ผลของภาวะเครียดจากซอร์บิทอลต่อปริมาณพอลิเอมีนในสายพันธุ์กลาย

Synechocystis sp. PCC 6803 ของยีน *sll1077* และ *sll0228*

นางสาวศุภาพิชญ์ เอกนิคม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF SORBITOL STRESS ON POLYAMINE CONTENTS IN *Synechocystis* sp. PCC 6803 MUTANTS OF *sll1077* AND *sll0228* GENES

Miss Supapid Eknikom

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Thesis Title	EFFECT OF SORBITOL STRESS ON POLYAMINE CONTENTS IN *Synechocystis* sp. PCC 6803 MUTANTS OF *sll1077* AND *sll0228* GENES
By	Miss Supapid Eknikom
Field of Study	Biotechnology
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 Sanit Piyapattanakorn, Ph.D.)

.....External Examiner
(Assistant Professor Wuttinun Raksajit, Ph.D.)

ศุภาพิชญ์ เอกนิคม : ผลของภาวะเครียดจากซอร์บิทอลต่อปริมาณพอลิเอมีนในสายพันธุ์กลาย *Synechocystis* sp. PCC 6803 ของยีน *sll1077* และ *sll0228* (EFFECT OF SORBITOL STRESS ON POLYAMINE CONTENTS IN *Synechocystis* sp. PCC 6803 MUTANTS OF *sll1077* AND *sll0228* GENES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. อรัญ อินเจริญศักดิ์, 101 หน้า.

จากการศึกษาผลกระทบของภาวะเครียดออสโมติกซึ่งเหนี่ยวนำด้วยซอร์บิทอลต่อการเจริญ ปริมาณคลอโรฟิลล์ เอ และคาโรทีนอยด์ การเกิดออกซิเจน ปริมาณพอลิเอมีน การแสดงออกของยีน และ แอคทิวิตีของแอกมาทิเนสในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 สายพันธุ์ปกติและสายพันธุ์กลายสองสายพันธุ์ที่ขาดยีนแอกมาทิเนส *sll1077* (Δ*sll1077*) และยีนอาร์จิเนส *sll0228* (Δ*sll0228*) ตามลำดับ แม้ว่าการเปรียบเทียบแบบแพร์ไวส์ของลำดับกรดอะมิโนของ *sll1077* และ *sll0228* แสดงความเหมือน 35.3% และเอกลักษณ์ 20.7% ผลของ phylogenetic tree บ่งบอกว่ายีนทั้งสองอยู่ในเครือบรรพบุรุษเดียวกันของแอกมาติเนสเมื่อเปรียบเทียบกับลำดับกรดอะมิโนของสายพันธุ์อื่นซึ่งทราบลำดับแล้ว เลี้ยงเซลล์ทั้งสามสายพันธุ์ของสายพันธุ์ปกติ สายพันธุ์ Δ*sll1077* และ สายพันธุ์ Δ*sll0228* ในอาหารสูตร BG_{11} ที่ประกอบด้วยความเข้มข้นของซอร์บิทอลที่ 250, 500 และ 700 มิลลิโมลาร์ ตามลำดับ อัตราการเจริญและปริมาณคลอโรฟิลล์เอและคาโรทีนอยด์ของสายพันธุ์ปกติและสายพันธุ์กลายไม่มีความแตกต่างกันภายใต้สภาวะซอร์บิทอล 250 และ 500 มิลลิโมลาร์ ขณะที่สภาวะซอร์บิทอล 700 มิลลิโมลาร์ ยับยั้งทั้งการเจริญและปริมาณคลอโรฟิลล์ เอ และคาโรทีนอยด์อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับผลภายใต้สภาวะปกติ เป็นที่น่าสนใจว่า เซลล์ Δ*sll1077* เจริญได้เล็กน้อยภายใต้สภาวะซอร์บิทอล 700 มิลลิโมลาร์ และสามารถรักษาปริมาณคลอโรฟิลล์ เอ และคาโรทีนอยด์ตลอด 14 วันของการทดสอบ การเกิดออกซิเจนจากกระบวนการสังเคราะห์ด้วยแสงของทั้งสามสายพันธุ์ไม่มีความแตกต่างภายใต้สภาวะซอร์บิทอล 0, 250 และ 500 มิลลิโมลาร์ ขณะที่สภาวะซอร์บิทอล 700 มิลลิโมลาร์ลดการเกิดออกซิเจนจากกระบวนการสังเคราะห์ด้วยแสงลงอย่างชัดเจน ปริมาณพอลิเอมีนรวมของเซลล์สายพันธุ์ปกติได้รับการเหนี่ยวนำอย่างชัดเจนโดยภาวะเครียดซอร์บิทอล 700 มิลลิโมลาร์ ขณะที่ Δ*sll1077* แสดงการลดลงบางส่วนของปริมาณพอลิเอมีนรวมภายใต้สภาวะเครียดทั้งหมด เป็นที่น่าประหลาดใจเมื่อพบว่า เซลล์ Δ*sll0228* มีปริมาณพอลิเอมีนรวมลดลงอย่างสมบูรณ์ ยิ่งกว่านั้นได้ค้นพบพอลิเอมีนที่ไม่พบทั่วไปชนิดนอร์สเปอร์มิดีนภายใต้ภาวะเครียดซอร์บิทอล แอคทิวิตีของแอกมาทิเนสในสายพันธุ์ปกติเพิ่มขึ้นโดยภาวะเครียดจากซอร์บิทอล ขณะที่สายพันธุ์ Δ*sll1077* มีแอกทิวิตีของแอกมาทิเนสที่ต่ำกว่าสายพันธุ์ Δ*sll0228* มีแอกทิวิตีของแอกมาทิเนสระดับน้อยมาก ในทางกลับกัน ระดับทรานสคริปต์ของ *sll1077* และ *sll0228* เพิ่มขึ้นภายใต้สภาวะซอร์บิทอล 700 มิลลิโมลาร์เมื่อเปรียบเทียบกับทั้งสายพันธุ์ปกติและ Δ*sll1077* นอกจากนั้น ระดับทรานสคริปต์ของยีน *N*-คาร์บาโมอิลพิวเทรสซีน อะมิโดไฮโดรเลส (*sll0601*) ในสายพันธุ์ Δ*sll1077* เพิ่มขึ้นภายใต้สภาวะซอร์บิทอล 700 มิลลิโมลาร์ ขณะที่มีการลดของระดับทรานสคริปต์ *sll0601* นี้ในสายพันธุ์ Δ*sll0228* สภาวะซอร์บิทอล 700 มิลลิโมลาร์ยังเหนี่ยวนำระดับทรานสคริปต์ของ *sll0873* ซึ่งเข้ารหัสคาร์บอกซีนอร์สเปอร์มิดีนดีคาร์บอกซีเลสในทุกสายพันธุ์ จากผลทั้งหมดนี้ การค้นพบของเราบ่งชี้ว่า แอกมาทิเนสมีบทบาทที่สำคัญสำหรับการตอบสนองและปรับตัวต่อภาวะเครียดของ *Synechocystis* ถึงแม้ว่ามันจะไม่ใช่เอนไซม์หลักของวิถีชีวสังเคราะห์พอลิเอมีน การขาดยีนที่คาดว่าน่าจะเป็นแอกมาทิเนส *sll1077* และ *sll0228* ไม่ส่งผลอันตรายต่อการเจริญของเซลล์ แต่มีผลทางอ้อมต่อกลไกการปรับตัวอื่นๆ ให้เพิ่มขึ้น เช่น การผลิตนอร์สเปอร์มิดีน และการแสดงออกของยีนที่คาดว่าจะเป็น *N*-คาร์บาโมอิลพิวเทรสซีนอมิโดไฮโดรเลส

