaine on the ApSHMT activity under salinity condition *in vitro*. When 50 mM of betaine was included in the assay medium, the activity was restored from 66 to 71%. With 100 mM betaine, the activity was restored from 55 to 68%. At higher concentrations, betaine efficiently restored the ApSHMT activity (Figure 4.10). These results indicate a distinctive feature of betaine in protecting the ApSHMT enzyme activity.
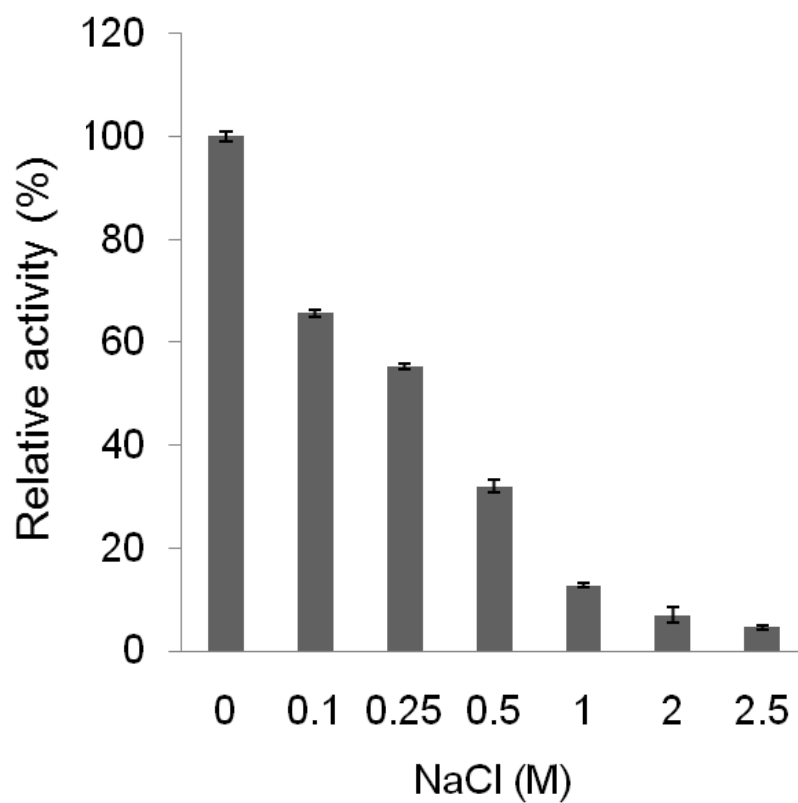
Figure 4.9 Effect of NaCl for ApSHMT (THF-dependent reaction).The ApSHMT activity measured in the absence of NaCl was taken as 100%.
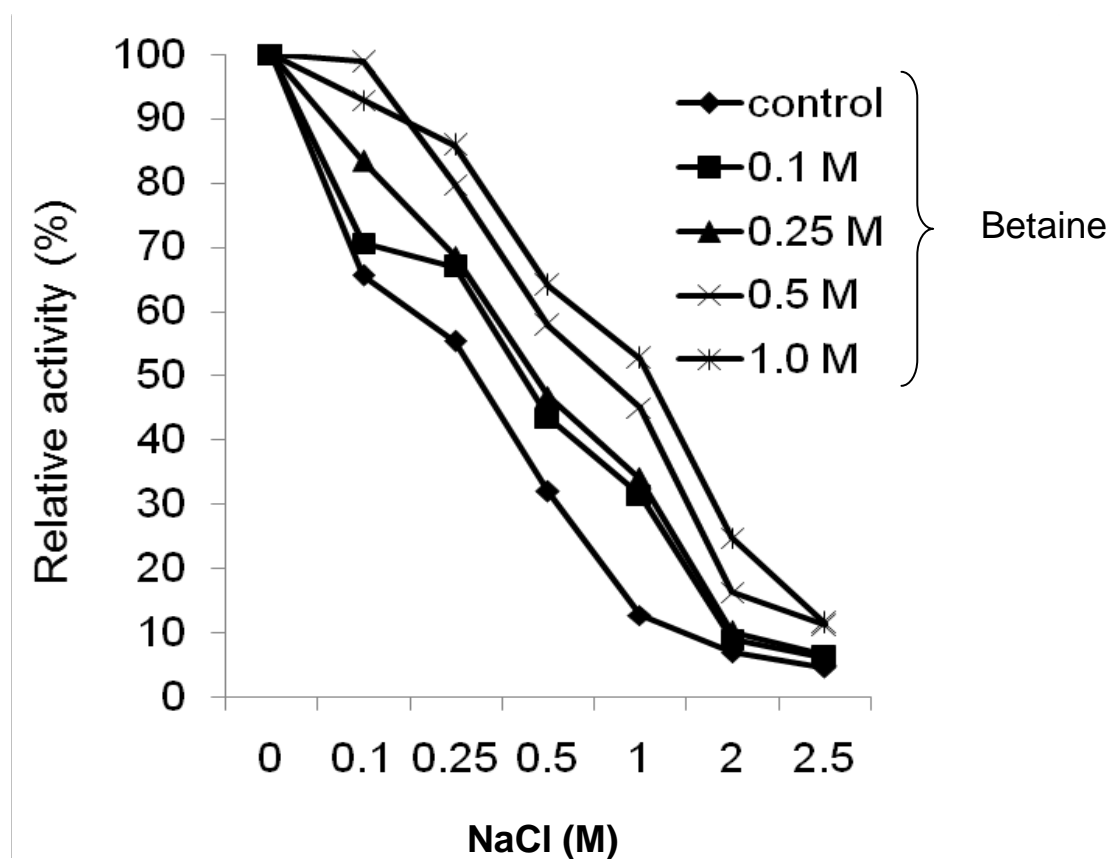
Figure 4.10 Influence of betaine on ApSHMT activity (THF-dependent reaction) in the present of various concentration of NaCl. The reaction product, glycine, was quantified by Amino Acid Analysis System. The ApSHMT activity measured in the absence of NaCl was taken as 100%.

