RESEARCH REPORT

A STUDY ON UTILIZATION OF CYANOBACTERIA TO PRODUCE VALUABLE SUBSTANCES : METABOLISM OF AMINO ACID AND POLYAMINE

การศึกษาเกี่ยวกับการใช้ประโยชน์จากไซยาโนแบคทีเรียในการผลิตสารอาหารที่มีคุณค่า : เมทาบอลิสมของกรดอะมิโนและพอลิเอมีน

BY

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บทคัดย่อ

โครงการวิจัยนี้เป็นการศึกษาความเปลี่ยนแปลงต่างๆทางสรีระวิทยาและทางชีวเคมีรวมถึงการนำเข้าของสารที่มีคุณค่า เช่น กรดอะมิโน และพอลิเอมีน ในไซยาโนแบคทีเรีย 2 ชนิด คือ อะฟาโนทีคี ฮาโลฟิทิคา และซิเนโคซิสทิส สายพันธุ์พีซีซี6803 รวมทั้งการศึกษาการตอบสนองต่อความเครียดจากสิ่งแวดล้อม พบว่า กรดอะมิโนกลูตาเมท พบมากในอะฟาโนทีคี ฮาโลฟิทิคา และมีปริมาณเพิ่มขึ้น เมื่อเลี้ยงเซลล์ในสภาวะที่มีความเครียดจากเกลือ นอกจากนั้น ยังพบว่ามีการเพิ่มขึ้นอย่างมีนัยสำคัญของไกลซีนบีเทนภายใต้สภาวะความเครียดจากเกลือเช่นเดียวกัน การนำเข้ากลูตาเมทที่มีอยู่ในอาหารเลี้ยงช่วยทำให้ปริมาณไกลซีนบีเทนในเซลล์เพิ่มขึ้น การศึกษาในซิเนโคซิสทิส พบว่าความเครียดจากเกลือหรือจากซอร์บิทอลทำให้เกิดการเพิ่มขึ้นของพอลิเอมีนภายในเซลล์ เมื่อศึกษาถึงผลของความเครียดจากแสงยูวี พบว่า ปริมาณพอลิเอมีนโดยรวมลดลงภายใต้แสงยูวีบีและยูวีซี เมื่อทำให้เซลล์ได้รับความเครียดจากออสโมติกและยูวีต่อเนื่องกัน ปรากฏว่า ปริมาณพอลิเอมีนยังคงเพิ่มขึ้น

การศึกษาการนำเข้ากลูตาเมทในอะฟาโนทีคี ฮาโลฟิทิคา พบว่าการนำเข้าเกิดขึ้นได้ดีกว่าในสภาวะที่มีโซเดียมคลอไรด์ 2.0 โมลาร์ เมื่อเทียบกับสภาวะ 0.5 โมลาร์ การนำเข้าต้องการใช้พลังงานและมีความจำเพาะต่อกลูตาเมท และ แอสพาร์เตท โดยที่มีโซเดียมช่วยให้เกิดการนำเข้าเพิ่มขึ้น

การศึกษาการนำเข้าพอลิเอมีนในซิเนโคซิสทิส ทำโดยใช้รีคอมบิแนนท์ไบน์ดิงโปรตีน(อาร์พอทดี) เป็นตัวอย่าง พบว่าอาร์พอทดีสามารถแสดงคุณสมบัติการจับกับพิวเทรสซีน สเปอร์มิดีน และสเปอร์มีนได้คล้ายคลึงกัน โดยที่มีการชอบในการจับกับพิวเทรสซีนน้อยที่สุด นอกจากนี้ยังพบอีกว่าการเติมพิวเทรสซีนหรือสเปอร์มิดีนลงในอาหารเลี้ยง ทำให้เซลล์มีการสะสมปริมาณพอทดีเพิ่มมากขึ้น ซึ่งบ่งชี้ว่าพอทดีมีบทบาทสำคัญเกี่ยวกับการขนส่งพอลิเอมีน

Abstract

The present project was undertaken to study physiological and biochemical changes including the uptake of valuable amino acids and polyamines as well as the responses to environmental stresses in two cyanobacteria, *Aphanothece halophytica* and *Synechocystis* sp. PCC 6803. Glutamate was the main amino acid inside A. *halophytica* and its content was increased by growing the cells under salt stress condition. Glycine betaine was also found to increase significantly by salt stress. The uptake of glutamate from the growth medium could also increase intracellular glycine betaine level. In *Synechocystis*, intracellular polyamines were also increased when growing the cells under salt stress or sorbitol stress condition. The short term stress by UV-B and UV-C radiation decreased total polyamine contents with spermidine representing the main type of polyamine inside the cells. When both osmotic and UV stresses were imposed in succession on *Synechocystis*, an increased level of polyamines still prevailed.

Studies on glutamate uptake in A. *halophytica* revealed that the uptake under high salt at 2.0 M NaCl was higher than at 0.5 M NaCl. The uptake was shown to be energy-dependent and highly specific for glutamate and aspartate. Sodium ion, but not other cations or anions, exerted strong stimulatory effect on glutamate uptake.

For the studies on the uptake of polyamines by *Synechocystis*, the recombinant polyamine binding protein (rPotD) was used to characterize the binding properties. Similar binding characteristics for putrescine, spermidine, and spermine were obtained with putrescine having the least affinity. *Synechocystis* cells grown in medium supplemented with putrescine or spermidine contained an elevated level of PotD protein implicating the role of PotD in mediating the transport of polyamines.

CONTENTS

PAGE

PAGE

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

PAGE

PAGE

LIST OF ABBREVIATIONS

^{o}C	degree Celsius
Ci	Curie
h	hour
kDa	kiloDalton
µg	microgram
µl	microliter
µM	micromolar
M	mole per liter (molar)
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometer
nmol	nanomol
OD	optical density
PAs	Polyamines
PotD	periplasmic polyamine-binding protein D
rpm	revolution per minute
sec	second
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume

CHAPTER I

INTRODUCTION

Increasing non-degradable chemical contaminants in environment and global warming are major problems for Thailand and the world. One possible way for combating these problems is using cyanobacteria capable of photosynthesis to convert carbon dioxide and water to bio-chemicals or bio-degradable products. Such a process also reduces atmospheric carbon dioxide and thus alleviating global warming effect. Cyanobacteria have economical advantages over other biochemical-producing organisms such as plants and fermentative bacteria because cyanobacteria are easy to grow using inexpensive minimum media and do not require fertile lands, starch and sugars for culturing. Biotechnology using cyanobacteria as a source of starting materials to produce various valuable substances and chemicals has received a lot of attention in the last decade. At least about 2,000 species of cyanobacteria are known and they exist in almost all habitats ranging from deep oceans to high mountains and even in diverse extreme conditions like hot spring or freezing temperature. The vast amount of knowledge obtained from research on cyanobacteria in many aspects has shown that cyanobacteria can be a rich source of several bioactive and valuable compounds.

In order to explore the possibility of using cyanobacteria to produce desired valuable compounds, we started this project by utilizing the potential of 2 cyanobacteria, namely *Aphanothece halophytica* and *Synechocystis* sp. PCC6803. Both are unicellular cyanobacterium. *A. halophytica* is an alkaliphilic halotolerant cyanobacterium. This cyanobacterium has a short cyclindrical shape covered with mucous membrane. Furthermore, it has the ability to grow under a wide range of salinity concentrations from 0.25-3.0 M NaCl

as well as in alkaline pH up to pH 11.0. *Synechocystis* sp. PCC6803 is a coccoid, or spherical cyanobacterium lacking gas vesicles or a sheath. This cyanobacterium is a non-nitrogen (N_2)-fixing cyanobacterium and a ubiquitous inhabitant of fresh water. Moreover, it is naturally transformable by exogenous DNA and grows heterotrophically at the expense of glucose. It was also the first phototrophic organism to be fully sequenced. The Morphology of both cyanobacteria is shown in Figure 1.1.

(A) (B)

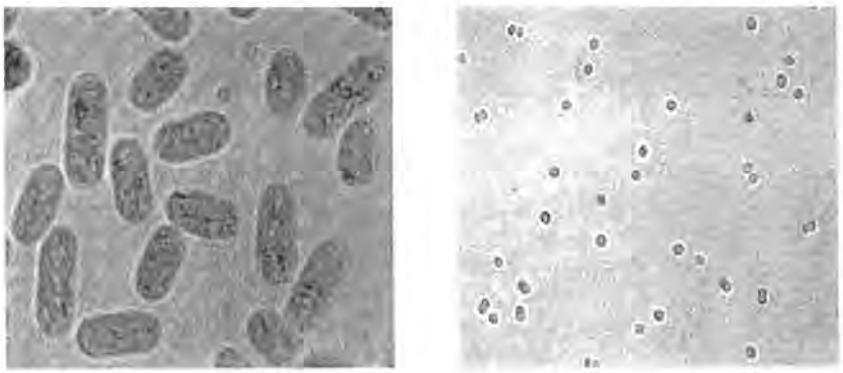


Figure 1.1 Morphology of *Aphanothece halophytica* (A) and *Synechocystis* sp. PCC 6803 (B).

The valuable compounds that we investigated were amino acids focusing on glutamate. The other compound was polyamine. In addition, another compound called phycocyanin was also briefly studied in terms of its contents in various species of cyanobacteria. Lastly, it should be pointed out that the investigation of this project emphasized the physiological and biochemical aspects which form the basis of possible applications in the long term.

CHAPTER II

METHODS

2.1 Organisms and growth conditions

2.1.1 Cyanobacterial strains

Two cyanobacterial strains and medium used in this work are listed in Table 2.1.

Table 2.1 Cyanobacterial strain and culture medium

Cyanobacterial strains	Culture medium
Aphanothece halophytica	BG11 + Turk Island Salt solution + 0.5 M NaCl
Synechocystis sp. PCC 6803	BG11

2.1.2 Growth conditions

2.1.2.1 Normal condition

Cyanobacterial cultures were grown in the medium as shown in Table 2.1. Cells were grown photoautotrophically at 30°C in medium under continuous illumination provided by warm white fluorescent tubes (3×30 W). The incident light intensity in the growth chamber was 50 μmol photons·m^{-2}·s^{-1}. Growth of liquid cultures was monitored by measuring the optical density at 730 nm (OD_{730}) with a Spectronic® Genesys^{TM}2 spectrophotometer.

2.1.2.2 Growth condition for glutamate uptake study

To study the effect of salt stressed on growth of *A. halophytica* cells, 1% (v/v) logarithmic cell culture (10 days) was transferred to new BG11 + Turk Island Salt solution containing 0.5 M NaCl (normal condition) and 2.0 M NaCl (stressed condition). The effect of glutamate concentrations (0 – 50 mM) on growth of *A. halophytica* cells under normal and salt stressed conditions were studied as mentioned above.

2.1.2.3 Growth condition for the study of effect of osmotic stress and UV on polyamine

Synechocystis sp. PCC 6803 were grown in BG_{11} medium (Rippka et al., 1979) with continuous filtered air-bubbling, under normal growth condition (2.1.2.1). Sodium chloride and sorbitol were adjusted to desired final concentrations. Cells at mid-logarithmic stage were harvested and resuspended in BG_{11} medium containing respective NaCl or sorbitol concentration. Cell suspension contained in flat bottom chamber was exposed to moderate UV radiations; UVA (365 nm; 11-13 $W.m^{-2}$ or 39.6 - 46.8 $kJ.m^{-2}.h^{-1}$), UVB (302 nm; 1.2-1.3 $W.m^{-2}$or 4.3 – 4.7 $kJ.m^{-2}.h^{-1}$) and UVC (254 nm; 1.0-1.3 $W.m^{-2}$ or 3.6 – 4.7 $kJ.m^{-2}.h^{-1}$), respectively. The UV irradiances were measured using UVX radiometer (UVP, Inc., Upland, CA).

2.2 Glutamate uptake

Transport assays were performed according to Waditee et al., (2002), *A. halophytica* cells were grown for 10 days in light at 30°C, in BG_{11} medium (pH 7.6). After 10 days of growth, cells were harvested, washed twice, and suspended in the desired buffer (the same salinity as original medium). Subsequently, the cells were incubated with shaking for 5 min at

37°C, and transport was initiated by the addition of 0.1 mM [1-^{14}C]glutamate. For K_m and V_{max} determinations, the concentrations of glutamate were varied from 0.01 to 5 mM. Glucose was added to a final concentration of 5 mM to energize the cells, and where indicated, salt (NaCl or KCl), sucrose, or sorbitol was added to the indicated concentrations. Cells were collected on 0.2-μm-pore-size cellulose nitrate filters. Filters were washed with 3 ml of buffer (the same salinity as assay buffer), and the radioactivity trapped in the cells was measured with a liquid scintillation counter. Competitions for glutamate uptake were performed in the presence of a 100-fold molar excess (10 mM) of competitors. Ionophore, protonophore and metabolic inhibitors were added according to desired concentration.

2.3 Determination of amino acid and glycine betaine

Cell pellet was extracted with 90% methanol. After centrifugation to remove cell debris, the supernatant was evaporated to dryness using rotary evaporator. The dried pellet was dissolved in MilliQ water followed by the addition of the same volume of chloroform. The mixture was centrifuged at 1,500 g for 5 min and the upper aqueous phase was filtered through a 0.2 μm membrane filter before analysis by an amino acid analyzer using a shim pack Li column (Shimadzu, Japan).

For glycine betaine determination, cell pellet was boiled with 80% (v/v) ethanol for 5 min. After centrifugation the pellet was re-extracted with the same solvent at 25 °C for 18 h. The combined supernatant was evaporated to dryness and the dried pellet was dissolved in distilled water. This solution was applied onto Dowex-50W column followed by elution with 2M NH_4OH. The eluate containing glycine betaine was dried by lyophilization. The dried pellet was dissolved in acidic KI_3 solution followed by the addition of 5 ml of 1,2-dichloroethane. After phase separation the lower organic layer was measured for A_{365}.

2.4 Extraction and determination of polyamines

Fifty ml of *Synechocystis* cell culture was harvested by centrifugation at 2,790g for 10 min. Cell pellets were then extracted by 5% cold perchloric acid. After one hour acid digestion on an ice bath, the broken cells solution was centrifuged at 2,790*g* for 20 min. Both supernatant and pellet fractions were continuously derivatized using benzoylation method (Redmond and Tseng, 1979). After that, high performance liquid chromatography (HPLC) was performed to detect the polyamines derivatives (Flores and Galston, 1982), using 1,6-hexanediamine as an internal standard. Authentic polyamine standards were similarly prepared and analyzed by HPLC device with inertsil®ODS-3 C-18 reverse phase column (5 μm; 4.6 x 150 mm) using UV-Vis detector (254 nm). The mobile phase was run at a flow rate of 0.5 ml/min using a methanol/water gradient of 60 – 100% (v/v) (Jantaro et al., 2011).

2.5 Extraction and preparation of total RNA

One hundred ml of *Synechocystis* cells culture at the mid-logarithmic phase was harvested by centrifugation and the pellet was immediately frozen in liquid nitrogen. The total RNA was extracted by the hot-phenol method (Mohamed and Jansson, 1989). The total RNA was kept at -80°C and was treated with RNase-free DNase before use. To determine concentration and purity of RNA, sample was diluted with RNA storage buffer before checking its concentration by measuring the optical density at 260 nm, and subsequently loading the sample onto 0.8% agarose gel.

2.6 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cDNA was generated using SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). The reaction mixture contained 5 μg RNA, 0.8 μl of 25 μM of 3'-primer,

namely, *adc1* (5'-ATATTACCTGCGACAGTGATGG-3' and 5'-GATCAAGGCTA ACTCCGTATGAC-3'), *adc2* (5'-ATATTACCTGCGACAGTGATG G-3' and 5'-TTAGCTGGTGTGGATGCCT-3') (see their locations in Figure 3) and *16S* rRNA (5'-AGTTCTGAC GGTACCTGATGA-3' and 5'-GTCAAGCCTTGGTAAGGTTCT-3'), all of which were designed from the Cyanobase sequence (http://genome.kazusa.or.jp/cyanobase). The cDNA were obtained by PCR technique; the initial denaturation at 95°C for 5 min was done, followed by 29 cycles of denaturation at 95°C for 1 min, annealing step of *adc1* and *16S* rRNA genes were performed at 50.7°C and 56°C, respectively while *adc2* gene was performed at 69°C, 4 cycles followed by 25 cycles of touchdown to 59°C (~0.5°C/cycle) for 1 min and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The PCR products were then analyzed by 0.8% agarose gel electrophoresis. Quantification was carried out using Syngene® Gel Documentation (Syngene, USA).