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SUPAPID EKNIKOM: EFFECT OF SORBITOL STRESS ON POLYAMINE CONTENTS IN *Synechocystis* sp. PCC 6803 MUTANTS OF *sll1077* AND *sll0228* GENES. ADVISOR: ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., 101 pp.

Effect of sorbitol-induced osmotic stress on growth rate, chlorophyll *a* and carotenoid pigment contents, oxygen evolution, polyamine contents, gene expression and agmatinase activity was investigated in cyanobacterium *Synechocystis* sp. PCC 6803 wild type and two mutants lacking putative agmatinase gene (*Δsll1077*) and putative arginase gene (*Δsll0288*), respectively. Although the pairwise alignment of *sll1077* and *sll10228* amino acid sequences showed 35.3% of similarity and 20.7% of identity, their phylogenetic tree demonstrated that they were in the same clade of agmatinase when aligned to known amino acid sequences of other species. All three strains of wild type, Δ*sll1077* and Δ*sll0228* were grown in BG_{11} media containing various sorbitol concentrations of 250, 500 and 700 mM, respectively. The growth rate, chlorophyll *a* and carotenoid contents of wild type and mutant strains were not different under 250 mM and 500 mM sorbitol conditions while 700 mM sorbitol condition significantly inhibited them compared to those under control condition. Interestingly, Δ*sll1077* cells slightly growed under 700 mM sorbitol condition and could maintain the chlorophyll *a* and carotenoid contents along 14 days of treatment. The O_2 evolution rates of those three strains were not significantly different under 0, 250 and 500 mM sorbitol conditions whereas 700 mM sorbitol condition obviously decreased their O_2 evolution rate. Total polyamine content of wild type cells was apparently induced by 700 mM sorbitol stress whereas Δ*sll1077* showed partial decreases of total polyamine contents under all stressed conditions. It was strikingly found that Δ*sll0228* cells completely diminished their total polyamines. Moreover, norspermidine, uncommon polyamine, was also found under sorbitol stress. The agmatinase activity of wild type was increased by sorbitol stress whereas Δ*sll1077* gave lower enzyme activity. The trace level of Δ*sll0228* agmatinase activity was clearly observed. On the other hand, the transcript levels of *sll1077* and *sll0228* under 700 mM sorbitol condition were up-regulated in both wild type and Δ*sll1077*. In addition, the transcript level of putative *N*-carbamoylputrescine amidohydrolase (*sll0601*) in Δ*sll1077* strain under 700 mM sorbitol condition was up-regulated whereas down-regulation of this *sll0601* transcript was observed in Δ*sll0228* strain. The 700 mM sorbitol condition also induced transcript levels of *sll0873* encoding carboxynorspermidine decarboxylase in all strains. Altogether, our findings are indicated that agmatinase has an important role for *Synechocystis* stress response and adaptation although it is not the key enzyme of polyamine-biosynthetic pathway. The lack of putative agmatinase genes, *sll1077* and *sll0228*, did not harm to cell growth but indirectly enhanced other adaptive mechanisms, such as norspermidine production and gene expression of putative *N*-carbamoylputrescine amidohydrolase.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

A	absorbance
bp	base pair
°C	degree Celsius
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
µg	microgram
h	hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
L	liter
µl	microliter
M	mole per liter (molar)
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometer
nmol	nanomol
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA

CHAPTER I

INTRODUCTION

All living organisms generally adapt themselves to grow and survive in various environmental stresses. The cells possess many mechanisms involved to prevention on their biological systems and homeostasis, which nevertheless depending on the variety of stresses. These mechanisms comprise of, osmolite production, the induction of antioxidant enzymes and gene expressions, the radical scavenger production, such as, polyamines and proline, for instance. In this study, we are focusing on polyamine function and biosynthesis which are ones of defensive responses to osmotic stress, one of severely abiotic stresses.

1.1 Abiotic stress

Stress is defined as the unsuitable condition that led living organisms facing unmaintainable and unbalanced capabilities on their usual life. Normally, environmental stress is classified into biotic and abiotic stresses. Biotic stress is mainly caused by living factors, for example, viral, bacterial and fungal pathogens, parasitic plants and insect herbivores (Pimentel 1991). On the other hand, abiotic stress is caused by the non-living factors, such as temperature (Went 1953, Wang and Zheng 2001), drought (Blum 1996, Farooq *et al.* 2009), high ion concentration (Munns and Termaat 1986, Kurban *et al.* 1999, Katerji *et al.* 2003), and radiation (Caldwell 1981, Kovacs and Keresztes 2002). These stresses gave mainly the biological effects leading to limit growth rate and reduce productivity of crop worldwide. The abiotic stress can trigger the changes on morphology, physiology, and metabolism of plant (Nilsen and Orcutt 1996). The initial stress signals of osmotic and ionic effects, trigger the downstream signaling process and transcription controls, such as up-regulating the

gene involved in stress response (Shinozaki and Yamaguchi-Shinozaki 1997, Kreps *et al.* 2002), generating the antioxidant (Scandalios 1990, Rao *et al.* 1996), and accumulating the osmoprotectant (Handa *et al.* 1986, Ashraf and Foolad 2007) to re-establish homeostasis and to protect and repair damaged proteins, as well as membranes towards to stress tolerance or resistance (Vinocur and Altman 2005).

