ผลของความเครียดจากความเป็นกรดด่างต่อปริมาณพอลิเอมีนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาวอภิญญาณ บุญประกอบกูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมีและชีววิทยาโมเลกุล ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

EFFECTS OF pH STRESS ON POLYAMINE CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

Miss Arpinyan Boonprakobkul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2013

Thesis Title	EFFECTS OF pH STRESS ON POLYAMINE CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803
By	Miss Arpinyan Boonprakobkul
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Assistant Professor Saowarath Jantaro, Ph.D.
Thesis Co-Advisor	Professor Aran Incharoensakdi, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

……... Chairman
(Professor Anchalee Tassanakajon, Ph.D.)

……..…... Thesis Advisor
(Assistant Professor Saowarath Jantaro, Ph.D.)

……..….. Thesis Co-Advisor
(Professor Aran Incharoensakdi, Ph.D.)

……. Examiner
(Assistant Professor Nuchanat Wutipraditkul, Ph.D.)

……. External Examiner
(Assistant Professor Saranya Phunpruch, Dr.rer.nat)

อภิญญาณ บุญประกอบกูล : ผลของความเครียดจากความเป็นกรดด่างต่อปริมาณพอลิเอมีนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (EFFECTS OF pH STRESS ON POLYAMINE CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร. อรัญ อินเจริญศักดิ์, 93 หน้า.

งานวิจัยนี้ได้ศึกษาผลของภาวะเครียดจากพีเอชต่อการเจริญ รงควัตถุภายในเซลล์และปริมาณพอลิเอมีนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 ภาวะเครียดระยะยาวจากพีเอชในช่วงพีเอช 6.5 – 8.5 ไม่มีผลกระทบต่อการเจริญของเซลล์ ปริมาณคลอโรฟิลล์ เอ และแคโรทีนอยด์ ขณะที่ภาวะที่มีพีเอช 5.5 ยับยั้งการเจริญของเซลล์และการสะสมของปริมาณรงควัตถุภายในเซลล์ สำหรับภาวะเครียดระยะสั้นภายในเวลา 4 ชั่วโมง นั้น ภาวะที่เป็นกรด ที่ พีเอช 2 และพีเอช 4 แสดงการเจริญของเซลล์ที่ลดลงอย่างมีนัยสำคัญเปรียบเทียบกับภาวะควบคุม นอกจากนี้ภาวะเครียดจากกรดที่พีเอช 2 ยังยับยั้งการสะสมของคลอโรฟิลล์ เอ อย่างสูง เช่นเดียวกันกับยับยั้งประสิทธิภาพการสังเคราะห์ด้วยแสงของเซลล์ *Synechocystis* ด้วย ผลการทดลองได้รับการยืนยันจากการลดลงของปริมาณทรานสคริปต์ของยีนคลอโรฟิลล์ เอ ซินเทส ซึ่งส่งผลกระทบต่อการเปลี่ยนสีของเซลล์ที่เพาะเลี้ยง สำหรับพอลิเอมีนมีการสะสมมากในรูปแบบอิสระ โดยเฉพาะสเปอร์มิดีนซึ่งปรากฏเป็นหลักในทุกภาวะที่ศึกษา นอกจากนี้ยังเกิดการรักษาระดับพอลิเอมีนในรูปแบบอิสระรวมอย่างสัมพันธ์กันภายใต้ภาวะเครียดทั้งกรดและด่าง ในขณะที่รูปแบบบาวน์ของพอลิเอมีนเหล่านั้นมีปริมาณขึ้นลงไม่แน่นอน โดยเฉพาะอย่างยิ่ง ภาวะเครียดจากด่างได้เหนี่ยวนำให้เกิดพอลิเอมีนรูปแบบบาวน์มากกว่าภาวะเครียดจากกรดอย่างมีนัยสำคัญ การทดลองของการเปลี่ยนค่าพีเอชบ่งชี้ถึงกลไกที่เกี่ยวข้องกับการปรับตัวของเซลล์ต่อภาวะเครียดจากพีเอช ตั้งแต่ที่ระดับพอลิเอมีนของเซลล์ที่เพิ่งประสบกับภาวะเครียดที่เป็นด่าง ถูกเหนี่ยวนำเพิ่มขึ้นในทันทีภายหลังที่เซลล์ได้ถูกเปลี่ยนมาเจอกับภาวะเครียดที่พีเอช 2 โดยที่พีเอชรอบนอกเซลล์ไม่มีการเปลี่ยนแปลงตลอดการทดลอง ทั้งนี้คำตอบของกลไกแรกที่ตอบสนองต่อภาวะเครียดจากกรดคือ การปรับสมดุลพีเอชภายในเซลล์ด้วยผลิตภัณฑ์จากกระบวนการแคแทบอลิซึมของอาร์จินีนในกรณีนี้คือพอลิเอมีน ในทางตรงกันข้าม เซลล์ *Synechocystis* ดูเหมือนว่าจะหลั่งพอลิเอมีนออกนอกเซลล์สูง ซึ่งพบว่าสอดคล้องกันกับการเพิ่มขึ้นของค่าพีเอชของอาหารเพาะเลี้ยงเซลล์ภายใต้ภาวะเครียดที่เป็นด่าง นอกจากนี้ได้ติดตามปริมาณของยีนทั้ง *adc* (รวมถึง *adc1* และ *adc2*) ที่เข้ารหัสอาร์จินีนดีคาร์บอกซิเลส และยีน *speB2* ที่เข้ารหัสแอ็กมาทิเนสซึ่งเกี่ยวข้องกับการสังเคราะห์พิวเทรสซีน โดยได้ตรวจวัดระดับทรานสคริปต์และโปรตีนด้วยเทคนิค RT-PCR และ Western blotting ตามลำดับ ระดับของยีนที่เกี่ยวข้องกับชีวสังเคราะห์ที่รวมถึง *adc* และ *speB*2 mRNAs ลดลงอย่างมากภายใต้ภาวะเครียดจากกรด ขณะที่เพิ่มขึ้นอย่างมีนัยสำคัญภายใต้ภาวะที่เป็นด่าง อย่างไรก็ตาม ระดับของโปรตีน ADC มีปริมาณลดลงเล็กน้อยภายใต้ภาวะเครียดจากกรด บ่งชี้ได้ว่าปริมาณพอลิเอมีนซึ่งถูกเหนี่ยวนำขึ้นโดยภาวะเครียดจากกรดอาจถูกควบคุมภายใต้ระดับโปรตีน สำหรับยีน *potD* ที่เกี่ยวข้องกับการขับพอลิเอมีนออกจากเซลล์แสดงการลดลงอย่างมากภายใต้ภาวะเครียดจากกรด ในขณะที่ยีน *pao* ที่เกี่ยวข้องกับการสลายพอลิเอมีนมีการเพิ่มขึ้นเล็กน้อยที่เวลาการทดลอง 120 นาที สำหรับภาวะเครียดจากด่างที่พีเอช 12 พบว่า ยีนที่เกี่ยวข้องกับการสังเคราะห์พอลิเอมีน ในที่นี้คือ *adc1* และ *adc2* mRNAs ถูกเหนี่ยวนำให้เพิ่มขึ้นอย่างเห็นได้ชัดที่เวลาการทดลอง 120 นาที ในขณะที่ปริมาณทรานสคริปต์ของ *speB2* น้อยกว่าชุดควบคุม การเพิ่มขึ้นอย่างสูงของปริมาณ mRNA ของ *potD* เกิดขึ้นอย่างมีนัยสำคัญภายใต้ภาวะเครียดจากด่าง ขณะที่ระดับ mRNA ของ *pao* ลดลงเมื่อเปรียบเทียบกับชุดควบคุม

ภาควิชา……ชีวเคมี……. ลายมือชื่อนิสิต……..…..……...…

สาขาวิชา…ชีวเคมีและชีววิทยาโมเลกุล….. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……..……..……...

ปีการศึกษา……2556...……... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม…….…....……

#5372377123: MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
KEYWORDS: POLYAMINES / INTRACELLUAR PIGMENTS/ pH STRESS / *Synechocystis* sp. PCC 6803

ARPINYAN BOONPRAKOBKUL : EFFECTS OF pH STRESS ON POLYAMINE CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. ADVISOR : ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., 93 pp.

Effect of pH stress on growth, intracellular pigments and polyamine contents in cyanobacterium *Synechocystis* sp. PCC 6803 was investigated. Long-term pH adapted condition in a range of pH 6.5 - 8.5 did not affect cell growth, chlorophyll *a* and carotenoid contents while pH 5.5 condition inhibited cell growth and the accumulation of intracellular pigments. For short-term stress of 4 h-period, acidic conditions at pH 2 and pH 4 influenced the significant decrease on cell growth compared to control. Moreover, acid stress at pH 2 highly inhibited the accumulation of chlorophyll *a*, as well as photosynthetic efficiency of *Synechocystis* cells. This result was corroborated by the decrease of chlorophyll *a* synthase gene transcript under acid stress treatment, thereby affecting on the changed color of cell culture. Free form-polyamine was found as a major form, and spermidine was present dominantly in all conditions. In addition, total free form-polyamines were maintained relatively under both tolerable acid and alkaline stresses whereas their bound form was mainly fluctuated. In particular, alkaline stress induced the titer of bound form-polyamines higher than acid stress did. The pH transition experiment was directed to the cellular adaptive mechanism against pH stress. Since the polyamine level of alkaline-stressed cells was immediately induced after transferring to pH 2 stress with unchanged ambient pH along treatment, the answer of first mechanism responded to acid stress was the internal pH balance of products from arginine catabolism, herein polyamines. In contrast, *Synechocystis* cells seemed to highly secrete polyamine in to culture medium corresponding to the increase of cell culture pH under alkaline condition. The amounts of both *adc* (including *adc1* and *adc2*) gene, encoding arginine decarboxylase, and *speB2* gene, encoding agmatinase, related to putrescine synthesis were determined. The transcript and protein levels were analyzed by RT-PCR and Western blotting, respectively. The level of biosynthetic genes including *adc* and *speB2* mRNAs were reduced strongly under acid stressed condition whereas they were induced significantly under alkaline condition. However, ADC protein level was slightly decreased under acid stress. It was suggestedthat polyamine content induced by acid stress might be regulated at protein level. The *potD* gene relating to polyamine secretion showed a high reduction by acid stress whereas *pao*gene, in polyamine degradation, was slightly increased after 120 min-treatment. For alkaline stress at pH 12, the polyamine biosynthesis genes of *adc1* and *adc2* mRNAs were induced obviously after 120 min-treatment whereas *speB2* transcript amount was lower than that of control. A high induction of *potD* mRNA amount was observed significantly under alkaline stress whereas *pao* mRNA level was decreased comparing with that of control.

Department : …...Biochemistry..…... Student's Signature…….....…....
Field of Study:…Biochemistry and... Advisor's Signature…….……...….
…Molecular Biology. Co-advisor's Signature……..…….....
Academic Year: ….....2013……....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Saowarath Jantaro, and my co-advisor Professor Dr. Aran Incharoensakdi, for their excellence guidance, encouragement, instruction and support throughout this thesis.

My gratitude is also extended to Professor Dr. Anchalee Tassanakajon, Assistant Professor Dr. Nuchanat Wutipraditkul and Assistant Professor Dr. Saranya Phunpruch for serving as thesis committee, for their available comments and useful suggestions.

My appreciation is also expressed to Dr. Nanthika Kongchareonporn for kindly providing the ADC antibodies.

Sincere thanks are also extended to all members and friends in Biochemistry department including Dr.Wanthanee, Bongkoj, Apiradee and Khanitha for their warm assistance and friendship.

The warmest gratitude is extended to my family and Dr. Surasak Laloknam for their understanding, patience, helping and support. My relatives and friends are also thanked for their lovely friendship and will support.

CONTENTS

PAGE

PAGE

PAGE

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

PAGE

PAGE

LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
bp	Base pair
BSA	Bovine serum albumin
^{o}C	Degree Celsius
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
g	Gram
HEPES	Hydroxyethyl piperazineethanesulfonic acid
kb	Kilo base
kDa	Kilo Dalton
h	hour
L	Liter
LHC	Light harvesting chlorophylla/b-protein complex
mM	Millimolar
min	Minute
µg	Microgram
µl	Microliter

ml	Milliliter
mM	Millimolar
M	Molar
nm	nanometer
OD	Optical density
PAs	Polyamines
PCR	Polymerase Chain Reaction
Put	Putrescine
PMSF	Phenylmethylsulfonyl fluoride
RT-PCR	Reverse transcription- Polymerase Chain Reaction
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
Spd	Spermidine
Spm	Spermine
TAE	Tris-acetate electrophoresis
TEMED	Tetramethylethylenediamine

CHAPTER I

INTRODUCTION

1.1 Abiotic stress

Abiotic stress is the most harmful stress generated by non-living factors influenced on living organisms, concerning growth and productivity. The most severe problem of abiotic stress as shown in Figure 1.1, such as temperature, pH, ionic strength, light and drought, is essentially unavoidable. Most plants have a tolerance or sensitivity limitation in response to abiotic stress. Some acclimation mechanisms have been intensively studied in *Arabidopsis thaliana*, *Synechocystis* sp. PCC 6803 and other photosynthetic organisms (Ohta *et al*., 2005).

1.1.1 pH stress

The pH stress encompasses both acidic and alkaline effects (Figure 1.1). Acid rain and acid soil are also considered as ones of most serious environmental problems. Presently, there are few studies about the acclimatization process to acid stress in plants, algae and cyanobacteria (Olson, 1993; Foster, 1995; Bearson *et al*., 1997). Acid stress responses have been focused widely in microorganisms such as *Escherichia coli* (Gale and Epps, 1942) and *Salmonella typhimurium* (Foster and Hall, 1990). The proposed strategies used to survive against acid stress are varied among the microorganisms observed. Some microbes utilize amino acid decarboxylases to neutralize the external environment (Gale and Epps, 1942), while others utilize arginine catabolism to increase and balance their intracellular pH (Casiano-Colon and Marquis, 1988; Rallu *et al*., 1996). Some microorganisms secreted a biofilm to slow

down the diffusion of molecules into the cell, while others were able to change their cell membrane to incorporate substances, such as fatty acids, that protected the cell. Another important way that microbes passively regulated their pH was the secretion of buffer molecules in order to raise pH (González-Toril *et al*., 2003). If cells are to survive in an alkaline environment, they must make their cytoplasm more acidic to buffer the alkalinity (Horikoshi, 1999). Under *in vitro* and *in vivo*, cyanobacteria have generally been reported to prefer neutral to slightly alkaline medium for optimum growth. However, in nature, cyanobacteria have the capacity to extend their distribution to pH values as low as 4 (Kurian *et al*., 2006). In 2002, Huang and colleagues found that *Synechocystis* sp. PCC 6308 (not 6803) did not grow at a pH below 4.4, and cell color had changed to blue, leading to the damage to photosystem II. Altogether, the survival mechanism coping pH stress in cyanobacteria is still unclear.

A)

B)

C)

Figure 1.1 Types of extreme environments such as acidic spring (A), alkaline lake (B) and salt lake (C). (Source: http://serc.carleton.edu/images/microbelife/extreme /acidic/Lemonade_Spring.jpg, http://serc.carleton.edu/images/microbelife/extreme /alkaline/Mono_Lake_Island_Spring.jpg, http://serc.carleton.edu/images/microbelife /extreme/hypersaline/Spiral_Jetty.jpg)

1.1.2 Polyamines and environmental stresses

The flexibility of the metabolism of microbes enables them to respond and adapt to environmental changes outside their range for optimal growth (Poole, 1999). Polyamine metabolism is responsive to wide arrays of environmental stresses (Bouchereau *et al.*, 1999; Incharoensakdi *et al*., 2010). The levels of polyamines are an integral response of living organisms to various stresses, such as osmotic, salinity, heat, drought and UV radiation. During salt stress, the pattern of polyamine contents was changed depending on tissue, species, salt concentration and duration of the treatment. Jantaro *et al*. (2003) reported that total polyamine content of *Synechocystis* sp. PCC 6803 was apparently increased under both salt and osmotic stresses. On the other hand, increased UV-B radiation resulting from ozone depletion is one of global environmental problems, which has dangerous effects to plant on the earth (Pang *et al*., 2007). Polyamines have been reported to play an important role in the protection of plants against UV-B damage and drought tolerance. In cucumber leaves, polyamine accumulation is one of the adaptive mechanisms to cope with the radiation of UV-B (An *et al*., 2004). Pothipongsa and colleagues (2012) found that intracellular polyamines of *Synechocystis* sp. PCC 6803 was increased about 2- and 4-fold under NaCl- and sorbitol-treatment, respectively, whereas polyamines content of irradiated cells with UV-B for 1 h were increased about 3-fold in NaCl-stressed but not sorbitol-stressed cells. Moreover, spermidine potentially delayed senescence in drought-stressed jack pine (Rajasekaran and Blake, 1999). Not only commonly short-chained polyamines, heat stress upon rice callus was also associated with the production of long-chained polyamines and several changes in polyamine metabolism (Roy and Ghosh, 1996).

1.2 Polyamines

1.2.1 Physiology

The aliphatic amines, namely polyamines, are naturally occurring substances in all organisms. The most common polyamines are triamine spermidine (1,8-diamino-4-azaoctane), tetramine spermine (1,12-diamino-4,9-diazadodecane) and their diamine precursor putrescine (1,4-diaminobutane). Their structures are shown in Figure 1.2. In cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) those three commons, with spermine representing a minor content, were found (Jantaro *et al*., 2003). Polyamines may occur not only as free molecules (free form) in cytosolic fraction, but also as cell wall bound or conjugated to many compounds (bound form), such as phenolics, inside the cell (Smith *et al*., 2001). Polyamines play important roles on cell differentiation and proliferation in eukaryotic and prokaryotic cells, as well as stress rescuers and indicators (Bouchereau *et al.,* 1999; Jantaro *et al.,* 2010). They are also implicated in a wide range of diverse environmental stresses, which include drought, salinity, low temperature, oxidative stress and metal toxicity (Hussain *et al*., 2011). Polyamines are basic amino acids which are positively charged at physiological pH that bind strongly *in vitro* to negatively charged compounds, such as nucleic acids (Feurstein and Marton, 1989; Bouchereau *et al.*, 1999), acidic phospholipids (Tadolini *et al*., 1985; Bouchereau *et al*., 1999) and many types of proteins, including numerous enzymes whose activities are directly modulated by polyamine binding (Carley *et al*., 1983; Bouchereau *et al.*, 1999). These ionic interactions are important in regulating the structure and function of biological macromolecules, as well as their synthesis *in vivo* (Jacob and Settler, 1989; Bouchereau *et al.*, 1999). In higher plants, polyamines were occurred dominantly in

free form, bound electrostatically to negatively charged molecules, and conjugated to small molecules and proteins (Martin-Tanguy, 1997). Moreover, polyamines are found in many organelles, such as nucleus, cytoplasm which probably indicates their role in the control of diverse fundamental cellular processes (Bouchereau *et al.*, 1999; Kaur-Sawhney *et al*., 2003).

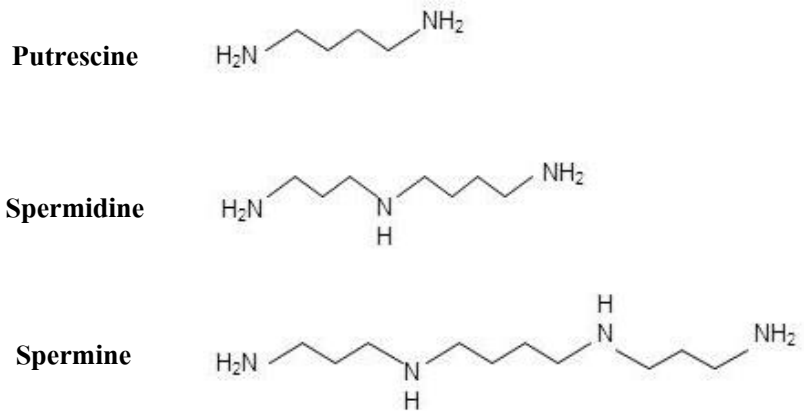

Figure 1.2 The structure of putrescine (Put; $NH_2(CH_2)_4NH_2$), spermidine (Spd; $NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine (Spm; $NH_2\ (CH_2)_3\ NH\ (CH_2)_4NH(CH_2)_3NH_2$) (Source: http//en.wikipedia.org/wiki)

1.2.2 Polyamine biosynthesis

The polyamine (PA) biosynthetic pathway is varied in different patterns depending on kingdom and/or species. In plants and bacteria, putrescine is formed either by direct decarboxylation of L-ornithine catalyzed by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17), or by decarboxylation of arginine by arginine decarboxylase (ADC; EC 4.1.1.19) via two important intermediates; agmatine and *N*-carbamoylputrescine, respectively, which is subsequently converted to putrescine (Figure 1.3). In mammals and fungi, only one pathway (ODC reaction) leads to putrescine formation (Walters, 2000). The organelles locating ODC in animals and plants are cytoplasm and nucleus (Voigt *et al*., 2000). For ADC, it was present in two different compartments: chloroplasts in leave (photosynthesizing organelle) and nuclei in roots (nonphotosynthesizing organelle), which may relate to specific functions of ADC in different cell compartments (Borrell *et al*., 1995; Bortolotti *et al*., 2004). Moreover, the spinach ADC was found the association with LHC of photosystem II. PAs synthesized in chloroplasts evidently stabilized photosynthetic complexes of thylakoid membranes under stress conditions (Legocka and Zaichert, 1999).

Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group onto putrescine and spermidine, respectively, catalyzed by enzyme spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively. The aminopropyl group is donated from decarboxylated *S*-adenosylmethionine (decarboxylated-SAM), which is catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Incharoensakdi *et al*., 2010). Aside from participating in numerous transmethylation reactions, as it does in

other organisms, SAM is also a precursor of the plant hormone ethylene. On the other hand, another type of polyamines found in some organisms is cadaverine. The diamine cadaverine is synthesized predominantly as the result of lysine decarboxylase (LDC; EC 4.1.1.18) activity from lysine. This diamine is not as widely distributed as putrescine and is mainly found in Leguminoseae and in the flowers of Arum lilies (Smith and Meeuse, 1976; Incharoensakdi *et al*., 2010).