2.7 Genomic organization and plasmid construction

The *slr0401* sequence encoding the putative periplasmic binding protein D of *Synechocystis* (PotD) (Kaneko et al., 1996) was amplified from the genomic DNA of *Synechocystis* using gene specific primers. *Nde*I and *BamH*I restriction sites (underlined) were added to *potD* forward primer (5'-CCA TAT GAA TTT ACC CTG CTA TTC CCG CCG-3') and *potD* reverse primer after the stop codon (5'-CGG GAT CCC TAA GCA CTC CGC ATG GTT T-3'). The PCR products were fractionated on agarose gel, and the DNA fragments were recovered and cloned into pET19b (Promega, USA) to construct pET*potD*. *E. coli* BL21 (DE3) was transformed with pET*potD* for protein expression.

2.8 Expression and purification of the recombinant *Synechocystis* PotD

The recombinant *E. coli* BL21 (DE3) cells harboring pET*potD* were grown at 37 °C in LB medium containing 100 μg/ml ampicillin until OD_{600} reached 0.6. Protein production was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant cells were harvested by centrifugation at 4,000*g*, 4°C for 15 min. The pellets were washed twice with 50 mM Tris-HCl buffer, pH 8.0, resuspended in the same buffer and disrupted by sonication with three short pulses of 30 s. After centrifugation at 18,000*g*, 4°C for 60 min, soluble and insoluble fractions were analyzed by SDS-PAGE.

His-tagged *Synechocystis* PotD protein (rPotD) was found in the pellet fraction as inclusion bodies, the pellet was subjected to purification by a Ni^{2+}-affinity chromatography column according to the manufacturer's suggestions for insoluble proteins (His GraviTrap™ kit, GE Healthcare, England). The purification was done according to Schlicke and Brakmann (2004). Briefly, inclusion bodies were washed twice in 50 mM Tris-HCl, pH 8.0 and dissolved in buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride and 8 M urea). The insoluble proteins were removed by centrifugation. The clear solution was applied onto a Ni^{2+}-Sepharose column equilibrated with three volumes of buffer A. rPotD was eluted by a modified buffer A containing 500 mM instead of 20 mM imidazole.

2.9 Protein refolding

rPotD was refolded by dialysis overnight at 4°C against 50 mM Tris-HCl buffer, pH 8.0 containing 0.1% Triton X-100 and 0.5 mM phenylmethanesulfonyl fluoride. The dialyzed protein sample was then centrifuged at 18,000*g* at 4 °C for 20 min to remove unfolded or aggregated proteins and analyzed by 15% (w/v) SDS-PAGE.

2.10 Binding of polyamine to the purified His-tagged *Synechocystis* PotD

The reaction mixtures containing 100 μg of purified rPotD protein, 50 mM Hepes-KOH, pH 7.6, 30 mM KCl and 50 μM each radioactively labeled polyamine (i.e. [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine) with a specific activity of 2 μCi/μmol were incubated at 37°C for 5 min. The reaction was stopped by rapid filtration through a filter. The filter was rinsed with 5 ml of cold buffer (50 mM Hepes-KOH, pH 7.6, 30 mM KCl). The radioactivity was counted with a liquid scintillation counter. The assay of competition by substrate analogs was done by adding 20-fold excess of unlabeled analogs to the assay mixture. The apparent dissociation constant (K_d) and the maximum binding (B_{max}) parameters were calculated from Scatchard plots as described previously (Kashiwagi et al., 1993).

2.11 Induction of PotD

PotD induction was studied in cells grown for 5 days in BG11 medium containing 0.5 mM each of putrescine, spermidine, spermine as well as 0.55 M NaCl, 0.3 M sorbitol, the combination of either NaCl or sorbitol with each polyamine. Soluble proteins (50 μg) were separated by SDS-PAGE using 15% (w/v) acrylamide gels. After electrophoresis, proteins were electroblotted to a polyvinylidene fluoride (PVDF) membrane, and subsequently probed with polyclonal anti-PotD. The detected bands were analyzed using Image Analysis Software.

2.12 Spermine uptake

Exponential cells were harvested by centrifugation (8,000 x g, 10 min, 4 °C), washed twice with 50 mM HEPES-KOH buffer pH 7.5 containing 0.4% glucose and suspended in the

same buffer. The cell suspension containing 0.1 mg cell protein was pre-incubated at 37°C for 5 min. The addition of 5 µl [^{14}C] spermine with a specific activity of 2 mCi/mmol at a final concentration of 50 µM was added to start the uptake experiment. The reaction was incubated, then, the cells were rapidly collected on membrane filters (cellulose acetate, 0.45 µm pore size. The filters were washed twice with 1 ml of cold buffer containing 1 mM specific polyamine to remove the adsorbed [^{14}C] spermine on the membrane. The radioactivity on the filter was determined with a liquid scintillation counter. Initial spermine uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of spermine taken up per min per milligram protein. For the effect of osmolality assay, cells were preincubated with either NaCl or sorbitol for 30 min at 37°C before the addition of [^{14}C] spermine. For the assay of inhibition by substrate analogues, cells were incubated with a mixture of labeled substrate and 20 fold excess of unlabeled analogue for 30 min at 37°C before the addition of [^{14}C] spermine.

2.13 Phycocyanin extraction

Fresh biomass was harvested after 7 days of cultivation by centrifugation at 5,000 rpm for 15 min and cells were washed twice with distilled water and suspended in potassium phosphate buffer (0.1 M, pH 7.0). The cells were disrupted by sonication for 60 sec. Repeated freezing at -20 °C and thawing at room temperature in the dark for 3 times, followed by centrifugation at 5,000 rpm for 15 min at 4 °C yielded a clear supernatant containing a mixture of phycocyanin.

2.14 Estimation of phycocyanin

The absorbance of phycocyanin (PC) containing supernatant was measured by spectrophotometer at wavelengths 620 and 652 nm. The concentration of PC was determined using the following equation (Bennett and Bogorad, 1973).

$$PC\ (mg/ml) = [A620-0.474(A652)/5.34$$

2.15 Other method

Protein was determined by Bradford's method as described previously (Bradford 1976). Cells number was determined using Haemacytometer.

CHAPTER III

RESULTS

3.1 Studies on amino acid metabolism

3.1.1 Contents of amino acid under normal and salt stress conditions

We first determined how salt and pH could affect growth of *A. halophytica*. Figure 3.1A shows that highest growth rate was observed in cells grown under normal condition (0.5 M NaCl) at pH 9.0. Growth was decreased at pH 7.6. A strong alkaline condition (pH 10.0) also reduced growth rate. Interestingly, *A. halophytica* could hardly grow at pH 5.0.

At optimal pH of 9.0, increasing the concentration of NaCl in the medium from 0.5M to 1.0 and 2.0M resulted in the decrease in growth rate (Figure 3.1B). On the other hand, growth rate was also lower at 0.25 M NaCl than at 0.5 M NaCl. The absence of NaCl or replacement of 0.5 M NaCl with 0.5M KCl led to growth inhibition. This suggested that *A. halophytica* requires Na^+ as a specific cation for growth.

Under salt stress condition, glutamic acid and glycine betaine were significantly increased (Figure 3.2). On the other hand, glutamine remained unchanged whereas glycine was drastically decreased.

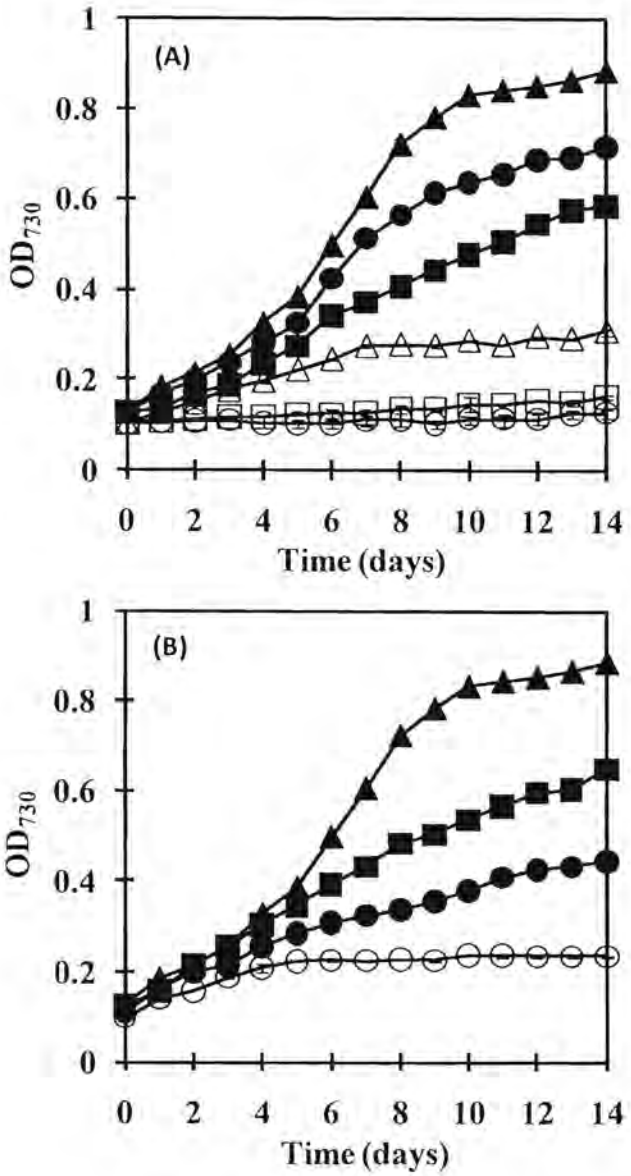


Figure 3.1 Effect of NaCl concentration and pH of the growth medium on *A. halophytica* growth. (A) Growth of *A. halophytica* grown in the growth medium containing various NaCl concentrations. 0 M NaCl (○), 0.25 M NaCl (■), 0.5 M NaCl (▲), 1.0 M NaCl (●), 2.0 M NaCl (△) and 0.5 M KCl (□) at pH 9.0. (B) Growth of *A. halophytica* grown in the growth medium containing 0.5 M NaCl at pH 5.0 (○), pH 7.6 (■), pH 9.0 (▲) and pH 10.0 (●). Each value shows the average of three independent measurements.

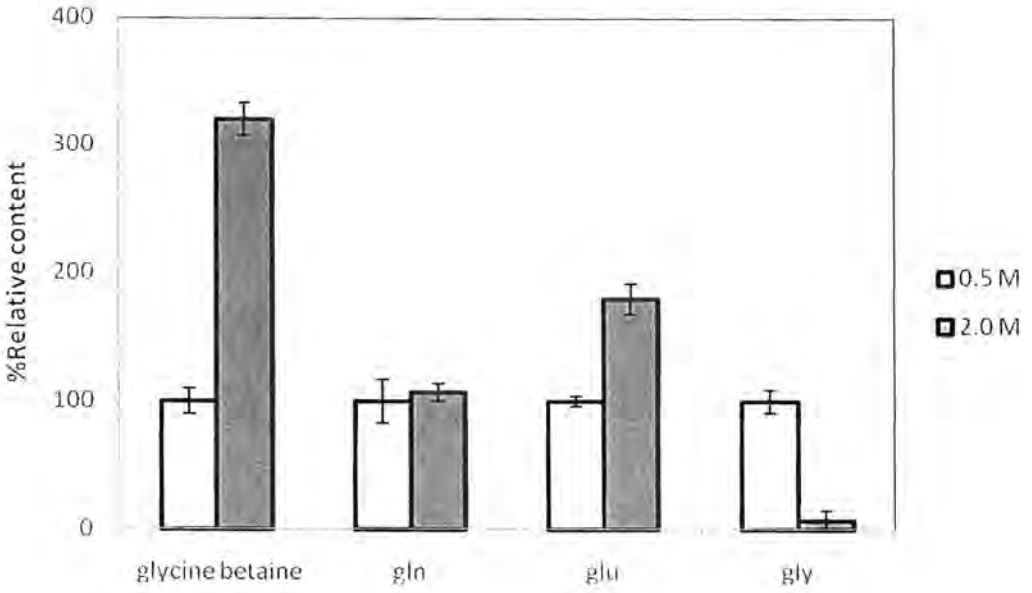


Figure 3.2 Glycine betaine, Glutamine (gln), Glutamic acid (glu) and Glycine (gly) contents at normal and stress condition.

3.1.2 Glutamate metabolism in *Aphanothece halophytica*

3.1.2.1 The growth of cells under normal and salt stress conditions

Growth of *A. halophytica* was studied in the medium containing NaCl at 0.5 M for normal condition and salt stress (2.0 M) condition. Figure 3.3 shows that addition of 0 – 50 mM glutamate could promote growth of *A. halophytica* under normal and salt stressed conditions. Growth at 2.0 M NaCl with supplemented glutamate (0 – 50 mM) was recorded and it was observed that glutamate could promote growth under salt stress condition. Maximal growth was found at 50 mM glutamate, suggesting not only growth stimulating but also its non-toxicity for the growth of *A. halophytica*.

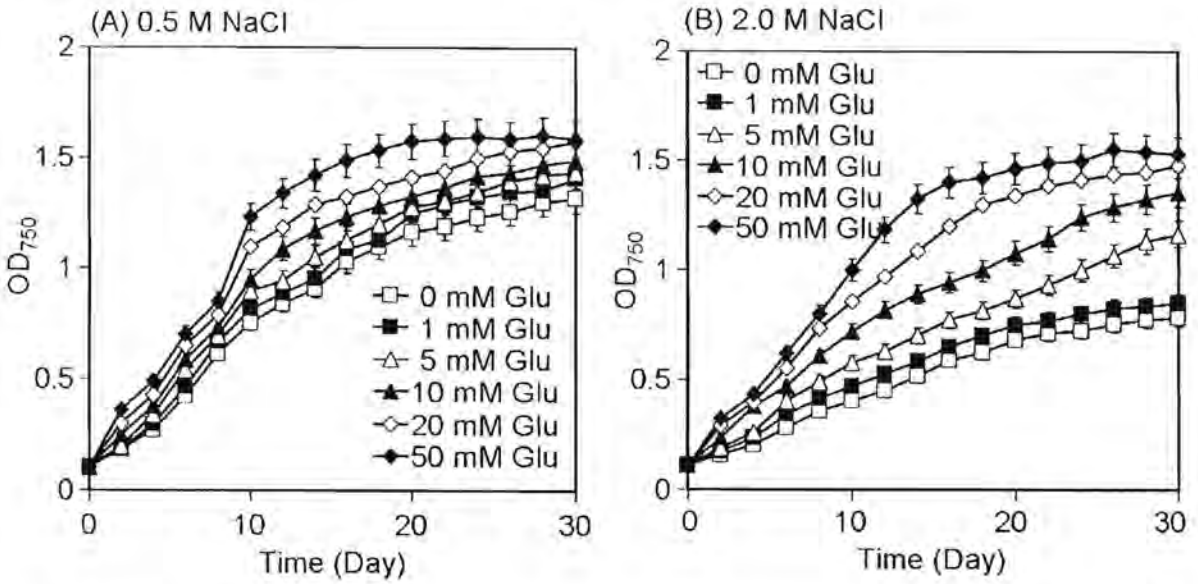


Figure 3.3 Effects of glutamate on the growth of *A. halophytica*. *A. halophytica* cells were grown under low (0.5 M NaCl) (A) and high salinity (2.0 M NaCl) (B) conditions with or without addition of glutamate at 0 – 50 mM.

3.1.2.2 Glutamate uptake in *A. halophytica* cells

Next, we examined glutamate uptake by *A. halophytica* cells. *A. halophytica* cells were grown in medium containing 0.5 M NaCl and harvested, after the cells were washed, glutamate uptake was measured under normal and salt stress conditions. As shown in Figures 3.4A and B, *A. halophytica* cells could actively take up glutamate from assay medium containing 0.5 M or 2.0 M NaCl. The V_{max} value for glutamate uptake for cells supplemented with 2.0 M NaCl was almost two fold higher than that for cells supplemented with 0.5 M NaCl (Table 3.1). However, the K_m values for glutamate uptake were slightly affected by the increase of salinity.