Stress-associated mechanisms effectively induce plant resistance to abiotic stresses by synergistically combining various expressions of genes, proteins and metabolic pathways. There are various kinds of metabolites have been identified, including amino acids (such as proline), amine groups (such as glycine-betaine and polyamines) and a variety of sugars and sugar alcohols (mannitol and trehalose) shown in Figure 1.1. Moreover, higher plants are able to gain their stress tolerance by gene manipulation technology, such as genetic engineering and conventional plant breeding, as well as, the use of molecular markers and quantitative trait loci (QTLs) (Vinocur and Altman 2005). Nowadays, the understanding of responsive mechanism can be expanded to create organisms with stress tolerance ability based on genetic engineering approach. Enhanced their tolerance by plant gene modification could enhance the protection and stability on function and structure of cellular components. Stress-induced gene expression can be applied to develop a transgenic with stress tolerance ability using single action gene approach (Kasuga *et al.* 1999). The modification of metabolite gene, for example, transgenic wheat plants that overexpressing a *P5CS* gene encoding Δ^1-pyrroline-5-carboxylate synthetase was increased on their tolerance to salt stress (Sawahel and Hassan 2002). Moreover, the overexpresstion of spermidine synthase gene in *Arabidopsis thaliana* enhanced their tolerance to various stresses including chilling, freezing, salinity, hyperosmosis, drought, and paraquat toxicity (Kasukabe *et al.* 2004). In addition, the engineering trehalose overproduction in rice increased tolerance response to abiotic stress and enhanced productivity through tissue-specific or stress-dependent overproduction (Garg *et al.*

2002). Likewise, overexpressing superoxide dismutase gene in chloroplasts of tobacco *Nicotiana tabacum* enhanced their tolerance to salt stress and water deficit condition which helped plants to survive under those stress conditions (Bhatnagar-Mathur *et al.* 2008).

1.1.1 Osmotic stress

The osmotic stress and/or water stress are results from the unsuitable level of water environment, generated by such as drought (water deficiency), high concentration of salt and low temperature. In this study, the osmotic stress was generated from osmotic pressure imbalance by water deficient condition. Drought problem is statistically predicted to spread out more than 50% around the world by year 2050 (Ashraf and Wu 1994). When cells have lack of water, their response mainly started depending on species or genotype specific, time period and stress intensity. The cellular water deficits relate to the concentration of solutes, loss of turgor, change in cell volume, denaturation of proteins and several physiological and molecular components as shown in Figure 1.2 (Griffiths and Parry 2002, Parry *et al.* 2002, Raymond and Smirnoff 2002). One of the most important physiological parameters for photosynthetic organisms which severely damaged by osmotic stress is photosynthetic efficiency. Causing a progressive and reduction in the CO_2 assimilation capacity potentially decreased in net photosynthetic rate that associated to a stomatal closure induced by a decline in leaf cell turgor. Then, cell turgor would reduce the production of PSII and induce photorespiration and H_2O_2 production (de Ollas *et al.* 2013). Moreover, the reactive oxygen species (ROS) which is a primary response to stress could be harmful to cells. Plants can handle this ROS by generating the antioxidant compounds such as ascorbic acid (vitamin C), glutathione (GSH), **α**-tocopherols (vitamin E), carotenoid and flavonoid (Ahmad *et al.* 2009). Furthermore, osmotic stress provoked the accumulation of amino acids, such as proline, valine, isoleucine, and

agmatine (polyamine precursor) and carbohydrate alcohols (Arbona *et al.* 2013). After rehydration, most plants could be restored to normal growth, but if the stress intensity was severe, it would not be recovered properly (Zingaretti *et al.* 2013).

Cyanobacteria were referred as the suitable systems for investigating environmental stress responses and used as models for plant (Apte 2001). Water stress generated from salt, sugar maltose, sugar alcohol sorbitol or drought condition had impact to the depletion of cellular water (Figure 1.3). Cyanobacteria had to react by cellular/molecular response which could promote growth and survive under stress condition, such as reduced damage of ROS by enzyme catalase (Gilichinsky *et al.* 1992), and generated the multiple genome copies in cyanobacteria leading to enhance their tolerance against DNA damage (Dadheech 2010). In the other hand, the cells were able to recovery turgor pressure by the accumulation of osmotically-active compatible solutes at high intracellular concentrations which increasing the internal osmolality and protecting itself from the decreasing osmotic potential in cytoplasm (Moore *et al.* 1987). Cyanobacteria were up-accumulated carbohydrate and organic solute compounds, such as, sucrose, glycine betaine, glucosylglycerol, and trehalose under osmotic stress (Reed *et al.* 1984, Warr *et al.* 1985, Hershkovitz *et al.* 1991). Therefore, this valuable knowledge from basic research was potentially used for transgenic construction, either in plant or cyanobacteria by genetic engineering approach. (Nelson *et al.* 2007). On the other hand, the sorbitol-induced nonionic compound of low molecular weight sugar alcohol (polyol), was found in higher plants (Moing *et al.* 1992) in most species of *Rosaceae*, such as, apple, pear and peach (Shiratake *et al.* 2008). However, sorbitol has been considered as a non-metabolite, because it was metabolically more inert than other saccharides (Wang *et al.* 1999) Then, many research used sorbitol as nonionic compound generating osmotic stress (Kaiser *et al.* 1981, Dove *et al.* 1997, Shen *et al.* 1999, Mikołajczyk *et al.* 2000, Jantaro *et al.* 2003, Pothipongsa *et al.* 2012).