4.7 Levels of glycine and serine in ApSHMT expressing cells.

We determined the amounts of amino acids, (glycine and serine), in control and ApSHMT expressing cells. The level of glycine in expressing cells ApSHMT was approximately 2-3-fold higher than that in the control cells when the cells were grown in the presence of 0-500 mM NaCl (Figure 4.11). The level of serine was also approximately 2-fold higher in the ApSHMT expressing cells more than control cells. Increase in the glycine and serine levels was much higher at high salinity conditions.
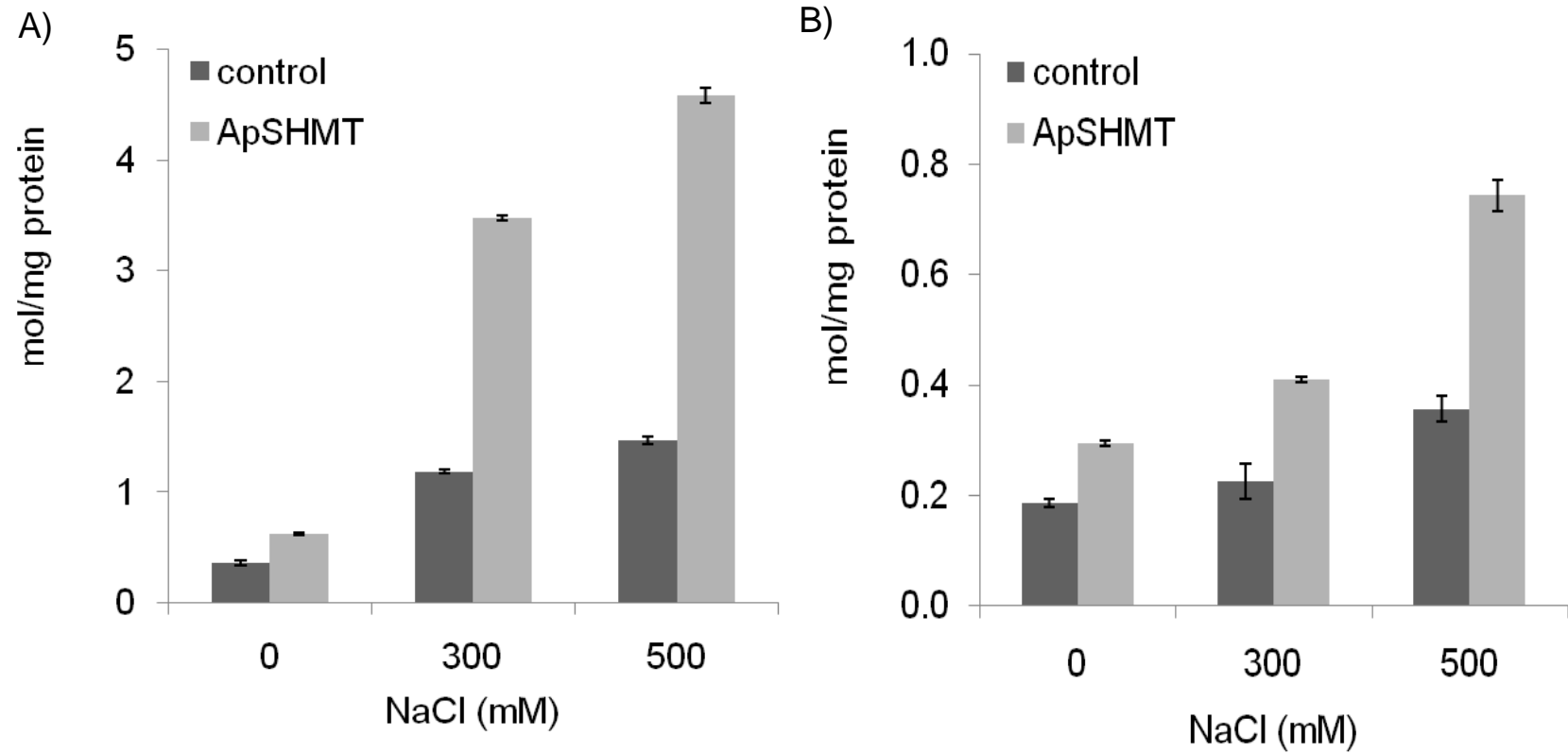
Figure 4.11 Glycine/serine content in control and expressing cells. Cells were grown in M9 minimal medium supplemented with 0-500 mM NaCl for overnight. Cell were harvested and used for amino acid analysis. A) Glycine, B) Serine.

4.8 Levels of choline and glycine betaine in ApSHMT expressing cells.

Betaine is synthesized from choline via two step oxidations in *E.coli*. Therefore, we further compared the levels of choline and glycine betaine in control and ApSHMT expressing cells. The control and ApSHMT expressing cells, grown in the minimum M9 medium with different concentration of NaCl 0, 300 and 500 mM NaCl respectively, were harvested and used to determined choline. Results showed that choline level increased to approximately 2-, 2.5-, and 5 fold in the ApSHMT expressing cells to their respective control cells when grown with 0, 300, and 500 mM NaCl, respectively (Figure 4.12). Betaine level also increased several folds higher in the ApSHMT expressing cells when cells were grown in M9 medium (Figure 4.13).
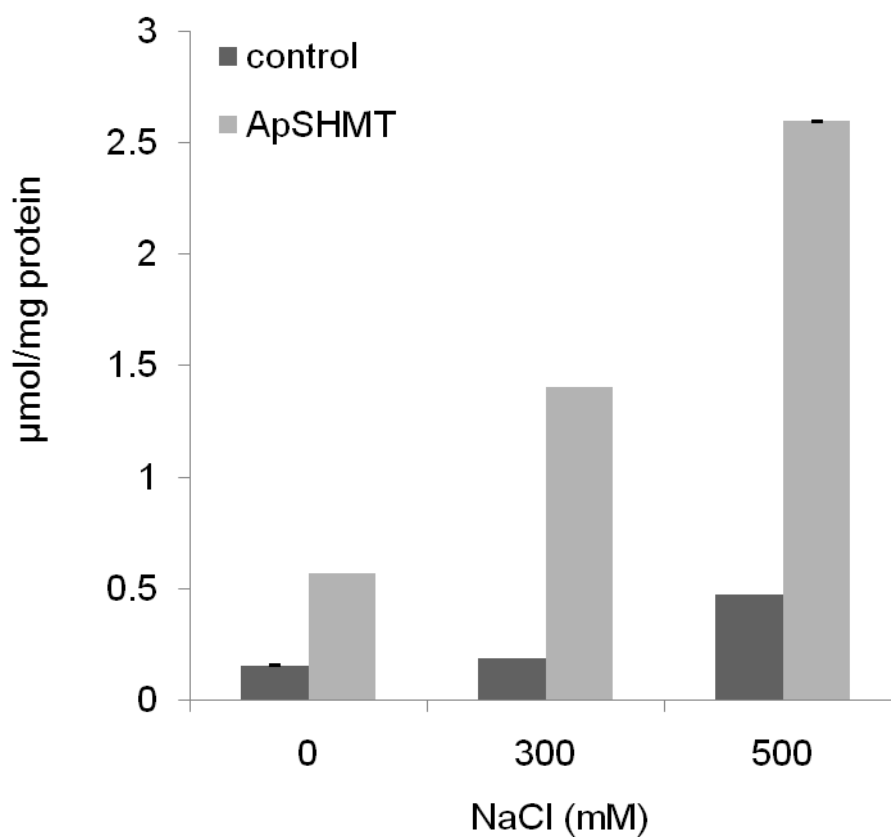
Figure 4.12 Choline content in control and expressing cells. Cells were grown in M9 minimal medium supplemented with 0-500 mM NaCl for overnight. Cells were harvested and used for choline analysis via TOF-MS.
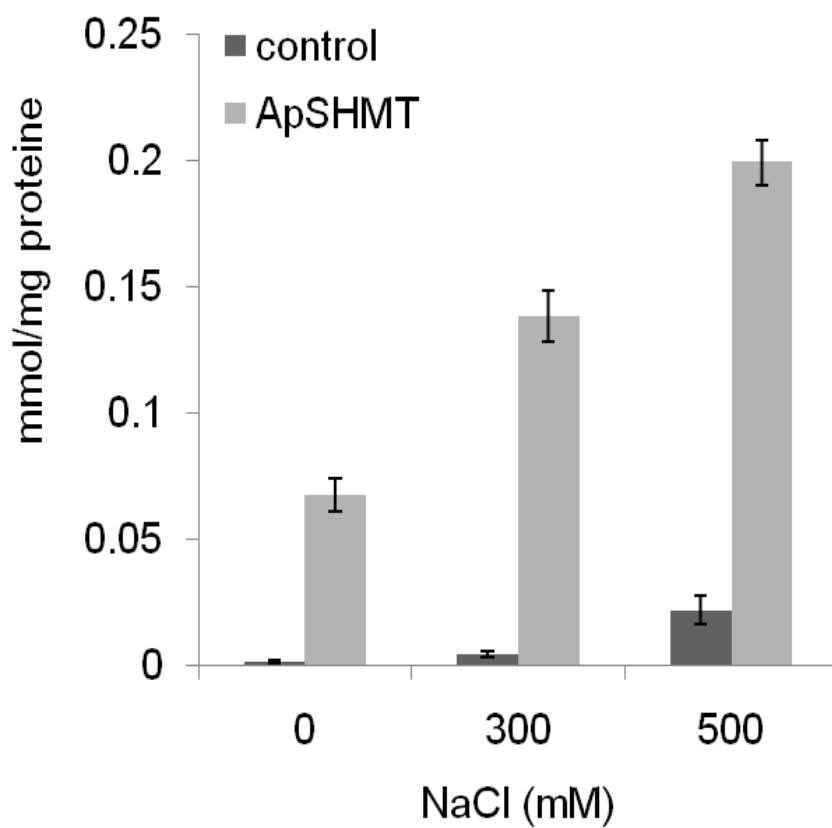
Figure 4.13 Betaine content in control and expressing cells. Cells were grown in M9 minimal medium supplemented with 0-500 mM NaCl for overnight. Cells were harvested and used for betaine analysis via TOF-MS.