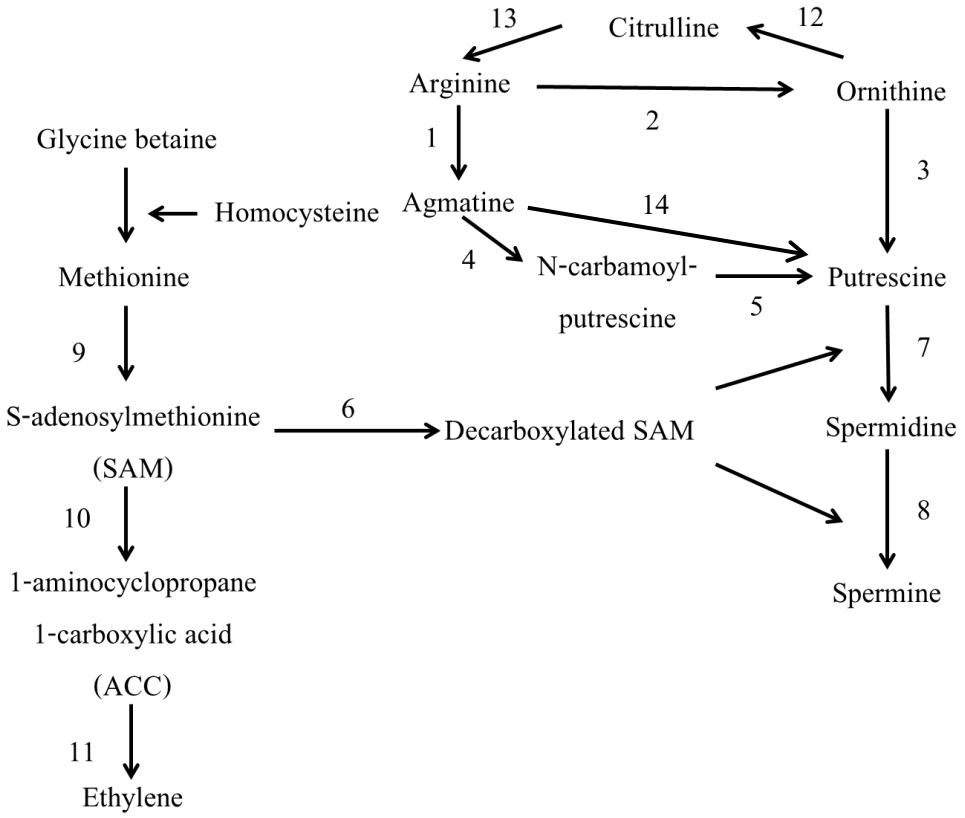

Figure 1.3 The common pathway of polyamine biosynthesis in plant and cyanobacteria (modified from Bouchereau *et al*., 1999; Incharoensakdi *et al*., 2010). **1**, Arginine decarboxylase (ADC); **2**, Arginase; **3**, Ornithine decarboxylase (ODC); **4**, Agmatine iminohydrolase; **5**, *N*-carbamoyl putrescine amidohydrolase; **6**, *S*adenosylmethionine decarboxylase (SAMDC); **7**, Spermidine synthase; **8**, Spermine synthase; **9**, SAM synthase; **10**, ACC synthase; **11**, ACC oxidase; **12**, Ornithine transcarbamylase; **13**, Arginine synthase; **14**, Agmatinase.

1.2.3 Polyamine degradation

Polyamines are oxidatively deaminated by action of amine oxidases (Figure 1.4). These enzymes include the copper containing diamine oxidases (DAO; EC 1.4.3.6), flavoprotein polyamine oxidase (PAO; EC 1.5.3.3). DAO was found to highly substrates specificity on diamine whereas PAO preferentially oxidizes spermidine and spermine at their secondary amino group (Bouchereau *et al*., 1999). The products of DAO reaction, from putrescine, are pyrroline, hydrogen peroxide and ammonia, whereas PAO yields pyrroline, 1,5-diazabicylcononane, respectively, diaminopropane (DAP) and hydrogen peroxide. Diaminopropane can be metabolized to β-alanine, while pyrroline can be converted to ɣ-aminobutyric acid (GABA) by pyrroline dehydrogenase (PDH). GABA can then be transaminated and oxidized to form succinic acid which is supplied to Krebs cycle (Flores and Filner, 1985). Also, these pathways reveal that the carbon and nitrogen elements resulting from putresine are recycled (Walters, 2000). Not only the elimination of polyamines inside the cells, but polyamine catabolism also involved in physiological processes (Bouchereau *et al*., 1999). Nevertheless, bacteria and fungi have different polyamines degradative enzymes (Bagni and Tassoni, 2001).

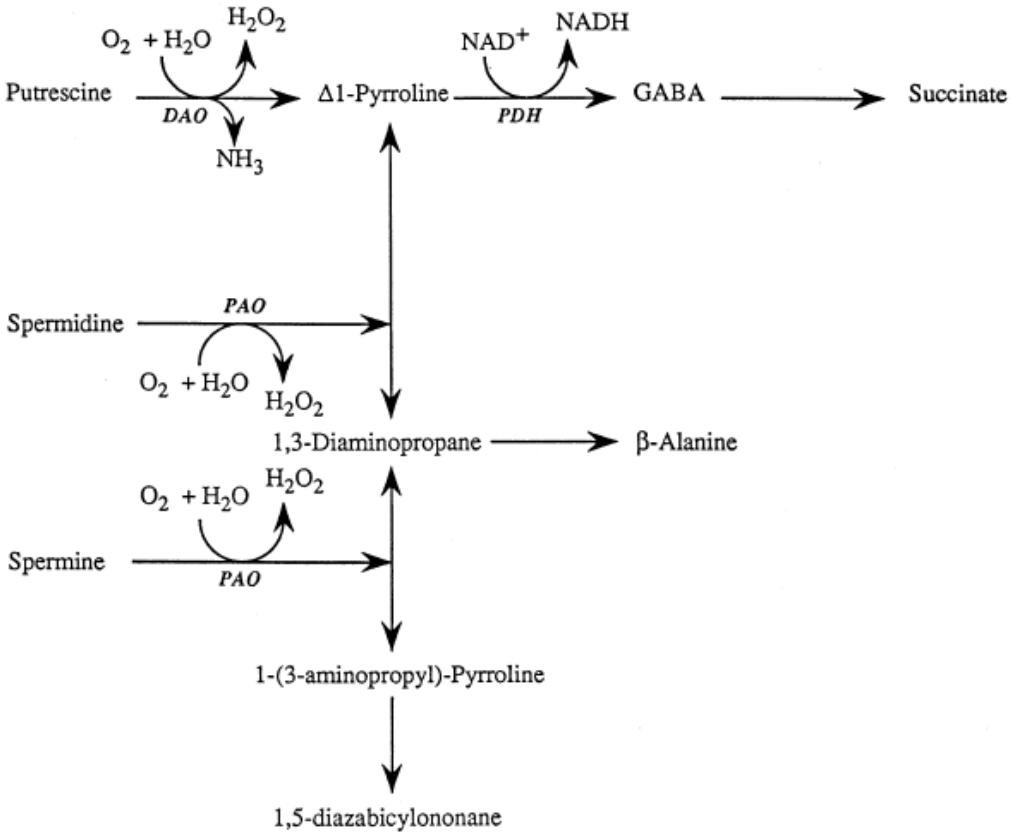

Figure 1.4 Schematic representation of polyamine degradation (Bouchereau *et al*., 1999). Amine oxidase. DAO, diamine oxidase; GABA, 7-aminobutyric acid; PAO, polyamine oxidase; PDH, pyrroline dehydrogenase.

1.2.4 Polyamine conjugation

In nature, polyamines often occur as free form, but they can also be associated with small molecules (so-called conjugated forms), such as, phenolic acids, and also to various macromolecules (namely, bound forms), such as protein and enzyme (Martin-Tanguy, 1997). The most common conjugated polyamines are covalently linked to cinnamic acids which have been observed in higher plants and are correlated with developmental phenomena. This conjugation occurs as water-soluble or water-insoluble forms. In the former, the single amine group of an aliphatic amine is linked with a phenolic cinnamic acid. The water-soluble forms can be divided into two classes. Firstly, each terminal amine group of an aliphatic amine is bound to cinnamic acid, while in the second class, the amine group of aromatic amine is linked to cinnamic acid. These amine conjugates were found in roots but were not normally found in shoots. In the root of tobacco, increases in water-soluble and water-insoluble conjugates were shown significantly before flowering (Martin-Tanguy *et al*., 1990) and occurred in shoot apices upon floral initiation (Havelange *et al*., 1996). The synthesis of polyamine conjugates was observed also in tobacco callus (Negrel, 1989). They were conjugated by the formation of an amide linkage, utilizing esters of CoA for the provision of the activated carboxyl groups (Negrel, 1989), which were formed by non-specific putrescine caffeyl-coenzyme A (CoA) transferase. Therefore, the synthesis of the water-insoluble polyamines, such as di-p-coumaroylputrescine, di-p-coumaroylspermidine, di-feruloylputrescine and di-feruloylspermidine has not been reported in higher plants (Martin-Tanguy, 1997). Furthermore, posttranslational covalent linkage of polyamines to protein was catalyzed by a class of enzymes known as transglutaminases (EC; 2.3.2.13) (Margosiak *et al*., 1990).

1.2.5 Polyamine transport

The transport of polyamines across the plasmalema of plant cells is energy-dependent and it is now clear that calcium is involved in the uptake mechanism. Indeed, calcium-activated putrescine uptake could be markedly reduced by treatments which reduced calmodulin action or activities of protein kinases or phosphatase (Antognoni *et al*., 1995). Polyamine-specific carriers were widely distributed in prokaryotes and eukaryotes and could replenish polyamine pools upon inhibition of the biosynthetic enzymes (Seiler *et al*., 1996). The transport of polyamines classified as ABC (ATP binding cassette)-type transports (Higgins, 1992) have been well characterized in bacteria, yeast, parasite, animals (Rinehart and Chen, 1984; Igarashi and Kashiwagi, 1999; Tassoni *et al*., 2002) and cyanobacterium *Synechocystis* sp. PCC 6803 (Raksajit *et al*., 2006). The ABC transporter consists of a membrane associated ATPase (PotA), two transmembrane proteins (PotB and PotC), and a periplasmic substrate-binding protein (PotD) (Igarashi and Kashiwagi, 1999). PotD is the periplasmic substrate-binding protein that recognize and facilitate the transport of the polyamines. PotD preferentially binds spermidine, but can also bind putrescine with a lower affinity. The uptake of putrescine and spermidine into *Synechocystis* cells was pH dependent with highest activity at pH 7.0 and 8.0, respectively. Moreover, the transport of putrescine and spermindine were energy-dependent (Raksajit *et al*., 2006; 2009).

1.3 *Synechocystis* sp. PCC 6803

Cyanobacteria can be classified in the Procaryota, Division of Cyanophyta and Cyanophyseae class. The bacteria and cyanobacteria lack mitochondria, chloroplast,

true vacuoles, endoplasmic reticular. There is no membrane bounded chloroplast; in cyanobacteria the photosynthetic lamellae are usually distributed in the peripheral cytoplasm. Cells of cyanobacteria are surrounded by the cytoplasmic membrane, the cell wall which contains an outer membrane and peptidoglycan layer, and in many cases, a glycocalyx layer (Figure 1.5). The outer membrane functions are more as a passive molecular sieve, whereas the cytoplasmic membrane serves as a true selective permeability barrier (Gantt, 1994). Among prokaryotes, cyanobacteria are the only organisms to engage in oxygenic photosynthesis, and there is an evidence to suggest that they are the progenitor(s) of plant plastids. Their phylogenetic position in the bacterial kingdom is still obscure, although recent analysis of ancient genes has indicated a genetic relationship with gram-positive bacteria (Xiong *et al*., 2000).

Synechocystis sp. PCC 6803 is a unicellular non-nitrogen (N_2)-fixing cyanobacterium and ubiquitous in habitat of fresh water. It has been one of the most popular organisms for genetic and physiological studies of photosynthesis with two major reasons; it is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982) and grows heterotrophically at the expense of glucose (Rippka *et al*., 1979; Pothipongsa, 2009). The entire genome of *Synechocystis* sp. PCC 6803 was sequenced in 1996 (Kaneko *et al*., 1996). It was also the first photoautotrophic organism whose genome was fully sequenced. *Synechocystis* has been defined as a unicellular coccoid, or spherical cyanobacterium lacking gas vesicles or sheath. They divide by binary fission at two or three successive plans (Ikeuchi and Tabata, 2001). There are four culture substrains of *Synechocystis* including PCC, ATCC, GT (Glucose-Tolerant) and Kazusa, all of them were derived from the Berkeley strain 6803, which was isolated from a fresh water in California by R. Kunisawa (Stanier *et*

al., 1971) (Figure 1.6). The circular genome was originally deduced to be 3,573,470 bp long (Figure 1.7). The average GC content is 47.7% (Kaneko *et al*., 1996). *Synechocystis* cells has several features that make this strain particularly suitable for studying stress response at molecular level (Glatz *et al*., 1999). The general assembly of photosynthetic membranes in cyanobacteria is similar to that of higher plants. Therefore, *Synchocystis* 6803 might serve as a powerful model for studying the molecular mechanisms of stress response and long-term adaptation (Lehel *et al*., 1993; Jantaro *et al*., 2003).

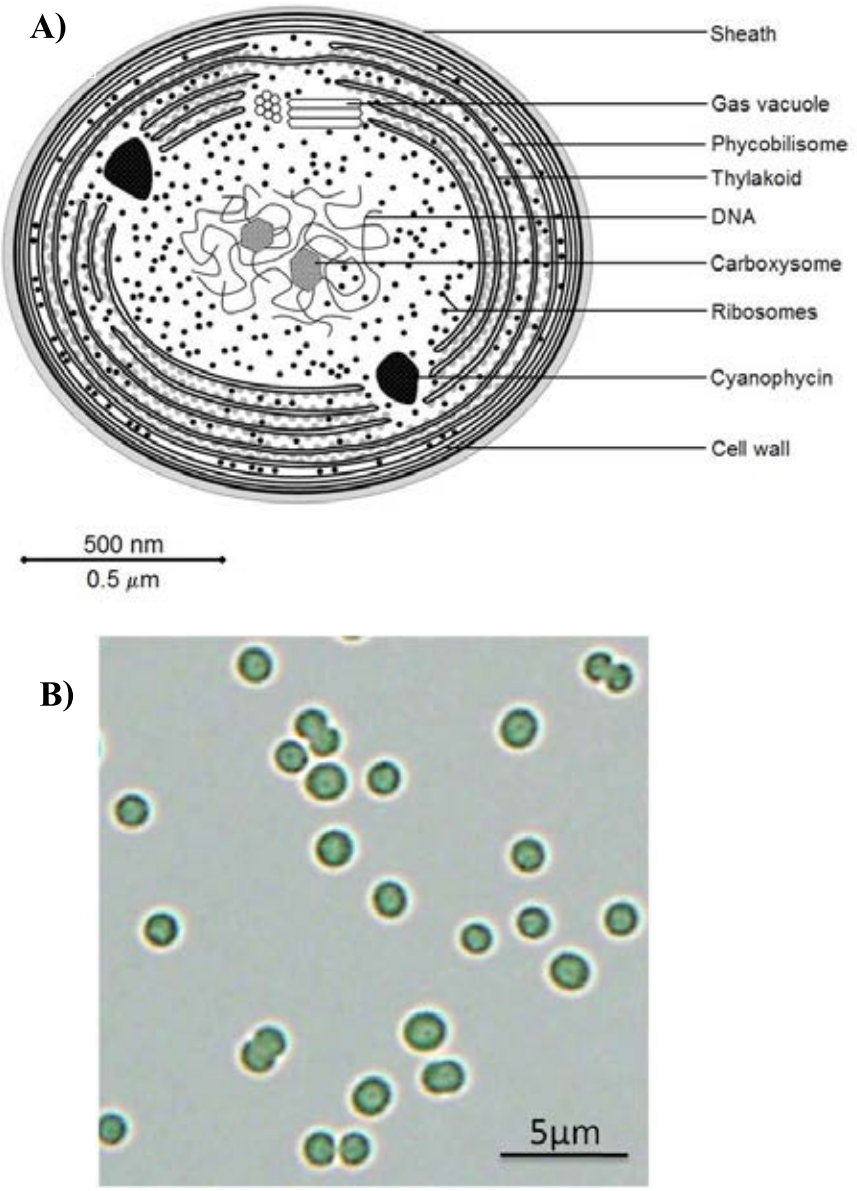

Figure 1.5 Ultrastructure of a cyanobacterial cell [Schematic representation of a thin section of a thin section of cyanobacteria cell (A) and *Synechocystis* sp. PCC 6803 cells (B)].

(Source:http://cronodon.com/BioTech/Cyanobacteria.html, http://www.emsl.pnl.gov/news/viewArticle.jsp?articleId=167).

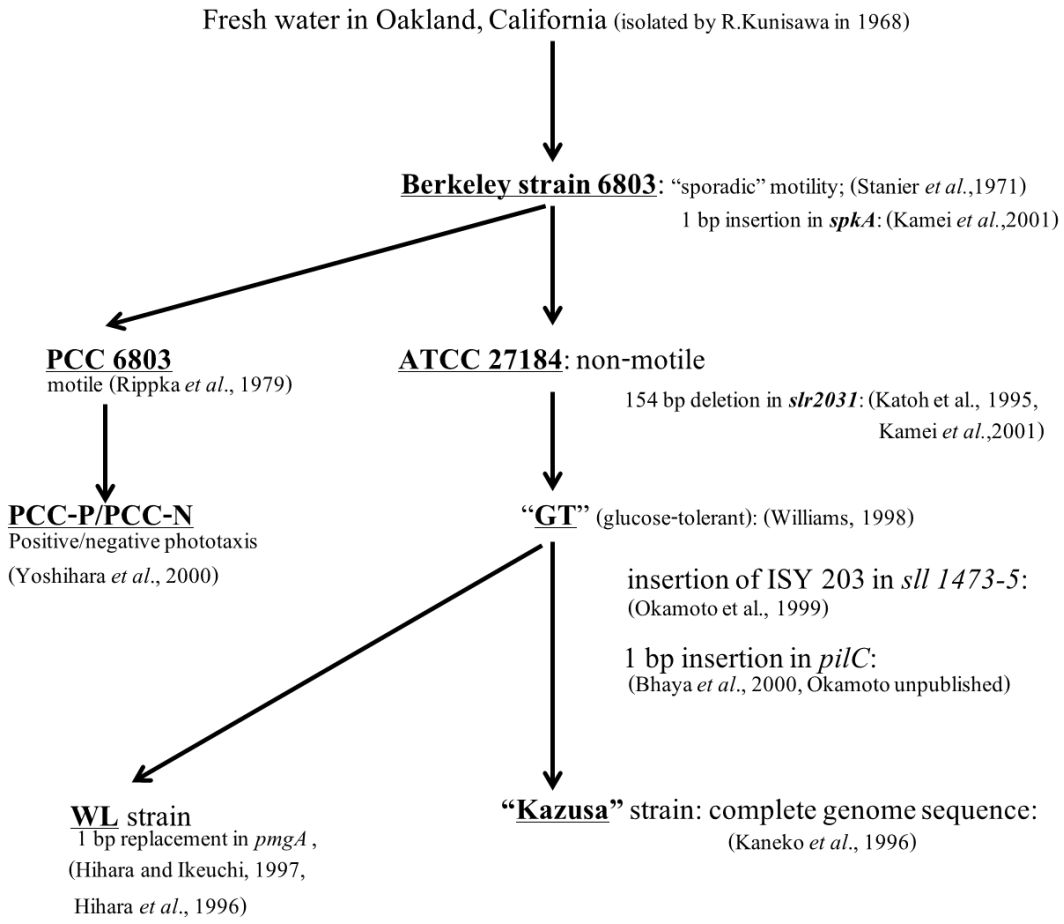

Figure 1.6 Strain history of *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001).

Cyanobateria has an important property on wide range distribution to remarkable capacities to adapt under various environmental conditions. A few data are available regarding adaptation of natural populations, most studies having been performed on a few model strains selected for specific adaptive capacities to a chosen environmental or stress factors as well as for solving agricultural problems (Ikeuchi and Tabata, 2001). Therefore, *Synechocystis* is useful tool in the study of the biochemistry and genetics of cyanobacteria (Joset *et al*., 1996).

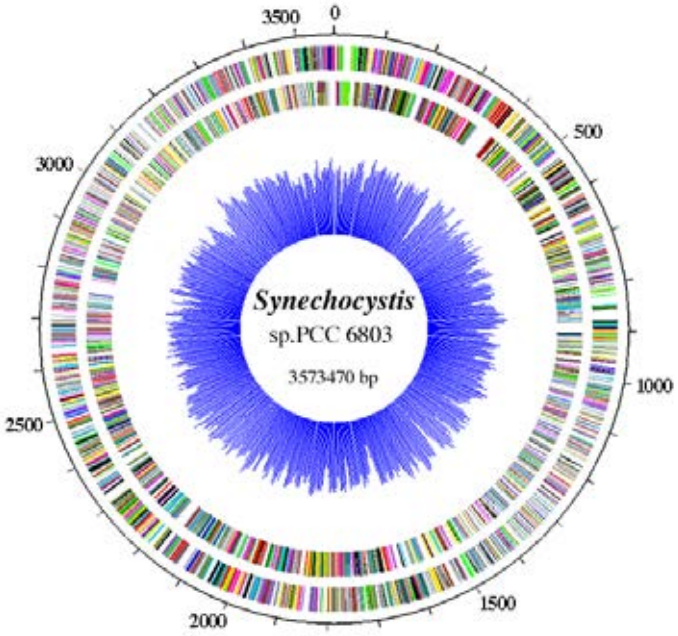

Figure1.7 The cellular genome of *Synechocystis* sp. PCC 6803 according to Cyanobase (Source: http://genome.microbedb.jp/cyanobase/Synechocystis/map/Chr).

1.4 OBJECTIVES OF THIS RESEARCH

1. To investigate the effect of pH stress on cell growth, intracellular pigments and polyamine contents in *Synechocystis* sp. PCC 6803.