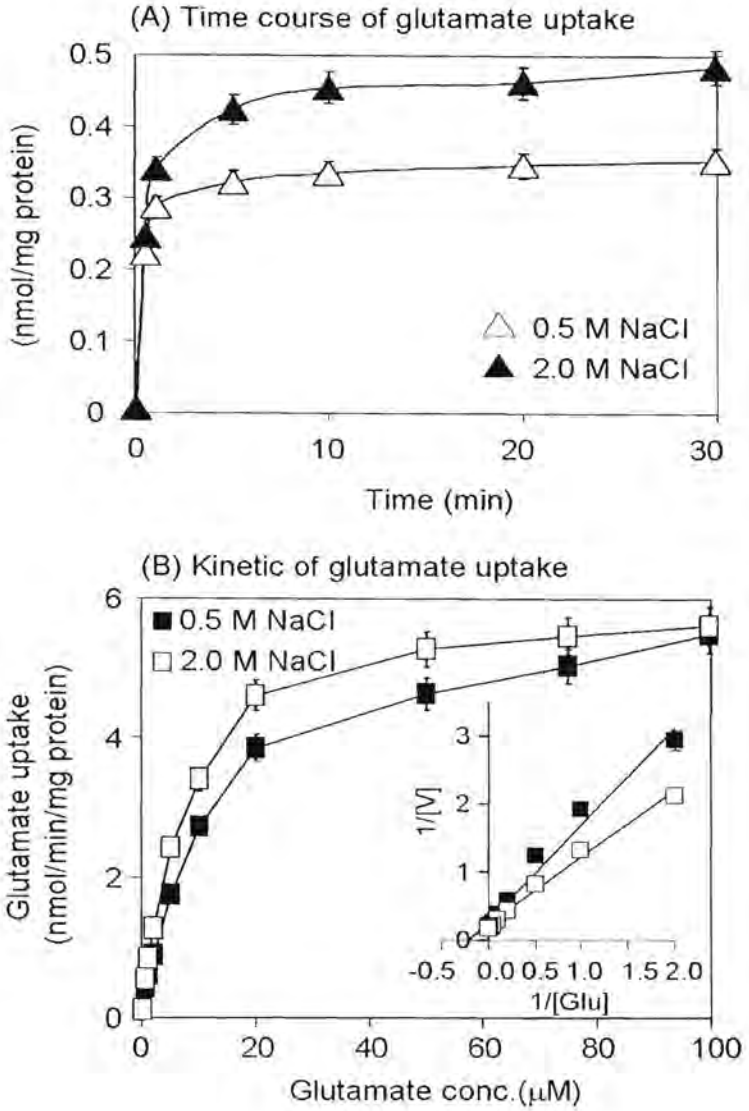


Figure 3.4 Kinetics of glutamate uptake into *A. halophytica* under normal and salt stress conditions. (A) Initial velocity of glutamate uptake and (B) Kinetics of glutamate uptake.

Table 3.1 Kinetic values of glutamate uptake into *A. halophytica*

Kinetic value	0.5 M NaCl	2.0 M NaCl
K_m (μM)	5.54	4.99
V_{max} (nmol/min/mg protein)	3.87	5.00

The effects of pH, ion specificity, and substrate specificity on glutamate uptake by *A. halophytica* were investigated. The optimum pH for glutamate uptake was 9.0 (Figure 3.5B). Also, Figure 3.5A shows that the uptake was stimulated by NaCl upto 2.0 M. The glutamate uptake was specific for Na^+ and showed a 2-3 fold increase compared to other ions (Figure 3.5C). Figure 3.6 shows that glutamate uptake into *A. halophytica* was not only specific for glutamate but also glutamine, asparagine and aspartate due to their inhibitory effect on glutamate uptake.

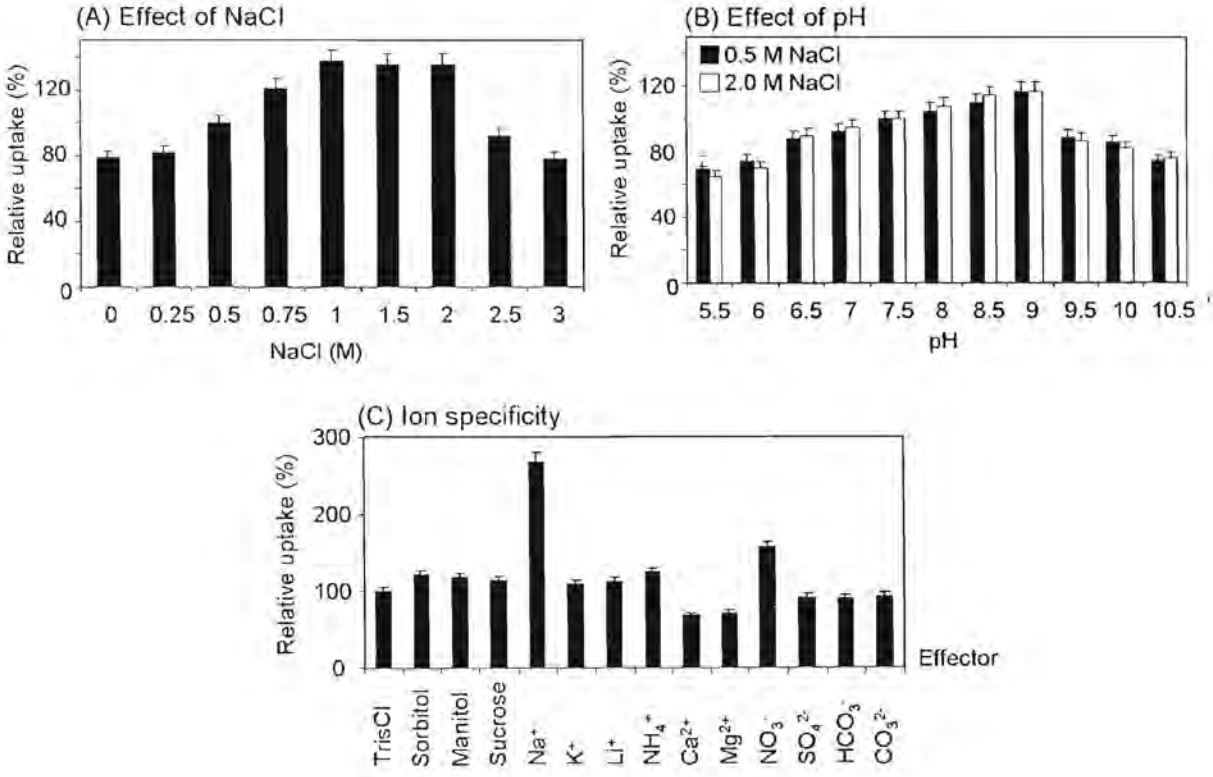


Figure 3.5 Effect of NaCl (A), pH (B) and ion specificity (C) of glutamate uptake into *A. halophytica*. The mid-log-phase cells were harvested, washed twice, and suspended to 1 mg protein/ml of 100 mM phosphate buffer. Subsequently, the cells were incubated for 10 min at room temperature, and transport was initiated by the addition of 10,000 CPM [1-14C] glutamate.

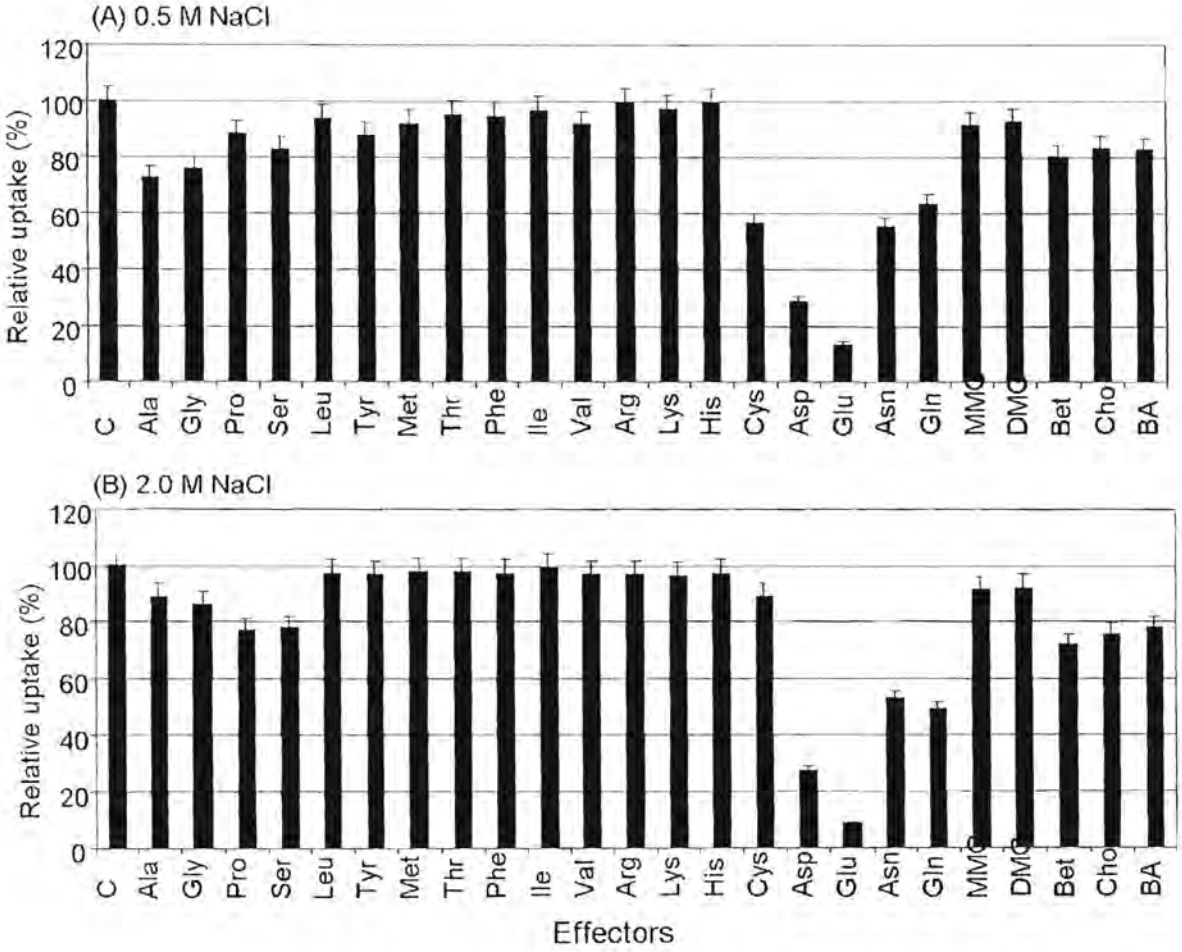


Figure 3.6 Substrate specificity of glutamate uptake into *A. halophytica* under normal and salt stress conditions.

3.1.2.3 Effect of different energy sources and metabolic inhibitors on glutamate uptake in *A. halophytica*

A. halophytica cells were starved to deplete endogenous energy sources. Glutamate uptake was then monitored after re-energization with glucose or lactate (Table 3.2). Both glucose and lactate could energize the uptake of glutamate in the starved cells. These results indicated that glutamate uptake is an energy-dependent process. The respiratory inhibitor, potassium cyanide (KCN), strongly inhibited glutamate uptake, either alone or together with glucose or lactate.

Table 3.2 Effect of energy sources on glutamate uptake[a]

Addition	Glutamate uptake (%)	
	0.5 M NaCl	2.0 M NaCl
None	100	100
Glucose (20 mM)	122	127
Lactate (10 mM)	110	116
KCN (20 mM)	68.14	75.23
Glucose + KCN	63.25	72.86
Lactate + KCN	56.23	62.15

[a]Cells were starved by suspending cells in the 100mM Tris-Cl buffer, pH 7.6 in the dark for 2 h. The starved cells were assayed for glutamate uptake in the presence of different energy sources or respiratory inhibitor. Starved cells were preincubated with the tested compound(s) in the dark for 30 min before the addition of [C-14] glutamate to initiate glutamate uptake. (100% at 0.5 M NaCl = 0.57 nmol/min/mg protein and 2.0 M NaCl = 0.72 nmol/min/mg protein)

3.1.2.4 Effect of external glutamic acid on changes in glycine betaine contents

Since glutamic acid could be taken up by *A. halophytica*, it is of interest to test whether the supply of external glutamic acid could influence the level of intracellular glycine betaine. Figure 3.7 shows that under both normal (0.5M NaCl) and salt stress (2.0M NaCl) conditions glutamic acid outside the cells could increase the level of glycine betaine. This suggested that glutamic acid could be converted to an intermediate in the pathway of glycine betaine biosynthesis.

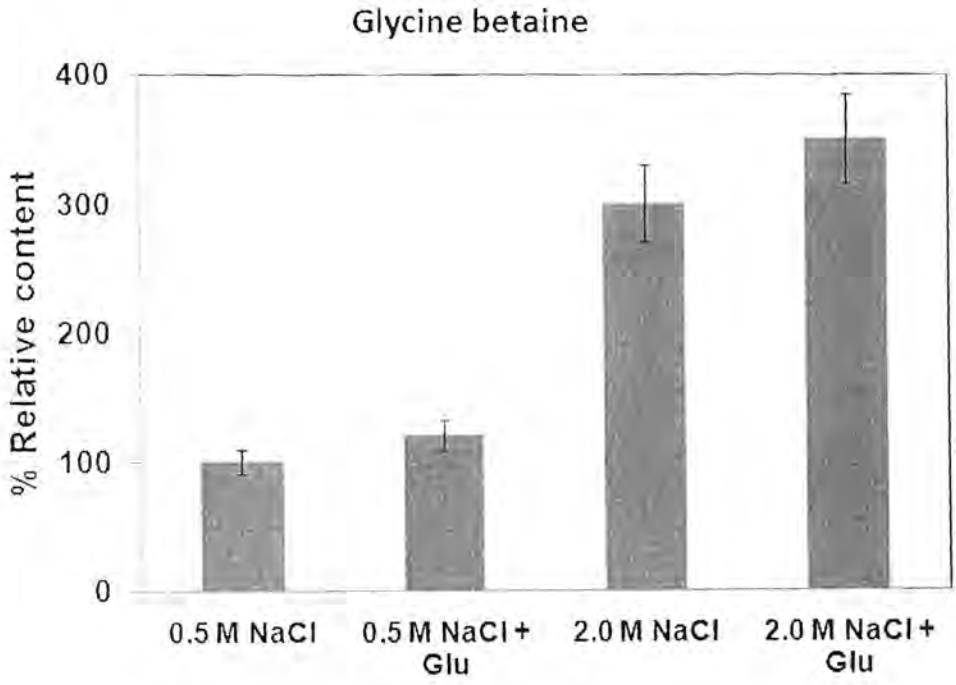


Figure 3.7 Betaine content in *A. halophytica* with or without external glutamic acid.

3.2 Studies on polyamine metabolism

3.2.1 Effect of salt and sorbitol stress on polyamine contents of *Synechocystis* sp. PCC 6803

Polyamine contents as affected by salt and sorbitol stress are shown in Table 3.3. Total polyamine was slightly increased by 550 mM NaCl but was significantly increased by 700 mM sorbitol. When examining individual polyamine it was found that spermine was greatly increased by NaCl stress whereas putrescine and spermidine were mainly the target of the increase by sorbitol stress.

Table 3.3 Effect of NaCl and sorbitol on polyamine contents

	Polyamine (nmol mg^{-1} protein)		
	Unstress	NaCl (550 mM)	Sorbitol (700 mM)
Putrescine	0.82 ± 0.05	1.1 ± 0.2	2.6 ± 0.8
Spermidine	1.7 ± 0.3	1.8 ± 0.2	4.1 ± 1.1
Spermine	0.3 ± 0.05	0.7 ± 0.06	0.5 ± 0.2
Total	2.8 ± 0.4	3.6 ± 0.4	7.2 ± 0.9

3.2.2 Effect of short-term UV-B and UV-C radiations on spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803

3.2.2.1 UV radiations affect polyamine accumulation

UV-A had no effect on total polyamine contents although there was an increase and a decrease in PCA-soluble and PCA-

insoluble polyamines, respectively (Figure 3.8). Both UV-B and UV-C reduced total polyamines by 25 and 50 %, respectively. The PCA-insoluble polyamine, represented mainly by Spd, was particularly affected by UV-B and UV-C. The levels of Put and Spm were extremely low, both accounting for about 0.01 nmol 10^{-8} cells whereas Spd levels were about 450-fold higher.