4.9 Salinity stress of expressing cells

Metabolic analysis clearly indicated that betaine level increased in ApSHMT expressing cell (Figure 4.13). We further investigated stress tolerance of *E.coli* expressing cells. Figure 4.14 showed growth rate of *E.coli* DH5α at difference concentration of NaCl. In this case, the growth rate in all conditions tested were the same. The results were similar when ApSHMT was expressed in *E.coli* BL21(DE3). Interestingly, when ApSHMT was expressed in *E.coli* BL21, the growth profile of control and expressing cells at high salinity were significantly different (Figure 4.16). At higher concentration (i.e., 500 mM NaCl), ApSHMT expressing cell grew faster and much better than control cells for about 2 folds.
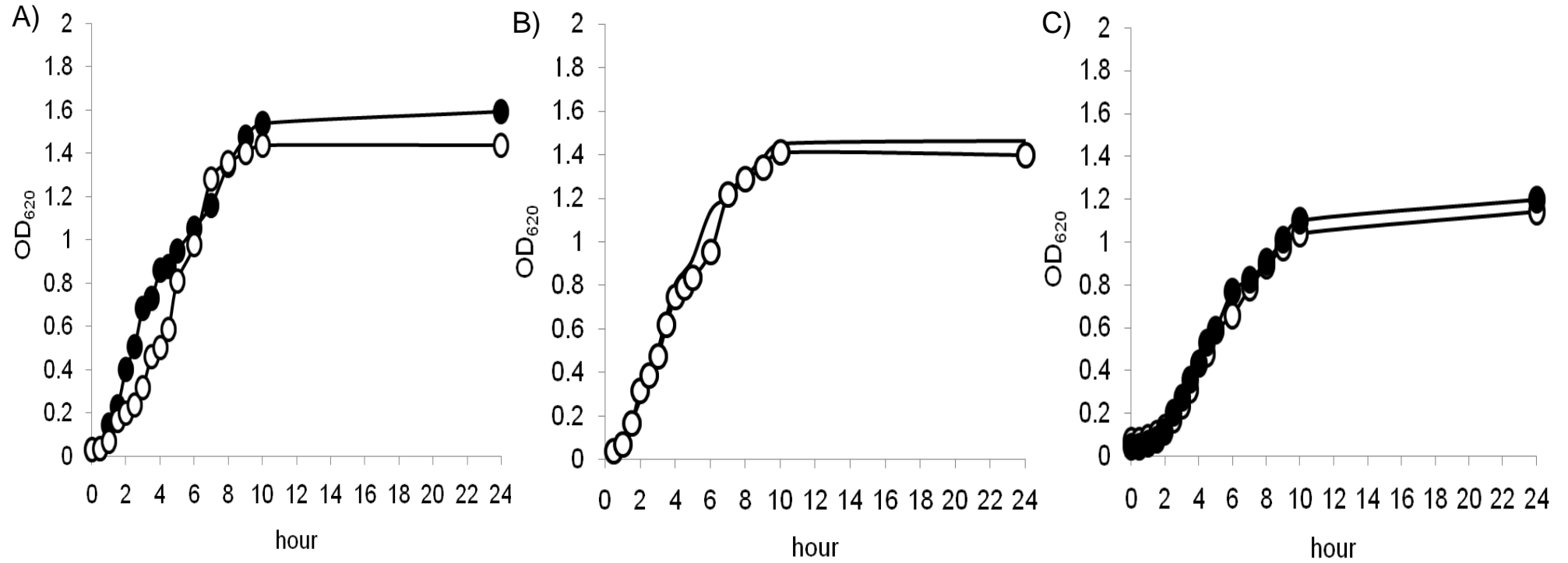
Figure 4.14 Growth profile of control and expressing cells (*E.coli* DH5α) in LB medium supplemented with 0-500 mM NaCl. A) 0 mM NaCl, B) 300 mM NaCl and C) 500 mM NaCl. (Control cells, open circle and expressing cells, closed circle).
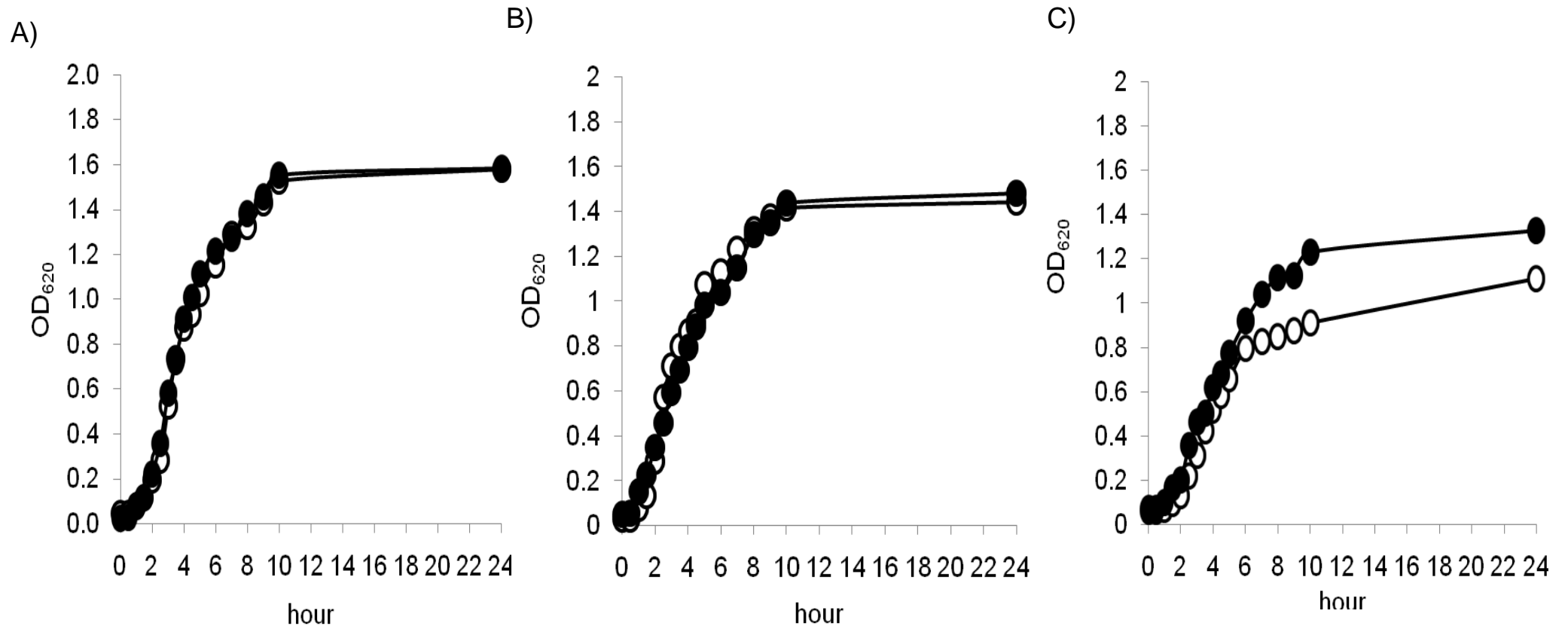
Figure 4.15 Growth profile of control and expressing cells (*E.coli* BL21(DE3)) in LB medium supplemented with 0-500 mM NaCl. A) 0 mM NaCl,