2. To investigate the gene expression of polyamine related gene and protein level of arginine decarboxylase (ADC) protein under pH stress in *Synechocystis* sp. PCC 6803.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave	Model HA-30, Hirayama Manufacturing Corporation, Japan
Balances	Model PB303-S METTLER TOLEDO, USA
	Model AB204-S METTLER TOLEDO, USA
	Model LC620S Sartorius, Germany
Cell disrupter	French® Press Thermo electron corporation, USA
Centrifuge	Mikro 220 R Hettich, Germany
	Sorvall Legend XTR, Thermo Scientific, USA
	5417C Eppendorf , Germany
C-18 column	4.6 × 150 mm intertsil® ODS-3 5µm i.d.,
Gel documentation	Syngene® Gel Documentation
HPLC	SPD-20A Shimadzu, Japan
Laminar flow	Boss Tech Scientific Instruments, Thailand
Mini Horizontal Gel Electrophoresis System	MJ-105-S Major Science, TAIWAN
Oxygraph System	Clark-type oxygen electrode, Hansatech Instruments, England
PCR apparatus	Master cycler gradient eppendorf, Germany
pH meter	METTLER TOLEDO, USA

Power supply	POWER PAC 1000 BIO-RAD, USA
	POWER PAC HC BIO-RAD, USA
Protein Electrophoresis	Mini-PROTEAN® Tetra Cell BIO-RAD, USA
Semi-Dry Electroblotting System	Owl HEF-1 Thermo Scientific, USA
Spectrophotometer	BIOMATE 3 Thermo Scientific, USA
Vortex	Model 232 Touch mixer Fisher Scientific, USA
Water bath	Bioer Technology, China

2.1.2 Chemicals

Acetic acid	Lab Scan, Ireland
Acrylamide	Scharlau Chemie S.A., Spain
Agarose	Invitrogen, USA
Ammonium ferric citrate	Ajax Finechem, Australia
Ammonium persulfate (APS)	Merck, Germany
Benzoyl chloride	Sigma, USA
BICINE	Sigma, USA
Boric acid	Scharlau Chemie S.A., Spain
Bromophenol blue	Sigma, USA
Calcium chloride dihydrate	Ajax Finechem, Australia
Citric acid	Ajax Finechem, Australia
Chloroform	Merck, Germany
Cobalt (II) chloride	Ajax Finechem, Australia
Cobalt (II) nitrate hexahydrate	Carlo Erba Reagents, France

Coomassie blue G-250	Fluka, USA
Coomassie blue R-250	Acros, Belgium
Copper(II) sulfate	Carlo Erba Reagents, France
Diethyl ether	Lab Scan, Ireland
Dimethylformamide	Lab Scan, Ireland
Dipotassium phosphate	Ajax Finechem, Australia
Dithiothreitol (DTT)	Sigma, USA
EDTA	Ajax Finechem, Australia
Ethanol	Burdick & Jackson®, USA
Ethidium bromide	Sigma, USA
Glycerol	Ajax Finechem, Australia
Glycine	Ajax Finechem, Australia
Hexanediamine	Sigma, USA
HEPES	Calbiochem®, Germany
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Manganese (II) chloride Tetrahydrate	Ajax Finechem, Australia
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate heptahydrate	Ajax Finechem, Australia
Mercaptoethanol	Sigma, USA
Methanol	Burdick & Jackson®, USA
Methylene-bis-acrylamide	Amersham Bioscience, Sweden
MES	Sigma, USA
Perchloric acid	Merck, Germany

Pyridoxal-5-phosphate	Sigma, USA
Phenol	Merck, Germany
PMSF	Sigma, USA
Putrescine	Sigma, USA
Sodium acetate	Ajax Finechem, Australia
Sodium bicarbonate	Ajax Finechem, Australia
Sodium chloride	Loba Chemie, India
Sodium dithionite	Ajax Finechem, Australia
Sodium dithiosulfate	Ajax Finechem, Australia
Sodium dodecyl sulfate	Loba Chemie, India
Sodium hydroxide	Ajax Finechem, Australia
Sodium molybdate	Sigma, USA
Sodium nitrate	Ajax Finechem, Australia
Sodium phosphate	Ajax Finechem, Australia
Sodium thiosulfate	Ajax Finechem, Australia
Spermidine	Sigma, USA
Spermine	Sigma, USA
TEMED	BIO-RAD, USA
Tris (hydroxymethyl)-aminomethane	USB Corporation, USA
Triton X-100	Packard, USA
Trizol® reagent	Invitrogen, USA
Tween-20	BIO-RAD, USA
Xylene cyanol FF	Sigma, USA
Zinc sulfate	Ajax Finechem, Australia

2.1.3 Kits

GeneRuler^{TM} 1 kb DNA Ladder	Fermentas, Canada
Prestained Protein Marker	Thermo Scientific, USA
RNase-Free DNase	Fermentas, Canada
SuperScript^{TM} III First-Strand Synthesis System	Invitrogen, USA
Taq DNA polymerase	Invitrogen, USA

2.1.4 Supplies

Nylon membrane filter	0.45 µm, 47 mm, Sartorius, Germany
Nylon membrane filter	0.45 µm, 13 mm, Sartorius, Germany
Immobilon-P membrane	Millipore Corporation, USA

2.1.5 Primers

Table 2.1 Sequences of the primers for RT-PCR

Target gene	Name	Sequences	Length in pairs (bp)
16S rRNA	forward-*16s*	5'-AGTTCTGACGGTACCTGATGA-3'	521
	reverse-*16s*	5'-GTCAAGCCTTGGTAAGGTTCT-3'	
adc1	forward-*adc1*	5'-ATATTACCTGCGACAGTGATGG-3'	315
	reverse-*adc1*	5'-GATCAAGGCTAACTCCGTATGAC-3'	
adc2	forward-*adc2*	5'-ATATTACCTGCGACAGTGATGG-3'	457
	reverse-*adc2*	5'-TTAGCTGGTGTGGATGCCT-3'	
speB2	forward-*speB2*	5'-GACTTGGTTGGCAAGTA-3'	456
	reverse-*speB2*	5'-GCTCATCCACTGCCATGTTC-3'	

2.1.6 Organism

Synechocystis sp. PCC 6803 wild type strain was obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biology, University of Turku, Finland.

2.2 Methods

2.2.1 Culture conditions

Synechocystis sp. PCC 6803 were grown in normal BG_{11} medium under white light (40-50 μmol photons/m^2s), continuously bubbling by filtered air at 30°C. For the study of buffer conditions, cells at mid-logarithmic phase were transferred to new BG_{11} medium containing various buffers; HEPES at pH 7.6, MES at pH 5.5, 6.5 and BICINE at pH 7.6 and 8.5, respectively. After that, growth of cell culture was monitored by measuring the optical density at 730 nm. The experiment of pH condition was generated initially by adjusting medium pH at 2, 7 and 12, respectively. *Synechocystis* cells, grown exponentially under normal growth condition, were harvested and centrifuged at 6,000 rpm (4,025 x g), 25°C for 15 min. Cell pellets was further adapted in pH-adjusted media at pH 2, 7 and 12. Cell density was monitored at interval times of 1, 60, 120 and 240 min, respectively. Each experiment was repeated three times. The pH treated-cells were used for further analysis.

2.2.2 Determination of intracellular pigment contents

One ml of cell culture was harvested by centrifuging at 10,000 rpm (17,507 x g) for 10 min. Cell pellets were further extracted with *N*,*N*-dimethylformamide by vortexing vigorously. After centrifugation at 10,000 rpm (17,507 x g) for 10 min,

extracted supernatant was transferred into a new tube. The optical density of that solution was then measured its absorbance at 461, 625 and 664 nm, respectively. The calculation of chlorophyll *a* and carotenoid contents was carried out according to following equations:

Chlorophyll *a* content (µg/cell)

$= [(12.1 \times OD_{664}) - (0.17 \times OD_{625})]$/total cells* (Moran, 1982)

Carotenoid content (µg/cell)

$= [(OD_{461} - (0.046 \times OD_{664})) \times 4]$/total cells* (Chamovitz *et al*., 1993)

Total cells* (cell/ml)

$= (OD_{730} / 0.25) \times 10^8$

2.2.3 Measurement of polyamine contents

2.2.3.1 Extraction and determination of polyamine content

Five percent (v/v) of cold $HClO_4$ (500 µl) (Perchloric acid or PCA) was used to extract cell pellets (from 50 ml of cell culture). The incubation time of extraction was 1 h on ice. Then, that extracted sample was centrifuged at 10,000 rpm (17,507 x g) for 20 min. Both supernatant (PCA-soluble) and pellet (PCA-insoluble) fractions representing free and bound forms of polyamines, respectively, were collected for polyamine determination using benzoylation and HPLC instrument. After 1 ml of 2 M NaOH was mixed with both supernatant and resuspended-pellet fractions, 10 µl of benzoyl chloride was added into each reaction. The mixture was vigorously vortexed for few seconds, and subsequently incubated for 20 min at room temperature. Saturated NaCl solution (2 ml) was added into that mixture in order to stop the reaction. The derivatives of benzoyl-polyamines were further separated by

solvent extraction of cold diethyl ether (2 ml). The ether phase containing polyamine derivatives was transferred into a new tube, and allowed evaporating to dryness. Finally, the dried pellet was dissolved in methanol (1 ml). The polyamine derivatives were then analyzed quantitatively by high performance liquid chromatography (HPLC) using a C-18 reverse phase column with a UV-Vis detector (254 nm). The mobile phase was run at a flow rate of 1.0 ml/min using a gradient of 60-100 % (v/v) methanol : water (Jantaro *et al*., 2010).

2.2.3.2 Extraction of total RNA

Synechocystis cell culture (50 ml) was harvested and subsequently centrifuged at 6,000 rpm (4,025 x g), 4°C for 15 min. The obtained pellet was then resuspended in Trizol® reagent (1 ml) in a tube with the addition of acid washed-glass beads (0.2 g) (Appendix D). Cell disruption was done by vortexing for 2-5 times until cells were completely broken. The disrupted sample was centrifuged at 12,000 rpm (21,009 x g), 4°C for 10 min, and transferred the supernatant fraction was then transferred into a new tube, and left at room temperature for 5 min. After that, chloroform (0.2 ml) was added into reaction tube, and was invertedly shaken by hand for 15 sec, and further incubated at room temperature of 3 min. The sample was later centrifuged at 12,000 rpm (21,009 x g), 4°C for 15 min. The upper aqueous phase containing RNA was transferred into a new tube, and consequently added with isopropanol (0.25 ml), followed by adding 0.25 ml of high salt precipitation solution (containing 0.8 M sodium citrate and 1.2 M sodium chloride). After mixing the reaction mixture, it was incubated at room temperature for 10 min, and centrifuged at 12,000 rpm (21,009 x g), 4°C for 15 min. The RNA precipitate obtained, formed a

gel-like pellet on the side and bottom of the tube, was washed with 75% (v/v) ethanol (2 ml). This RNA washing step was performing by vortexing in few seconds, and RNA pellet was separated by using centrifugation at 12,000 rpm (21,009 x g), 4°C for 15 min. Next step, the solvent phase of ethanol was removed, the RNA was further dissolved in RNase-free water, and incubated at 55°C for 5 min (Agervald *et al*., 2008). The RNA concentration was determined by measuring absorbance of RNA solution at 260 nm, and calculated using the formula below:

RNA concentration (µg/µl RNA) = OD_{260} × 0.04 × dilution factor

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

Total RNA of *Synechocystis* cells obtained from 2.2.3.2 was used as the template to generate cDNA by SuperScript™ III First-Strand Synthesis System. Each reaction was added as followings; 1 µg of total RNA treated before with RNase-free DNase, 1 µl of Random hexamers and 1 µl of 10 mM dNTP mix, and adjusted volume with DEPC water up to final volume of 10 µl. The reaction was incubated at 65°C for 5 min, then placed on ice for 1 min. The 10 µl of cDNA Synthesis Mix (containing 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ and 1 µl of SuperScript™ III Reverse transcriptase) was added into the mixture and incubated the tube was at 25°C for 10 min, followed by incubation at 50°C for 50 min. The termination of cDNA synthesis reaction was done at 85°C incubation for 5 min. After that RNaseH (1 µl) was added into reaction, and the reaction was incubated at 37°C for 20 min in order to remove RNA. The synthesized cDNA was further amplified by PCR method. PCRs were performed at various steps; the initial denaturation at 94°C for 3 min, followed by 24, 25, 25 and 24 cycles of

three reactions including denaturation at 94°C for 30 sec, annealing step of *adc1*, *adc2*, *speB2* and *16S* rRNA genes performed at 53°C, 53°C, 53°C and 55°C, respectively, for 30 sec and extension at 72°C for 50 sec. The last step was final extension at 72°C for 5 min. The PCR products were checked by electrophoresis of 1.5 % agarose gel in 1X TAE buffer (Appendix C). Quantification was carried out using Syngene® Gel Documentation.

2.2.3.4 Protein Extraction

Synechocystis cells (400 ml culture) were harvested and separated using centrifugation at 6,000 rpm (4,025 x g), 4°C for 15 min. After that, cell pellet was resuspended in 4 ml of extraction buffer (Appendix E), and disrupted under the pressure of 700 psi using French® Press cell disrupter. The disrupted sample was centrifuged at 6,000 rpm (4,025 x g), 4°C for 15 min, and further transferred the supernatant into a new tube. The total protein content was measured spectrophotometrically for the absorbance at 595 nm (Bradford, 1976).

2.2.3.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Total proteins from *Synechocystis* cells were extracted as shown in 2.2.3.4. To analyze the extracted proteins, total proteins (60 µg) in sample buffer (Appendix F) with the ratio of 4:1 of total proteins: sample buffer were denatured by boiling for 5 min. SDS-polyacrylamide gel consisted of 8% separating gel and 5% stacking gel (Appendix F) was prepared. After that, the polymerized SDS-polyacrylamide gel was placed into electrophoresis chamber and samples were loaded

into each well of SDS-gel. The SDS-PAGE was performed at a constant current of 20 mA per gel for 50 min. After that, the protein gel was stained with staining solution containing coomassie blue (Appendix F).

2.2.3.6 Western blot analysis

The 4×6 cm SDS-polyacrylamide gels performed in 2.2.3.5 were transferred onto the immobilon^{TM} PVDF transfer membrane by blotting solution (Appendix G). That polyvinylidene fluoride (PVDF) membrane was activated with 100% methanol for 30 min before use by continuously shaking. Blotting was done at 50 mA for 1 h. After that blotting membrane was washed with 1XTBS (Appendix G) and subsequently blocked with blocking solution (Appendix G) for 1 h at room temperature. After washing twice with TBS-T (Appendix G) for 5 min, the membrane was incubated with first antibody (diluted 1:1500) in TBS containing 3% skim milk (w/v) for 6 h at room temperature, and further washed 4 times with TBS-T for 5 min. After that, the membrane was incubated accordingly with second antibodies of anti-Horseradish peroxidase – Goat antimouse, HRP-GAM (diluted 1:3000) at 4°C, for overnight in TBS buffer containing 3% skim milk (w/v). Peroxidase activity was visualized by staining with substrate mixture consisting of 0.025% DAB, 0.01% H_2O_2 and 0.001% $CoCl_2$ in TBS buffer. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

2.2.4 Measurement of oxygen evolution

An aliquot of cell culture containing 10 µg of chlorophyll *a* was harvested and centrifuged at 6000 rpm (4,025 x g). Cell pellet was further resuspended in 1 ml of TE

buffer**.** The oxygen evolution was measured as light-saturated rate of oxygen evolution by the Clark-type oxygen electrode (Oxygraph, Oxygen electrode Clark-type oxygen electrode, Hansatech Instruments, England) at 25°C.

CHAPTER III

RESULTS

3.1 Effect of long-term pH stress on cell growth and intracellular pigments of *Synechocystis* sp. PCC 6803

3.1.1 The growth of *Synechocystis* cells under various pH buffers

Axenic cells of *Synechocystis* grown in BG_{11} media varying pH adjusted by various buffers, including HEPES at pH 7.6, MES at pH 5.5 and 6.5, and BICINE at pH 7.6 and 8.5, respectively, for 16 days were determined for their cell growth. The result shows that the mid-logarithmic stage of cell growth ($OD_{730} \sim 0.5$) in BG_{11} media containing various buffers was observed at Day 5-6 of cultivation. Cells grown in BG_{11} media containing HEPES at pH 7.6, MES at pH 6.5, BICINE at pH 7.6 and BICINE at pH 8.5, were continuously increased whereas MES at pH 5.5 obviously inhibited cell growth since Day 1 of treatment (Figure 3.1A). On the other hand, the different types of buffer chosen at the same pH treatment, between HEPES and BICINE of pH 7.6, did not show any effects on cell growth.

3.1.2 The contents of intracellular pigments under various pH buffers

Synechocystis cells grown under normal growth condition in BG_{11} media containing various buffers, including HEPES at pH 7.6 as control, MES at pH 5.5 and 6.5, and BICINE at pH 7.6 and 8.5, respectively, were harvested and determined for the intracellular pigments of chlorophyll *a* and carotenoid contents. The results are shown in Figure 3.1B that chlorophyll *a* contents from starting cells on pH treatment

at Day 0 were similar at about 1.28 μg chl *a*/10^8cells. After 1 day-cultivation, the chlorophyll *a* contents of cell cultures under three conditions of MES at pH 6.5, BICINE at pH 7.6 and 8.5 were moderately increased while that of cells grown under MES at pH 5.5 condition was slightly decreased compared to that of control (HEPES at pH 7.6). However, cells grown in BG_{11} with BICINE at pH 8.5 gave the highest content of chlorophyll *a*. Moreover, the condition of HEPES at pH 7.6 induced the accumulation of chlorophyll *a* higher than that of BICINE at the same pH in last 16 days of cultivation. Although, the pattern of carotenoid contents (Figure 3.1C) showed as similar as that of chlorophyll *a*, their content at day 0-treatment of 0.33 μg/10^8 cells was lower than the content of intracellular chlorophyll *a*. Buffers used with BICINE at both 7.6 and 8.5 in growth medium decreased the accumulation of carotenoid contents after Day 9-treatment compared to those contents under HEPES and MES at pH 7.6 and 6.5 conditions, respectively. However, cells grown in BG_{11} containing MES at pH 5.5 showed the severe inhibition either on cell growth or intracellular pigments.

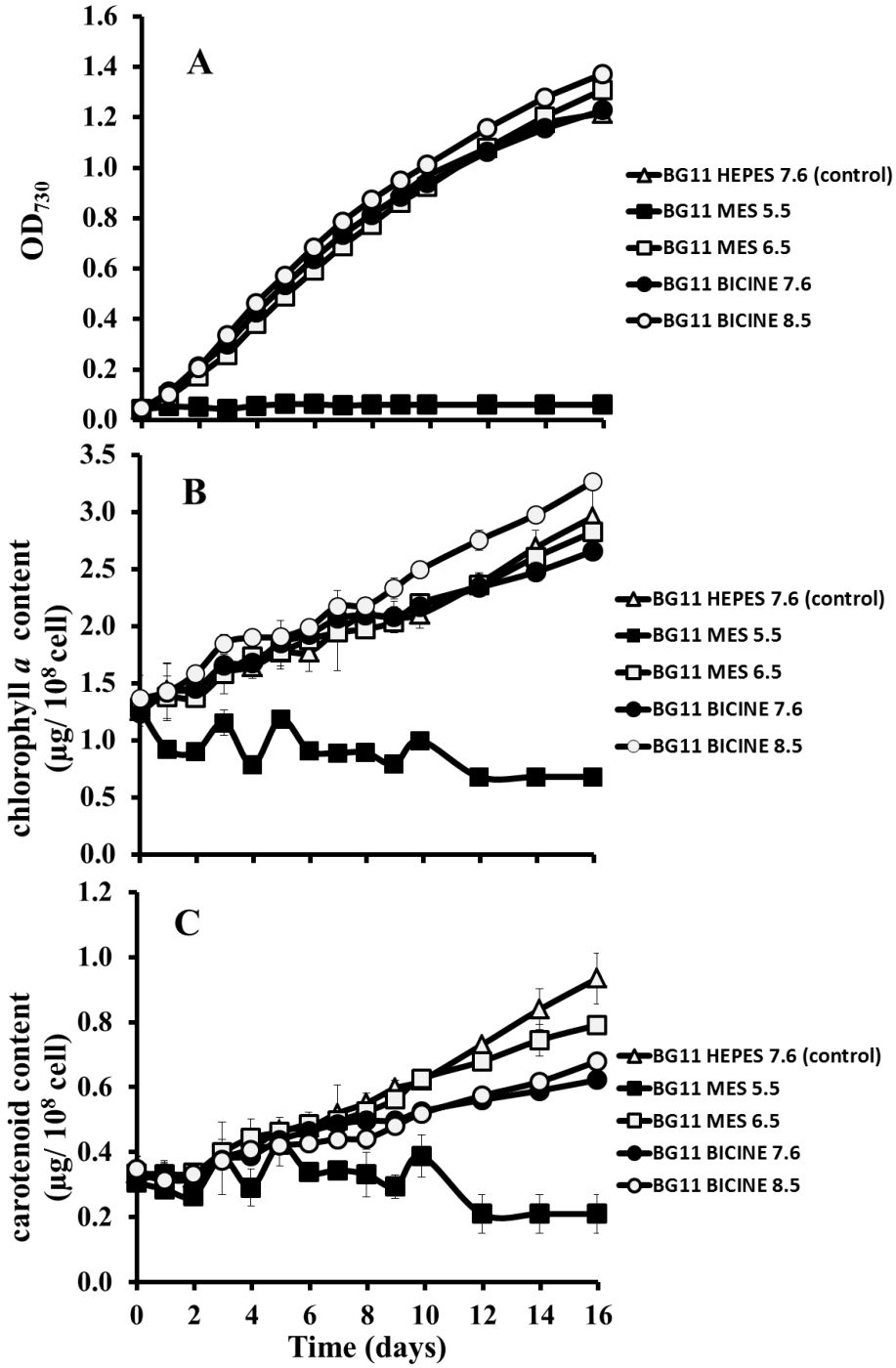

Figure 3.1 Effect of various pH buffers on *Synechocystis* cell growth (A), chlorophyll *a* content (B) and carotenoid content (C). Cells were grown in BG_{11} medium under normal growth condition for 16 days in the buffers used in BG_{11} media including HEPES at pH 7.6 as control, MES at pH 5.5 and 6.5, and BICINE at pH 7.6 and 8.5, respectively. Data are mean ± SD., n = 3.

3.2 Effect of short-term pH stress on *Synechocystis* sp. PCC 6803 cells

3.2.1 The growth of *Synechocystis* cells under short term-pH stress

Axenic cells of *Synechocystis* growing to exponential phase were adapted in various pH conditions of BG_{11} media adjusted pH in a range of 2-12, for 0-240 min. The growth of cells was measured for the optical density at 730 nm. The results are shown in Figure 3.2A, acid conditions (pH 2, 4), neutral conditions (pH 7, 8) and alkaline conditions (pH 10, 12) slightly decreased the growth of *Synechocystis* cell at 1-60 min-treatment. However, *Synechocystis* cell growth in each treatment was stable after 120 to 240 min-treatment.