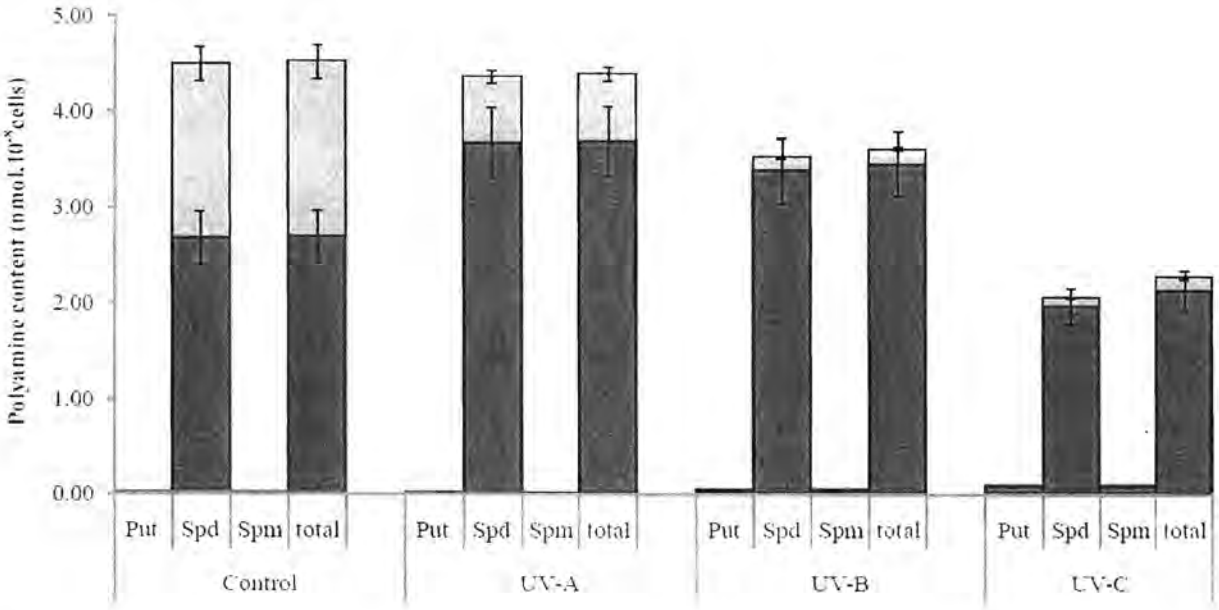


Figure 3.8 The contents of polyamines including putrescine (Put), spermidine (Spd) and spermine (Spm) from *Synechocystis* cells (A) which were under different UV-B radiations for 1 hour. B and C represent the enlarged scales from A of the trace amounts of Put and Spm accumulations under the three UV radiations, respectively. Cells grown under normal growth light represent a reference control. The open bar represents insoluble-PCA content of polyamines whereas the soluble-PCA content or free form of polyamines is shown in solid bar. The experimental data are shown as means ± S.D., n =3.

3.2.2.2 UV radiations affect *adc* transcripts and ADC levels

Two transcripts of arginine decarboxylase, *adc1* and *adc2*, were detected by RT-PCR after one hour exposures to either normal white light or UV radiations (Figure 3.9A). The *adc1* transcript was increased significantly by UV-A while no changes were observed by UV-B and UV-C (Figure 3.9B). The three UV radiations decreased *adc2* transcripts. The total *adc* transcripts were markedly decreased by UV-C followed by UV-B whereas UV-A had little or no effect. As shown in Figure 3.9C, ADC protein was detected using ADC-specific antibody. All three UV radiations increased the level of ADC protein (Figure 3.9D)

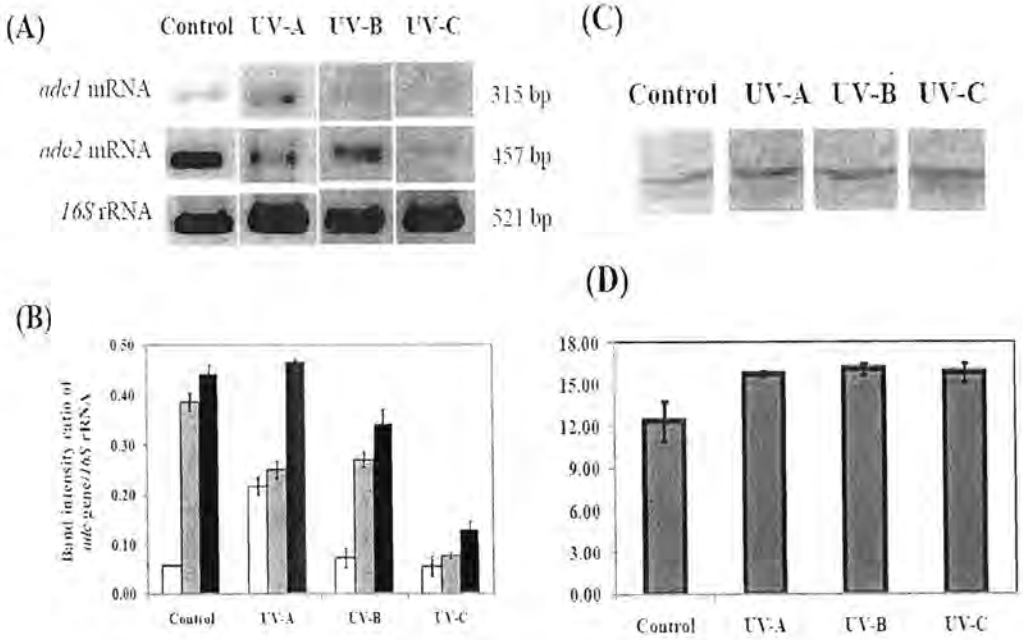


Figure 3.9 RT-PCR analyses (A) of *adc1* and *adc2* mRNA levels and Western blot analysis (C) of ADC protein levels in *Synechocystis* sp. PCC 6803 under different UV radiations for one hour.

3.2.2.3 Effect of UV-radiation on growth

In control cells grown under normal growth light for 3 h, growth inhibition was seen at high concentration of 350 mM NaCl and 500 mM sorbitol (Figure 3.10 A, E). At low concentration, 125 mM NaCl and 250 mM sorbitol, growth was similar to that without added salt and sorbitol. Upon treatment with UV-radiations, salt-stressed cells showed no growth during 3h-exposure to UV-A (Figure 3.10 B), UV-B (Figure 3.10 C), and UV-C (Figure 3.10 D). Sorbitol-stressed cells also exhibited similar growth profiles to those of sale-stressed cells after 3h-exposure to UV radiations (Figure 3.10 F,G,H). The detrimental effects of UV-A and UV-C on sorbitol-stressed cells (Figure 3.10 F,H) were more pronounced than those on salt-stressed cells (Figures 3.10 B,D) especially at 500 mM sorbitol.

3.2.2.4 Changes in polyamines by osmotic and UV radiation stresses

As shown in Figure 3.11A, a slight increase of PAs by salt-stressed cells was observed whereas salt-stressed cells in combination with either UV-B or UV-C had large increases of PAs. A 5-fold and 4-fold increase was caused by UV-B and UV-C, respectively. On the other hand, only a slight increase and a 2-fold increase in PAs was observed in sorbitol-stressed cells by UV-B and UV-C respectively, whereas under normal growth light and UV-A a small decrease of PAs was detected (Figure 3.11B). The effects of UV-radiations on changes in relative contents of putrescine and spermidine are shown in Figure 3.11C. The control (unstress) cells had lower spermidine:putrescine after exposure to UV-radiations. In contrast, the three UV-radiations caused an increased spermidine:putrescine for both salt-stressed and sorbitol-stressed cells. The only exception was in the case of UV-C treatment on sorbitol-stressed cells with about 50% decrease in spermidine:putrescine.

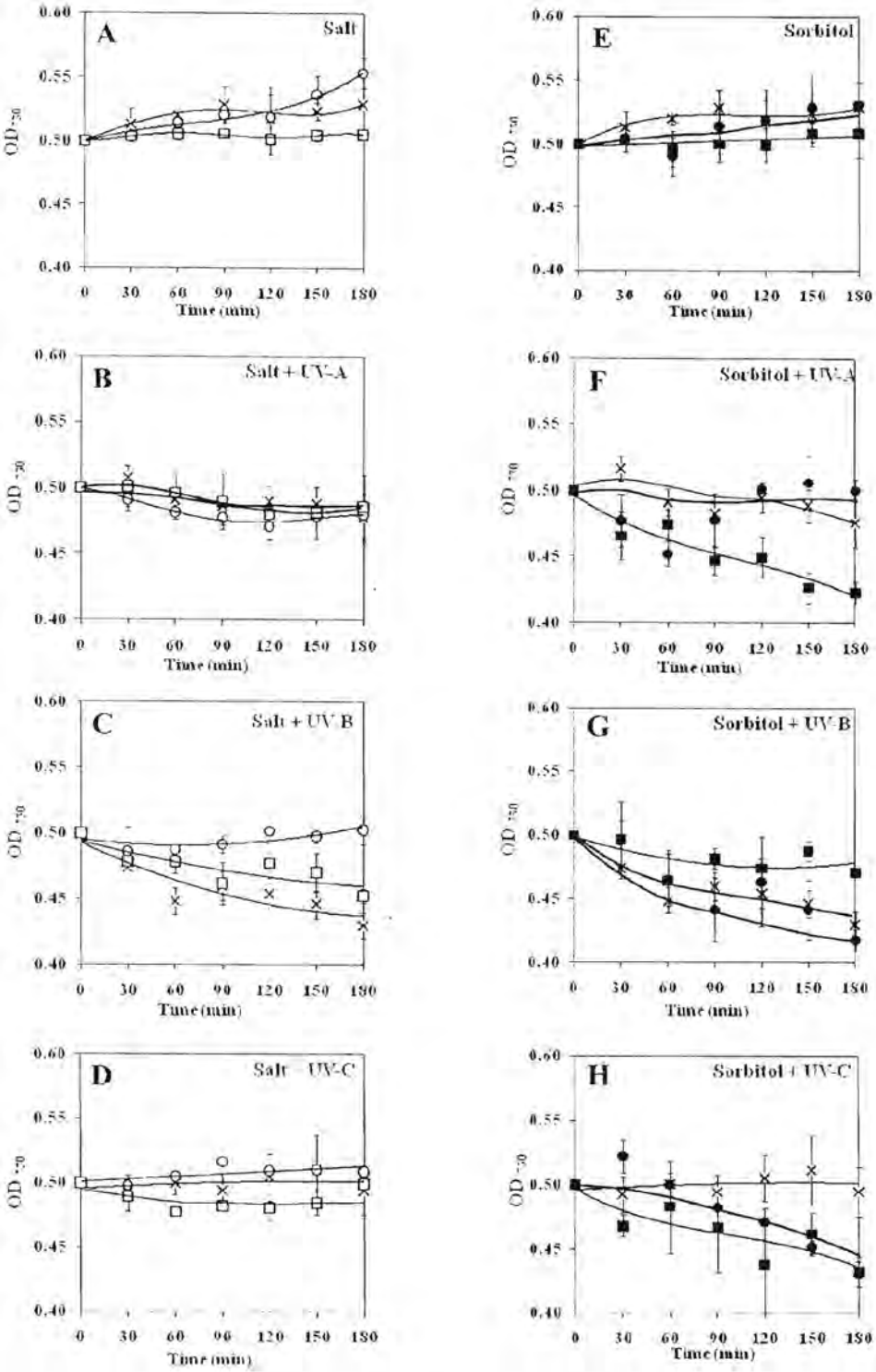
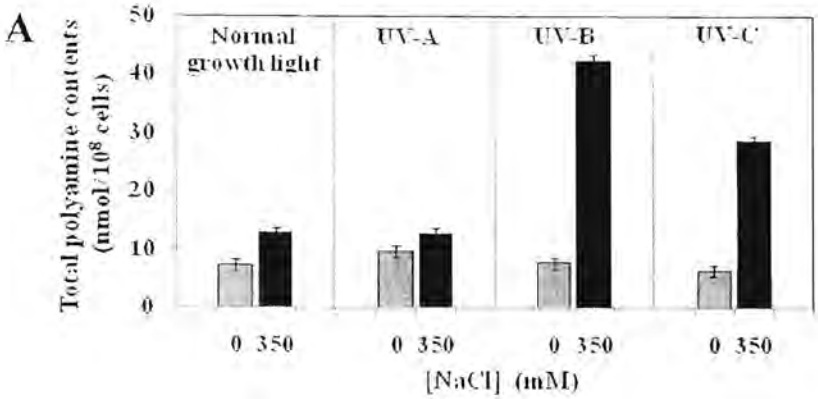
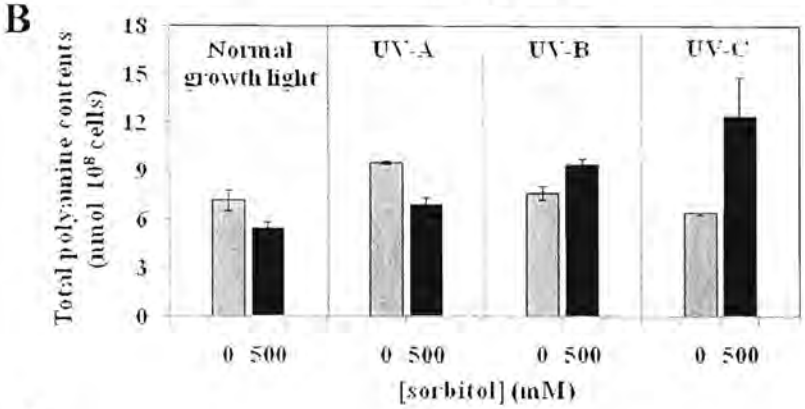


Figure 3.10 Effect of UV radiations on the growth of *Synechocystis* cells in BG_{11} medium containing various concentrations of NaCl (A-D) and sorbitol (E-H). The concentration of NaCl are 0 mM (x, control), 125 mM (○), and 350 mM (□), respectively. The concentration of sorbitol are 0 mM (x, control), 250 mM (●) and 500 mM (■), respectively. Results are the mean of three replicates ± SD.





C

	Ratio of Spd/Put content		
	Control	Salt stress (350 mM NaCl)	Sorbitol stress (500 mM sorbitol)
Normal growth light	9.94 – 10.07	18.40 – 19.16	14.04 – 14.15
UVA	5.51 - 5.84	20.98 – 22.49	30.18 – 34.22
UVB	6.49 – 6.82	38.49 – 40.77	27.39 – 31.04
UVC	8.11 – 8.34	25.77 – 25.98	7.13 – 7.30

Figure 3.11 Effect of UV radiations on polyamine contents of *Synechocystis* cells stressed with NaCl (A) or sorbitol (B) with 1h exposure to UV-radiations. The data represent means ± SD., n = 3. The ratio of Spd/Put content is shown in (C).

3.2.2.5 Responses of adc1 and adc2 transcripts to osmotic and UV stresses

The pattern of *adc* transcript levels, including *adc1* and *adc2*, was investigated in salt-stressed and sorbitol-stressed *Synechocystis* under different UV-radiation exposure. The unstress cells grown under normal growth light showed *adc2* transcript level, about 7-fold higher than that of *adc1* transcript (Figure 3.12 A). The osmotic stress imposed by 350 mM NaCl, 500 mM or 700 mM sorbitol had little or no effect on *adc2* transcript; however, an approximately 6-fold increase of *adc1* transcript was observed in salt- and sorbitol-stressed cells (at 700 mM sorbitol) (Figure 3.12 A,B,C). The osmotic stress by NaCl or by sorbitol (at 700 mM) elicited about 2-fold higher total *adc* transcript than that without osmotic stress. In contrast to an increased *adc* transcript seen in osmotic-stressed cells, the irradiation by UV-A on unstress cells caused an unchanged level of total *adc* transcript (Figure 3.12 A,B,C). UV-B on unstress cells caused little effect on total *adc* transcript; however, a large decrease of total *adc* transcript was observed by UV-C exposure. In salt-stressed cells, UV-radiations reduced the *adc* transcripts with highest reduction seen by UV-C (Figure 3.12 A). For sorbitol-stressed cells (at 500 mM), an approximately 1.5-fold increase in *adc* transcripts occurred as a result of UV-A and UV-B exposures; however, about a 2-fold decrease of *adc* transcripts was brought about by UV-C treatment (Figure 3.12 B). Increasingly, under high sorbitol stress at 700 mM, UV-C caused an increase in *adc* transcripts with only minor changes in *adc* transcripts by UV-A and UV-B (Figure 3.12 C).