B) 300 mM NaCl and C) 500 mM NaCl. (Control cells, open circle and expressing cells, closed circle).
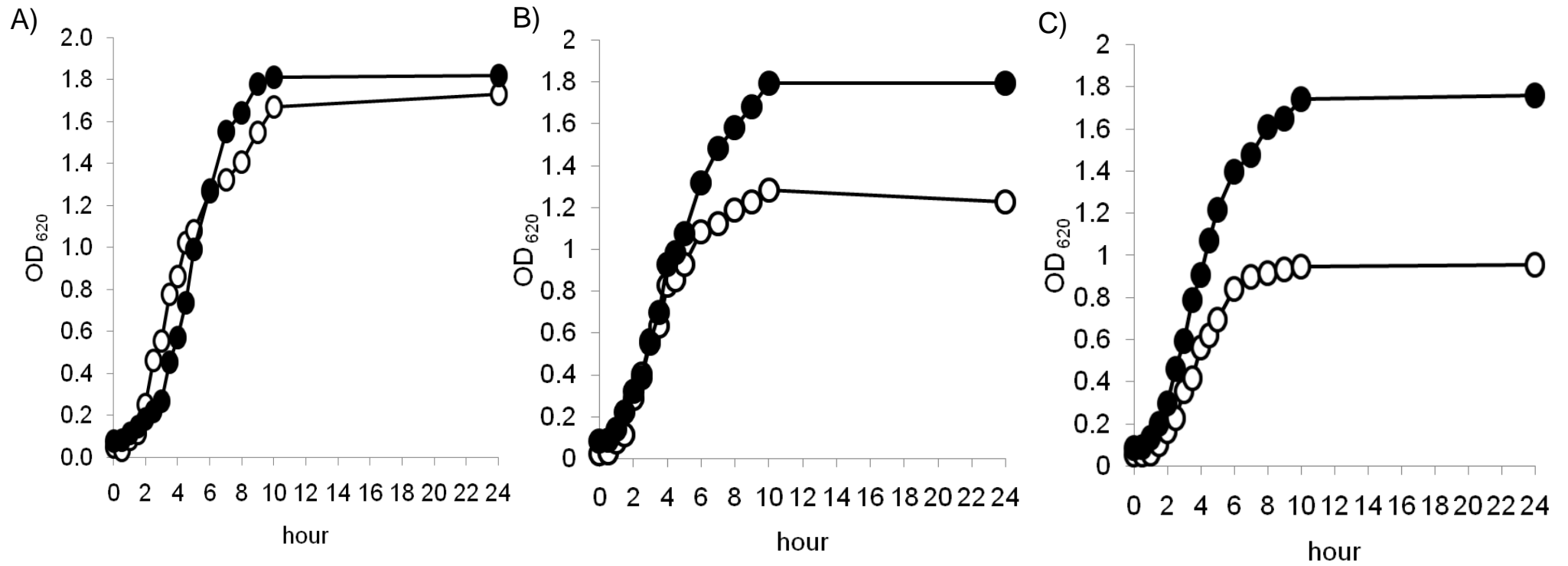
Figure 4.16 Growth profile of control and expressing cells (*E.coli* BL21) in LB medium supplemented with 0-500 mM NaCl. A) 0 mM NaCl, B) 300 mM NaCl and C) 500 mNaCl. (Control cells, open circle and expressing cells, closed circle).

4.10 Oxidative stress of expressing cells

Control and expressing ApSHMT cells (strain BL21) were grown in LB medium in the presence difference concentrations of rose bengal (0.02% and 0.2%). The couple reaction of SHMT produces an antioxidant, namely THF. Therefore we tested whether expressing cells could enhance oxidative stress response. The resulted shown in Figure 4.17 indicated that the growth profile at log phase within 6 hours of expressing cells BL21 were faster than those of control cells. This result indicated expressing cells conferred oxidative-stress tolerance from rose bengal.
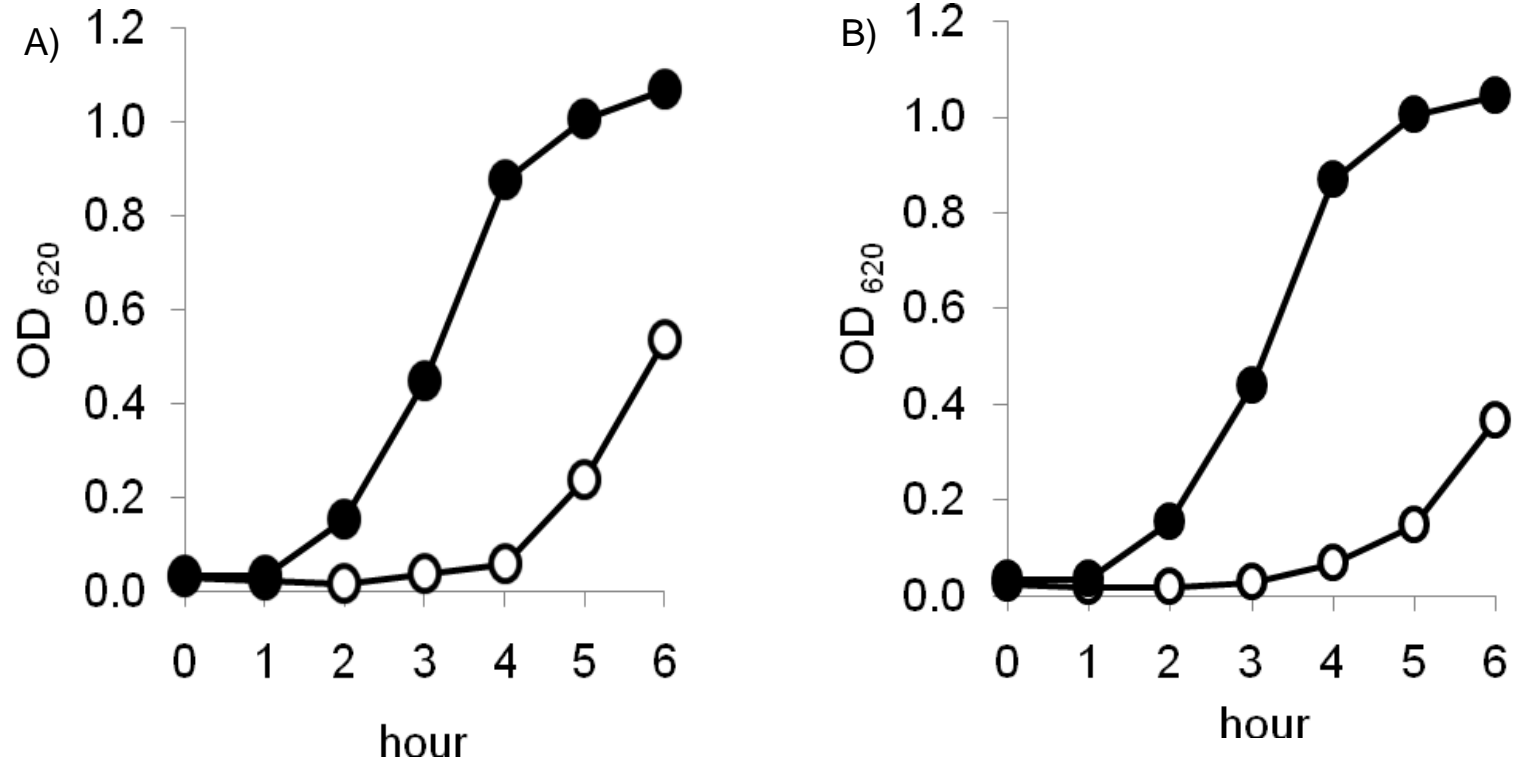
Figure 4.17 Growth profile of control and expressing cells (*E.coli* BL21) in LB medium supplemented with rose bengal. A) 0.02% rose bengal,

B) 0.2% rose Bengal, (Control cells, open circle and expressing cells, closed circle).