3.2.2 The content of intracellular pigments under short term-pH stress

Synechocystis cells growing to exponential phase were adapted in various pH conditions of BG_{11} media adjusted pH in a range of 2-12 by 12M HCl and 10M NaOH, for 0-240 min. Cells were harvested and determined for the intracellular pigments of chlorophyll *a* and carotenoid contents. The results show that acid stress condition of pH 2, the chlorophyll *a* content was rapidly decreased at first 1 min and gradually decreased until 240 min compared to other conditions (Figure 3.2B). The pattern of chlorophyll *a* accumulation in each pH, except pH 2, showed the same tendency which the constant level was maintained during first 0-120 min, and the marked decrease was observed after that up to 240 min. On the other hand, the carotenoid content was not affected in all pH conditions, except at pH 2, within 240 min (Figure 3.2C). The color of cell culture (Figure 3.3) was changed from green to yellowish green at pH 2 after 120 min of adapted time whereas there was no color changes of cell culture in other pH conditions.

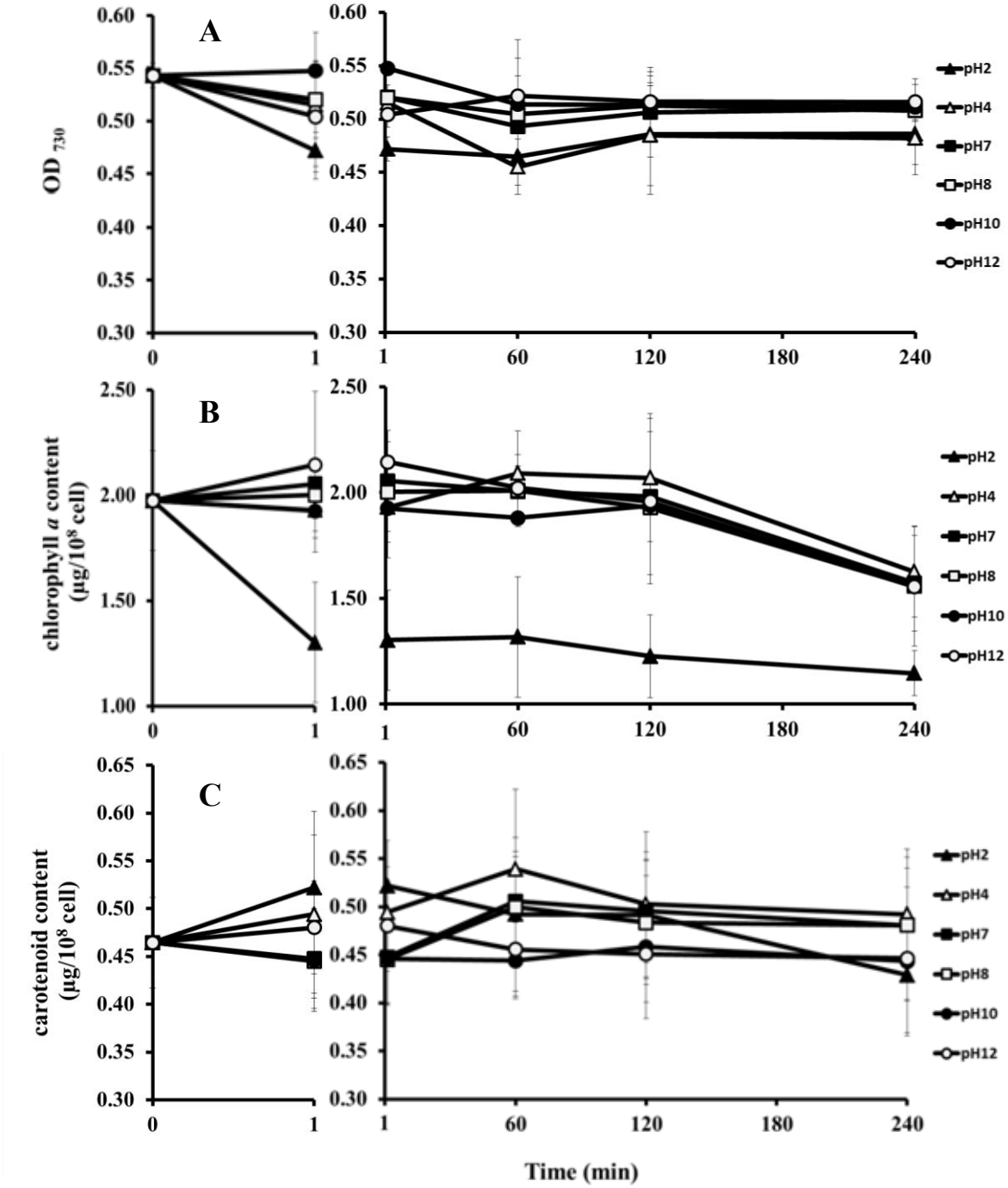

Figure 3.2 Effect of short term-pH stress on *Synechocystis* cells growth (A), chlorophyll *a* content (B) and carotenoid content (C). Cells were adapted in pH-adjusted media ranging from pH 2-12 under white light for 240 min. Data are mean ± SD., n = 3.

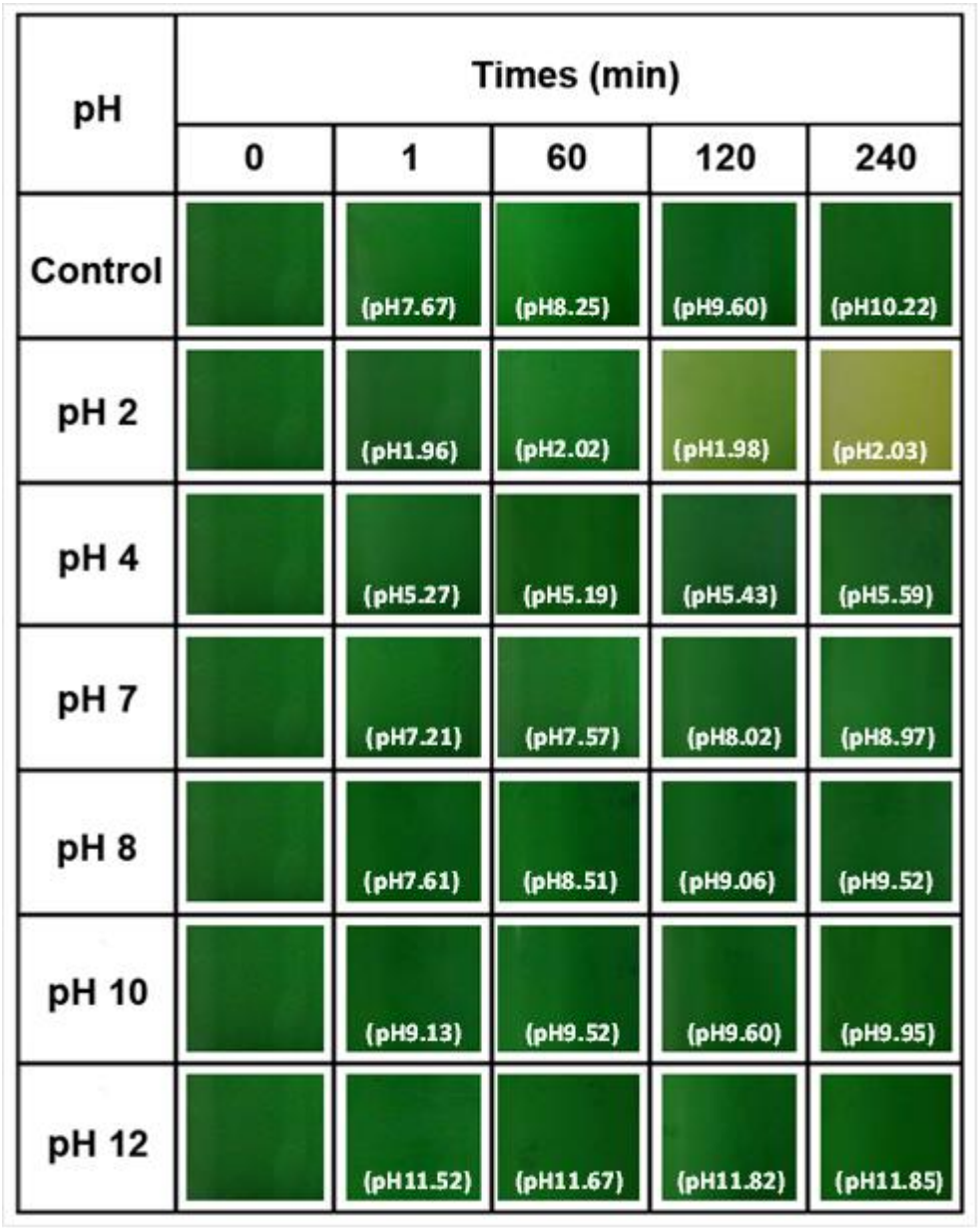

Figure 3.3 Optical appearance of *Synechocystis* cell culture adapted in pH-adjusted media at pH 2-12, for 240 min.

3.2.3 Effect of pH stress on oxygen evolution of *Synechocystis* cells

In order to measure the oxygen evolution of cell representing the photosynthetic efficiency, various pH adapted *Synechocystis* cells at pH7, pH 2 and pH12 conditions for 240 min were determined for their oxygen evolution. The result is shown in Figure 3.4 that the oxygen evolution of *Synechocystis* cells under control condition at pH 7 was not changed significantly, even showed their highest tendency at 120 min-treatment. Interestingly, the photosynthetic efficiency under alkaline stress at pH 12 was maintained along 240 min-treatment. In contrast, it was obviously shown that the acid stress condition at pH 2 completely inhibited the oxygen evolution in *Synechocystis* cells, in particular at first minute of treatment upto 240 min.

3.2.4 The polyamine contents under short term-pH stress

The contents of intracellular polyamines of *Synechocystis* cells adapted in various pH conditions of pH 2 and pH 4 (Figure 3.5), pH 7 (control) and pH 8 (Figure 3.6), and pH 10 and pH 12 (Figure 3.7) were investigated. Total polyamine contents under pH 2 condition (Figure 3.5A) was decreased after 1 min-treatment, and constantly accumulated during 1-120 min-treatment. The apparent decrease under pH 2-condition was observed at 240 min-treatment. The unchanged level of polyamine accumulation during 0-240 min-treatment was observed under each pH condition of pH 4, pH 7, pH 8, and pH 10, respectively (Figure 3.5B, 3.6AB, 3.7A). On the other hand, a slight increase of total polyamine content under pH 12-condition was noted within 240 min-treatment (Figure 3.7B). It was due to bound form-Spd content which was induced significantly whereas free form-Spd content showed a slight decrease at

240 min-time point. Since, the strong acid stress at pH 2 and strong alkaline stress at pH 12 apparently affected polyamine levels, they was further selected for studying on the aspect of pH transition.

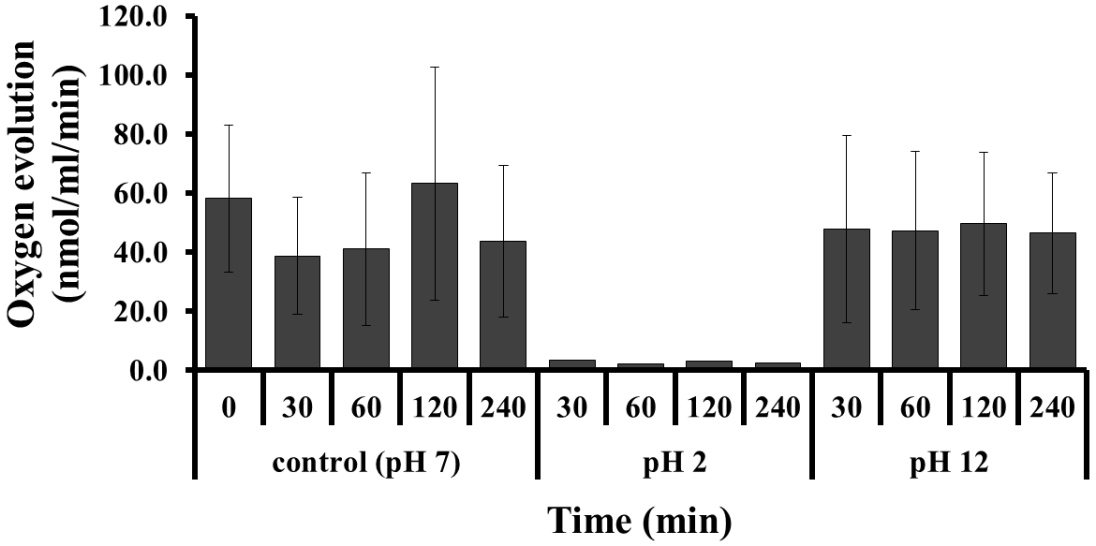

Figure 3.4 Effect of pH stress on oxygen evolution of *Synechocystis* sp. PCC 6803. The oxygen evolution was measured in Clark type oxygen electrode under saturating light intensity at 25°C. Data are mean ± SD., n = 3.

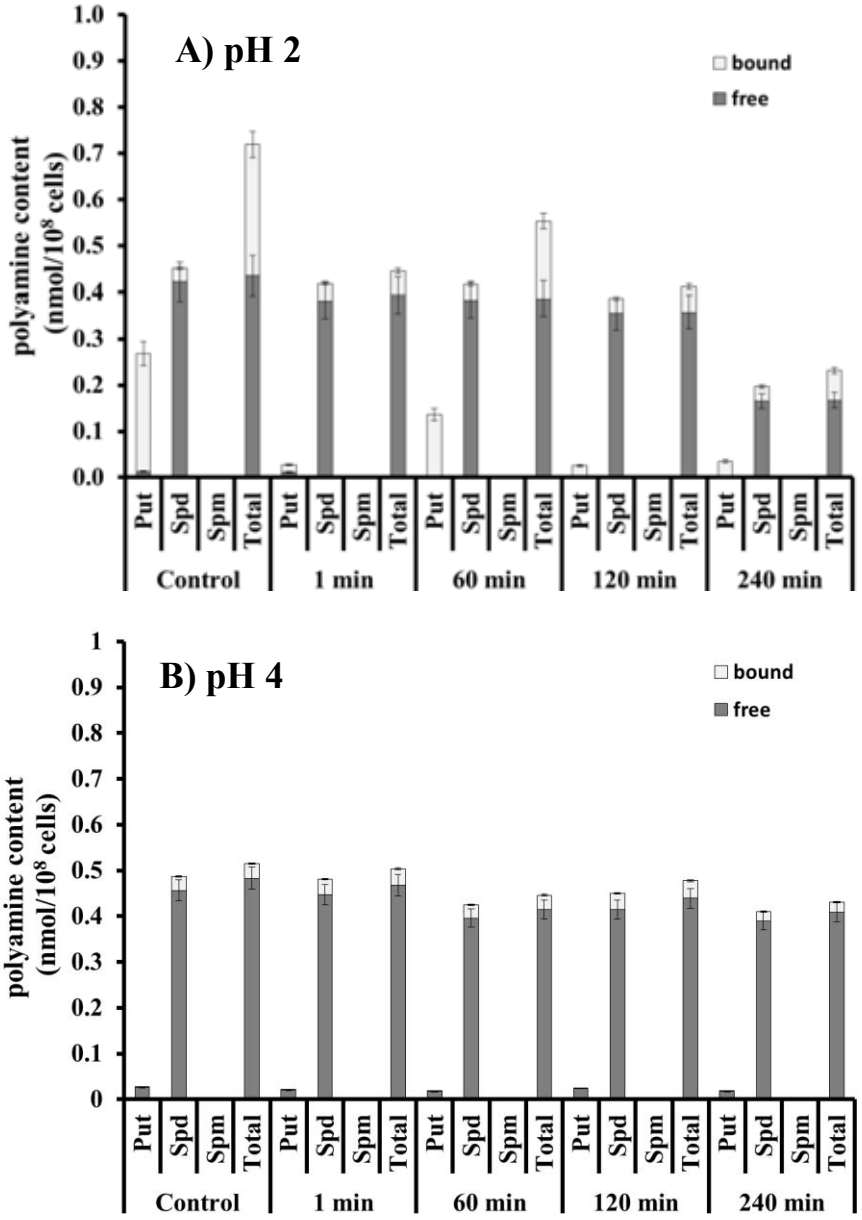

Figure 3.5 Polyamine contents of *Synechocystis* sp. PCC 6803 under short-term pH stress conditions of (A) pH 2 and (B) pH 4 within 240 min-treatment. Data are mean ± SD., n = 3.

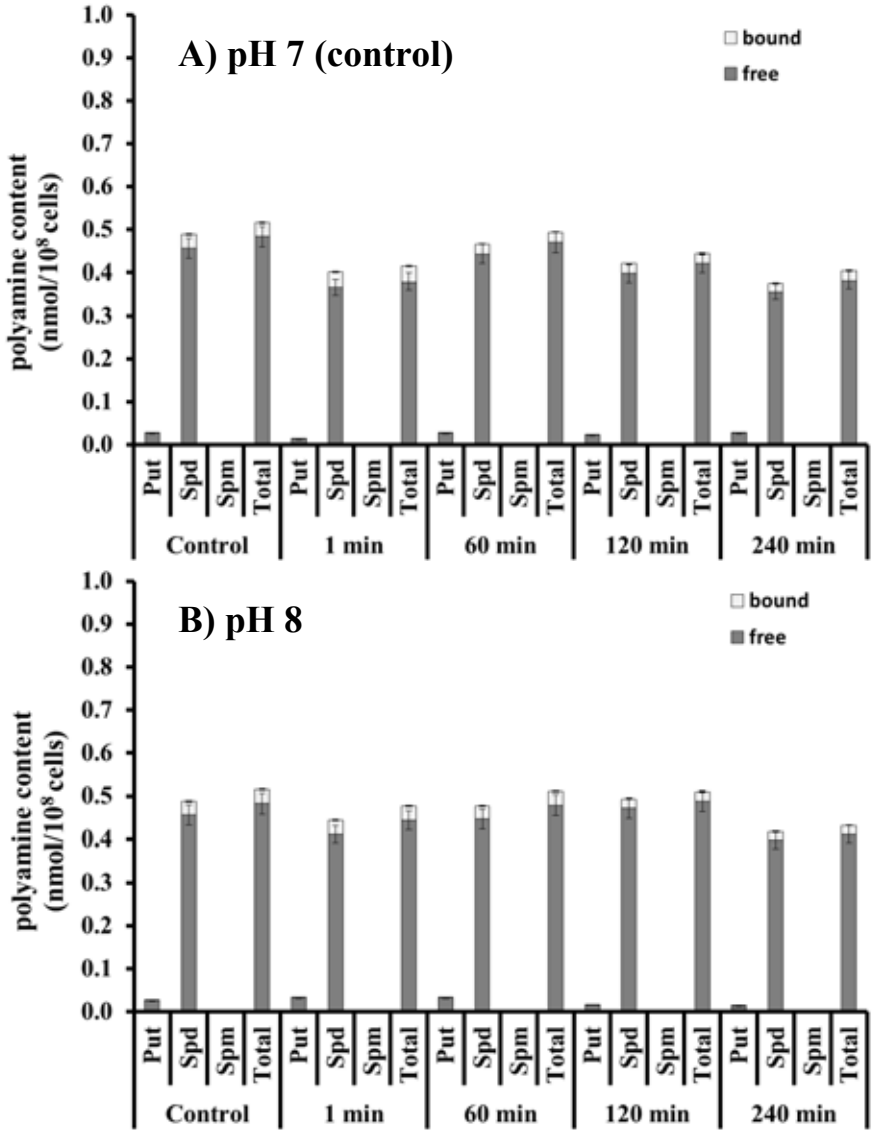

Figure 3.6 Polyamine contents of *Synechocystis* sp. PCC 6803 under short-term pH stress conditions of (A) pH 7 (control) and (B) pH 8 within 240 min-treatment. Data are mean ± SD., n = 3.

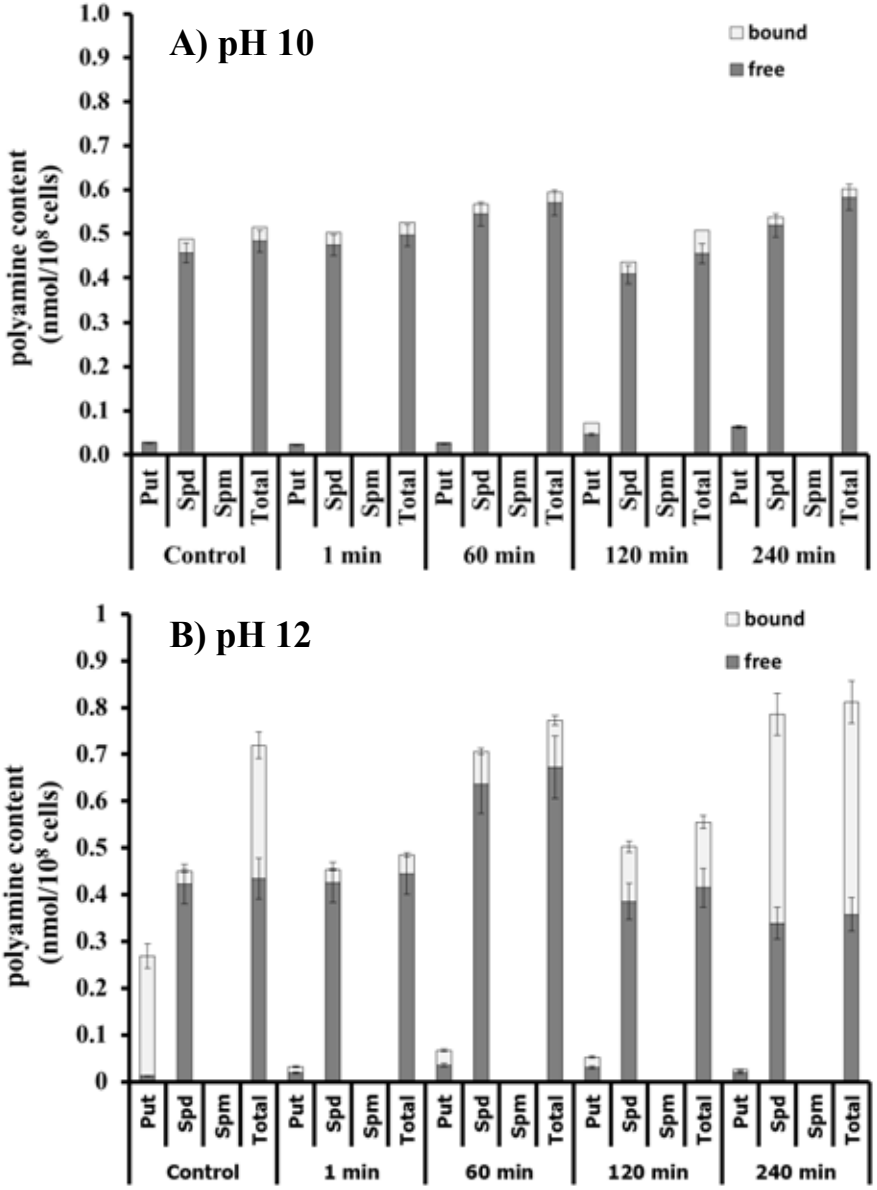

Figure 3.7 Polyamine contents of *Synechocystis* sp. PCC 6803 under short-term pH stress conditions of (A) pH 10 and (B) pH 12 within 240 min-treatment. Data are mean ± SD., n = 3.