When examining the relation changes of *adc1* and *adc2*, it was found that salt-stressed cells and sorbitol-stressed cells (at 700 mM) had significantly increased *adc1* but not *adc2* (Figure 3.12 A, C, left panels). On the contrary, *adc2* was mainly affected by UV-radiations, a small decrease by UV-A and UV-B and a large decrease by UV-C (Figure 3.12 A).

Among the three UV-radiations, UV-C conferred the most drastic effects on *adc* transcripts. Significant reduction of the transcript was observed in UV-C treated salt-stressed

and 500 mM sorbitol-stressed cells (Figure 3.12 A,B). In contrast, UV-C radiation caused a decreased and an increased transcript in unstressed and 700 mM sorbitol stressed cells, respectively (Figure 3.12 A,B,C).

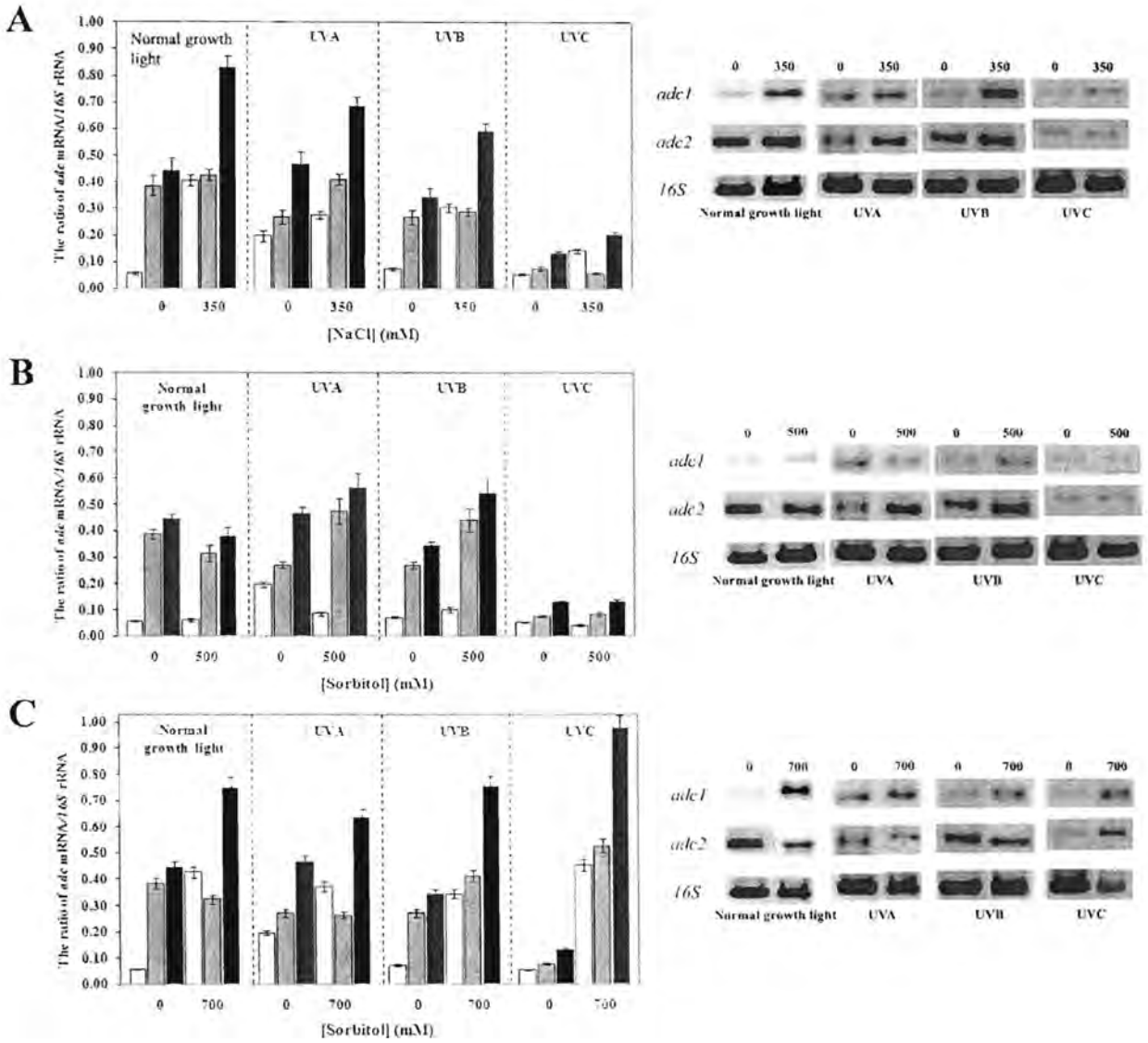


Figure 3.12 RT-PCR analysis of arginine decarboxylase (*adc1* and *adc2*) mRNA levels in salt-stressed (A) or sorbitol-stressed (B,C) *Synechocystis* sp. PCC 6803 under exposure to UV-radiations for 1 h. The *16S* rRNA was used as an internal reference.

3.2.3 Study on polyamine-binding protein

3.2.3.1 Expression and purification of the His-tagged *Synechocystis* PotD

The *potD* gene was inserted into a pET19b vector to construct pET*potD* (Figure 3.13A), and subsequently expressed in *E. coli* BL21 (DE3) cells. Whole cells and a crude extract from sonicated cells were analyzed by SDS-PAGE. The induction of an approximate 43 kDa recombinant protein occurred after 1 h of induction by IPTG and gradually increased over the 5 h induction (Figure 3.13B). rPotD content induced for 5 h was 10-fold higher than that of the non-induced. The rPotD was found in pellet fraction as inclusion bodies. By Ni^{2+}-affinity chromatography purification, rPotD was detected in the fraction eluted with 500 mM imidazole. The recombinant PotD was purified to homogeneity as determined by SDS-PAGE (Figure 3.13C). An analysis by immunoblotting with anti-His monoclonal antibody and polyclonal PotD antiserum indicated that rPotD had an apparent molecular mass of 43 kDa, corresponding to the size of the predicted rPotD (Figure 3.13C).

Affinities for polyamine binding. The binding of putrescine, spermidine or spermine to rPotD was saturable (Figure 3.14A). Scatchard plots showed linear transformation of the binding of all three polyamines to rPotD (Figure 3.14B). The values of the dissociation constant (K_d), calculated from the reciprocal of the slope for putrescine, spermidine and spermine *in vitro* were 13.2, 7.8, and 8.3 μM, respectively (Table 3.4). The maximum binding (B_{max}) values, the X-intercepts of the Scatchard plot, of putrescine, spermidine and spermine were 0.74, 1.4 and 0.13 mol/mol rPotD, respectively (Table 3.4). The data suggested that the three polyamines bind to rPotD with a 1:1 binding stoichiometry based on the molecular mass for rPotD of 43 kDa. The seemingly low values of less than 1 for putrescine and spermine might be due to the dissociation of the complex occurring during

the filtration/washing of the membrane. Another possibility is that a portion of improper refolded rPotD was present in the final preparation, that was incompetent to bind polyamines.

The changes in the polyamine binding capacity as a function of external pH were observed (Figure 3.14C). The specific binding of all types of polyamines to rPotD displayed an optimum at pH 8.0. It is noted that rPotD had the highest binding capacity for spermidine at all pH values tested.

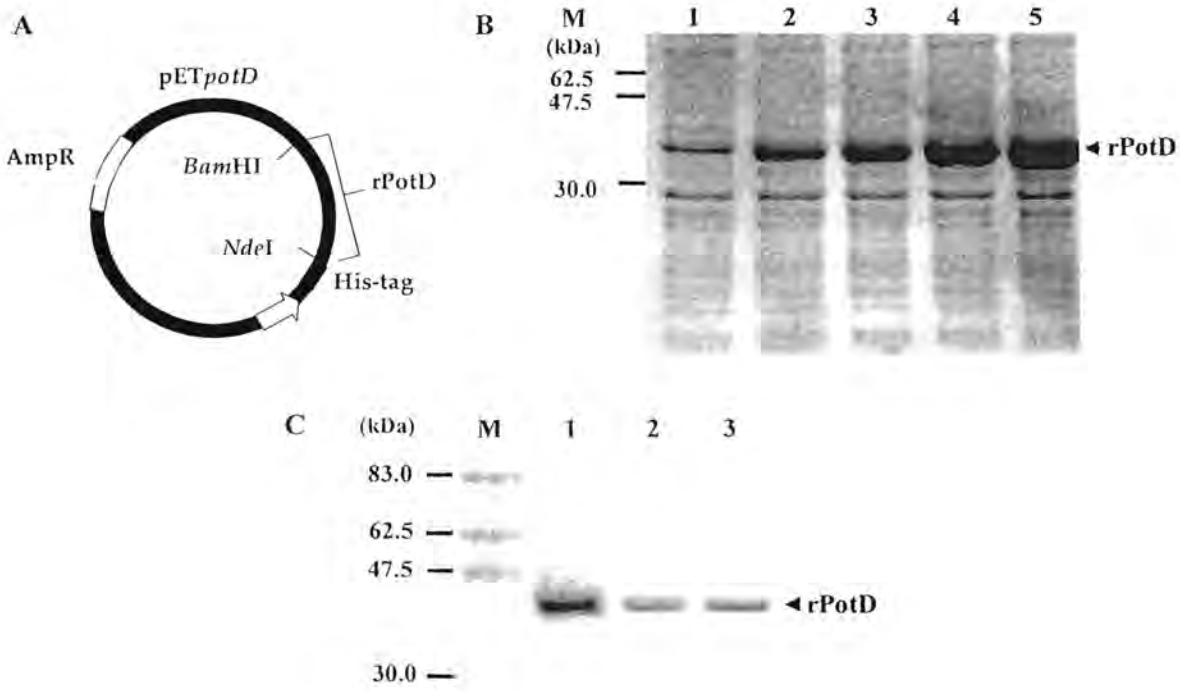


Figure 3.13 Expression of recombinant *Synechocystis* PotD in *E. coli* BL21 (DE3). (A) Schematic view of the recombinant plasmid. (B) Coomassie-stained gel for analysis of expression of His-tagged *Synechocystis* PotD (rPotD) in *E. coli* from the vector pET19b. (C) 15% SDS-PAGE analysis of eluted fraction from Ni^{2+}-chromatography purification of rPotD from *E. coli* and immunoblotting analysis.

Table 3.4 Parameters for rPotD binding to different polyamines

Polyamine	K_d (mM)	B_{max} (mol/mol)
Putrescine	13.2 ± 0.1	0.74 ± 0.05
Spermidine	7.8 ± 0.1	1.4 ± 0.4
Spermine	8.3 ± 0.1	0.13 ± 0.03

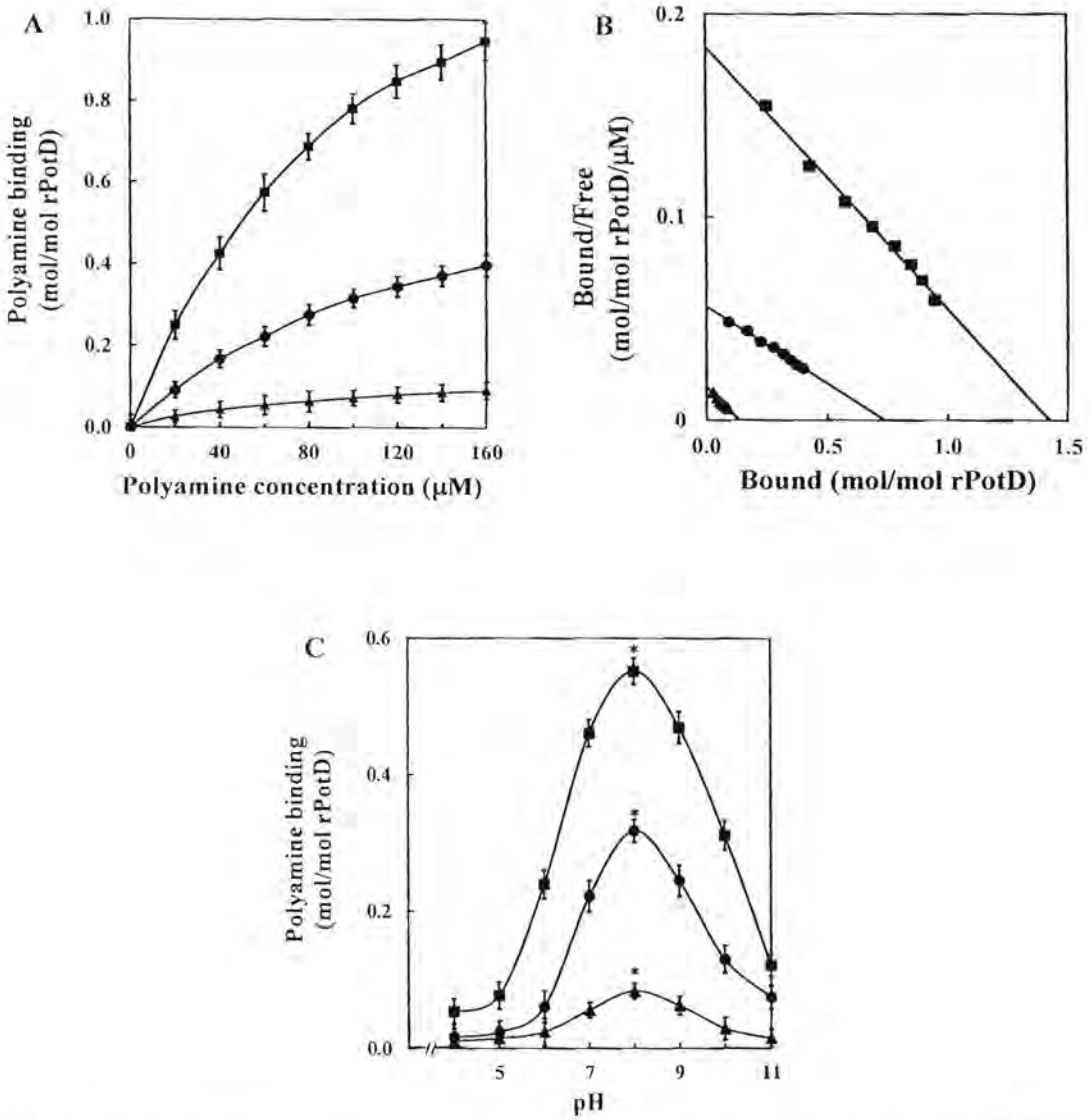


Figure 3.14 Putrescine (●), spermidine (■), and spermine (▲) binding. (A) Different amounts of each polyamine were added to give different external polyamine concentrations. The data represented three independent biological replicates. (B) Scatchard analysis of the data. The line drawn is derived from regression analysis of the data. K_d and B_{max} were obtained from the reciprocal of the slope and the intercept on the X-axis, respectively. (C) Effect of external pH on polyamine binding.

3.2.3.2 Specificity of polyamine binding

Experiments were carried out to determine if the interactions between the rPotD and polyamines are specific. As shown in Table 3.5, the binding of each radioactively labeled polyamine to rPotD was not inhibited by compounds structurally related to polyamines, including L-arginine, L-asparagine, L-glutamic acid, L-lysine, and L-ornithine. Binding of [^{14}C]spermidine to rPotD was inhibited by non-labeled spermine and putrescine by 35 and 42%, respectively. Binding of [^{14}C]spermine to rPotD was inhibited by non-labeled putrescine and spermidine by 39% and 58%, respectively. Similarly, binding of [^{14}C]putrescine to rPotD was inhibited by non-labeled spermine and spermidine by 38% and 58%, respectively. These results proved that rPotD is able to bind specifically to the three polyamines putrescine, spermidine and spermine.