4.11 pH stress of expressing cells

Control and expressing cells (strain BL21) were grown in LB medium at different pH values (pH 4-10). As shown in Figure 4.18 A-D, at pH 9.0 expressing cells grew much better and faster than control cells and both expressing cells and control cells cannot grow at pH 10 and from this result, it is likely expressing cells confer alkaline-stress tolerance.
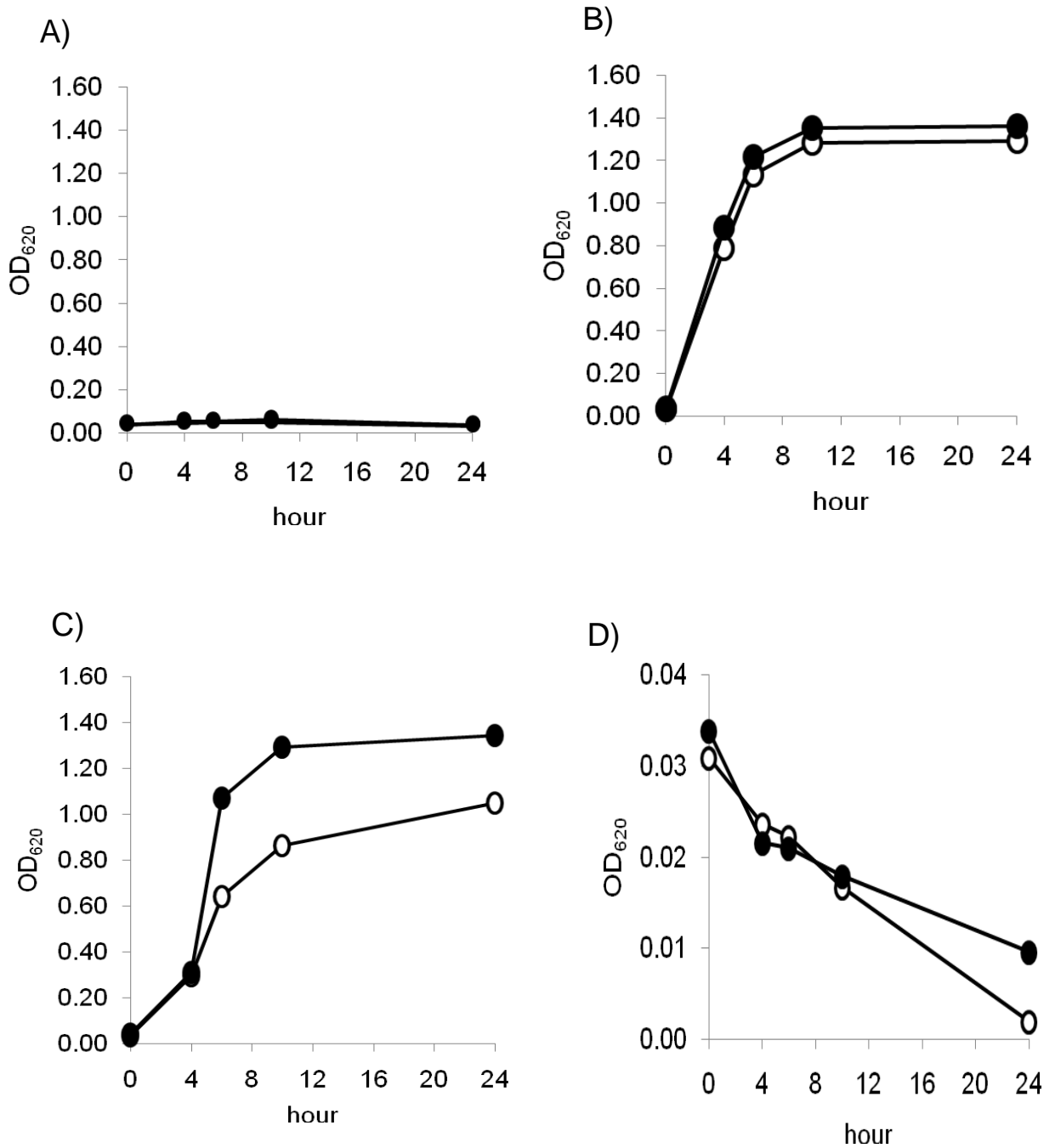
Figure 4.18 Growth profile of control and expressing cells (strain BL21) in LB medium at various pHs. A) pH 4, B) pH 7, C) pH 9 and D) pH 10, (Control cells, open circle and expressing cells, closed circle).

4.12 RT-PCR

The expression of *ApSHMT* was monitored by RT-PCR using the total RNA extracted from NaCl treated up- and down-shocked cells. As a constitutive control, the *RNase P* gene, *AprnpB*, was used. The NaCl up-shock condition caused a rapid induction in the *ApSHMT* transcript expression (Figure 4.19). Induced expression of *ApSHMT* was detectable within 1 h in the up shocked cells and that increased in abundance within 12 h. There was no obvious change in *ApSHMT* transcripts under NaCl down-shock conditions (data not shown).
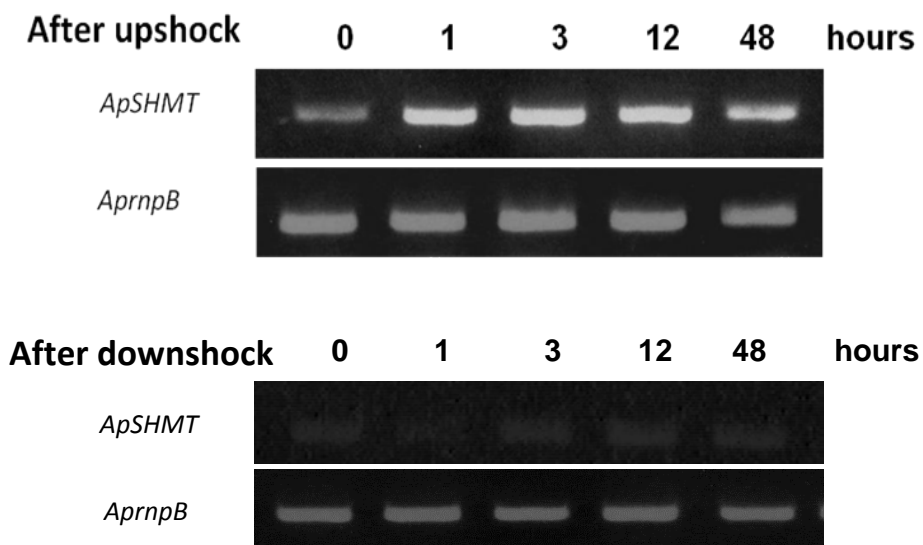
Figure 4.19 Semiquantitative RT-PCR with *ApSHMT* specific primers. *AprnpB* primer was used as a positive control.