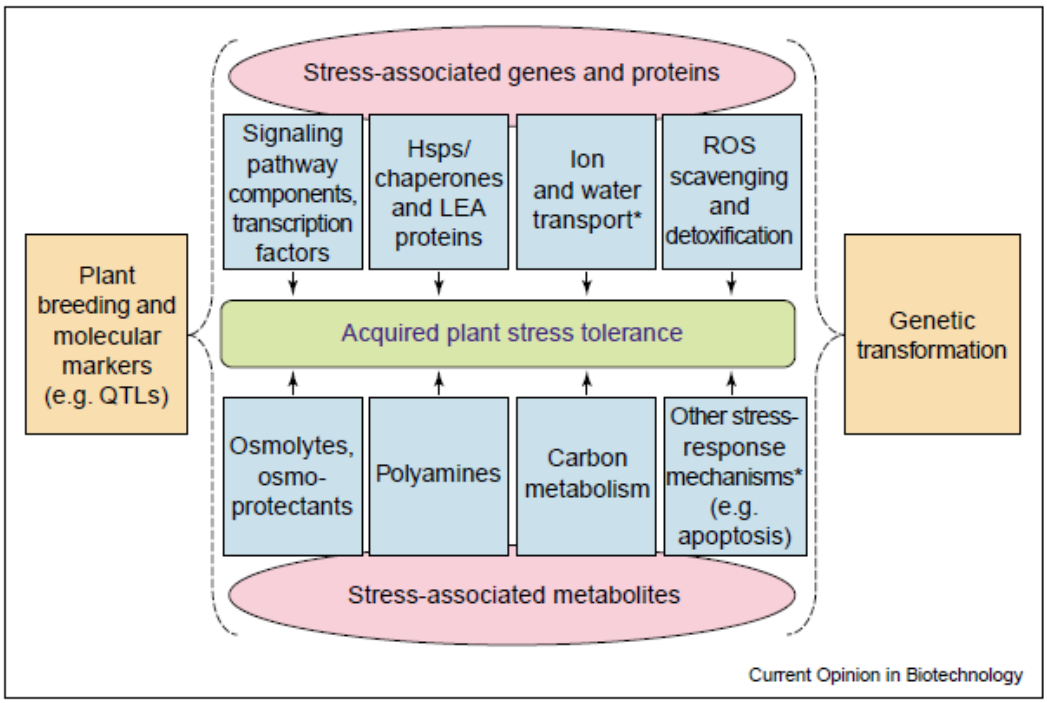

Figure 1.1 Acquired plant stress tolerance can be enhanced by manipulating stress-associated genes and proteins, as well as by overexpression of stress associated metabolites (Vinocur and Altman 2005).

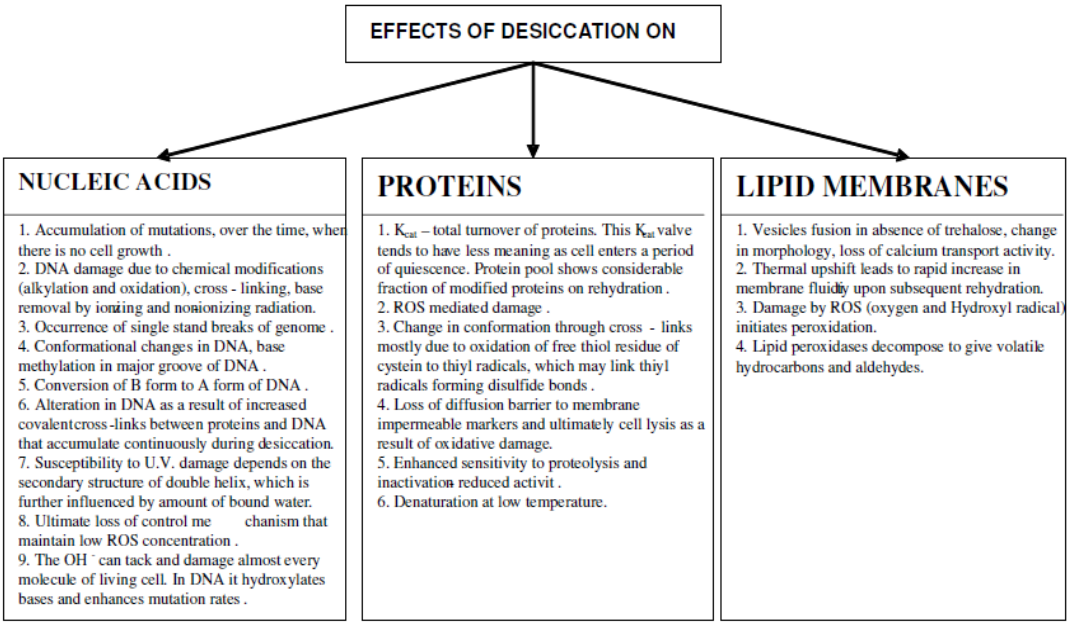

Figure 1.2 The effect of desiccation on various biomolecules and its mechanism of tolerance in cyanobacteria (Dadheech 2010).

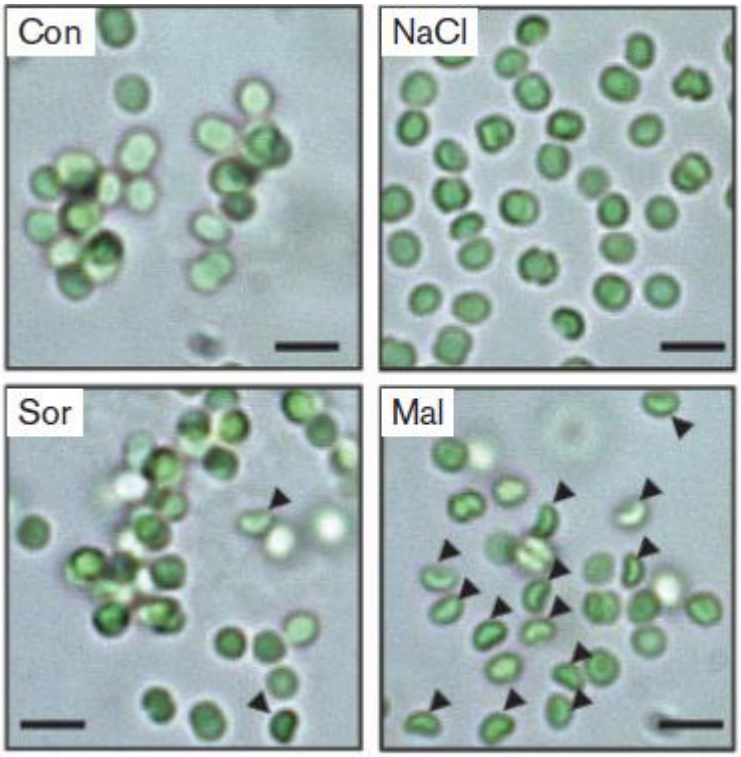

Figure 1.3 *Synechocystis* cells under light microscope control (Con) and 2 min after adapted to osmotic stress by addition of 500 mM NaCl (NaCl), sorbitol (Sor) or maltose (Mal) (Marin *et al.* 2006).

1.2 Polyamines

1.2.1 Polyamine physiology and function

Polyamines are polycationic compounds which contained more than one of amine groups on hydrocarbon chain (Takahashi and Kakehi 2010). They are found in all living organisms. The three common types of polyamines are diamine putrescine, triamine spermidine and tetraamine spermine, less common polyamines are diaminopropane and cadavarine (Figure 1.4). The other types of polyamines were found in some organisms, for example, thermophilic bacteria, *Thermus thermophilus* which had long chain and branch polyamines (Oshima 2007). Polyamines generally existed in either free form or conjugated or bound form. Conjugated form mainly binds to low molecular weight compounds, such as phenolic acids, whereas bound form is bound to macromolecules, such as proteins and nucleic acids (Kaur-Sawhney *et al.* 2003). These existed forms lead polyamines involving in a cellular processes including DNA replication and transcription (Childs *et al.* 2003, Wallace *et al.* 2003, Terui *et al.* 2005) , RNA modification (Raina and Cohen 1966), protein synthesis and function scavenging (Drolet *et al.* 1986), cell-cycle regulation (Tabor and Tabor 1985), and signal transduction (Bachrach *et al.* 2001, Pignatti *et al.* 2004).