Table 3.5 Effect of polyamine analogs on specific binding activity of *Synechocystis* rPotD

Analog	Polyamine binding (%)		
	[^{14}C]putrescine	[^{14}C]spermidine	[^{14}C]spermine
None	100 ± 2	100 ± 2	100 ± 2
Putrescine	-	58 ± 2[a]	61 ± 3[a]
Spermidine	42 ± 6[a]	-	42 ± 4[a]
Spermine	62 ± 3[a]	65 ± 5[a]	-
L-arginine	94 ± 3	92 ± 3	93 ± 2
L-asparagine	96 ± 6	95 ± 3	96 ± 5
L-glutamic acid	96 ± 5	98 ± 3	97 ± 3
L-lysine	90 ± 5	94 ± 6	92 ± 4
L-ornithine	91 ± 4	91 ± 5	93 ± 5

The data shown are the means of three independent experiments, representing the percent binding activity relative to the control (100%) for putrescine, spermidine and spermine, which were 0.33 ± 0.03, 0.55 ± 0.18, and 0.05 ± 0.01 mol/mol, respectively.

[a] statistically significant differences (Student's *t*-test, $P < 0.05$, n = 3) with respect to the control.

3.2.3.3 Induction of PotD

Growth of *Synechocystis* cells in the medium containing either 0.5 mM putrescine or spermidine led to an increase in PotD, about 1.6- and 2.8-fold, respectively (Fig. 3.15A). A slight decrease of PotD was observed in the presence of 0.5 mM spermine. As previous studies showed an increase in polyamines uptake under osmotic stress [10, 11], we further tested whether PotD levels could change under osmotic stress. A small increase of PotD by about 40 and 30% was observed when *Synechocystis* cells were stressed with 0.55 M NaCl and 0.3 M sorbitol, respectively (Figure 3.15B). There was no synergistic effect on the increase of PotD by the simultaneous presence of polyamine and either NaCl or sorbitol (Figure 3.15C).

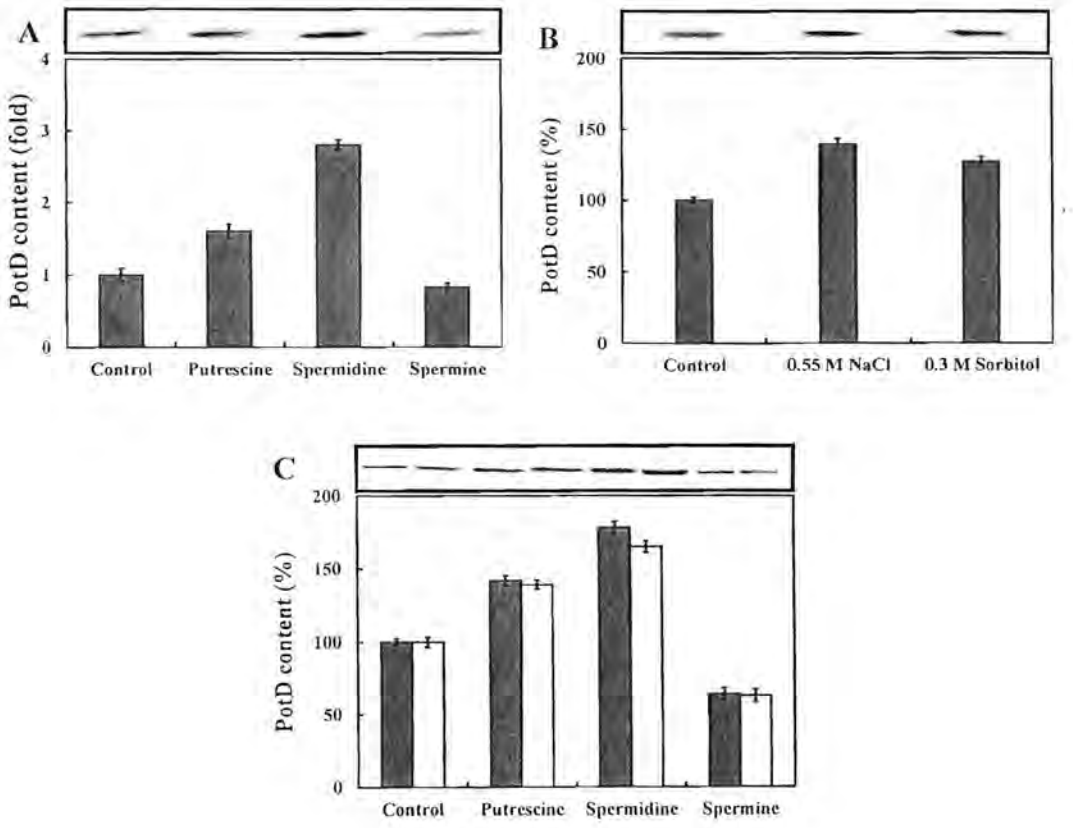


Figure 3.15 Western blot analysis of PotD induction by 0.5 mM polyamine (A), osmotic stress either 0.55 M NaCl or 0.3 M sorbitol (B), and the combination of polyamine and osmotic stress imposed by 0.55 M NaCl (■) or 0.3 M sorbitol (□) (C).

3.2.4 Studies on spermine uptake

3.2.4.1 Effect of external spermine on growth of *Synechocystis* sp. PCC6803

Synechocystis cells were grown in BG11 medium without or with various concentrations of spermine from 0.1, 0.3 and 0.5 mM. The growth of cells cultured with 0.1 and 0.3 mM spermine could reach an early stationary phase at day 5th whereas growth of cells cultured without spermine normally could reach this phase at day 9th. However, the addition of 0.5 mM spermine retarded the growth rate. It should be noted that the increase of the external concentration of spermine resulted in decreasing growth rate (Figure 3.16).

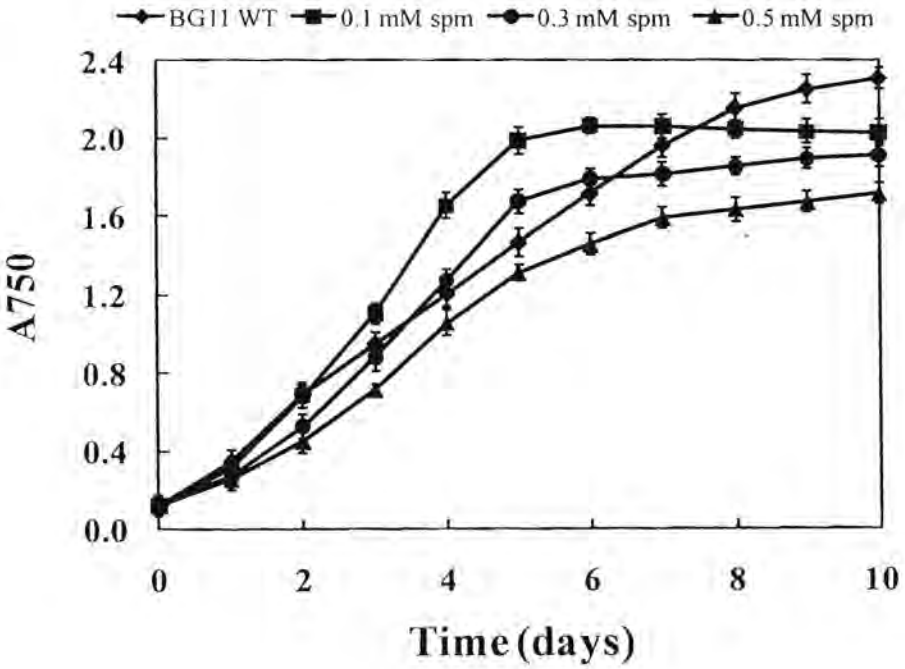


Figure 3.16 Effect of exogenous spermine on growth of *Synechocystis* sp. PCC6803. Cell were grown in BG11 medium (♦), 0.1 mM spermine (■), 0.3 mM spermine (●) and 0.5 mM spermine (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

3.2.4.2 Kinetics of spermine uptake

Incubation of *Synechocystis* cells with increasing concentration of spermine up to 500 mM resulted in a saturable initial uptake rate (Figure 3.17). A Lineweaver-Burk transformation of the data yielded a straight line typical of Michaelis-Menten kinetics. The apparent affinity constant (K_m) value of 141.42±10 μM and the maximal velocity (V_{max}) value of 0.31±0.02 nmol/min/mg protein were obtained.

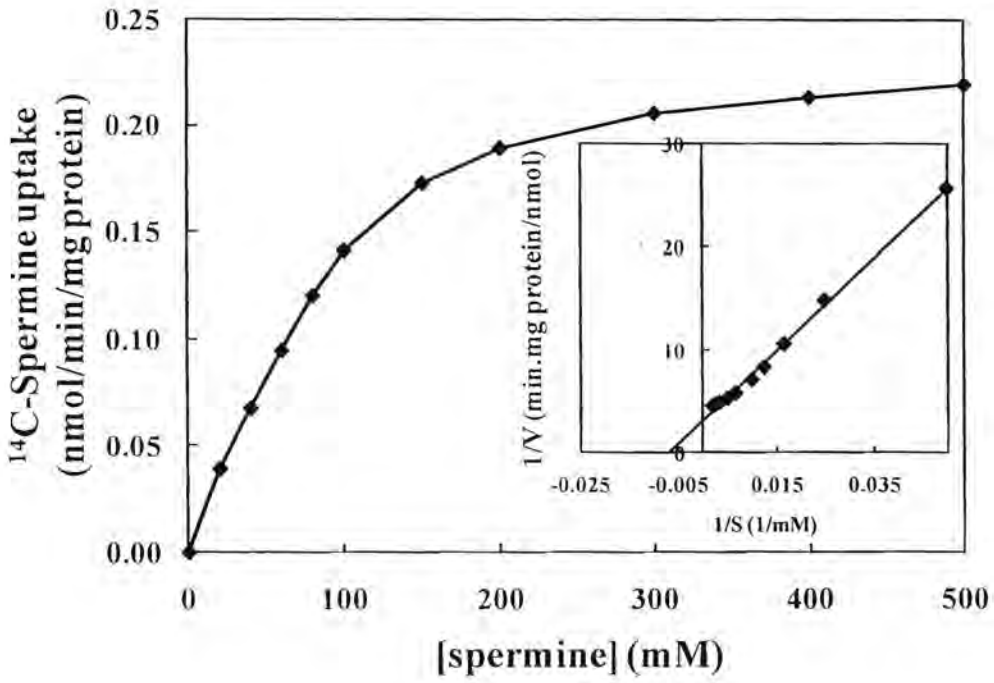


Figure 3.17 Kinetics of spermine uptake by *Synechocystis* sp. PCC6803 incubated with 0-500 μM spermine. Initial rates were determined with 1 min incubation time. Inset represents a Lineweaver-Burk transformation of the data. The line drawn is that derived from regression analysis of the data and the points shown are typical of this result from triplicate experiments.

3.2.4.3 Effect of external pH on spermine uptake

To determine whether the extracellular pH influences the spermine uptake activity, *Synechocystis* cells were incubated at different extracellular pHs and the uptake of spermine was determined. Increasing the pH up to 7.0 increased uptake activity of spermine. The optimum pH of spermine uptake activity was at 7.0 and the uptake was decreased at alkaline pH (Figure 3.18).

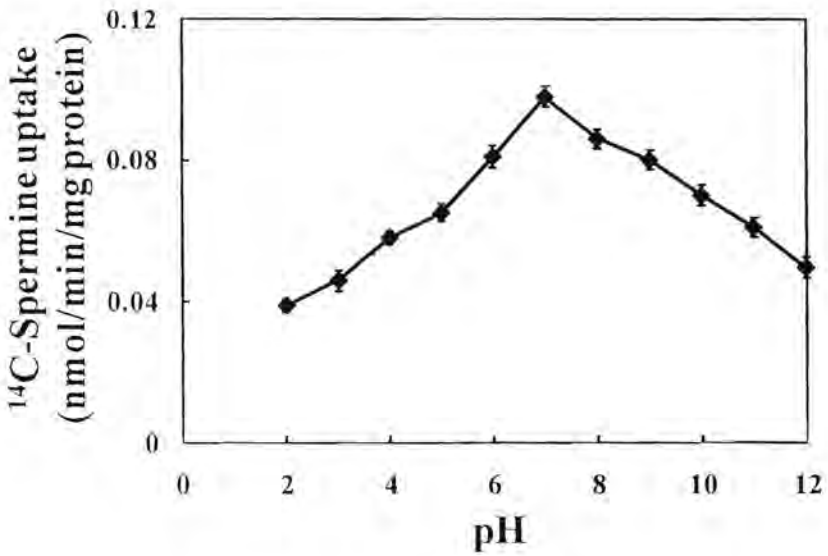


Figure 3.18 Effect of external pH on spermine uptake by *Synechocystis* sp. PCC6803. Spermine uptake was assayed at different pH, where 50 mM potassium citrate was used for buffer pH 4.0-6.0; 50 mM Hepes-KOH for buffer pH 7.0-8.0; 50 mM glycine-KOH for buffer pH 9.0-12.0. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

3.2.4.4 Effect of external osmolality on spermine uptake

The effect of external osmolality generated by either NaCl or sorbitol on spermine uptake was examined in order to investigate whether the spermine uptake in *Synechocystis* cells is affected by the osmotic upshift. Changes in spermine uptake activity were observed when changing NaCl or sorbitol concentration (Figure 3.19). Increasing NaCl concentration to 10 mM (18.3 mosmol/kg) resulted in the highest spermine uptake activity.

However, the higher concentrations of 50 mM NaCl (43.1 mosmol/kg) caused a decrease of the uptake. Similar results were observed upon changing concentration of sorbitol. The elevation of sorbitol concentration up to 20 mM (22.1 mosmol/kg) promoted the spermine uptake. Nevertheless, the decrease of spermine uptake was detected when sorbitol concentration was higher than 50 mM. (47.5 mosmol/kg) These observations indicated that high concentration of NaCl and sorbitol perturbed the uptake of spermine in *Synechocystis* cells.

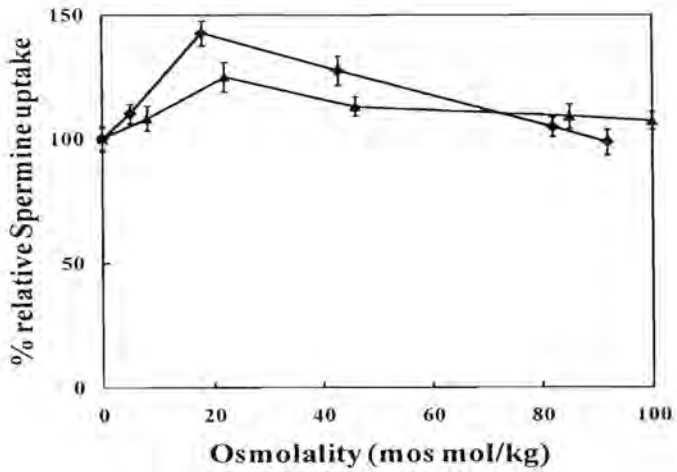


Figure 3.19 Effect of external osmolality on spermine uptake by *Synechocystis* sp. PCC6803. Initial uptake rates were determined in the presence of increasing osmolality generated by either NaCl (♦) or sorbitol (▲). The data are means from three independent experiments representing the percent uptake rate relative to the control rate, which was 0.12±0.02 nmol/min/mg protein.

3.2.4.5 Effect of amino acids on spermine uptake

In order to investigate whether *Synechocystis* cells bind specifically to spermine, the competitive substrates, such as amino acids, were added into the reaction and measured for the uptake rate. The results showed that serine, lysine and asparagine had no effect on spermine uptake (Table 3.6). The presence of alanine and cadaverine, one of polyamines found in plant, slightly inhibited spermine uptake rates. On the contrary, spermine uptake rate was strongly inhibited by 47 and 50% in the presence of spermidine and putrescine, respectively These results suggested that *Synechocystis* cell has a high specificity for polyamine and not amino acid uptake.

Table 3.6 Effect of polyamine analogs on the spermine uptake of *Synechocystis* cells.