CHAPTER V

DISCUSSION

SHMT is one of important enzymes in the pathway for the interconversion of C-1 which catalyzes reversible reaction of serine and THF to glycine and *N5 N10*-methylene-THF. The resultant compound from SHMT catalytic reaction, namely 5, 10-methylene THF, is the precursor of purine biosynthesis in all living organisms (Agrawal et al., 2003). Besides a crucial function in purine biosynthesis, we thought that SHMT might also play a pivotal role in glycine-serine cycles because precursor deficiency of choline was observed for the accumulation betaine (Nomura et al., 1998) and serine is a precursor for choline (Rontein et al., 2002).

Phylogenetic analysis revealed this enzyme is highly conserved during evolution. In higher plants, multiple *SHMT* genes which are predicted to different compartments were found. (The Arabidopsis Genome Initiative, 2000). But physiological roles of SHMT in different organisms are not well known except for photorespiratory role in mitochondria (Jamai et al., 2009; Voll et al., 2005; Summerville and Ogren, 1981). In cyanobacteria, only single gene encoding SHMT was found. This suggests cyanobacteria SHMT must play multiple roles. In deed, the complete segregation of mutant for SHMT could not be isolated (Eisenhut et al., 2006).

In this study, we have successfully produced recombinant ApSHMT using pCold I as expression vector. In our tested conditions, following pColdI's protocol when IPTG was added at mid log phase ApSHMT produced very small amount. But, we have successfully induced a large amount using the cells grown at stationary phase. We investigated molecular properties of cyanobacterium SHMT which has never been

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examined. Results presented here clearly indicated the ApSHMT is up-regulated by NaCl. *In vitro* analysis revealed that cyanobacterium SHMT catalyzes the interconversion of glycine-serine cycles. We also demonstrated here that heterologous expression of ApSHMT caused elevated levels of choline and glycine betaine in *E.coli*.

Photorespiration is a high-flux pathway and has a major impact on cellular metabolism, particularly under high light, CO_2 or water deficits. In cyanobacteria, catalytic reaction of SHMT, which provides amino acids (glycine and serine), is important for primary metabolism and providing C-1 unit for many biosynthetic reactions. Besides these physiological functions, SHMT is an integral part of photorespiratory pathway. Analysis of photorespiratory metabolism in *Synechocystis* sp. PCC6803 gives evidence that cyanobacteria possess a complete photorepiratory metabolism via glycerate-glyoxylate cycles (Eisenhut et al., 2006). In addition to photorespiratory pathway, SHMT in *A. halophytica* must play a unique role because its cells accumulate a large amount of betaine under salt-stress conditions. It should be mentioned that *A. halophytica* synthesizes glycine betaine from glycine via three step methylation (Waditee et al., 2003) whereas other organisms mostly synthesize glycine betaine via choline oxidation. The present data clearly indicate that, expression of *ApSHMT* is up-regulated by NaCl and increased the accumulation of glycine betaine. These results suggest that *A. halophytica* has a conventional metabolic pathway that provides amino acids (glycine and serine) including Adomet for glycine betaine synthesis.

Biochemical analysis of the recombinant ApSHMT showed catalytic activity towards DL-threo-3-phenylserine and L-serine. The apparent Km value of ApSHMT for DL-threo-3-phenylserine was 0.2 mM with Vmax 3,500 nmol/min/mg. This Km value is significantly lower when compared with SHMT from other organisms,

PvSHMT (8.6 mM) and Sheep (84 mM) (Sopitthummakhun et al., 2009 ; Ulevich and Kallen, 1977). The apparent Km values of ApSHMT for L-serine and THF were 0.1 mM (Vmax 800 umol/min/mg) and 0.25 mM (Vmax 750 umol/min/mg), respectively which were similar, 0.1-1 mM range (for L-serine) and 0.02-0.8 mM range (for THF), to those of other organisms (*Plasmodium vivax*, *Escherichia coli*, *Bacillus stearothermophilus*, Sheep, Rabbit and human) (Di Salvo et al., 1998; Jala et al., 2002; Sopitthummakhun et al., 2009, Schirch et al.; 1985; Ulevitch and Kallen, 1977). Higher affinity of ApSHMT to DL-threo-3-phenylserine would suggest some physiological function of ApSHMT which remains to be clarified.

In vitro protection of the ApSHMT activity by betaine under high salinity conditions is worthwhile to be mentioned. Figure 4.10 suggested that *in vivo*, glycine betaine protects ApSHMT under high salinity conditions because *A. halophytica* can grow even in the presence of 2,500 mM NaCl. Expression of ApSHMT in *E.coli* resulted in changes in quantitative increasing amino acids (glycine, serine). This is interesting because the amino acid L-serine is required for pharmaceutical purposes, and the availability of a sugar-based microbial process for its production is desirable (Stolz et al., 2007). The total annual demand for L-serine is estimated to be 300 tons. The production processes currently used still rely on the extraction of L-serine. The present data suggest the possibility to use ApSHMT for the production of serine and also for metabolic engineering to increase betaine level in target microorganisms.

CHAPTER VI

CONCLUSIONS

I) Amino acid sequence showed ApSHMT has high homology with cyanobacterial SHMTs, but far from those of bacteria and higher plants.

II) ApSHMT could be produced in high amount as soluble form in expression vector, pColdI and the highest expression level was obtained in *E.coli* BL21.

III) Biochemical characterization suggests that ApSHMT has catalytic activity for both THF‑dependent and THF‑independent reactions.

IV) Expression of ApSHMT in *E.coli* leads to increase levels of glycine, serine, choline and betaine.

V) *ApSHMT* is a salt‑inducible gene.

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APPENDICES

APPENDIX A


```
                                    lacZα ATG
        M13 Reverse Primer                                    Hind III              Kpn I        Sac I  BamH I      Spe I
   CAG GAA ACA GCT ATG ACC  ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA
   GTC CTT TGT CGA TAC TGG  TAC TAA TGC GGT TCG AAC CAT GGC TCG AGC CTA GGT GAT

                               BstX I  EcoR I                                        EcoR I
   GTA ACG GCC GCC AGT GTG CTG GAA TTC GCC CTT [PCR Product] AAG GGC GAA TTC TGC
   CAT TGC CGG CGG TCA CAC GAC CTT AAG CGG GAA              TTC CCG CTT AAG ACG

       EcoR V              BstX I      Not I      Xho I              Nsi I  Xba I        Apa I
   AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
   TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
                                                                                          ↑

       T7 Promoter                        M13 Forward (-20) Primer
   AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC
   TCA CTC AGC ATA ATG TTA AGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC CTT TTG
```

pCR®2.1-TOPO®
3.9 kb

+1 Plac lacZα f1 ori Kanamycin Ampicillin pUC ori

(Invitrogen, USA)

APPENDIX B

cspA 3' UTR
multiple cloning site (MCS)
Factor Xa site
His Tag
TEE
cspA 5' UTR
lac operator
cspA promoter

M13 IG

Amp

pCold I

4407 bp

lacI

ColE1 ori

(Takara, Japan)

APPENDIX C

Modified BG11 medium

Trace element solution

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

Dissolved all compositions with distilled water to 1 liter.

BG11 solution

$NaNO_3$	1.5	g
K_2PO_4	40	mg
$MgSO_4.7H_2O$	75	mg
$CaCl_2.H_2O$	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
$MgSO_4.7H_2O$	6.92	g
$MgCl_2.6H_2O$	5.50	g
$CaCl_2.2H_2O$	1.47	g

Modified BG11 medium was prepared by dissolved all compositions with distilled water to 1 liter, adjust pH to 8.2.