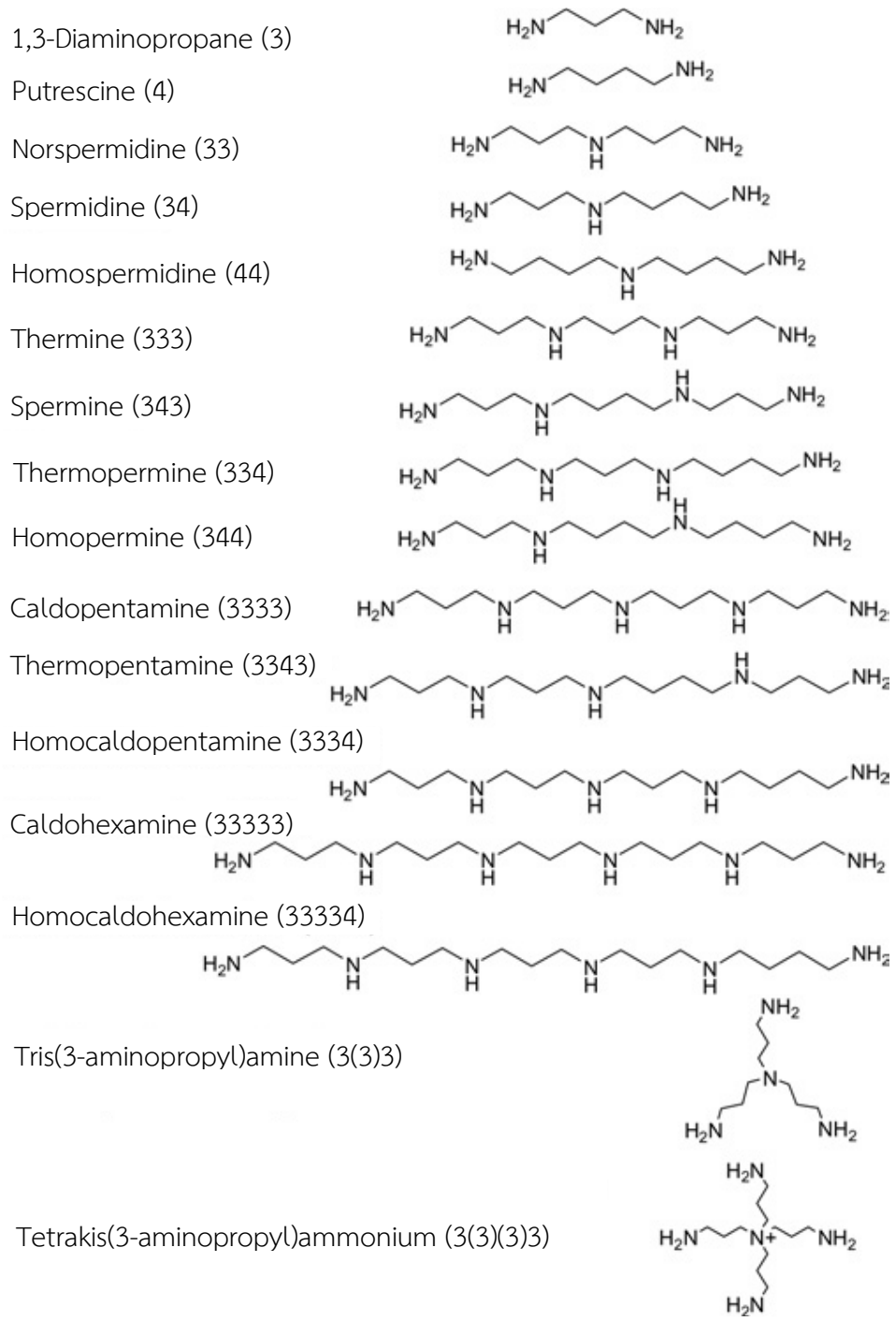

Figure 1.4 structures of common and unique polyamines, The number shown the carbon atoms separating the amino or aza groups (Terui et al. 2005).

1.2.2 Polyamine biosynthetic pathway

Pathway of polyamine biosynthesis exists differently among plant, animal and bacteria (Figure 1.5A). The biosynthesis starting from L-arginine, one of the main amino acids, can be divided into two ways to produce putrescine. The first pathway, putrescine can be synthesized via arginine decarboxylase (EC 4.1.1.19) to agmatine and later converted to putrescine via agmatinase (EC 3.5.3.11) or agmatine iminohydrolase (EC 3.5.3.12), and *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53), respectively. The other pathway, arginase (EC 3.5.3.1) converts L-arginine to L-ornithine and produces putrescine via ornithine decarboxylase (EC 4.1.1.17). After that, putrescine is converted to spermidine and then spermine via spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively, using an aminopropyl residue from decarboxylated *S*-adenosylmethionine (AdoMet), which results from decarboxylation of AdoMet by AdoMet decarboxylase (EC 4.1.1.50) (Kusano *et al.* 2007).

The polyamine biosynthetic pathway in cyanobacteria *Synechocystis* sp. PCC 6803 (Figure 1.5B) based on Cyanobase (genome.microbedb.jp/cyanobase) showed only one pathway leading to putrescine formation by arginine decarboxylase (*slr1312* and *slr0662*) and agmatinase (*sll1077*). The information of genes encoding ornithine decarboxylase, spermidine synthase and spermine synthase were unknown when compared with known other species. In contrast, the bioinformatics analysis from *Schriek* et al. (2007) reported three genes encoding arginine decarboxylase (*sll1683*, *slr0662*, *slr1312*) and two genes encoding agmatinase (*sll1077*, *sll0228*) (Table 1.1). Interestingly, these results opposed to Cyanobase information which predicted *sll0228* gene as a putative arginase instead of agmatinase.