3.3 Effect of pH transition on *Synechocystis* sp. PCC 6803 cells

3.3.1 The optical density of *Synechocystis* cells under pH transition treatment

The optical density of *Synechocystis* cells under pH transition is shown in Figures 3.8 and 3.9. The acid stress at pH 2 did not harm cell density within 240 min-treatment (Figures 3.8A). However, it was found a 20% decrease of cell density after changing the ambient pH from 2 to 12 (Figure 3.8A). The pH 2 to 7-transition did not affect cell density within 240 min-treatment (Figure 3.8B). On the other hand, *Synechocystis* cell density was slightly decreased under pH 12-treatment (Figure 3.9). It was found that the immediate change from pH 12 to pH 2 did not affect the density of cells (Figure 3.9A), whereas an apparent slightly decrease of optical density under pH transition from 12 to 7 was observed (Figure 3.9B).

3.3.2 The content of intracellular pigments under pH transition treatment

The contents of intracellular pigments, including chlorophyll *a* and carotenoids, of *Synechocystis* cells under pH transition were investigated. The chlorophyll *a* content was decreased obviously at acid stress (pH 2) by starting to decrease immediately at first 1 min time point and stable at the similar level up to 240 min (Figure 3.10) while alkaline stressed cells showed no apparent change of chlorophyll *a* levels along 240 min-treatment (Figure 3.11). The chlorophyll *a* content showed a slight increase at pH 2 to 12-transition whereas it was not changed at the transition from pH 2 to 7 (Figure 3.10A, B). At alkaline stress, the chlorophyll *a* content was dramatically decreased at pH 12 to 2-transition whereas it did not affect at pH 12 to 7-transition (Figure 3.11A, B). On the other hand, carotenoid content

under pH 2 condition was slightly decreased within 240 min-treatment (Figure 3.10). The small increase was observed at 1 min-treatment of pH 12 after treated by pH 2 and gradually decreased (Figure 3.10A). In contrast, the constant level of carotenoids of pH 2 to 7-stressed cells was demonstrasted (Figure 3.10B). Moreover, carotenoid accumulation inside *Synechocystis* cells stressed by pH 12-condition showed unchanged level within 240 min-treatment (Figure 3.11). It was clearly depicted that pH 2 condition slightly inhibit the carotenoid content in pH 12-treated cells (Figure 3.11A) whereas pH 12 to 7-transition did not (Figure 3.11B).

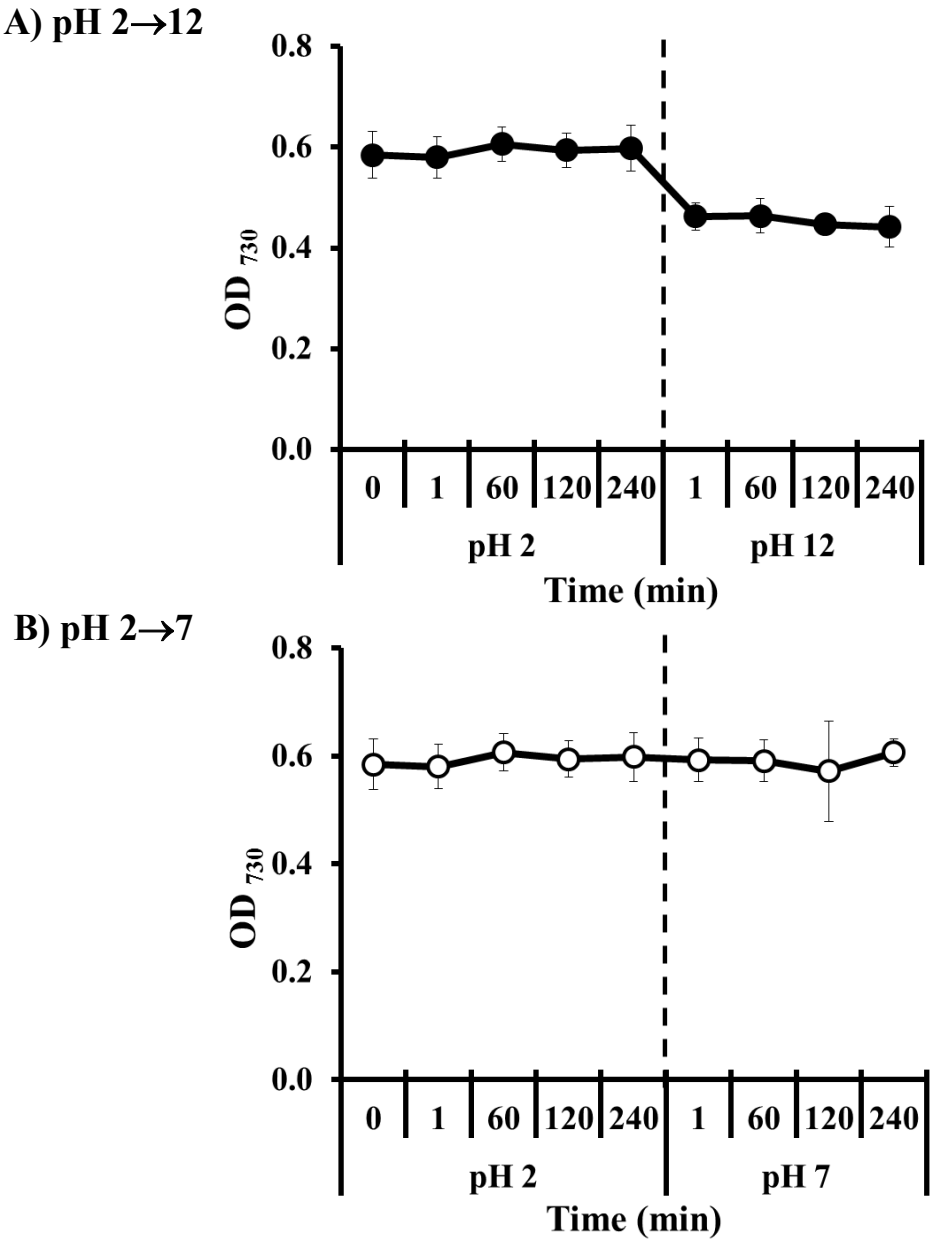

Figure 3.8 The optical density of *Synechocystis* cells under acid stress conditions for 0-240 min. The transition was performed by transferring cells from pH 2→12 (A) and pH 2→7 (B) condtitions. Cells were monitored on their density by measuring OD at 730 nm. Data are mean ± SD., n = 3.

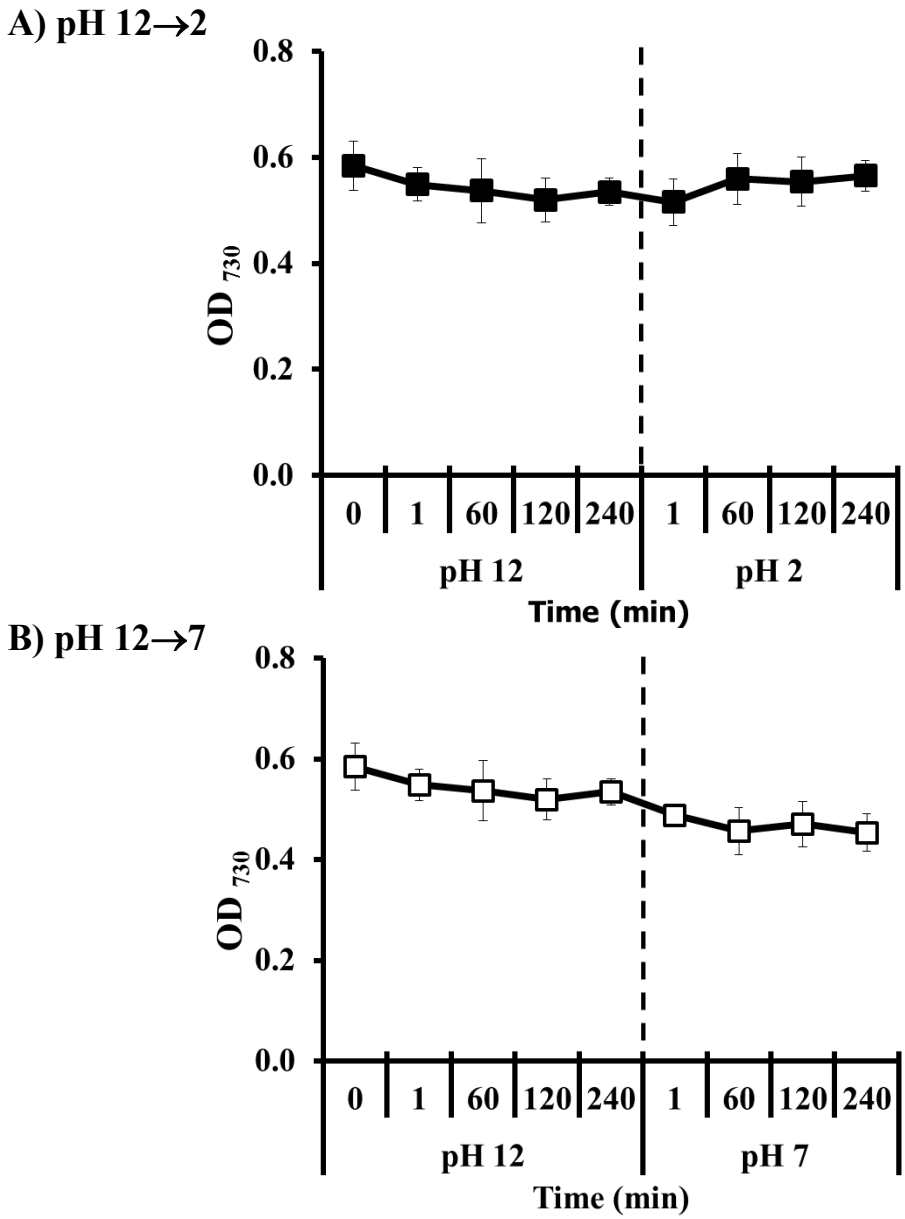

Figure 3.9 The optical density of *Synechocystis* cells under alkaline stress conditions for 0-240 min. The transition was performed by transferring cells from pH 12→ 2 (A) and pH 12→7 (B) conditions. Cells were monitored on their density by measuring OD at 730 nm. Data are mean ± SD., n = 3.

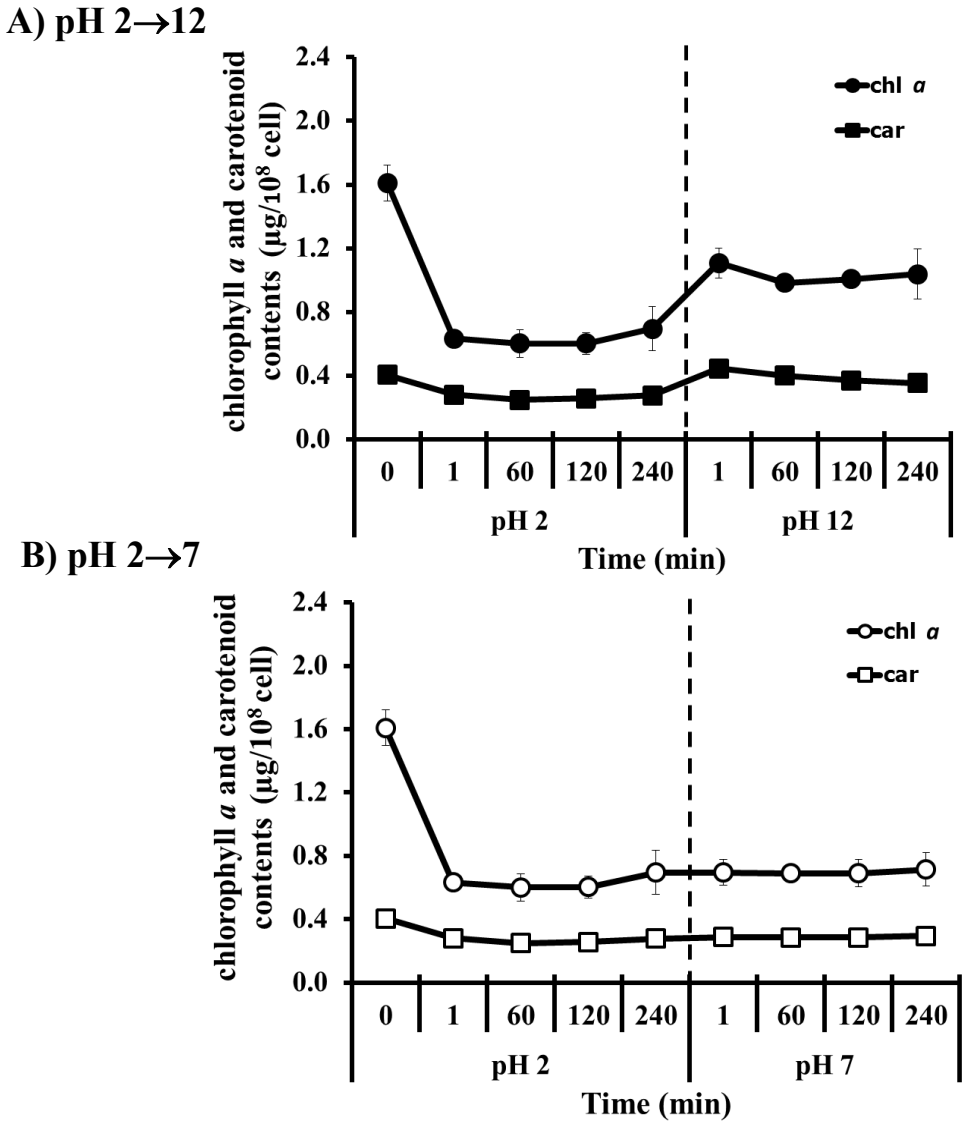

Figure 3.10 The contents of intracellular pigments of *Synechocystis* cells under pH transitions of pH 2→12 (A) and pH 2→7 (B) conditions. Data are mean ± SD., n = 3.

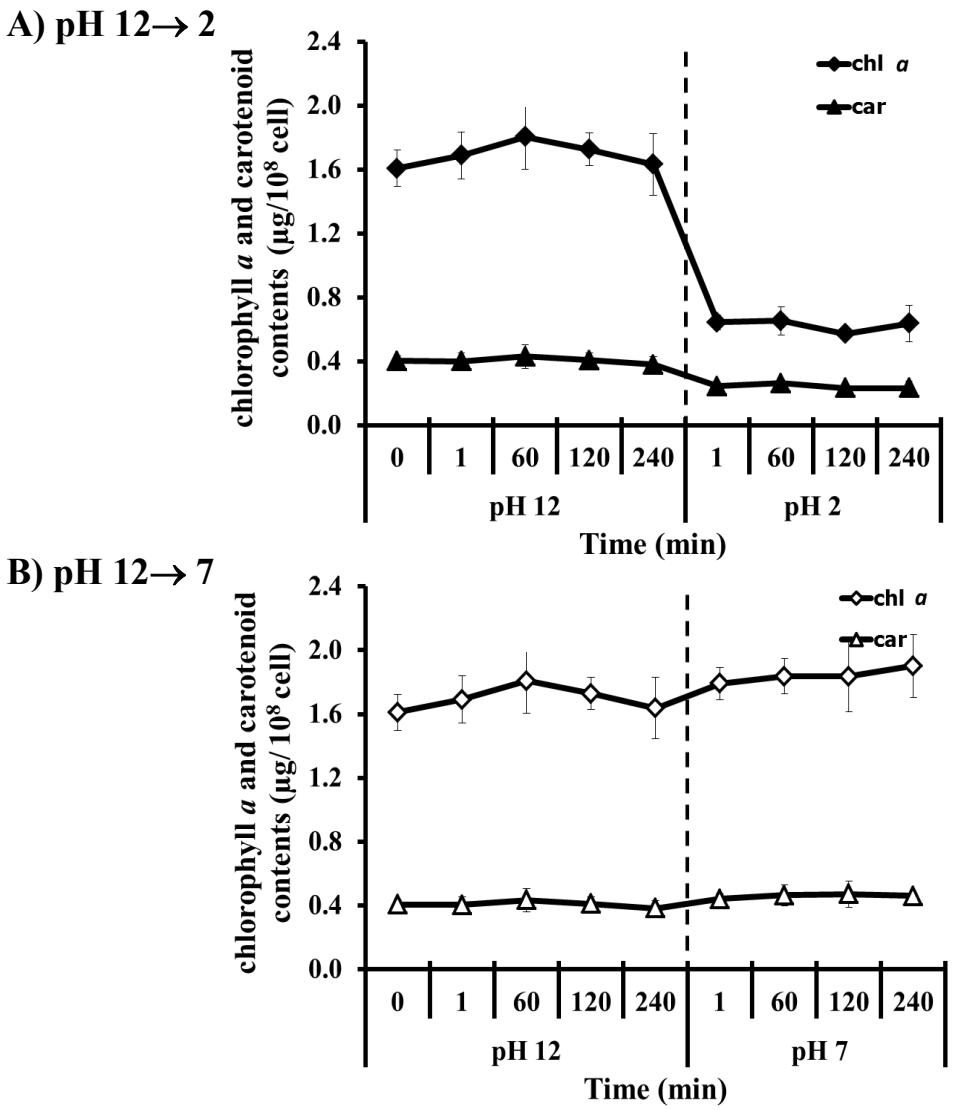

Figure 3.11 The contents of intracellular pigments of *Synechocystis* cells under pH transitions of pH 12→ 2 (A), pH 12→7 (B). Data are mean ± SD., n = 3.

3.3.3 The polyamine contents under pH transition treatment

The polyamine contents of *Synechocystis* cells under each pH transition were investigated (Figures 3.12-3.15). In Figure 3.12A, left panel, free form-Spd content of acid stressed cells (pH 2) was not decreased significantly under 0-120 min-treatment but obviously decreased at 240 min-treatment. The free form of polyamines was severely diminished after dramatically transferring pH 2-treated cells into pH 12 condition. However, the decrease of free form-polyamines was affected on external pH value of reaction treatments. In contrast, bound form-polyamines was decreased by pH 2 stress up to 240 min-treatment but apparently up-regulated after 240 min-treating with pH 12 condition, in particular Put level (Figure 3.12B). It was found that pH 7 condition of control could maintain the residual of free form-polyamines of pH 2 stressed-cells, especially Spd level, along 240 min-treatment (Figure 3.13A). Interestingly, bound form-Spd content was up-regulated temporarily after facing pH 7 condition for 60 min and down-regulated at 120 and 240-min treatments (Figure 3.13B).

On the other hand, alkaline stress at pH 12 mostly maintained free form-polyamines, except the induction of free form-polyamine was observed only at 60 min-treatment (Figure 3.14A, left panel). The sharp increase of free form-Spd content was demonstrated immediately after transferring to pH 2 condition at 1 min-treatment (Figure 3.14A, right panel). However, free form-polyamine amounts were decreased gradually along 240 min-treatment in pH 2 condition. In contrast, bound form-polyamine content was apparently increased at 240 min-treatment, in particular Spd (Figure 3.14B, left panel). After transferring pH 12 stressed-cell to acid stress, bound form-polyamine content was decreased significantly (Figure 3.14B, right panel).

Furthermore, pH 7 condition enabled to induce the accumulation of free form-polyamines in pH 12 stressed-cells (Figure 3.15A, right panel) whereas it temporarily increased bound form-Put level at 1 min-treatment (Figure 3.15B, right panel).

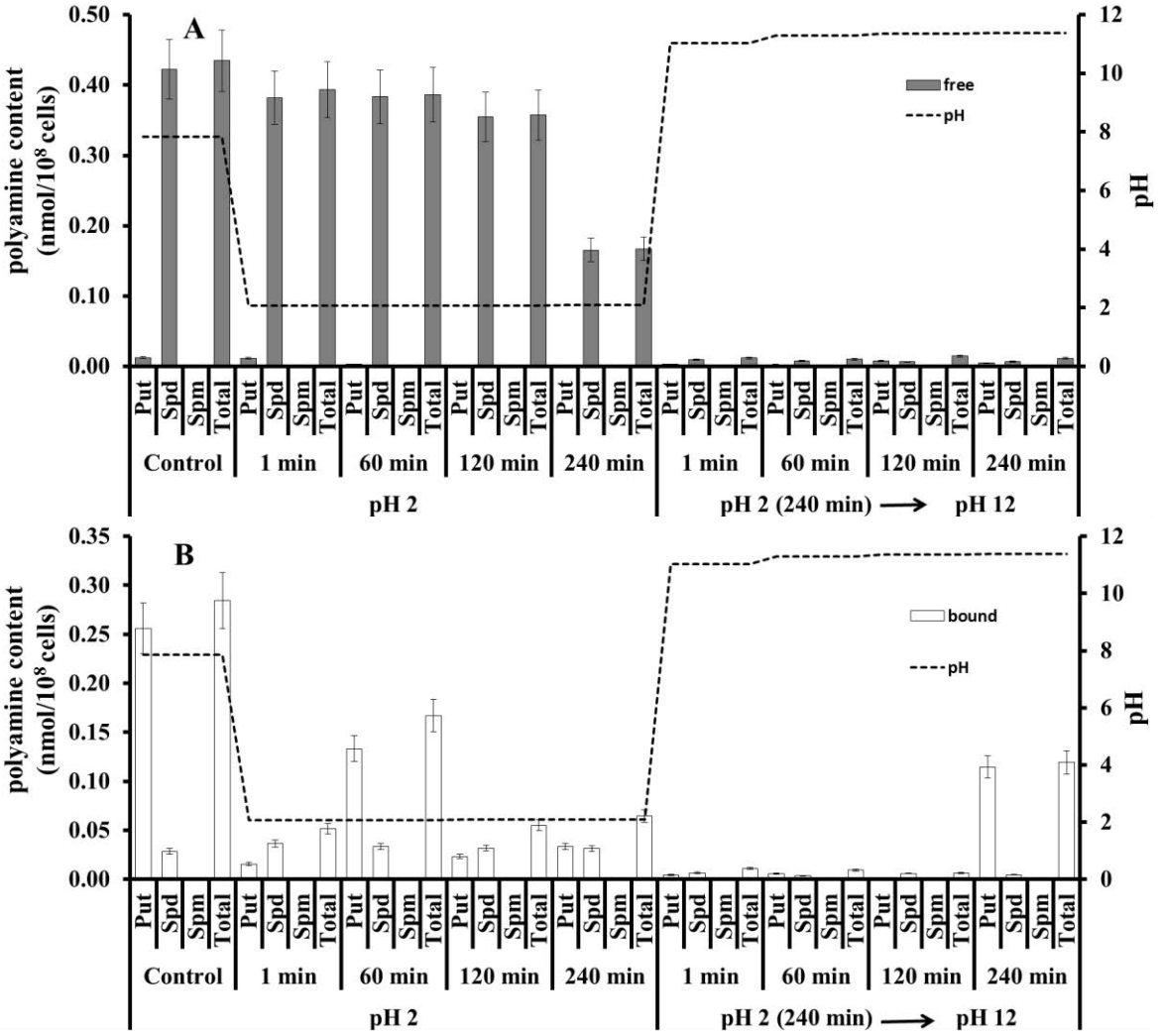

Figure 3.12 Polyamine contents including free form (A) and bound form (B) of *Synechocystis* cells under pH transition of pH 2→ 12. Data are mean ± SD., n = 3.