Analogs	Spermine uptake[a] (%)
None	100 ± 3
Alanine	90 ± 3
Asparagine	96 ± 3
Cadaverine	85 ± 2
Lysine	98 ± 2
Serine	99 ± 4
Putrescine	50 ± 3
Spermidine	53 ± 3

[a]Cells were incubated in the mixture containing 1 mM unlabeled analog and 50 μM [^{14}C]spermine. The data shown are the means of three independent experiments representing the percent of spermine uptake rate relative to the control rate which was 0.12± 0.02 nmol/min/mg protein.

3.2.4.6 Effect of metabolic inhibitors on spermine uptake

We next determined the energy source for spermine uptake by testing the effect of the inhibitors. The summarized results are shown in Table 3.7. Chloramphenicol inhibited spermine uptake, indicating that protein synthesis is required for functional spermine transport. *p*-chloromercurisulfonic acid (PCMS), which is the protein structure modifier, moderately inhibited spermine uptake. The inhibitors for ATP formation, sodium arsenate and sodium fluoride clearly decreased uptake activity. This suggests directly the requirement of ATP for spermine transport. The transmembrane potential was disrupted in order to assess the role of electrochemical gradient on spermine transport. Potassium cyanide, an inhibitor on the electron transport chain, caused effective inhibition on spermine uptake. Transport uncouplers, gramicidin D and DNP, which dissipate proton motive force, could significantly inhibit spermine uptake. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nigericin, an ionophore which abolishes transmembrane proton gradient (ΔpH) and the proton motive force (Δp), respectively, showed strong inhibition of spermine uptake. Moreover, the inhibition of uptake rate occurred in the presence of amiloride, a potent inhibitor of many Na^+-coupled transport systems including Na^+/H^+ antiporter. Reagents, which interfere with the transmembrane sodium ion gradient such as monensin for a sodium ionophore and ouabain for an inhibitor of the plasma membrane Na^+/K^+-ATPase, caused an effective reduction of spermine uptake. The sensitivity of spermine transport to various types of inhibitors suggests that the uptake system was ATP-dependent requiring proton motive force.

Table 3.7 Effect of metabolic inhibitors on the spermine uptake of *Synechocystis* cells.

Inhibitors	Concentration	Spermine uptake[a]
None	-	100 ± 2
Chloramphenicol	1 μg/ml	61 ± 2
PCMS	1 mM	62 ± 2
Sodium fluoride	1 mM	70 ± 5
Sodium arsenate	1 mM	65 ± 3
KCN	1 mM	53 ± 2
DNP	1 mM	62 ± 3
Gramicidin D	100 μM	76 ± 4
CCCP	100 μM	52 ± 2
Nigericin	10 μM	59 ± 4
Amiloride	100 μM	56 ± 3
Ouabain	1 mM	60 ± 2
Sodium ionophore	100 μM	67 ± 4

[a]Cells were preincubated with inhibitors for 30 min before the addition of 50 μM [^{14}C]spermine to initiate the uptake. The data shown are the means of three independent experiments representing the percent of spermine uptake rate relative to the control rate which was 0.12±0.02 nmol/min/mg protein.

3.3 Studies on phycocyanin accumulation

3.3.1. Screening of cyanobacteria for high production of phycocyanin

The results in Figure 3.20 showed that phycocyanin extraction from fresh mass of all cyanobacteria gave higher phycocyanin content than the extraction from dried mass. The cynabacterium isolated from Kood Island (KK) showed the highest phycocyanin content, followed by *A. siamensis*.

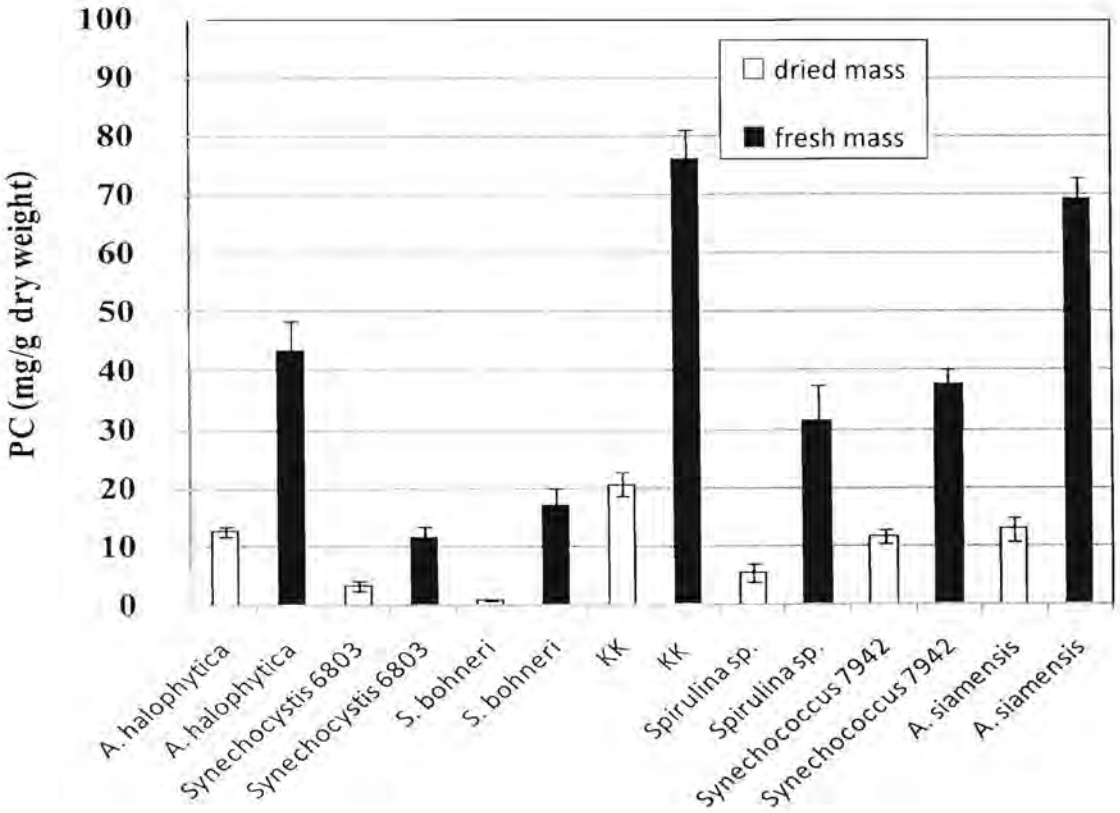


Figure 3.20 Phycocyanin contents in various cyanobacteria after extraction using dried mass or fresh mass

3.3.2. Effect of extractant on phycocyanin content

We extracted phycocyanin from *A. siamensis* and KK with various extractants such as distilled water, 0.1 M potassium phosphate buffer pH 7.0 and 0.1 M $CaCl_2$. It was found that the extraction of phycocyanin from both *A. siamensis* and KK with 0.1 M potassium phosphate buffer pH 7.0 gave the higher phycocyanin content than the extraction with distilled water and 0.1 M $CaCl_2$ (Figure 3.21)

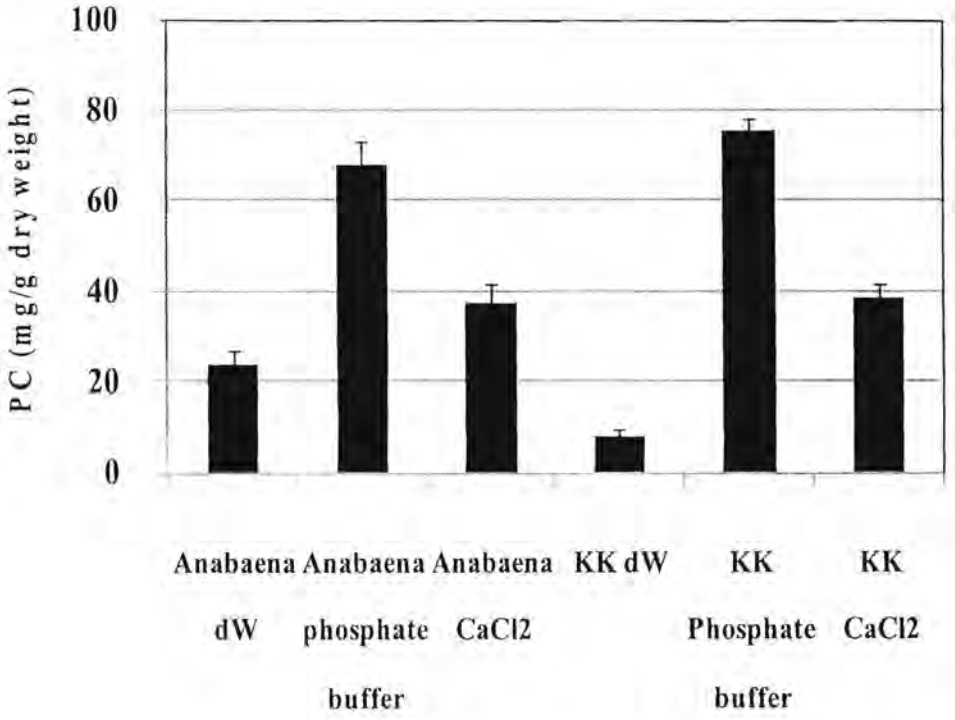


Figure 3.21 Phycocyanin contents in *A. siamensis* and KK using different extractants

3.3.3. Effect of culture times on phycocyanin content

High phycocyanin contents were obtained from cells grown for at least 7 days (Figure 3.22). A slight decrease of phycocyanin was detected at longer culture time.

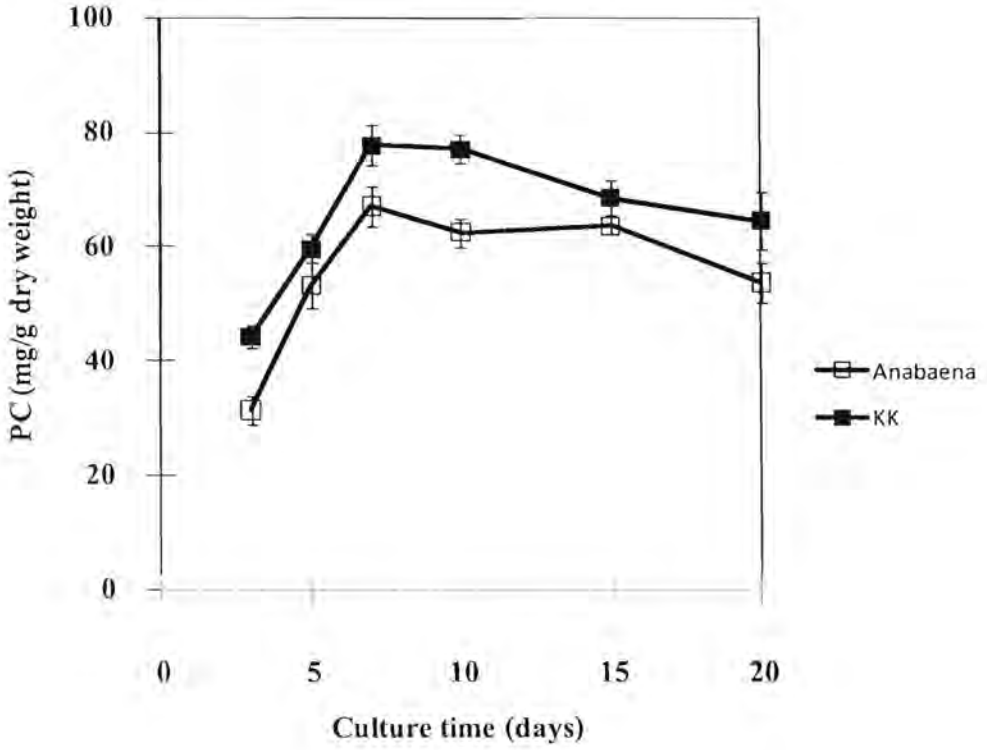


Figure 3.22 Effect of culture time on phycocyanin contents

CHAPTER IV

DISCUSSION

4.1 Amino acid metabolism

Aphanothece halophytica is an alkaliphilic halotolerant cyanobacterium that can grow at high pH. Optimal concentration of NaCl to yield maximum growth was at 0.5M (Figure 3.1). Increasing NaCl to 2.0M resulted in considerable reduction of the growth rate. This is probably due to the energy needed for growth is diverted to the initial adjustment of cellular volume by regulating the influx and efflux of certain ions. The results in Figure 3.2 clearly indicate that salt stress causes an increase of intracellular glycine betaine. Glutamate was also increased under salt stress; however, its increase was less than that of glycine betaine. These results suggest that glycine betaine is the main compatible solute which plays a role in the regulation of turgor pressure of *A. halophytica* under salt stress condition.

It is interesting to note that the external supply of glutamate to the growth medium under salt stress condition (2.0 M NaCl) could promote the growth of *A. halophytica* (Figure 3.3B). The efficiency of glutamate to promote the cell growth was concentration-dependent with the highest growth rate at 50 mM glutamate. This phenomenon was also observed in cells grown under unstress condition (0.5 M NaCl) (Figure 3.3A), although the effect was less drastic than that under salt stress condition. Glutamate uptake occurred at a higher rate under salt stress than unstress condition, with V_{max} values of 5.00 and 3.87 nmol/min/mg protein, respectively. It is possible that the higher amount of glutamate taken up by salt stress cells could contribute to the better growth-promoting effect of glutamate on salt-stressed cells. This contention can be further supported by the evidence that the external supply of

glutamate could increase the intracellular glycine betaine in both salt-stressed and unstressed cells (Figure 3.7).

4.2 Polyamine metabolism

We have shown that the increase in the cellular level of polyamine in *Synechocystis* cells was induced by long-term osmotic stress, and to a lesser extent by salt stress. Furthermore, the main classes of polyamine induced by osmotic stress and salt stress were Spd and Spm, respectively. Put appeared to be unaffected by osmotic stress. It is likely that the Put level would rise initially in *Synechocystis* cells aided by the increased activity of ADC and would subsequently be converted to the more preferably accumulated Spd. The levels of polyamines accumulated in *Synechocystis* cells after salt and osmotic stress treatments were too low to account for any osmotic importance. The increase of various classes of polyamines observed inthis study represents a steadystate level reflecting the acclimatization of the cells toward the stress rather than the response mechanism.

Short-term exposure to all three UVs did not kill the cyanobacterial cells in this study. This was in contrast to what was observed in bacteria where UV-C illumination killed bacteria within a minute-time period (Bank et al., 1990). The cell wall of *Synechocystis* cells showed the ruffled appearance, mostly caused by UV-A exposure generating an indirect oxidative damage caused by reactive oxygen species (ROS)-induced lipid peroxidation of the membrane (Shimizu et al., 2006). The cell wall of cyanobacteria *Synechocystis* strain was identified as gram negative-like consisting of neutral sugars, amino sugars, proteins, and lipids. This suggested that the cell wall-disarrangement occurred as a result of the damage of the biochemical composition such as proteins and lipids of *Synechocystis* cell wall structure by UV-A-induced ROS. Thus, we could observe that during 60 min of UV-exposure, UV-A

likely changed the physiological appearance and reduced the survival ability of *Synechocystis* cells stronger than those by other shorter wavelength-UVs.

Chlorophyll a levels were lowered by all UVs treatments as compared to that by white light treatment. In particular during the first 15 min, UV radiations attacked *Synechocystis* cells with the resultant loss of about 20 - 25 % chlorophyll a from the initial level. This indicated that UV radiations, especially UV-A and UV-B quickly target the intracellular pigment chlorophyll a of *Synechocystis*. These results are in agreement with the reduction of chlorophyll content and photosynthetic efficiency in chloroplast of higher plants in response to UV-B radiation (Barbato et al., 2000; Smith et al., 2001). The pattern of the changes of carotenoids contents in response to UV radiations was similar to that observed for chlorophyll, a rapid decrease in the first 15 min and remained relatively unchanged up to 60 min. This indicated that under UV radiations the maintenance of both chlorophyll and carotenoids levels was crucial for the survival of *Synechocystis*. The slight reduction of growth rate under UV treatments observed in Figure 3.10 supports this contention.