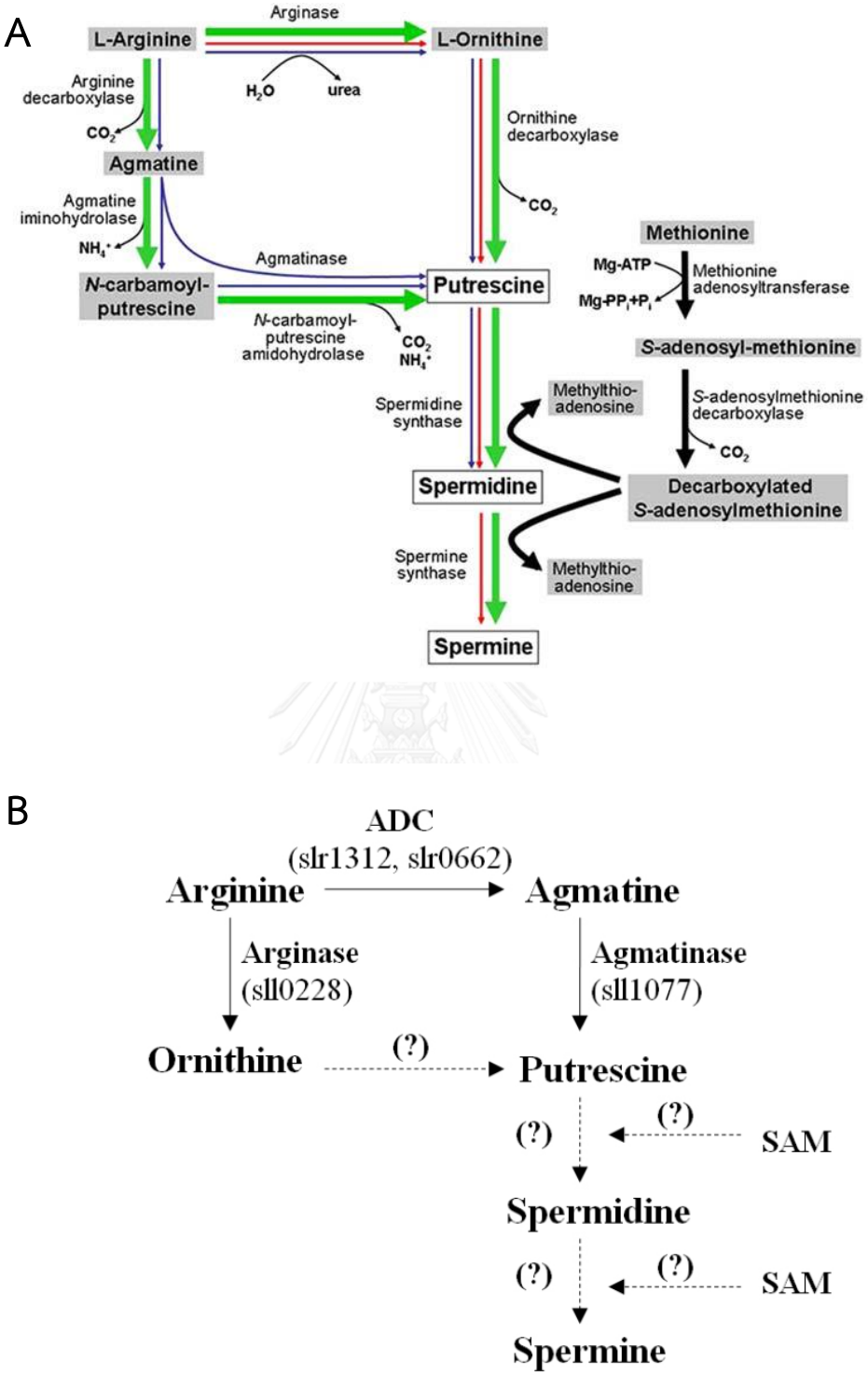

Figure 1.5 Polyamine biosynthetic pathways (A) in plant (green arrow), animal (red arrow) and bacteria (blue arrow) (Kusano et al. 2008) and (B) in cyanobacterium *Synechocystis* sp. PCC 6803 based on Cyanobase database (Incharoensakdi et al. 2010).

Table 1.1 Database entries of genes encoding putative L-arginine decarboxylases (A1), agmatinases (A2.1), agmatine deiminases (A2.2), *N*-carbamoylputrescine amidohydrolases (A2.3), putrescine oxidases or putrescine transaminases (A3), and 4-aminobutyraldehyde dehydrogenases (A4) of the L-arginine decarboxylase pathway (Schriek et al. 2007).

Enzyme	A1	A2.1	A2.2	A2.3	A3	A4
Freshwater species						
Synechococcus elongatus sp. PCC 6301	Syc0823_d, Syc0510_c	n.d	SYC1703_c, SYC1643_d	Syc1946_d, Syc1745_c	n.d	Syc1030_d
Synechococcus elongatus sp. PCC 7942	Synpcc7942_0707, Synpcc7942_1037	n.d	Synpcc79422402 Synpcc79422461	Synpcc79422145 Synpcc79422358	n.d	Synpcc7942_0489
Synechococcus Yellowstone sp. JA-3-3-AB	CYA_1002, CYA_0128	CYA_0859	n.d	CYA_0558	n.d	CYA_0364
Synechococcus Yellowstone sp. JA-2-3Ba (2-13)	CYB_2779, CYB_0482	CYB_1744	n.d	CYB_1181	n.d	CYB_0715, CYB_1893
Thermosynechococcus elongatus BP-1	Tlr1866, Tll1807	n.d.	Tlr0111	Tlr0112, Tll0920	n.d	Tlr0221
Synechocystis sp. PCC 6803	Sll1683, Slr0662, Slr1312	Sll1077, Sll0228	n.d	Sll0601, Sll1640	n.d	Sll1495, Slr0370
Gloeobacter violaceus PCC 7421	Gll4070, Gll3478	n.d	Glr1681	Glr1682, Glr2043	n.d	Gll2207, Gll1504, Glr3848, Gll2805
Nostoc sp. PCC 7120	All3401, All4887	Alr2310	n.d	Alr2001	n.d	Alr2826, Alr3771, All3556, All5022
Nostoc punctiforme PCC 73102	Npun02000556, Npun02000612	Npun02002114	n.d	Npun02002053	n.d	Npun02003427, Npun02002895, Npun02002692, Npun02003702
Anabaena variabilis ATCC 29413	Ava_2157, Ava_3423	Ava_0127	n.d	Ava_5061	n.d	Ava_1107, Ava_1554, Ava_3534, Ava_2258

N.d. = not detected.

1.2.3 Agmatinase

Agmatinase (agmatine ureohydrolase) is a binuclear manganese metalloenzyme and belongs to the ureohydrolase superfamily which also includes arginase, formiminoglutamase, and proclavaminate amidinohydrolase (Ouzounis and Kyrpides 1994, Sekowska *et al.* 2000). It can be found in bacteria (Salas *et al.* 2004, Goda *et al.* 2005, Schneider and Wendisch 2010), cyanobacteria (Schriek *et al.* 2007, Burnat and Flores 2014), and mammals (Sastre *et al.* 1996, Iyer *et al.* 2002). Agmatinase plays important regulatory roles by modulating the cellular levels of agmatine, the product of arginine decarboxylation (Figure 1.5), which is a metabolic intermediate in the biosynthesis of putrescine and higher polyamines (Ahn *et al.* 2004). Moreover, agmatinase plays several roles in mammalian tissues, including neurotransmitter /neuromodulatory actions in the brain (Sastre *et al.* 1996).