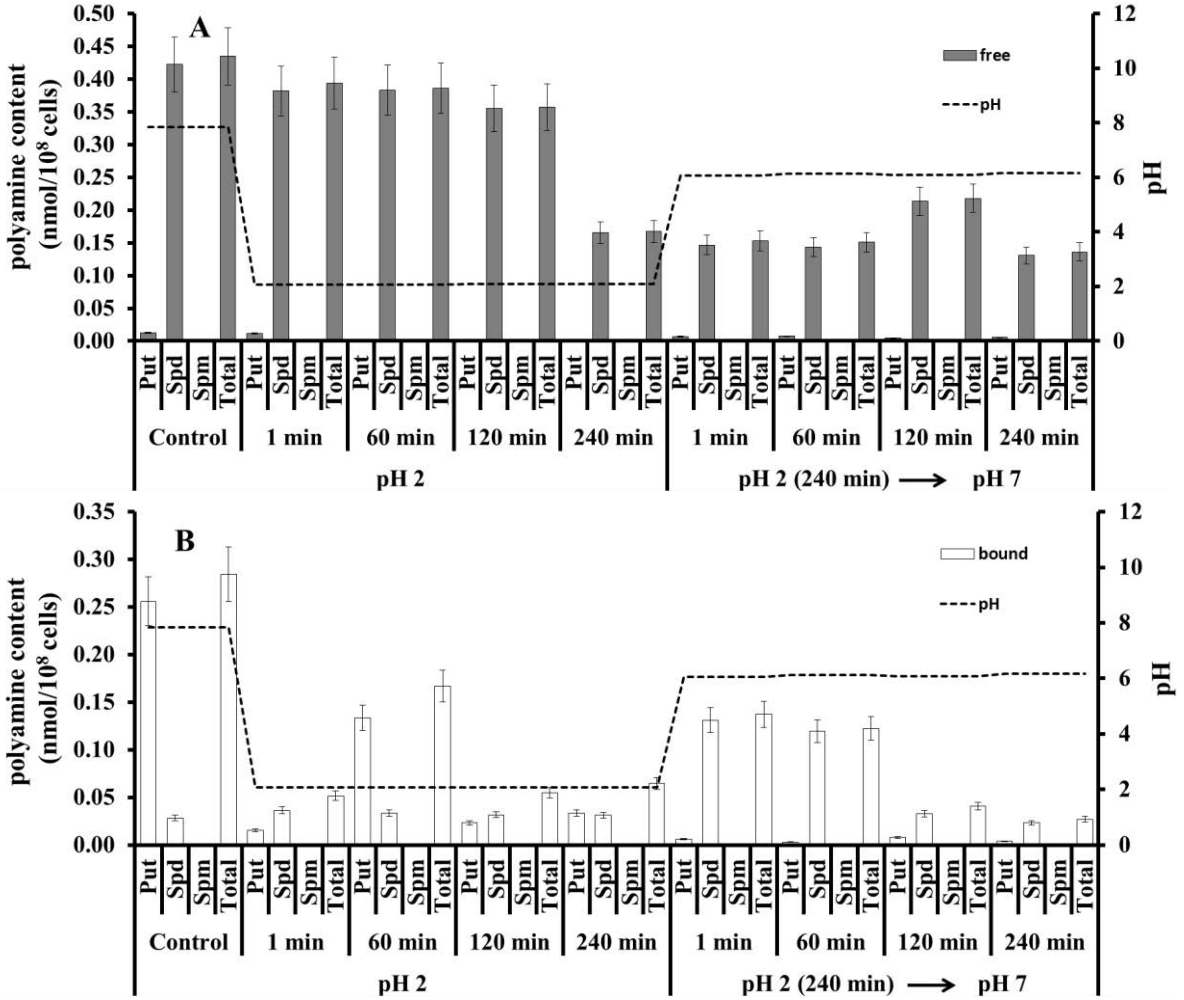

Figure 3.13 Polyamine contents including free form (A) and bound form (B) of *Synechocystis* cells under pH transition of pH 2→7. Data are mean ± SD., n = 3.

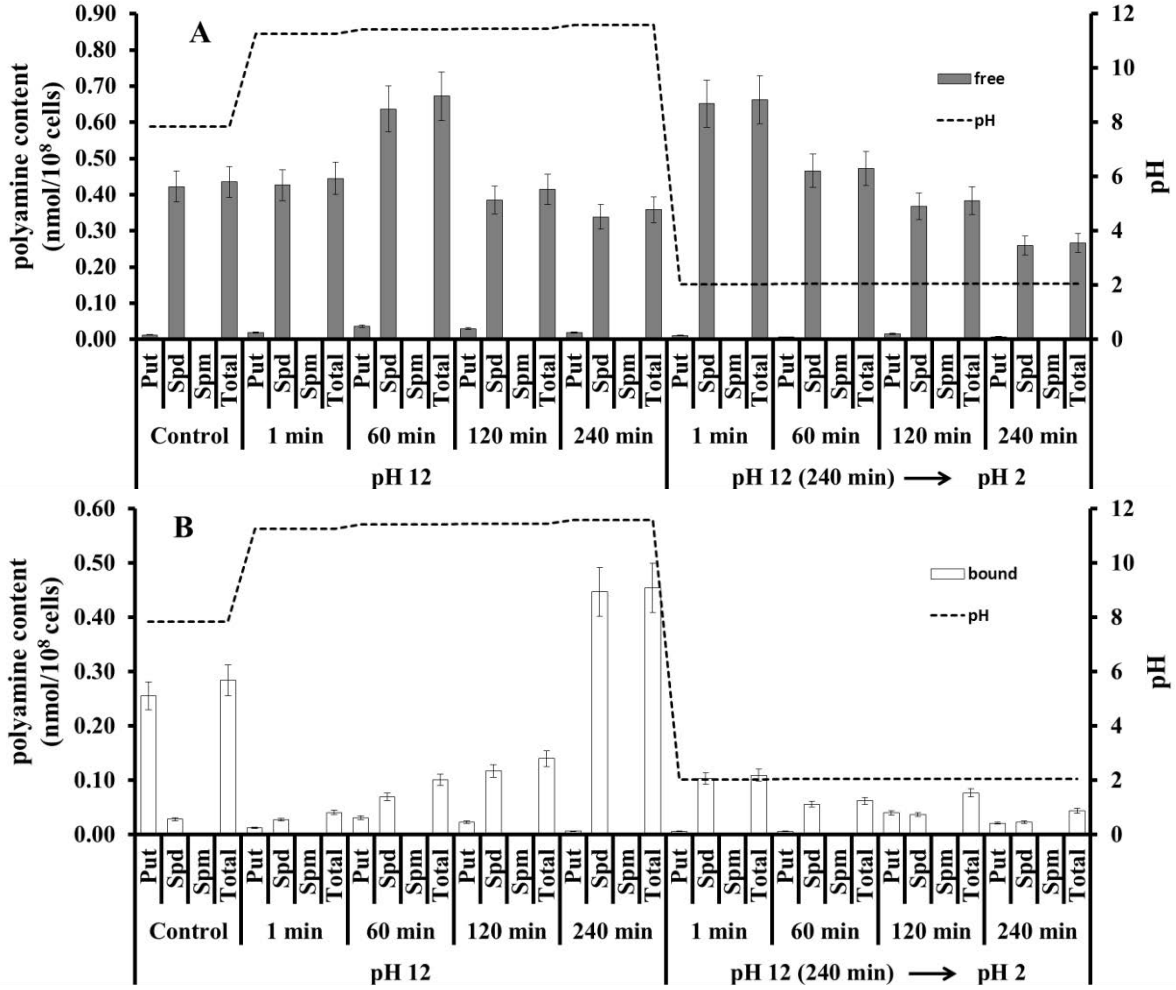

Figure 3.14 Polyamine contents including free form (A) and bound form (B) of *Synechocystis* cells under pH transition of pH 12→2. Data are mean ± SD., n = 3.

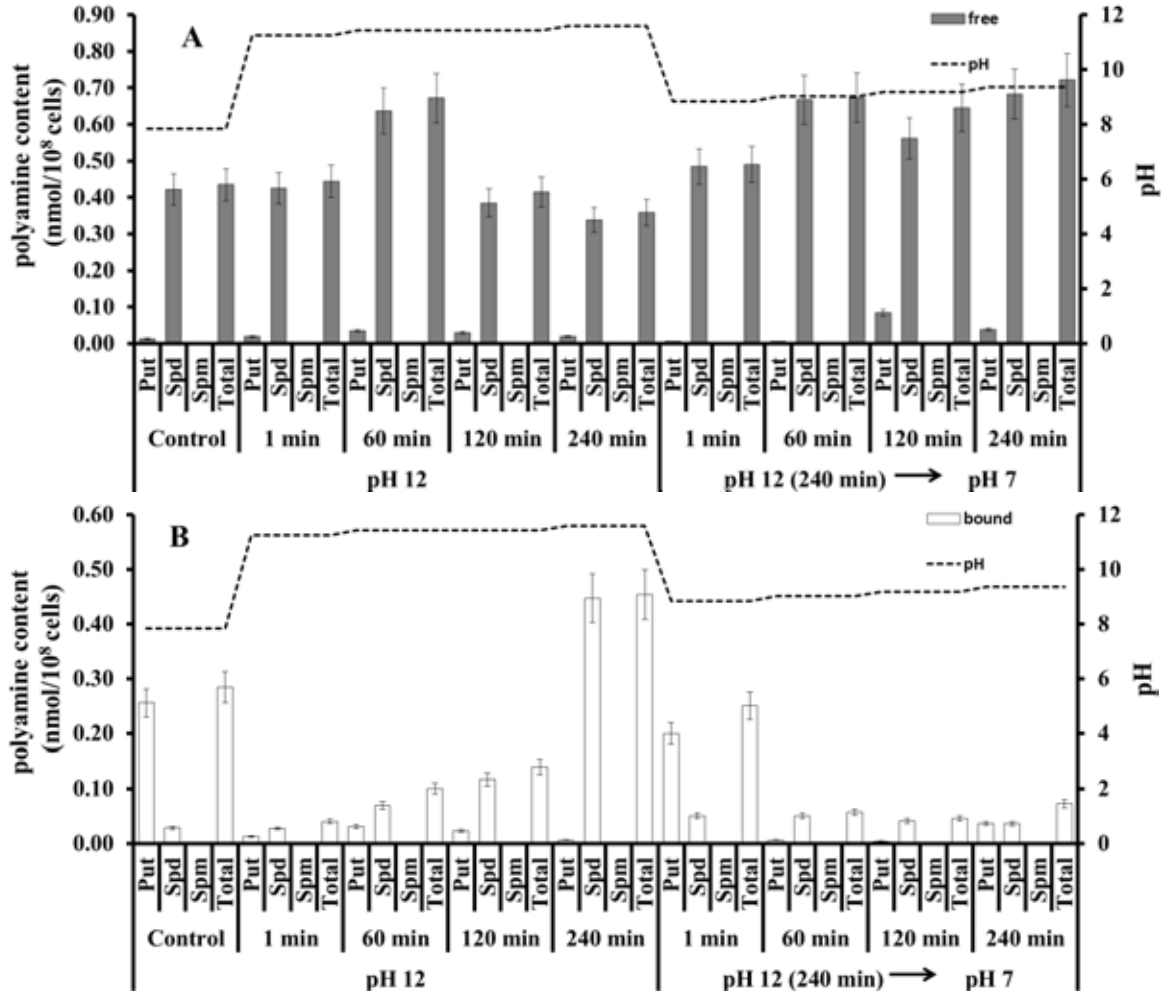

Figure 3.15 Polyamine contents including free form (A) and bound form (B) of *Synechocystis* cells under pH transition of pH 12→7. Data are mean ± SD., n = 3.

3.4 Effect of short term-pH stress on mRNA levels in *Synechocystis* cells

Total RNAs of *Synechocystis* cells adapted in normal BG_{11} (control condition, pH 7), BG_{11} adjusted pH at 2 (acid condition) and 12 (alkaline condition) were isolated and used as template for RT-PCR amplification. The transcript levels and the ratio of band intensity (gene/*16s* rRNA) are shown in Figure 3.16. As the result, the *adc* gene enconding arginine decarboxylase. Under normal condition, *adc1* and *adc2* mRNA levels was slightly increased within 240 min-treatment whereas both *adc* mRNA amounts of acid stressed cells (pH 2) was obviously decreased. For alkaline condition (pH 12), the levels of *adc1* and *adc2* mRNAs were significantly increased and gave the highest level at 120 min-treatment.

On the other hand, the *speB2* gene encoding agmatinase, which catalyzes the conversion of an intermediate agmatine to putrescine, was also detected. The level of *speB2* mRNA under pH 2 condition was significantly decreased compared to that of control condition (pH 7). Similarly, the alkaline condition at pH 12 affected the amounts of *speB2* transcript at lower levels in all time-treatments compared to those of control. Moreover, the level of *ChlG* mRNA encoding chlorophyll *a* synthase was investigated. Under normal condition, the constant level of *ChlG* mRNA was observed whereas acid stress at 120 and 240 min-treatments markedly decreased *ChlG* transcripts. In contrast, *ChlG* mRNA under alkaline stress was slightly increased at 120 min-treatment and constant later that to last 240 min-tratment.

The transcriptional level of *potD* gene, which encodes periplasmic polyamine-binding protein, and *pao* gene, encoding polyamine oxidase in degradation pathway, was also investigated. Interestingly, *potD* transcripts showed moderate amounts under normal condition, but were highly reduced under acid stress condition. In contrast,

alkaline condition enabled to increase the accumulation of *potD* transcripts at all time treatments. The amount of *pao* mRNA was apparently decreased after treating with acid and alkaline conditions at last 240 min. However, at 120 min-treatment between pH 2 and pH 12 conditions, the slight increase of *pao* transcripts was observed comparing to that of control at the same time point. The results for *16S* rRNA in samples derived from the same amount of total RNA was also included as an internal standard.

3.5 Effect of pH stress on ADC protein in *Synechocystis* cells

Total proteins of *Synechocystis* cells under various pH conditions (pH 7 as control , pH 2 and pH 12) were extracted and separated onto SDS-PAGE analysis. The proteins were transferred from SDS gel onto PVDF membrane which was further probed with ADC antibody. After incubation of PVDF membrane with antibody, two protein bands with the molecular weights of about 75 and 45 kDa were found. Since the molecular weight of ADC predicted from amino acid sequences was about 74,461 Da or 74.4 kDa (using program from EnCor Biotechnology Inc.), the upper band on our obtained blotting membrane was indicated as the ADC protein band. ADC protein levels (upper band) under control condition at pH 7 were slightly increased at 120 min-treatments and further decreased at 240 min-treatment (Figure 3.17A) while ADC protein levels (lower band) were constant at both 1 and 120 min-treatments, and further decreased at 240 min-treatment. The pH 2 treatment at 120 and 240 min-treatment reduced the accumulation of ADC proteins (both two bands) compared with that at 1 min-treatment (Figure 3.17B). On the other hand, alkaline condition at pH 12

could induce the amounts of ADC proteins, in particular at 240 min-treatment (Figure 3.17C).

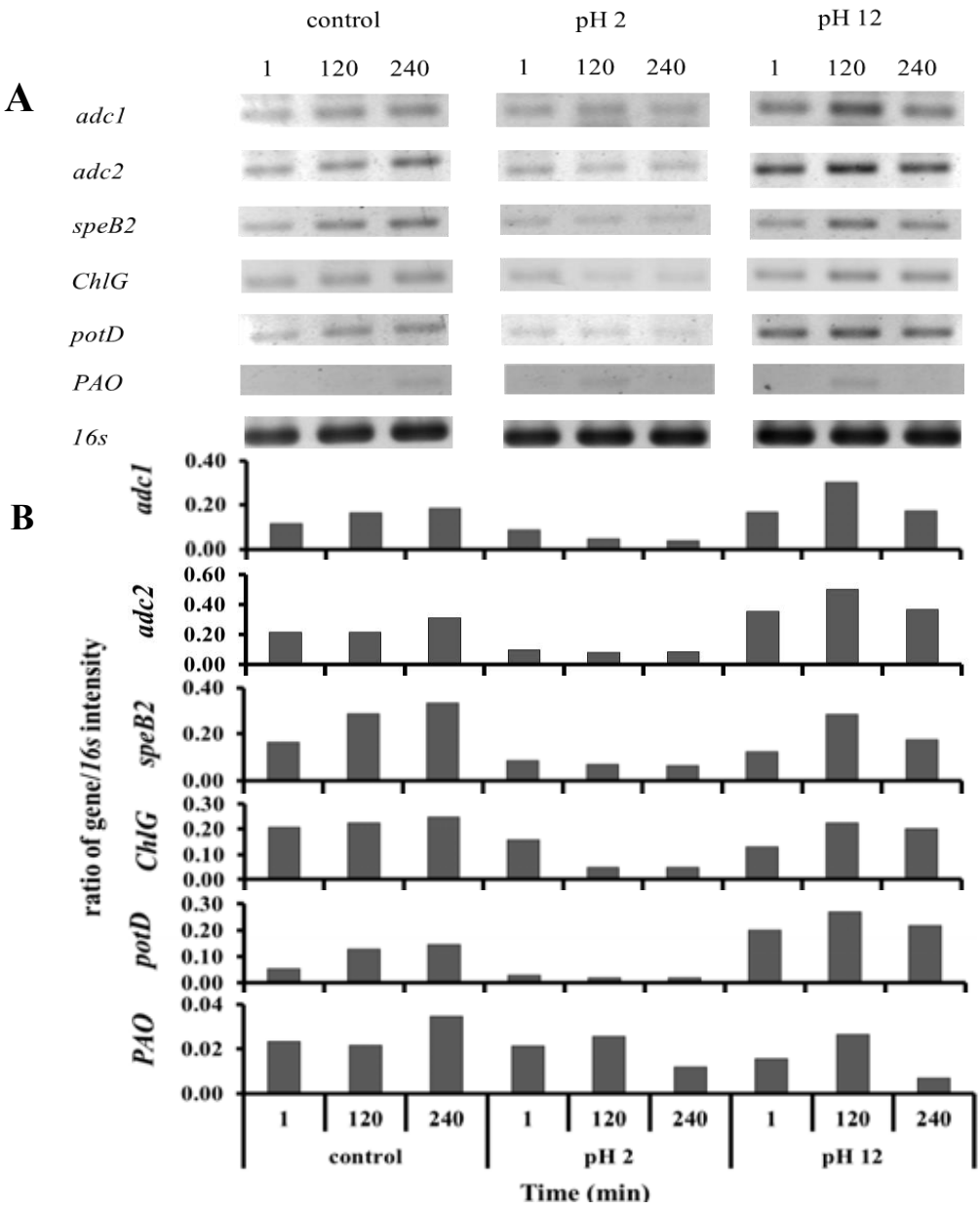

Figure 3.16 Transcription levels of *adc1*, *adc2*, *speB2*, *ChlG*, *potD* and *pao* genes in *Synechocystis* sp. PCC 6803 under various pH conditions. Cells were grown in BG_{11} until mid-log phase and further adapted in pH-adjusted media of pH 2, 7 and 12. RT-PCR products were checked by agarose gel electrophoresis (A) and their quantification was carried out using Genesnap program from Syngene® Gel Documentation. The *16S* rRNA was used as a reference on relative abundance, and depicted as graphs in

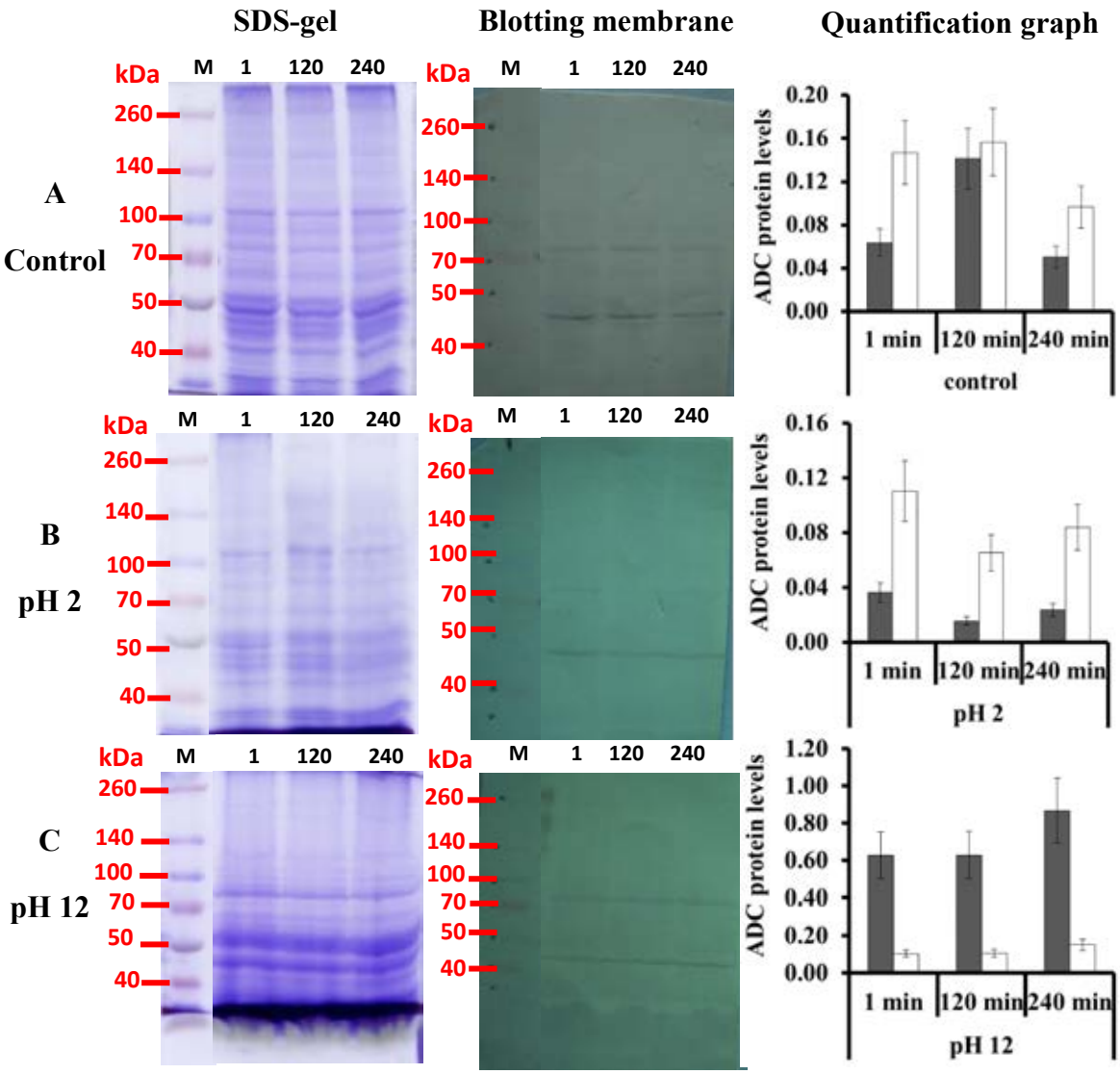

Figure 3.17 Western blot analysis of arginine decarboxylase (ADC) under various pH conditions (control, pH 2 and pH 12) from *Synechocystis* cells. Total protein extract (60 µg) was loaded onto the SDS-gel and further electrophoretically tranferred onto PVDF membrane with probing for 4 h at room temperature with 1:1500 dilution of ADC antibody. The images of SDS-gel, blotting membrane and quantification graph with intensities of upper band (solid bar) and lower band (open bar) are shown: (A) control at pH 7, (B) pH 2 and (C) pH 12 conditions. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation. Data are mean ± SD., n = 3.