APPENDIX D

Chlorophyll analysis

Determination of chlorophyll (Giusy, 2006)

Pigments were extracted from cell suspensions and total cells with 90% (v/v) acetone and incubated in dim light for overnight. After centrifugation at 10.000 x g for 10 min at 4°C, chlorophyll *a* contents were calculated from the absorbance of the acetone extract at 652 and 665 nm using the equation:

chlorophyll (μg/ml) = 16.82 x A_{665} – 9.28 x A_{652}.

APPENDIX E

Transformation

1. Preparation of competent cells (CaCl$_2$ method), (adapted from Xiaowei, 2010)

A single colony of E.coli was incubated to 2 mm of LB medium and incubation at 37 C overnight with vigorous shaking. This culture was reinoculated to fresh LB medium and incubated 37 $^{\circ}$C with vigorous shaking for 3-4 hours until the OD$_{620}$ reach 0.4-0.6. The culture was stand on ice for 10 min and centrifuged at 4000 rpm for 10 min at 4 $^{\circ}$C. Cell pellet was resuspend in 0.05 of 15% of glycerol in CaCl$_2$ and stand on ice 10 min. This cell suspension was dispensed in 100 μl aliquots into 1.5 ml microcentrifuge tubes and stored at -80 $^{\circ}$C.

2. Transformation

One hundred microlitres of competent cells was thawed on ice prior to the addition of 1-3 μl of plasmid DNA or ligation mixture. The transformation mixture was flicked 2-3 times and stand on ice 10 min. Subsequently, the mixture was heated to 42 $^{\circ}$C for 90 second futher on ice 5 min. The mixture was diluted with 900 of LB medium and gently shaking at 37 $^{\circ}$C for hour. Cell suspension was spread on selection medium as desired.

APPENDIX F

Agarose gel electrophoresis

1. Agarose gel electrophoresis for DNA

To measure the size and the amount of DNA in the sample, 0.8-1.5% agarose gel (consist of 0.08 µl/ml Gel green) in 1X TBE buffer (89 mM Tris Cl, 89 mM boric acid and 25 mM EDTA pH 8.3). The DNA sample was mixed with 1/5 volume of loading dye (0.25% bromphenol blue, 0.25% cyanolFF and 30% glycerol in water) before loading into the well of gel which submerged in the 1X TBE buffer in an electrophoretic chamber. An appropriate amount of DNA ladder was also load to the gel to serve as a DNA marker. Generally, the gel was run at 50-100 volts until bromphenol blue migrated to the other edge. The DNA band was visualized under UV light and photograph. The concentration and molecular weight of DNA sample were estimated by comparing with the intensity and relative mobility.

APPENDIX G

Preparation for polyacrylamide gel electrophoresis (Laemmli, 1970)

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 8.8 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 8.8 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 6.8	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% Amonium sulfate	50	μl
TEMED	10	μl

5% stacking gel

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

Sample buffer

1M 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 litre

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

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

APPENDIX H

Standard protein (Bradford, 1986)

$y = 0.103x$

$R^2 = 0.995$
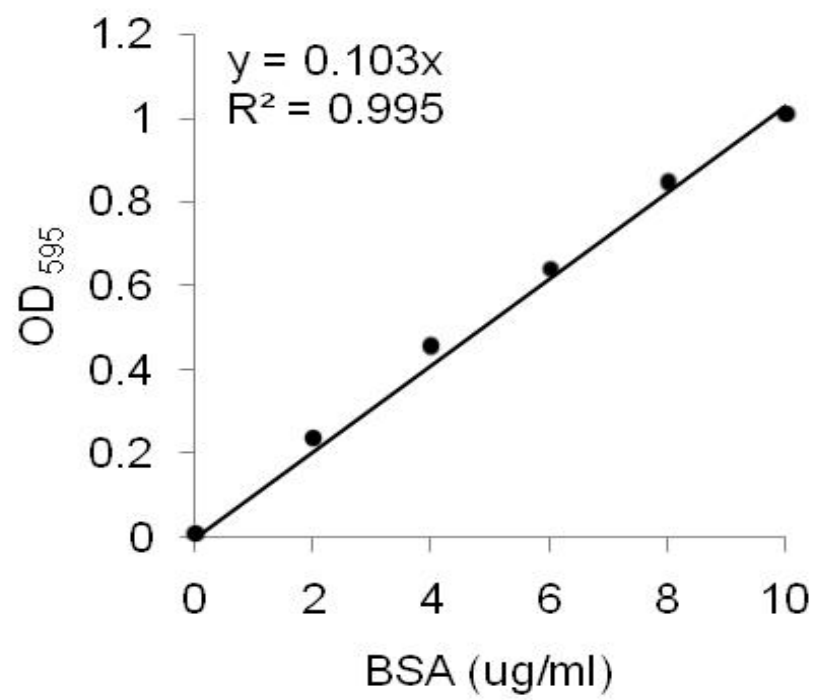
APPENDIX I

Purification solution

Purification buffer for purification under native protein conditions (GE healthcare manufacture)

Lysis buffer

NaH_2PO_4	6.90	g
NaCl	17.54	g
Imidazole	0.68	g

Adjust pH to 8.0 using NaOH and adjust volume to 1 liter.

Wash buffer

NaH_2PO_4	6.90	g
NaCl	17.54	g
Imidazole	1.36	g

Adjust pH to 8.0 using NaOH and adjust volume to 1 liter.

Elution buffer

NaH_2PO_4	6.90	g
NaCl	17.54	g
Imidazole	17.00	g

Adjust pH to 8.0 using NaOH and adjust volume to 1 liter.

Stripping buffer

Sodium phosphate	20	mM
NaCl	500	mM
EDTA	50	mM

Adjust pH to 8.0 using NaOH and adjust volume to 1 liter.

APPENDIX J

Buffer for western blotting

PBS buffer (Phosphate-buffer-saline)

Final concentration per 1 lite

10	mM	sodium phosphate pH 7.4
150	mM	NaCl

Blocking buffer

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

Blotting transfer buffer

Final concentration per 1 liter.

39	mM	glycine
48	mM	Tris base
0.037	%	SDS
20	%	methanol

Detection reagent for western blotting

150 mM Barbital pH 9.6	18	ml
0.1% NTB (Nitro Blue Tetrazolium)	2	ml
1M $MgCl_2$	80	µl
0.5% BCIP (5-bromo-4-chloro-3-indolyl phosphate)	200	µl

Detection reagent for western bloting should be freshly prepared and used within 30 min. When the bands are desired intensity, wash the nitrocellulose membrane with deionized water 2-3 times.

APPENDIX K

Amino acid analysis

Solution for analysis (Lithium column)

4N LiOH

LiOH.H$_2$O	42.0	g

Dissolved with distilled water to 250 ml and store in plastic bottom.

MA solution

Trilithium citrate tetrahydrate	14.1	g
Ethyleneglycol monoethylether	70	ml
60 % HClO$_4$	13	ml
H$_2$O	800	ml

Adjust pH 2.6 using 60 % HClO$_4$ then fill up water to 1000 ml and then filtration MA solution using whatman no.1.

MB solution

Trilitium citrate tetrahydrate	28.2	g
Borate	12.4	g
4 N LiOH	30	ml
H$_2$O	800	ml

Adjust pH 10.0 using 4 N LiOH then fill up water to 1000 ml and filtration MB solution using whatman no.1.

MC solution

LiOH.H$_2$O	4.2	g
H$_2$O	450	ml

Fill up water to 500 ml and filtration MC solution using whatman no.1.