1.2.4 The role of polyamine response to abiotic stress

There are a lot of studies reported that polyamine accumulation was occurred under abiotic stresses. The higher levels of polyamines was observed in plant under environmental stresses, for example, under osmotic and salt stresses which found an increase of putrescine accumulation in leaf segments from 8 wheat varieties (Erdei *et al.* 1990). Also, in cereal leaves, putrescine and spermidine were accumulated according to the increase in ADC activity in osmotically stressed tissues (Flores and Galston 1984). In addition, tomato showed increased of putrescine and spermine levels under salinity (Santa-Cruz *et al.* 1999). Polyamine supplement in *Zea mays* L. could promote chilling tolerance (Songstad *et al.* 1990). Also in cucumber, polyamines played important roles by prevention ROS generation and acting as oxidative machinery against chilling injury for chilling tolerance (Shen *et al.* 2000, Zhang *et al.* 2009), and conferred short-term salinity tolerance through inducing antioxidant enzymes and osmoticants (Duan *et al.* 2008). In recent years, the study of molecular

and genetic engineering with mutants and transgenic plants involved in the biosynthesis of polyamines was used to a better understanding of functional role of polyamines in plants. Moreover, transgenic eggplant increased in ADC enzyme, has showed polyamine accumulation which exhibited increased tolerance levels to multiple abiotic stresses, such as salinity, drought, low and high temperatures, and heavy-metal and also resistance against fungal wilt disease caused by *Fusarium oxysporium* (Prabhavathi and Rajam 2007). Transgenic *Arabidopsis* which constitutively expressing arginine decarboxylase two genes showed high level of putrescine and more tolerate to drought stress than wild type (Alcázar et al. 2010). In addition, transgenic rice, *Oryza sativa* L., which overexpressing *S*-adenosylmethionine decarboxylase gene showed increasing of polyamine level and sodium chloride-stress tolerance (Roy and Wu 2002).

Moreover, in order to understand polyamine roles in organisms, the genetic approach will be employed for analyzing biological functions of polyamine metabolism under stress response, such as the response of mutants deficient on polyamine biosynthetic genes. There are many studies focusing the function of polyamines in plant model organism *Arabidopsis thaliana*. For example, transgenic mutant which cannot produce spermine showed higher sensitivity to high salt stress than wild type plants while an addition of exogenous spermine effectively cured that mutant strain, but not by the other polyamines of putrescine and spermidine (Yamaguchi *et al.* 2006). The mutant lacking of spermidine synthase affected plant on the reduction of embryo development (Imai *et al.* 2004). Moreover, the reduction of arginine decarboxylase activity decreased fresh weight, chlorophyll content, and *Arabidopsis* photosynthetic efficiency and finally reduced salt tolerance (Kasinathan and Wingler 2004). Additionally, mutants deficient in putrescine biosynthetic genes (*adc*1, *adc*2) displayed a reduced freezing tolerance (Cuevas *et al.* 2008).

1.2.5 Norspermidine

Norspermidine is unique polyamine which has a similar structure to common spermidine (Figure 1.4). Norspermidine was found in some species of plants (Rodriguez-Garay *et al.* 1989, Hamana *et al.* 1998), bacteria (Yamamoto *et al.* 1979), and algae (Hamana and Matsuzaki 1982). Interestingly, norspermidine was detected as a major component in *Vibrio* and *Beneckea* (Yamamoto *et al.* 1979). On the other hand, it was found as a minor component in extreme thermophile, *Thermus Thermophilus* only under high temperature (Oshima 1983). Norspermidine was also developed for use as an antitumor medicine in cancer treatment (Prakash *et al.* 1987, Sunkara *et al.* 1988). Norspermidine biosynthetic pathway was separated from normal polyamine biosynthetic pathway. It was synthesize from aspartic β-semialdehyde and 1,3-diaminopropane which first reduced by a NADPH-dependent enzyme to yield carboxynorspermidine and turned to decarboxylated by a pyridoxal phosphate dependent enzyme carboxynorspermidine decarboxylase to form norspermidine (Yamamoto *et al.* 1986). However, the function of norspermidine still unclear although the addition of low concentrations of norspermidine could replace the role of the related and natively produced polyamine, spermidine, during biofilm formation in *Bacillus subtilis* (Cairns *et al.* 2014).

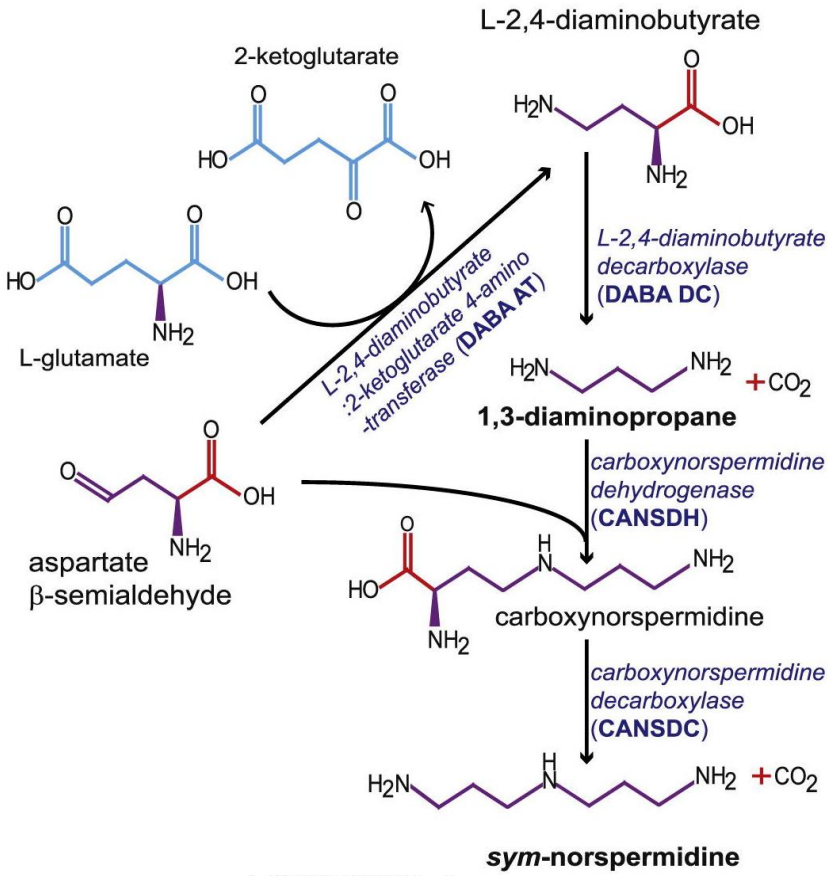

Figure 1.6 Norspermidine biosynthetic pathway of *V. cholera* (Hobley et al. 2014).