CHAPTER IV

DISCUSSION

We have demonstrated that 5-6 day culture with cell density at OD_{730} about 0.5-0.6 of *Synechocystis* sp. PCC 6803 under various pH buffers was at mid-logarithmic phase. The 16 day-cultivation treatment was represented as a long-term pH effect on cell growth and intracellular pigments (Figure 3.1). *Synechocystis* cells had acclimated well in pH capacity more than 6.5, since the growth and pigment contents of cells grown in BG_{11} media containing HEPES at pH 7.6, MES at pH 6.5, BICINE at pH 7.6 and BICINE at pH 8.5, were continuously increased along 16 days. Long term acidic stress of pH 5.5 (MES buffer) severely inhibited both growth and intracellular pigments. The different types of buffers chosen at the same pH treatment, between HEPES and BICINE of pH 7.6, gave no effect on cell growth but they had a decreasing effect on intracellular pigments. It was found that HEPES buffer was more suitable to cell culture than BICINE buffer. This might be due to the potential physiological pH maintainance of HEPES despite changes of ambient CO_2 for air and cell respiration, compared to other buffers. However, HEPES, MES and BICINE are included in "Good's buffers" list as described by Norman Good and co-workers (Good *et al*., 1966). Coincidently, Kurian *et al*. (2006) reported that the growth of *Synechocystis* sp. PCC 6803 under acidic conditions was significantly retarded at pH 6.0 (MES) in 8 day culture and severely impaired at pH 5.5 (MES and HOMOPIPES) in 6 day culture.

In this study, we also demonstrated that the short-term pH stress of 240 min-treatment effect on both growth and intracellular pigment contents of *Synechocystis* cells compared to cells grown under normal condition (pH 7) (Figure 3.2). Our results show that cells could maintain their growth rate within 240 min-treatment (short-term effect), even at pH 2. This result suggests that the cellular defensive mechanism would enable cells to survive and acclimate against harsh pH environments, herein both strong acid stress at pH 2 and alkaline stress at pH 12. A previous study by Huang *et al*. (2002) reported that *Synechocystis* sp. PCC 6308 (which not 6803) was able to grow into exponential phase after acclimating in a long-lag phase at acid stress with pH between 4.4 and 7.7 for 24 h. *Synechocystis* PCC 6308 cultures in pH-stress condition at below pH 4.4 were not grown and become blue color, as well as, damaged on their photosystem II. It is consistent with this study, the chlorophyll *a* content at acid stress (pH 2) was rapidly decreased at first 1 min-treatment, and continuously decreased until 240 min-treatment (Figure 3.2) corresponding to the change of cell color. The color of cell culture at pH 2 was obviously changed from green to yellowish green after 120 min-treatment (Figure 3.3). Interestingly, when we determines the transcript level of *ChlG* mRNA gene, encoding chlorophyll *a* synthase in chlorophyll synthesis, of pH 2-stressed cells, the amount of *ChlG* mRNA was significantly decreased at 120 and 240 min-treatment (Figure 3.16B). This finding suggests that the decrease of photosynthetic efficiency induced by acid stress might be due to the decrease of chlorophyll *a* content and *ChlG* transcripts. Similarly with previous study, *Synechocystis* sp. PCC 6308 stressed at pH 3.6 had significantly reduced amount of chlorophyll *a* content as compared to cell stressed at pH 4.4 and pH 5.6. Cells stressed below pH 3.5 turned yellow (Huang *et al*., 2002). On the other

hand, few studies reported about the transcript level of gene related to chlorophyll synthesis, such as, the expression of chlorophyll synthase gene (*CS*) in the cucumber plants was increased during treating with abscisic acid (ABA) compared with that in control plants under low-nitrogen condition, though the expression was barely affected under sufficient-nitrogen condition (Oka *et al*., 2012).

Moreover, we also determined the oxygen evolution that represented the photosynthetic efficiency of cells stress at pH 7, pH 2 and pH 12 for 240 min. The results demonstrate that the oxygen evolution of *Synechocystis* cells under control condition at pH 7 was unchanged along 240 min-treatment and showed the highest level at 120 min-treatment whereas alkaline stress at pH 12 significantly increased their oxygen evolution, compared to control condition. Strikingly, acid stress condition at pH 2 completely inhibited the photosynthetic efficiency of *Synechocystis* cells along 240 min-treatment (Figure 3.4). This result is consistent with the chlorophyll *a* content and *ChlG* transcripts as described above. It was reported previously in *Anabaena* sp. strain PCC 7120 cells that about 60% inhibition on *Anabaena* photosynthesis capacity was observed under pH condition below pH 6.5 for 3-4 days whereas a high reduction of their photosynthesis capacity was shown only after 1 day of pH 5.4 treatment (Giraldez-ruuiz *et al*.,1997). However, pH stress of this study in all ranges (pH 2-12) did not affect much on growth and carotenoid content. *Synechocystis* cells seemed to maintain intracellular carotenoid level under pH stress in this study. Previously, we also found that the carotenoid content was constant even under UV radiation (Jantaro *et al*., 2010).

In this study, we investigated the effect of a rapid change of ambient pH (called pH transition) on growth and intracellular pigments in *Synechocystis* cells in order to figure out cellular defensive mechanism under harsh pH stress. We found the decrease of cell density about 20% after changing the ambient pH from 2 to 12 (Figure 3.8A). The pH transition from harsh acidic pH to alkaline pH seemed to harm cell growth. Moreover, chlorophyll *a* pigment was mainly destroyed by pH stress rather than carotenoids, since a 56% reduction of chlorophyll *a* was observed at pH 12 to 2-transition, compared to that of carotenoids (Figure 3.11A). When cells have faced the environment stress, they potentially acclimate themselves towards resistance by various response/defense mechanisms depending on stress period and severity. Recently, the growth acclimation of *Synechocystis* sp. PCC 6803 cells to pH 7.5 to 11.0 transition was reported (Zhang *et al*., 2009). It was found that the growth of the alkaline pH stressed cells was maintained in low level during first 24 h, and slightly increased after 48 h-treatment, followed by recovering gradually upto 148 h, but the cellular mechanism was still unclear. However, these observations suggest that the external pH of acidic stress (pH 2 in this study) generates deleterious effect to *Synechocystis* cells rather than alkaline pH stress.

Generally, in order to acclimatize against pH stress some microorganisms utilize amino acid decarboxylases to neutralize the extreme environment (Gale and Epps, 1942), while others utilize arginine catabolism to increase and balance internal pH (Casiano-Colon and Marquis, 1988; Rallu *et al*., 1996). In this study, we found that there was no external pH change under acid stress (pH 2) whereas a slight increase of external pH was observed under alkaline stress (pH 12) along 240 min-treatment (Figure 3.12). These results suggest that whether the coping mechanism

under acid stress would go to the intracellular arginine catabolism which synthesizes some alkaline compounds for internal pH balance. Consequently, one of important products of arginine catabolism of interest is polyamine. Our findings in this study indicate that free form level of polyamine was maintained constantly inside the cells facing acid stress at most 120 min-treatment (Figure 3.12). When we immediately changed the ambient pH of acid stressed cells into harsh alkaline stress (pH12), the polyamine amount was dramatically decreased. This result had confirmed us towards the different mechanism of *Synechocystis* cell adaptation between acid and alkaline pH stresses. Moreover, after pH 12 stressed cells were changed their surrounding pH into acidic condition at pH 2, free form-polyamines was obviously induced at first 60 min-period (Figure 3.14). Thus, our results suggest that a defensive mechanism enabled *Synechocystis* cells to survive against short term of pH stress by intracellular balance, especially due to intracellular polyamine titers, products or intermediates of arginine utilization. However, different species may take advantages on coping mechanism in different characters, such as, *Synechocystis* sp. PCC 6308 (not 6803) showed the capacity of alkaline compound secretion to neutralize external pH stress, especially acid stress (Huang *et al*., 2002)

We also demonstrate the polyamine level under various pH conditions, as well as transcripts and protein levels of polyamine-related pathways. Our findings indicate that *Synechocystis* cells efficiently maintained their polyamine levels, especially free form, under pH conditions in a range of 4-10 (Figure 3.5B, 3.6A and B, 3.7A). Polyamine amounts were changed obviously under strong acidic and alkaline stresses (pH 2 and pH 12, respectively) (Figure 3.5A and 3.7B). This might be due to their internal adjustment to survive under extreme environments. The main types of

polyamines existing in *Synechocystis* 6803 cells under both normal and pH stressed cells was Spd. A precursor diamine Put was induced in little amount by any pH stress whereas Spm was present in trace amount. Moreover, we found that bound form of polyamine (PCA-insoluble) was increased markedly by alkaline stress (pH 12) (Figure 3.7B). This result suggests that the induced bound form of polyamines might relate to membrane stabilization or arrangement against strong alkaline stress. However, there are many reports studied about the level of each polyamine type in other organisms. Previously, it was also studied in higher plant, wheat leaves; the Put content was induced by pH 3.5 condition higher than that by pH 6 condition while Spd levels were not changed under all pH conditions (Shen *et al*., 1994). On the other hand, in other stresses studied, intracellular polyamines increased by about 2- and 4-fold in NaCl and sorbitol-stressed cells especially Spd and Put in *Synechocystis* 6803 cells (Pothipongsa *et al*., 2012). In addition, when they applied UV-B radiation upon NaCl-stressed cells, it highly induced Spd level, at least 3 fold. On the other hand, we also demonstrated transcripts and protein levels in this study. In first aspect, we focused on polyamine biosynthesis by investigating 2 genes including *adc* and *speB2* genes encoding arginine decarboxylase and agmatinase, respectively. The *adc1* and *adc2* transcripts were up-regulated clearly by alkaline stress (pH 12) whereas down-regulation was observed markedly under strong acid stress (Figure 3.16). It was surprised since we have found earlier that pH 2 stress could maintain polyamine level inside the cells, but the *adc* mRNAs was less. However, the data evidence clearly showed later that the ADC protein level was highly induced by pH 2 stressed condition compared with those of control and pH 12 at 240 min-treatment (Figure 3.17). The polyamine production in response to strong acid stress indicated from those

results might then depend on translational level of ADC. Interestingly, strong alkaline stress for 120 min-treatment enhanced both *adc1* and *adc2* transcript levels, especially they are consistent with their ADC protein level. Polyamine accumulation under alkaline stress was then existed due to the regulation of both transcript and protein levels. For other environmental stresses, it was also found the induction consequence on polyamine biosynthetic genes in the same and different patterns in each species. The combined stress was recently performed and reported that NaCl- or sorbitol-stressed cells contained about 5-fold higher level of *adc1* transcript than the unstressed cells after 1 h irradiation with UV-B (Pothipongsa *et al*., 2012). Both salt and osmotic stresses mainly caused the significant increase in *adc* mRNA (Jantaro *et al*., 2003). Moreover in term of enzyme level, the acid adapted cells showed higher relative expression levels for arginine decarboxylase and lysine decarboxylase, which demonstrates that the induction of specialized pH homeostatic systems plays an important role in *S. Typhimurium* protection against acid stress (Álvarez-Ordóñez *et al*., 2010). On the other hand, two protein bands obtained from this experiment were quantified including upper and lower bands with about 75 kDa and 45 kDa, respectively, in *Synechocystis*. The upper band with about 75 kDa was indicated as the ADC protein band due to the corresponding data from predicted size of ADC at 74.4 kDa. Although the homology of amino acid sequences between higher plants and cyanobacteria gave no tight correlation, the molecular mass of both ADC protein were similar. In flowers, leaves, stems and roots of tobacco found the ADC protein with the molecular mass of 77 kDa whereas another distinct band of ADC protein found in leaves and stems had an approximate molecular mass of 54 kDa (Bortolotti *et al*., 2004). Moreover, we also show the mRNA level of *speB2* which encoded agmatinase

relating to polyamine biosynthesis pathway. Agmatinase gene was up-regulated by strong alkaline stress whereas decreased significantly under strong acid stress which was consistent with *adc* mRNA patterns. On the other hand, in aspect of polyamine transport, we monitored the transcript level of *potD* gene encoding periplasmic polyamine-binding protein. Strikingly, we found that *potD* mRNA was highly induced by strong alkaline stress while was decreased by strong acid stress. Previously, our colleagues in research group found that long-term (3 days) treatment, such as light intensity, salt, osmotic, temperature and nutrient availability, resulted in slightly increased amounts of the *potD* transcripts (Brandt *et al*., 2010), and PotD might relate to the polyamine secretion rather than uptake. Consequently, our finding might indicate that *Synechocystis* cells secret the excess polyamine out of cells in order to protect themselves from high/excess polyamine accumulation, especially under alkaline stress. Moreover the degradation aspect of polyamine was also investigated by determine *pao* transcript levels (encoding polyamine oxidase). It was demonstrated that both strong acid and alkaline stresses slightly increased the *pao* mRNA at 120 min-treatment compared to that of control (Figure 3.16). However, *pao* mRNA amounts was mainly unaffected by pH stress. Polyamine degradation was then seemed to be not the main response under pH stress as polyamine biosynthesis and transport did. In *Arabidopsis*, *AtPAO2* and *AtPAO4* transcripts and activities of PAOs were both induced by ethylene, plant hormone. In transgenic *Arabidopsis* plants over-expressing *AtPAO2* and *AtPAO4*, stomatal movement was more sensitive to ethylene treatment and H_2O_2 production was also significantly induced as side products of reaction (Hou *et al*., 2013).

CHAPTER V

CONCLUSIONS

Based on the results, the following specific conclusions were drawn:

1. The long-term pH stress to *Synechocystis* cells grown in BG_{11} containing various pH buffers, including pH 6.5 (MES), pH 7.6 (HEPES and BICINE) and 8.5 (BICINE), did not affect their growth, chlorophyll *a* and carotenoid contents whereas pH 5.5 (MES) showed the severe inhibition both on cell growth and intracellular pigments.
2. Short term (4 h) of acid stress (pH 2) rapidly decreased the content of chlorophyll *a*, in particular at first 1 min-treatment and gradually decreased upto 240 min-treatment whereas all of pH 7 and 12 conditions did not affect the carotenoid content.
3. The color of cell culture was changed from green to yellowish green in a short term of acid stress at pH 2 whereas there was no color changes of cell culture in other pH conditions studied.
4. The oxygen evolution of *Synechocystis* cells was inhibited severely by strong acid stress (pH 2). It was consistent with both the reduction of chlorophyll *a* content and the decrease of *ChlG* transcript.
5. Free form-polyamines were found as major forms, and free form-spermidine was present dominantly in all conditions whereas putrescine and spermine showed in trace amounts.

6. *Synechocystis* cells could maintain the level of polyamine accumulation along 240 min-treatment in pH conditions ranging from pH 4 – 10.
7. The pH 2 and pH 12 stresses apparently decreased free form-polyamines at 240-min treatment. Bound form-polyamines were not changed significantly by strong acid stress whereas strong alkaline stress at pH 12 highly induced bound form-polyamines.
8. The pH transition from pH 2 $\rightarrow$ 12 and the increase of ambient pH along treatment indicated the adaptive mechanism of *Synechocystis* cells directing to polyamine secretion against strong alkaline stress.
9. The pH transition from pH 12 $\rightarrow$ 2 and the constant of ambient pH along treatment indicated the adaptive mechanism of *Synechocystis* cells directing to internal balance on the level of arginine catabolism producs, namely polyamines.
10. The transcript levels of polyamine biosynthetic genes, including *adc1, adc2* and *speB2*, were decreased under acid stress whereas strong alkaline stress increased their transcript levels.
11. The *potD* transcript, related mainly to polyamine secretion, was highly induced by alkaline stress (pH 12) while it was decreased during acid stress (pH 2).
12. Strong acid and alkaline stresses slightly increased the *pao* mRNA, related to polyamine degradation, at 120 min-treatment.
13. Polyamine level under acid stress was consistent only with the level of ADC protein whereas polyamine content under alkaline stress was correlated with both the levels of *adc1,2 and speB2* mRNAs and the amount of ADC protein.

REFERENCES

Agervald, Å., Stensjö, K., Holmqvist, M. and Lindblad, P. Transcription of the extended *hyp*-operon in *Nostoc* sp. strain PCC 7120. BMC Microbiology 8 (2008) : 69-80.

An, L. Z., Liu, G. X., Zhang, M. X., Chen, T., Liu, Y. H., Feng, H. Y., Xu, S. J., Qiang, W. Y. and Wang, X. L. Effect of enhanced UV-B radiation on polyamine content and membrane permeability in cucumber leaves. Russian Journal of Plant Physiology 51(5) (2004) : 658-662.

Antognoni, F., Pistocchi, R., Casali, P. and Bagni, N. Does calcium regulate polyamine uptake in carrot protoplasts?. Plant Physiology and Biochemistry 33 (1995) : 701-702.

Bagni, N. and Tassoni, A. Biosynthesis oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids 20 (2001) : 301-317.

Bearson, S., Bearson, B. and Foster, J.W. Acid stress responses in enterobacteria**.** FEMS Microbiology Letters 147(2) (1997) : 173-180.

Borrell, A., Culianez-Macia, F.A., Altabella, T., Besford, R.T., Flores, D. and Tiburcio, A.F. Arginine decarboxylase is localized in chloroplasts. Plant Physiology 109 (1995) : 771-776.

Bortolotti, C., Cordeiro, A., Alcázar, R., Borrell, A., Culiañez-Macià, F.A., Tiburcio, A.F. and Atabella, T. Localization of arginine decarboxylase in tobacco plants. Physiologia Plantarum 120 (2004) : 84-92.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J. Polyamines and environmental challenges: recent development. Plant Science 140 (1999) : 103–125.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 (1976) : 248 – 254.

Carley, E., Wolosisuk, R.A. and Hertig, C.M. Regulation of the activation of chlorophast fructose-1,6-bis phosphate (E.C.3.1.3.11). Inhibition by spermidine and spermine. Biochemical and Biophysical Research Communications 115 (1983) : 707-710.

Casiano-Colon, A. and Marquis, R.E. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Applied and Environmental Microbiology 54 (1988) : 1318-1324.

Chamovitz, D., Sandmann, G. and Hirschberg, J. Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a ratelimiting step in carotenoid biosynthesis. Journal of Biological Chemistry 268 (1993) : 17348–17353.

Cohen, S.S. A Guide to the Polyamine. Oxford: Oxford University Press, 1998.

Feurstein, B.G. and Marton, L.G. Specificity and binding in polyamine/nucleic acid interaction, in: Bachrach,U., Heimer, Y.M. (Eds.), The Physiology of Polyamines, Vol. 1, pp. 109-120. CRC Press, Boca Raton, FL, 1989.

Flores, H.E. and Filner, P. Polyamine catabolism in higher plants: characterization of pyrroline dehydrogenase. Plant Growth Regulation 3 (1985) : 277–291.

Foster, J.W. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Critical Reviews in Microbiology 21 (1995) : 215-237.

Foster, J.W. and Hall, H.K. Adaptive acidification tolerance response of *Salmonella typhimurium.* Journal of Bacteriology 172 (1990) : 771-778.

Gale, E.F. and Epps, H.M.R. The effects of the pH of the medium during growth on the enzymatic activities of bacteria (*E. coli* and *M. lysodiekticus*) and the biological significance of the changes produced. Biochemistry 36 (1942) : 600-619.

Gantt, E. Supramolecular membrane organization. In: Bryant, D.A. (Ed), The Molecular Biology of cyanobacteria, pp. 119-138. Dordrecht, The Netherlands : Kluwer Academic, 1994.

Giraldez-Ruiz, N., Mateo, P., Bonilla, I and Fernandez-Piñas, F. The Relationship between Intracellular pH, Growth Characteristics and Calcium in the Cyanobacterium Anabaena sp. Strain PCC7120 Exposed to Low pH. New Phytologist 137 (1997) : 599-605.

Glatz, A., Vass, I., Los, D.A. and Vígh, L. The *Synechocystis* model of stress: From molecular chaperones to Membranes. Plant Physiology and Biochemistry 37(1) (1999) : 1-12.

González-Toril, E., Llobet-Brossa, E., Casamayor, E.O., Amann, R., and Amils, R. Microbial Ecology of an Extreme Acidic Environment, the Tinto River. Applied and Environmental Microbiology 69 (2003) : 4853-4865.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. Hydrogen ion buffers for biological research. Biochemistry 5(2) (1996) : 467-477.

Grigorieva, G. and Shestakov, S. Transformation in the cyanobacterium *Synechocystis* sp 6803. FEMS Microbiology Letters 13 (1982) : 367-370.

Hassain, S.S., Ali, M., Ahmad, M. and Siddique, K.H.M. Polyamine: Natural and engineered abiotic and biotic stress tolerance in plants. Biotechnology Advances 29 (2011) : 300-311.