Spermidine is a dominant polyamine in *Synechocystis* cells whereas only trace amounts of Put and Spm were observed (Figure 3.8). However, the dominant polyamine in each organism can be either the same or different pattern depending on strains, stress tolerance, and their changing environments. Our results are in line with a previous report showing an outstanding level of Spd in *Synechocystis* cells under ionic and osmotic stresses (Jantaro et al., 2003), and also with that from the leaves of *Nicotiana tabacum* L. cv Bel W3 but not with Bel B in response to 7-d exposure to UV-B radiation (Lütz et al., 2005). On the contrary, high Put level was found in UV-B treated plant *Phaseolus vulgaris* L. (Smith et al., 2001), and in tobacco (*Nicotiana tabacum* L. cv. *Petit Havana*) callus cultures under UV-C stress (Zacchini and Agazio, 2004). This indicated that the change of total polyamines in

response to environmental stress reflected the change of each dominant polyamine *in vivo* (Bouchereau et al., 1999; Groppa and Benavides, 2008; Incharoensakdi et al., 2010).

Long term stress by 350 mM NaCl and 500 mM sorbitol, respectively, did not affect growth of *Synechocystis* cells; however when coupled with UV radiation, a reduction in the growth rate occurred (Figure 3.10). Previously, we found that sorbitol at 700 mM had a more growth-inhibitory effect than NaCl stress (Jantaro et al., 2003). The growth of cells of cucumber (*Cucumis sativum*) and other dicotyledonous seedlings also showed different responses to three UV wavebands of short wavelengths UV-C, long wavelength UV-B and UV-A (Shinkle et al., 2004). Although, synergistic effect of Osmotic-UVC decreased the growth of cells within 3 h-treatment rather than either UVC or Ionic-UVC synergistic effect, *Synechocystis* growth was not declined completely. Many survival strategies of cell mechanisms are unknown, however, the changes of intracellular polyamine levels has been proposed to play a role (Chattopadhyay et al., 2003; 2006; Groppa and Benavides, 2007; Kusano et al., 2008).

Total intracellular polyamines are composed of both free form (PCA-soluble) and bound form (PCA-insoluble) of three common polyamines; Put, Spd and Spm. We found free polyamines as the major form in *Synechocystis*. Most synergistic stresses increased the accumulation of total PAs contents except for single osmotic stress at 500 mM sorbitol or osmotic-UVA synergistic stress which showed significant decreases of total PAs amounts. Existing Spd contents were highest among those three common polyamines which mainly sensed environmental stresses. Especially, Spd was conspicuously increased under high salinized conditions (Jantaro et al., 2003; Maiale et al., 2004; Tassoni et al., 2008). It has been previously reported in plants that high titers of spermidine and/or spermine, but not putrescine, are correlated with the response to acclimatize under long-term salinity (Sanchez et al., 2005; Jiménez-Bremont et al., 2007). Moreover, environmental stresses, either single

stress or concomitant stresses, accelerated apparently polyamines biosynthesis and the ratio of Spd/Put (Figure 3.11). In *Synechocystis*, arginine decarboxylase (ADC) is the first key enzyme to produce Put. To track the polyamine biosynthesis, transcript, enzyme and protein levels were monitored. Previously, our findings indicated that ADC enzyme activity and ADC protein levels were constant and did not tightly connected to *adc* transcript levels against salinity stress (Jantaro et al., 2003; 2006). The transcriptional level of *adc* genes seemed to be the main regulatory factors giving rise to total polyamines content in response to environmental stresses in *Synechocystis*, at least salinity and UV stresses.

The significant expressions of two genes encoding ADC (*adc*1 and *adc*2) in *Synechocystis* in response to single and synergistic stresses were different. Consistent to the polyamine contents, expression patterns suggested that polyamines titer was plausibly regulated under transcriptional level. The amount of *adc2* transcripts dominantly existed in *Synechocystis*. Long term ionic stress, generated by 350 mM NaCl in this study, conspicuously elicited the *adc1* increase with 6 times higher (Figure 3.12). *Synechocystis adc1* is considerably inducible by salt stress whereas *adc2* was constant, or slightly increased. Long term osmotic stress at 500 mM sorbitol, which slightly inhibited cell growth, caused an insignificant decrease on *adc* transcripts while the higher osmotic stress at 700 mM sorbitol, which extremely inhibited growth (Jantaro et al., 2003), induced *adc1* transcript amounts. These results also suggest that inducible *adc1* was expressed only by harmful hyperosmotic stress. Recently, Urano et al. (2004) found that the *Arabidopsis adc2* (*AtADC2*) is a key gene under salinity stress, which was induced by both ionic and osmotic stress. When the gene identity in cyanobase is concerned, *Synechechocystis adc1* has been mentioned in comparison to *Arabidopsis adc2*. Moreover, it is likely that *adc2* is a constitutive gene due to the lack of changes in *adc2* under salinity stress. This consideration is consistent with *AtADC1* gene expression which occurred at constant levels either under normal condition or salinity stress

condition (Urano et al., 2004). It was more interesting that the levels of *adc2* transcripts correlated to Put amounts whereas *adc1* transcripts did not, at least under ionic and osmotic stresses. In contrast with plants, ADC is found to be regulated at the post-translational level (Watson and Malmberg, 1996). The ratio of Spd/Put is somewhat related to the expressed pattern of *adc1* mRNA. However, all of these findings potentially indicated that the ADC was tightly connected to the salt stress response.

To gain more understanding on *adc* gene expression, synergistic stress-treated cells were used which encompassed concomitant osmotic and UV stress. The synergistic stress of salt-UVs apparently increased the amounts of a*dc1* transcripts. This was also the case for a slight increase of *adc1* mRNA by salt-UVC stress (Figure 3.12). It is worth mentioning that the induction of *adc1* transcripts under the salt-UVs combination was due mainly to ionic stress rather than UV irradiation. This might suggest that *Synechocystis adc1* gene is strongly salt stress-inducible, even though some transcript amounts were reduced by UVs, especially UVB and UVC. On the other hand, the changes due to sorbitol-UVs suggest intricate expressions, varying in each UV and sorbitol concentration. Osmotic-UVA and –UVB stresses at 500 mM sorbitol, with the exception of osmotic-UVC, enhanced *adc2* transcripts higher than *adc1* transcripts. This finding indicates that higher polyamines accumulation under 500 mM sorbitol-inducing stress synergized with UVC may be regulated at translational level instead of transcriptional level. Furthermore, osmotic (700 mM sorbitol)-UVB and –UVC synergistic stresses strikingly increased both *adc1* and *adc2* transcripts levels whereas osmotic (700 mM sorbitol)-UVA increased only *adc1* transcript level. Altogether, *Synechocystis adc1* gene is mainly inducible sensing environmental stresses, in particular salt stress, growth harmful- hyperosmotic stress, ionic-UVs and osmotic-UVs. Mostly, *adc2* gene is constitutive and unlikely changed, but clearly inducible by synergistic conditions of harsh environments; osmotic (500mM sorbitol)-UVA, -UVB, osmotic (700mM

sorbitol)-UVB and –UVC. These changes in *adc* gene expression have been reported previously in plants and were implicated in processes such as cell division, cell expansion or stress response (Bouchereau et al., 1999), all of which are important for cell growth and survival (Kusano et al., 2008).

We reported previously that *Synechocystis* sp. PCC6803 is able to transport putrescine and spermidine into the cells (Raksajit et al., 2006; Raksajit et al., 2009). In this study, we found that *Synechocystis* has the ability to transport spermine into the cell as well. *Synechocystis* cells grown in the presence of low spermine concentration showed faster initial growth than that lacking spermine. However, high amount of spermine decreased the growth rates. It should be noted that small amount of spermine act as the growth promoter. On the other hand, high spermine content is able to disrupt several metabolic functions within the cells by inhibition of protein biosynthesis accompanied by an irreversible dissociation of ribosomes (Ramakrishna et al., 1978).

Kinetic analysis of spermine uptake revealed that the uptake was saturable with Michaelis-Menten relationship. The spermine transport kinetics of *Synechocystis* indicated that the transport is a carrier-mediated process. However, the spermine uptake showed the low affinity around 141 μM. A similar situation was observed for putrescine and spermidine transport (Raksajit et al., 2006; Raksajit et al., 2009). These results suggested *Synechocystis* cells need small amount of polyamine to induce cell growth. Corresponding with the growth rates, the high polyamines concentration inhibited the cells growth. The induction of the spermine uptake was detected at low concentration of either NaCl or sorbitol. Increasing concentration caused a gradually declined uptake activity. Noticeably, cells required polyamine to protect cells against osmotic stress (Flores *et al.*, 1984, Soyka and Heyer, 1999).

In addition, we have studied the *in vitro* binding capacity with respect to polyamines of the binding protein of *Synechocystis* in order to clarify the characteristics of polyamine transport. Sets of genes involved in polyamine uptake have been identified in the annotated genome of *E. coli* so far (Pistocchi, 1993). Unlike *E. coli* and other bacterial species genes, the gene encoding the binding component subunit of *Synechocystis* (PotD) are not physically linked to those encoding the hydrophobic components, suggesting alternative regulatory pathways controlling the balanced expression of the transport system components (Pistocchi, et al., 1993). The transporters in cyanobacteria consist of several polypeptide subunits but their structure is not much related to the corresponding *E. coli* ones. Moreover, the low sequence identity between the PotD in *E. coli* and *Synechocystis* subunits does not allow identification of the *Synechocystis* ones using homology searches. Hence, it is necessary to clarify the details of the transport mechanism in *Synechocystis* since it is not obvious that the functions of PotD are identical in *E. coli* and *Synechocystis*.

The *potD* gene (*slr0401*) was cloned and overexpressed in *E. coli* as a recombinant His-tagged protein (rPotD). The SDS-PAGE gel showed an overexpression of the His-tagged PotD, with an apparent molecular mass of 43 kDa, which is slightly higher than the theoretical full-length native form of PotD (41 kDa) previously reported in other bacteria (Shah and Swiatlo, 2006). rPotD is capable of binding putrescine, spermidine as well as spermine although the affinity for putrescine is about 2-fold lower than that for spermidine. The K_d values for polyamine binding reported here are different from those previously reported by Brandt et al. (Brandt et al., 2010) using surface plasmon resonance (SPR) method. SPR measurements involved immobilization of rPotD on the surface of modified gold and, as a result, the protein could not maintain its native form (Oskarsson and Holmberg , 2006). In contrast, the binding studies using radioactive polyamines are quite sensitive and

the native form of rPotD was maintained during the experiment. Polyamines can effectively bind to the binding site of rPotD giving rise to reliable K_d values.

L-Amino acids such as arginine, asparagine, glutamic acid or lysine hardly inhibited spermidine binding. Spermidine showed higher inhibition (58%) of spermine binding activity than did spermine on spermidine binding activity (35%) (Table 3.5). This lent further support for the preference of rPotD to bind spermidine rather than spermine. The less favorable binding of spermine to rPotD might be related to the four positively charged nitrogen atoms leading to the hindrance structure of spermine.

Spermidine was more effective than putrescine in the induction of PotD in *Synechocystis*. In *E. coli*, spermidine was also shown to better induce the transcription of TPO5 gene than putrescine (Tachihara et al., 2005). The protein TPO5 had a role in the excretion of polyamine with higher capacity for putrescine than spermidine. For *Synechocystis*, the increase in PotD content by spermidine is necessary for the acclimation of cells to external spermidine, accompanied by uptake and accumulation of spermidine. Since high intracellular spermidine is inhibitory to cell growth (Raksajit et al., 2009), we found that the increase of PotD is beneficial to the cells due to the ability of PotD to excrete spermidine into the medium (data not shown). This would enable the cells to maintain a low level of intracellular spermidine when exposed to high external concentration of spermidine.

CHAPTER V

CONCLUSION

We have undertaken physiological and biochemical studies of some useful and valuable compounds in two unicellular cyanobacteria, *Aphanothece halophytica* and *Synechocystis* sp. PCC 6803. The compounds under study are amino acids, glutamate and glycine betaine in particular, as well as polyamine using *A. halophytica* and *Synechocystis* as specimen, respectively. A considerable part of the study is devoted to metabolism of polyamine in *Synechocystis* with the emphasis on environmental effects on changes in polyamine contents, the characteristics of polyamine binding as well as the transport of spermine are also investigated. Such investigation has resulted in two international publications. Another possible publication concerning the combined effect of osmotic and UV stresses on polyamine contents is now in the preparation stage.

For glutamate metabolism, we have been able to characterize the changes in the contents in *A. halophytica* under salt stress. The kinetic studies on glutamate uptake can partly explain its role for salt stress tolerance in *A. halophytica*. The findings in glutamate uptake study are now organized to make another international publication. In conclusion, this project has proved to be successful in terms of the output as well as providing basic knowledge necessary for further improvement of the production of amino acids and polyamine using cyanobacteria.

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APPENDIX

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2. Yodsang, P., Raksajit, W., Brandt, A.M., Salminen, T.A., Mäenpää, P. and Incharoensakdi, A. 2011. Recombinant polyamine binding protein of *Synechocystis* sp. PCC 6803 specifically binds to and is induced by polyamines. **Biochemistry (Moscow)** 76(6): 713-719.
3. Characterization of glutamate transport in a halotolerant cyanobacterium *Aphanothece halophytica* (in preparation)
4. Effect of UV-B and UV-C radiation on polyamine content in osmotically-stressed *Synechocystis* sp. PCC 6803 (in preparation)

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Education

1976	B.Sc.	Biochemistry, Victoria University of Wellington, New Zealand
1979	M.Sc.	Biochemistry, Mahidol University, Thailand
1986	Ph.D.	Plant Biochemistry, Nagoya University, Japan
2000, 2002		Postdoctoral, Meijo University, Nagoya, Japan

Research Interests

- Mechanism of salinity tolerance in cyanobacteria
- Biochemical responses to osmotic stress in cyanobacteria
- Signal transduction in cyanobacteria under environmental stress
- Polyamines metabolism in cyanobacteria
- Solutes transport in cyanobacteria
- Biohydrogen as an alternative energy source
- Biotechnology utilizing cyanobacteria as starting materials

Publications in the last 5 years

1. Baebprasert W, Karnchanatat A, Lindblad P, **Incharoensakdi A.** (2011) Na^+-stimulated nitrate uptake with increased activity under osmotic upshift in *Synechocystis* sp. strain PCC 6803. World J. Microbiol. Biotechnol. (in press)

2. Baebprasert W, Jantaro S, Khetkorn W, Lindblad P, **Incharoensakdi A.** (2011) Increased $H(_2)$ production in the cyanobacterium Synechocystis sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. Metab Eng. 13:610-616.

3. Yodsang P, Raksajit W, Brandt A-M, Salminen T.A, Mäenpää P, **Incharoensakdi A.** (2011) Recombinant polyamine-binding protein of *Synechocystis* sp. PCC 6803 specifically binds to and is induced by polyamines. Biochemistry (Moscow) 76: 713-719.

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11. Baebprasert W, Lindblad P, **Incharoensakdi A** (2010) Response of H_2 production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Int. J. Hydrogen Energy. 35: 6611-6616.

12. Bualuang A, Soontharapirakkul K, **Incharoensakdi A** (2010) Na^+/H^+ exchange activity in the alkaliphile halotolerant cyanobacterium *Aphanothece halophytica*. J. Appl. Phycol. 22: 123-129.

13. Brandt AM, Raksajit W, Mulo P, **Incharoensakdi A**, Salminen TA, Mäenpää P (2009) Transcriptional regulation and structural modeling of the FutC subunit of an ABC-type iron transporter in *Synechocystis* sp. strain PCC 6803. Arch. Microbiol. 191: 561-570.

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Professional Experiences

1. Editorial Board Member of ScienceAsia since June 2008 (Formerly, Journal of Science Society of Thailand), now listed in ISI-Web of Science
2. Member of Japanese Society of Plant Physiologists
3. Recipient of research awards (2002, 2006, 2009) from the National Research Council of Thailand
4. Recipient of research awards (2001, 2005) from Chulalongkorn University (CU)
5. Head of Department of Biochemistry (CU) 2004-2008
6. Outstanding researcher award from Chulalongkorn University (2008)

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Research Interests

- Cyanobacteria biochemistry
- Adaptation to stress responses

Publications in the last 5 years

1 Baebprasert W, **Jantaro S**, Khetkorn W, Lindblad P, Incharoensakdi A.(2011) Increased $H(_2)$ production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. Metab Eng. 13:610-616.

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