Remark : MA, MB and MC solution store at 4 °C at least 3 weeks.

Buffer for preprartion RA and RB solution.

Na_2CO_3	40.7	g
K_2SO_4	18.8	g
H_3BO_3	13.6	g
H_2O	830	ml

Dissolve complete step by step and fill up water to 1000 ml.

Brij -35 10%

Brij-35	10	g
H_2O	50	ml

Fill up water to 100 ml**.**

RA solution

Mixed buffer with 400 µl NaClO and then filtration RA solution using whatman paper no.1.

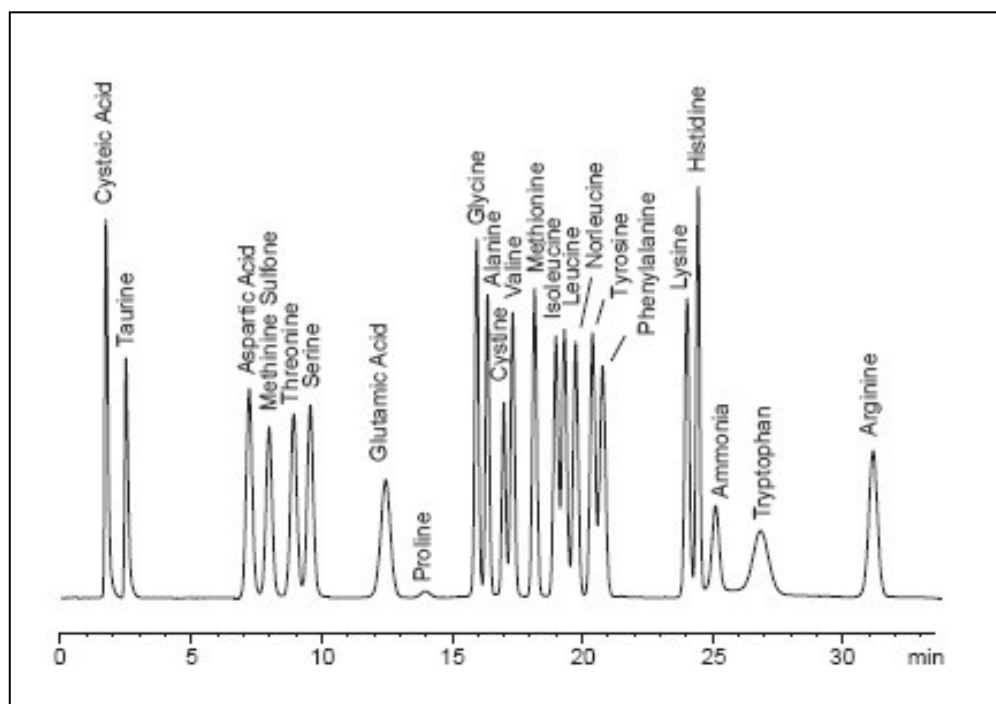**RB solution**

O-pathaldehyde	400	mg
Ethanol	7	ml
2-mercaptoethanol	1	ml
Brij-35 10%	2	ml
Buffer	490	ml

Rinse 10-25 ml ethanol in flask before use.Put O-pathaldehyde 400 mg resolved with ethanol then Pore above solution into 450 buffer.Add 2-mercaptoethanol

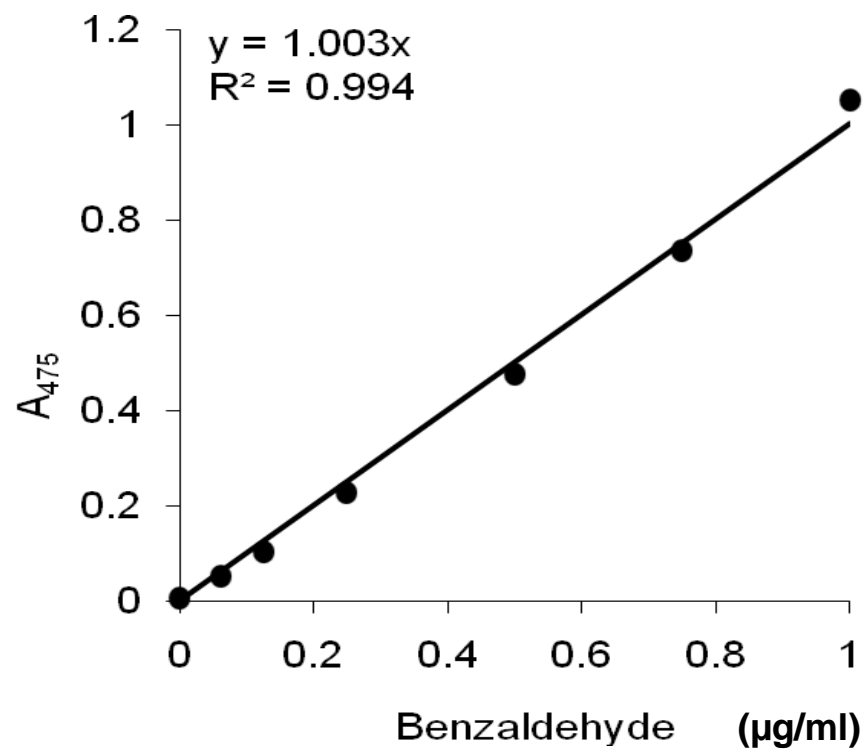1 ml and Brij-35 10% 2 ml and fill up buffer to 500 ml.Filtration RB solution using whatman no.1.

Standard of amino acid profile

APPENDIX L

Standard benzaldehyde (Misano, 2005)

$y = 1.003x$
$R^2 = 0.994$

APPENDIX M

M9 minimal medium

5X M9 solution

$Na_2HPO_4.7H_2O$	64	g
KH_2PO_4	15	g
NaCl	2.5	g
NH_4Cl	5	g

5X M9 salts is made by dissolving the following salts in deionized water to a final volume of 1 liter. Devide the 5X M9 salts 200 ml and sterile by autoclaving for 121 °C 15 min.

M9 minimal medium

5x M9 solution	200	ml
1 M $MgSO_4$	2	ml
20 % glucose	20	ml
1 M $CaCl_2$	0.1	ml
Sterilize deionized water	980	ml

Prepare the $MgSO_4$ and $CaCl_2$ solutions separately, sterilize by autoclaving, and add the solution diluting the 5X M9 salts to 980 ml with deionized water. Sterilize the glucose by passing it through a 0.22 μm filter before it is added to the diluted M9 salts. When *E.coli* DH5α cells were used, the M9 minimal medium added supplement 0.01% thiamine.

APPENDIX N

Luria-bertani Medium

LB medium (Luria-bertani Medium) Per liter

To 950 ml of deionized H_2O , add

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for $121^{\circ}C$ for 15 min.

APPENDIX O

Time of flight mass spectrophotometer analysis

Matrix solution

3,5 Dimethoxy-4-hydroxy Cinnamic acid	10	mg
Acetonitile	400	μl
TFA	0.6	μl

Dissolved with Milli Q water to 1 ml.

For TOF-MS analysis, mixed 1 μl sample with 1 μl internal standard and 1 μl metrix then blow dry mixer solution and detected amino acid and metabolites by TOF-MS. Internal standard for detection choline and glycinebetaine are d9-choline and d11-betaine, respectively.

BIOGRAPHY

Miss Duangjai Sittipol was born on March 9, 1987 in Prachinburi, Thailand. She graduated with a Bachelor of Science degree in Industrial Microbiology from King Mongkut Institute of Ladkrabang University in 2008. She has further studied for the Master of Science degree in Microbiology Department, Chulalongkorn University since 2009.