Havelange, A., Lejeune, P., Bernier, G., Kaur-Sawhney, R. and Galston, A.W. Putrescine export form leaves in relation of floral transition in *Sinapis alba*. Physiologia Plantarum 96 (1996) : 59-65.

Higgins, C.F. ABC transporters: from microorganisms to man. Annual Review of Cell Biology 8 (1992) : 67-113.

Horikoshi., K. Alkaliphiles: Some Applications of Their Products for Biotechnology. Microbiology and Molecular Biology Reviews 63(4) (1999) : 735-750.

Hou, Z., Liu, G., Hou, L., Wang, L. and Liu, X. Regulatory Function of Polyamine Oxidase-Generated Hydrogen Peroxide in Ethylene-Induced Stomatal Closure in *Arabidopsis thaliana*. Journal of Integrative Agriculture 12(2) (2013) : 251-262.

Huang J. J., Kolodny N. H., Redfearn J. T. and Allen M. M. The acid stress response of the cyanobacterium *Synechocystis* sp. Strain PCC 6308. Archives of Microbiology 177 (2002) : 486-493.

Igarashi, K. and Kashiwagi, K. Polyamine transport in bacteria and yeast. Biochemical Journal **344** (1999) : 633-642.

Ikeuchi, M and Tabata, S. *Synechocystis* sp. PCC 6803 - a useful tool in the study of the genetics of Cyanobacteria. Photosynthesis Research 70 (2001) : 73-83.

Incharoensakdi, A., Jantaro, S., Raksajit, W. and Mäenpää, P. Polyamines in cyanobacteria: biosynthesis, transport and abiotic stress response. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (2010) : 23-32.

Jacob, S.T. and Stetler, D.A. Polyamines and RNA synthesis, in Bachrach,U., Heimer, Q.M. (Eds.), The Physiology of Polyamines, Vol. 1, pp. 133-140. CRC Press, Boca Raton, FL, 1989.

Jantaro, S., Mäenpää, P. and Incharoensakdi, A. Content and biosynthesis of polyamines in salt and osmotically stressed cells of *Synechocystis* PCC 6803, FEMS Microbiology Letters 228 (2003) : 129-135.

Jantaro, S., Pothipongsa, A., Khanthasuwan, S. and Incharoensakdi, A. Short-term UV-B and UV-C radiations preferentially decrease spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803. Current Microbiology 62 (2010) : 420-426.

Joset, F., Jeanjean, R. and Hanemann, M. Dynamics of the response of cyanobacteria to salt stress: Deciphering the molecular events. Physiologia Plantarum 96 (1996) : 738-744.

Kaneko, T., Sato, S., Kotani H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S.,Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. II. Sequence determination of the entire genome

and assignment of potential protein-coding regions. DNA Research 3 (1996) : 109-136.

Kaur-Sawhney, R., Tiburcio, A.F., Altabella, T. and Galston, A.W. Polyamines in plants: an overview. Journal of Cell and Molecular Biology 2 (2003) : 1-12.

Kurian, D., Phadwal, K. and Mäenpää, P. Proteomic characterization of acid stress response in *Synechocystis* sp. PCC 6803. Proteomics 6 (2006) : 3614-3624.

Legocka, J. and Zaichert, J. Role of spermidine in the stabilization of apoprotein of the light-harvesting Chlorophyll a/b-protein complex of Photosystem II during leaf senescence process. Acta Physiologia Plantarum 21 (1999) : 127-137.

Lehel, Cs., Gombos, Z., Török, Zs. and Vígh, L. Growth temperature modulates thermo tolerance and heat shock response of cyanobacterium *Synechocystis* PCC 6803. Plant Physiology and Biochemistry 31 (1993) : 81-88.

Margosiak, S.A., Dharma, A., Gonzales, A.P., Louie, D. and Kuehn, G.D. Identification of the large subunit of ribulose 1,5-bisphosphate/ carboxylase/oxygenase as a substrate for transglutaminase in plants. Plant Physiology 92 (1990) : 88-96.

Martin-Tanguy, J., Tepfer, D., Paynot, M., Burtin, D., Heister, L. and Martin, C. Inverse relationship between polyamine level and the degree of phenotypic alteration induced by root inducing left hand T-DNA from *Agrobacterium rhizogenes*. Plant Physiology 92 (1990) : 912-918.

Martin-Tanguy, J. Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiologia Plantarum 100 (1997) : 675-688.

Moran, R. Formulae for Determination of Chlorophyllous Pigments Extracted with N,N-Dimethylformamide. Plant Physiology 69(6) (1982) : 1376-1381.

Negrel, J. The biosynthesis of cinnamoyl putrescines in callus tissue cultures of *Nicotiana tabacum*. Phytochemistry 28 (1989) : 477-481.

Ohta, H., Shibata, Y., Haseyama, Y., Yoshini, Y., Suzuki, T., Kagasawa, T., Kamei, A., Ikeuchi, M. and Enami, I. Identification of genes expressed in response to acid stress in *Synechocystis* sp. PCC 6803 using DNA microarrays. Photosynthesis Research 84 (2005) : 225-230.

Oka, M., Shimoda, Y., Sato, N., Inoue, J., Yamazaki, T., Shimomura, N. and Fujiyama H. Abscisic acid substantially inhibits senescence of cucumber plants (*Cucumis sativus*) grown under low nitrogen conditions. Journal of Plant Physiology 169(8) (2012) : 789-96.

Olson, E.R. Influence of pH on bacterial gene expression. Molecular Microbiology 1 (1993) : 5-14.

Pang, X.M., Zhang, Z.Y., Wen, X.P., Ban, Y. and Moriguchi T. Polyamines, All-purpose players in response to environment stresses in plants. Plant Stress 1 (2007) : 173-188.

Poole, R. K. Bacterial response to pH. Introduction. Novartis Foundation Symposium 221 (1999) : 1-3.

Pothipongsa, A., Jantaro, S. and Incharoensakdi, A. Polyamines induced by osmotic stress protect *Synechocystis* sp. PCC 6803 cells and arginine decarboxylase transcripts against UV-B radiation. Applied Biochemistry and Biotechnology 6 (2012) : 1476-88.

Pothipongsa, A. Changes of polyamines content under osmotic and UV-radiation stresses in cyanobacterium *Synechocystis* sp. PCC 6803. Master Thesis, Depatment of Biochemistry, Faculty of Science, Chulalongkorn University, 2009.

Rajasekaran, L. R. and Blake, T. J. New plant growth regulators potect photosynthesis and enhance growth under drought of jack pine seedlings. Journal of Plant Growth Regulation 18 (1999) : 175-181.

Raksajit, W., Mäenpää, P. and Incharoensakdi, A. Putrescine transport in a cyanobacterium *Synechocystis* sp. PCC 6803. Journal of Biochemistry and Molecular Biology 39 (2006) : 394-399.

Raksajit, W., Yodsang, P., Mäenpää, P. and Incharoensakdi, A. Characterization of spermidine transport system in a cyanobacterium *Synechocystis* sp. PCC 6803. Journal of Microbiology and Biotechnology 19(5) (2009) : 447-454.

Rallu, F., Gruss, A. and Maguin, E. *Lactococcus lactis* and stress. Antonie van Leeuwenhoek 70 (1996) : 243-251.

Rinehart, C.A., Jr. and Chen, K.Y. Characterization of polyamine transport system in mouse neuroblastoma cells. Journal of Biological Chemistry 259 (1984) : 4750-4756.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. Generic assignments, stain histories and properties of pure cultures of cyanobacteria. Journal of General and Applied Microbiology 111 (1979) : 1-61.

Roy, M. and Gosh, B. Polyamines, both common and uncommon, under heat stress in rice (*Oryza sativa*) callus. Physiologia Plantarum 98 (1996) : 196-200.

Seiler, N., Delcros, J.G. and Moulinoux, J.P. Polyamine transport in mammalian cells: an update. The international Journal of Biochemistry & Cell Biology 28 (1996) : 843-861.

Shen, H.J., Xie, Y.F. and Li, R.T. Effects of acid stress on polyamine levels, ion efflux, protective enzymes and macromolecular synthesis in cereal leaves. Plant Growth Regulation 14 (1994) : 1-5.

Smith, B.N. and Meeuse, B.J.D. Production of volatile amines in some Arum lily species. Plant Physiology 41 (1966) : 343-347.

Smith, J., Burritt, D. and Bannister, P. Ultraviolet-B radiation leads to a reduction in free polyamines in *Phaseolus vulgaris* L. Plant Growth Regulation 35 (2001) : 289-294.

Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriological Reveiws 35 (1971) : 171-205.

Summerfield, T.C. and Sherman, L.A. Global Transcriptional response of the alkali-tolerant cyanobacterium *Synechocystis* sp. strain PCC 6803 to a pH 10 environment. Applied and Environmental Microbiology 74(17) (2008) : 5276–5284.

Tadolini, B., Cabrini, L., Varani, E. and Sechi, A.M. Spermine binding and aggregation of vesicles of different lipid composition. Biogenic Amines 3 (1985) : 87-92.

Tassoni, A., Napire, R.M., Franceschetti, M., Venis, M.A. and Bagni, N. Spermidine-binding proteins. Purification and expression analysis in maize. Plant Physiology 128(4) (2002) : 1303-1312.

Xiong, J., Fischer, W.M., Inoue, K., Nakahara, M. and Bauer, C.E. Molecular evidence for the early evolution of photosynthesis. Science 289 (2000) : 1724-1730.

Walters, D.R. Polyamine in plant-microbe interactions. Physiological and Molecular Plant Pathology 57 (2000) : 137-146.

Walters, D.R. Polyamines and plant disease. Phytochemistry 64 (2003) : 97-107.

Zhang, L.F., Yang, H.M., Cui, S.X., Hu, J., Wang, J., Kuang, T.Y., Norling, B and Huang, F. Proteomic analysis of plasma membranes of cyanobacterium *Synechocystis* sp. strain 6803 in response to high pH stress. Journal of Proteome Research 8 (2009) : 2892-2902.

APPENDICES

APPENDIX A

BG-11 medium

		Solid medium	**Liquid medium**
	100 ml	1,000 ml	1,000 ml
$CaCl_2.2H_2O$	3.6 g	1 ml	1 ml
Citric acid	0.6 g	1 ml	1 ml
$MgSO_4.7H_2O$	7 g	1 ml	1 ml
Na-EDTA	0.1 g	1 ml	1 ml
Na_2CO_3	2 g	1 ml	1 ml
K_2HPO_4	3.048 g	1 ml	1 ml
Trace element*		1 ml	1 ml
Ammonium ferric citrate	0.6 g	1 ml	1 ml
1 M HEPES pH 7.6	23.83 g	10 ml	10 ml
$NaNO_3$	15 g	10 ml	10 ml
30% $Na_2S_2O_3$	30 g	10 ml	-
1.5% Agar		15 g	-
H_2O		962 ml	972 ml

1,000X Trace element*

	1,000 ml
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.221 g
$Na_2MoO_4.2H_2O$	0.390 g
$CuSO_4.5H_2O$	0.080 g
$Co(NO_3)_2.6H_2O$	0.049 g

APPENDIX B

Chromatogram of standard curve of polyamines

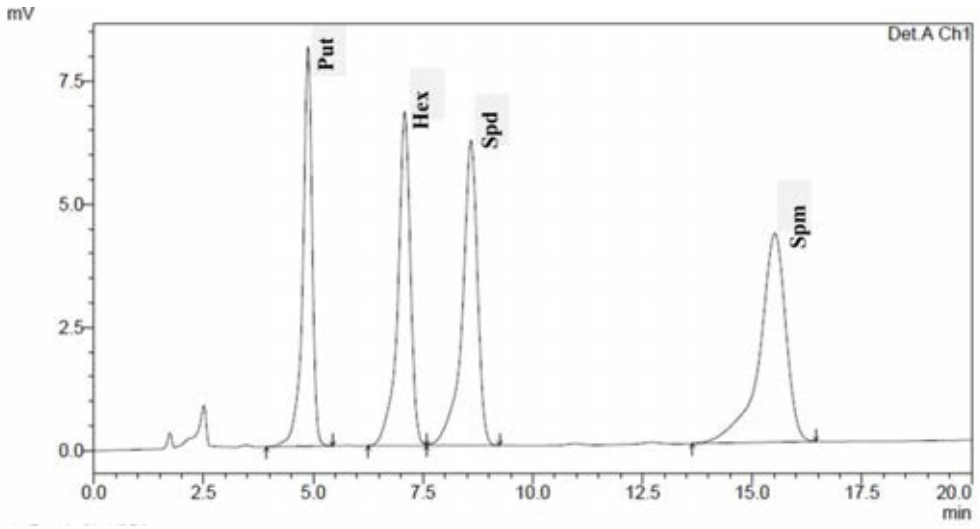

Figure B.1 Chromatogram of standard polyamines (Put = Putrescine, Spd = Spermidine, Spm = Spermine, respectively) and internal standard (Hex = 1,6-Hexanediamine) Retention time of Put, Hex, Spd and Spm are 4.748, 7.017, 8.488 and 15.256, repectively.

Standard curve of polyamines

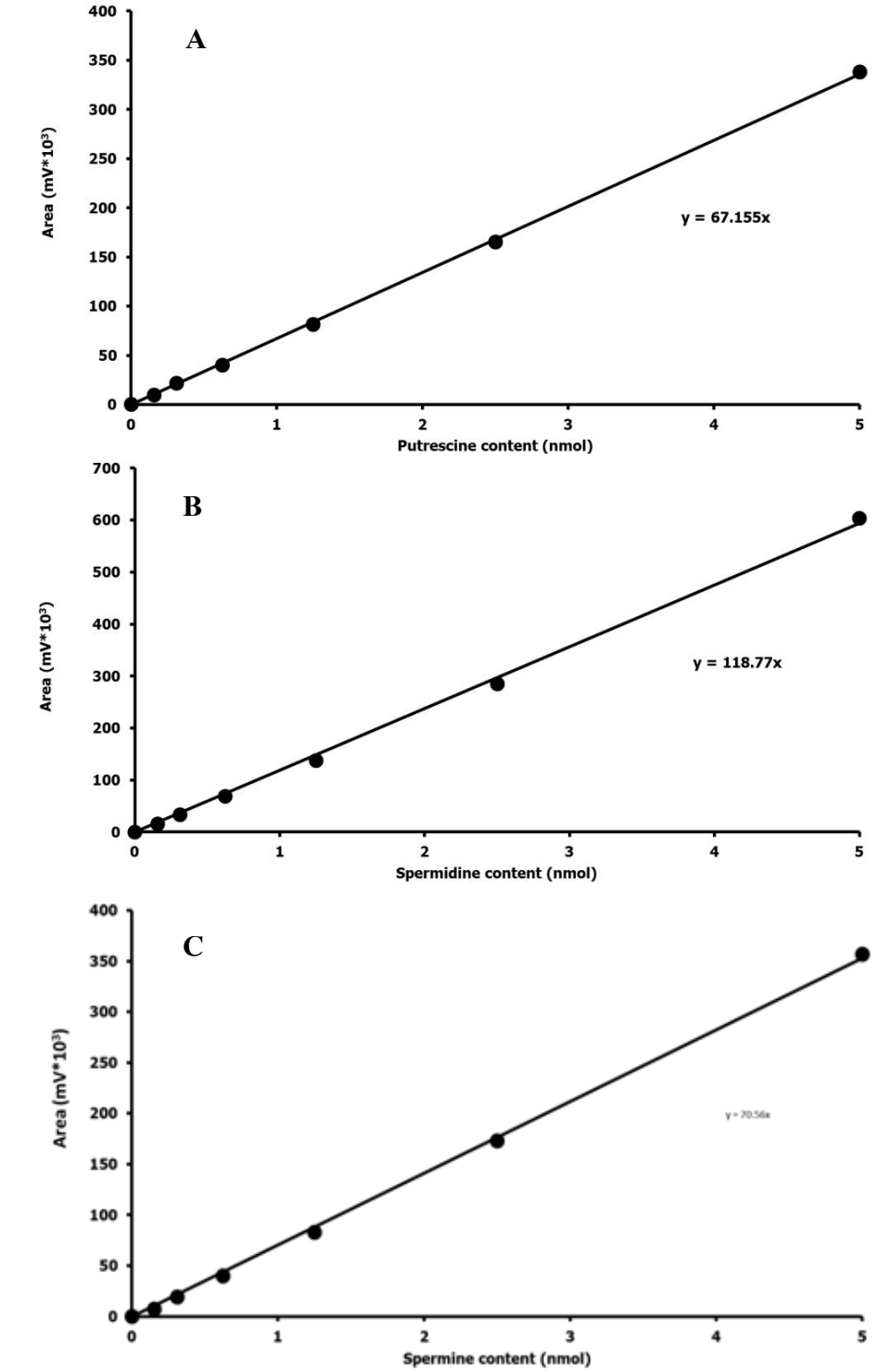

Figure B.2 Standard curve of polyamines (A = Putrescine, B = Spermidine, C = Spermine).

APPENDIX C

50X TAE buffer

Tris base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Add distilled water to a total volume of 1 liter.

APPENDIX D

Acid washed glass beads

1. Weigh 50 g of 0.5 mm glass beads into a 100 ml of Erlenmeyer flask, the volume of glass beads should not be more than 1/5 the volume of the flask that is used.
2. Add enough 5.8 M HCl to cover the glass beads.
3. Swirl the flask to wash the glass beads.
4. Carefully pour 5.8 M HCl into another bottle
5. Add distilled water to wash the glass beads by volume of distilled water should be at least 5X the volume of the glass beads in flask. Swirl 10 sec to stir up the glass beads.
6. Pour off the distilled water wash and repeat wash steps for 10 times to reduce the acid to below 10 mM
7. Move the glass beads to a beaker, cover with foil and autoclave the beads for 20 min.
8. Dry the glass beads at 50°C overnight.

APPENDIX E

Protein extraction buffer containing:

50 mM Tris-HCl (pH 8.0)

1 mM EDTA

50 µM Pyridoxal phosphate

5 mM DTT

0.5 mM PMSF

10% (v/v) Glycerol

0.2% Triton X-100

APPENDIX F

Preparation of polyacrylamide gel electrophoresis

Stock solution

Acrylamide solution, 100 ml (30% Acrylamide, 0.8% bis-acrylamide)

Acrylamide	29.2 g
N,N'-methylene-bis-acrylamide	0.8 g

Add distilled water to make 100 ml and stirred until completely dissolved.

2 M Tris-HCl, pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjust pH to 8.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

1 M Tris-HCl, pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane	12.1 g

Adjust pH to 8.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

10% Sodium dodecyl sulfate (SDS), 100 ml

Sodium dodecyl sulfate	10 g

Add distilled water to make 100 ml and stored at room temperature.

10% Ammonium persulfate (APS), 1 ml

Ammonium persulfate	0.1 g

Add distilled water to a total volume of 1 ml.

50% Glycerol, 100 ml

100% Glycerol	50 ml

Add distilled water to a total volume of 100 ml

1% Bromophenol blue, 10 ml

Bromophenol blue	0.1 g

Add distilled water to a total volume of 10 ml

10X Electophoresis buffer

Tris (hydroxymethyl)-aminomethane	30.2 g
Glycine	144 g
SDS	10 g

Add the distilled water to a total volume of 1 liter (Final pH is approximately 8.3, do not adjust pH with acid or base)

Coomassie Gel Stain, 1 liter

Coomassie blue R-250	1 g
Methanol	450 ml
Glacial acetic acid	100 ml

Add distilled water to a total volume of 1 liter

Coomassie Gel Destain, 1 liter (20% Methanol, 10% Acetic acid)

Methanol	200 ml
Glacial acetic acid	100 ml

Add distilled water to a total volume of 1 liter

Working solution

Solution B (for SDS-PAGE), 100 ml

2 M Tris-HCl, pH 8.8	75 ml
10% SDS	4 ml

Add distilled water to a total volume of 100 ml.

Solution C (for SDS-PAGE), 100 ml

1 M Tris-HCl, pH 6.8	50 ml
10% SDS	4 ml

Add distilled water to a total volume of 100 ml.

5X Sample buffer, 10 ml

1 M Tris-HCl, pH 6.8	0.6 ml
50% Glycerol	5 ml
10% SDS	2 ml
Mercaptoethanol	0.5 ml
1% Bromophenol blue	1 ml

Add distilled water to a total volume for 10 ml. Stored at 4°C for week or -20°C for months.

SDS-PAGE

8% Separating gel (for 2 gel)

40 % Acrylamide solution	2 ml
Solution B	2.5 ml
Distilled water	5.5 ml
10% APS	75 µl
TEMED	7.5 µl

5% Separating gel (for 2 gel)

40 % Acrylamide solution	1.25 ml
Solution C	2.5 ml
Distilled water	6.25 ml
10% APS	75 µl
TEMED	7.5 µl

APPENDIX G

Western blotting buffer

1X TBS (Tris-buffer-saline), 1 liter

20 mM Tris-HCl, pH 7.5	2.423 g
0.5 M NaCl	29.22 g

Add distilled water to a total volume 1 liter.

TBS-T

1X TBS

0.05% Tween-20

Blotting buffer, 100 ml (Fresh preparation)

Tris (hydroxymethyl)-aminomethane	0.582 g
Glycine	0.293 g
Methanol	20 ml
Distilled water	80 ml

Blocking solution

5% Skim milk in TBS

Antibody solution

3% Skim milk in TBS

Development solution

DAB	3 mg
30% H_2O_2	10 µl
1% $CoCl_2$	25 µl
TBS	10 ml

BIOGRAPHY

Miss Arpinyan Boonprakobkul was born on April 10, 1987 in Bangkok, Thailand. She graduated with Bachelor of Science degree in General Science, Faculty of Science, Chulalongkorn University in 2009. In 2010, she further studied for the Master of Science degree in Biochemistry, Faculty of Science, Chulalongkorn University.

Academic experience:

A staff of the 17th Biological Sciences Graduate Congress, 8-10 December 2012, Chulalongkorn University, Bangkok Thailand.

Outputs from research:

1. **Boonprakobkul, A.**, Incharoensakdi, A., and Jantaro, S. Effect of pH transition on intracellular pigment and polyamine contents in cyanobacterium *Synechocystis* sp. PCC 6803. The 13th FAOBMB Congress, 25-29 November 2012, Bangkok International Trade and Exhibition Center (BITEC) Bangna, Bangkok Thailand (Poster presentation; proceeding).

2. **Boonprakobkul, A.**, Incharoensakdi, A., and Jantaro, S. Effect of pH on intracellular pigments and polyamine content in cyanobacterium *Synechocystis* sp. PCC 6803. The 20th The Science Forum 2012: Science-Preparation for the Asean Economic Community, 19-20 April 2012, Chulalongkorn University, Bangkok Thailand (Oral presentation).