1.3 *Synechocystis* sp. PCC 6803 as model organism

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Figure 1.7) (hereafter *Synechocystis*) is unicellular coccoid or spherical gram negative bacteria which lack of nitrogen fixing ability and gas vesicles. It was inhabitant in fresh water using binary fission for reproduction (Ikeuchi and Tabata 2001). The genome sequence has been known since 1996 by Kazusa DNA Research Institute as the first genome of photosynthetic organism which is access in CyanoBase (http://genome.microbedb.jp /cyanobase) containing the entire 3.9 Mb genome sequence in six circular genomic molecules (chromosome and plasmids) with a total of 3725 genes (Nakao *et al.* 2009). Therefore, the circular chromosome is 3,573,470 bp and had 47.7% of average GC contents (Kaneko *et al.* 1996) as well as closely related to *Microcystis aeruginosa* (unicellular spherical cyanobacterium with gas vesicles) than *Synechocystis* groups by using a phylogenetic tree based on *16S* rRNA sequences (Honda *et al.* 1999). *Synechocystis* had four culture substrains of PCC, ATCC, GT (glucose-tolerant) and Kazusa, which all derived from the Berkeley strain 6803 (Stanier *et al.* 1971) which grouped together under the name of *Synechocystis* sp. PCC strain number 6803 (Rippka 1992). However, the entire genome sequence was determined in the Kazusa strain only (Kaneko *et al.* 1996). On the other hand, the four substrains show differences in each phenotype (Figure 1.8). For example, the Berkeley and PCC strains are motile, PCC and ATCC strains are sensitive to glucose, excepted GT strain (Williams 1988). The Kazusa strain is not able to natural transformation while the other strains are naturally transformable (Grigorieva and Shestakov 1982). Due to the capability of genetic engineering, this strain is the standard of cyanobacteria which used as a model in various areas of research, such as photosynthesis, stress response and metabolism (Ikeuchi and Tabata 2001).

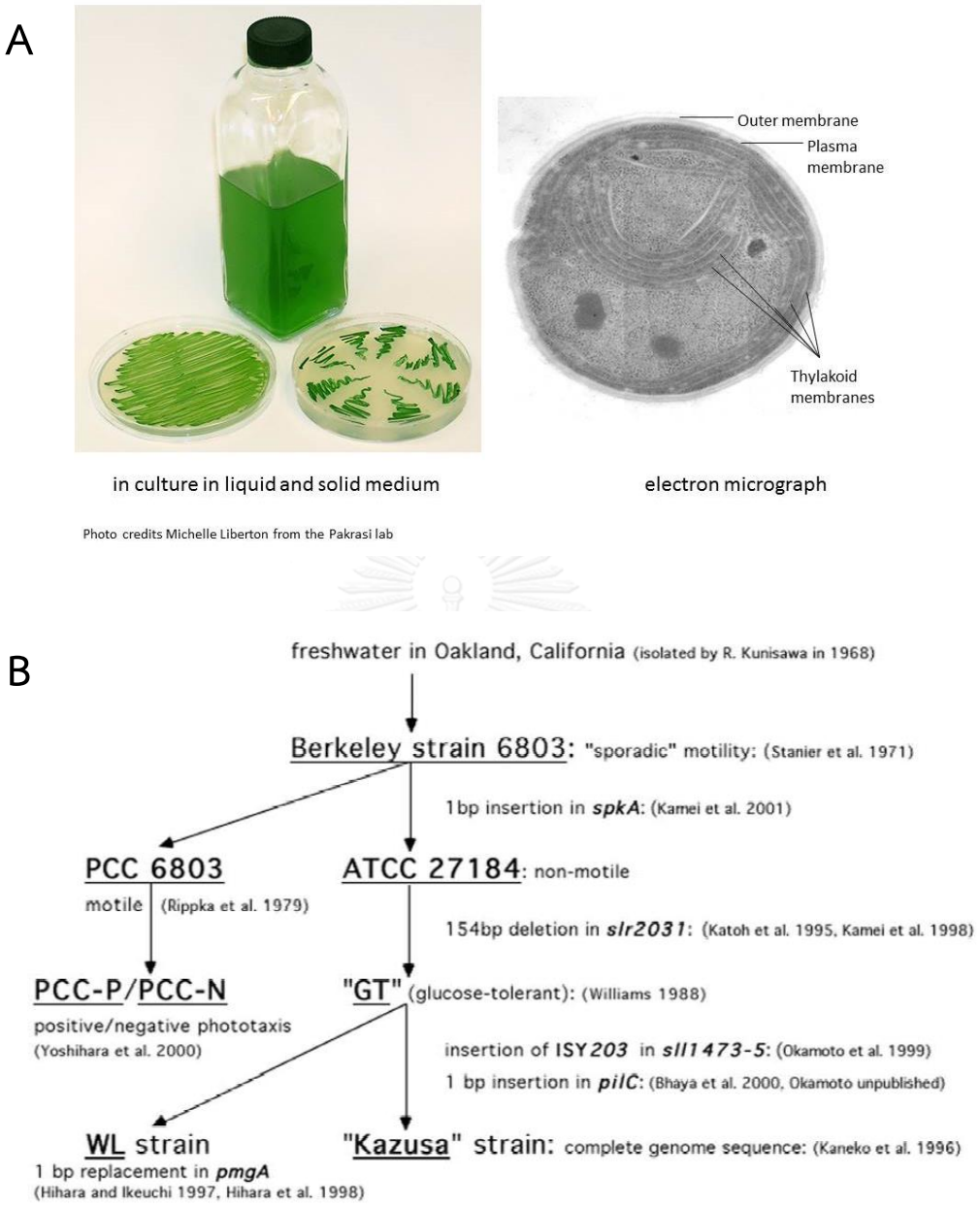

Figure 1.7 (A) *Synechocystis* cells in liquid and solid media (left) and under electron micrograph (right). (https://newunderthesunblog.wordpress.com/thebasics/cyanobacteria) (B) The strain history of *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata 2001).

1.4 Gene knockout construction in *Synechocystis* cells

In this study, knockout mutant which are one of the useful tools in the studies was employed on metabolisms and physiology of polyamines *in vivo*. Gene knockout or gene inactivation is a mutation that inactivated a gene function. It was a useful tool for genetic study of genomic DNA function. Genetic recombination in *Synechocystis* sp. PCC 6803 was carried out by homologous double recombination which is essential for integrating the gene sequence of interest to the target site (Grigorieva and Shestakov 1982, Williams 1988). Figure 1.8 shows a scheme of homologous double recombination for inserting DNA into the target site in the genome of cyanobacteria. Recombination will occur only between incoming DNA and genomic DNA with similar sequences (Thiel 2004). The length of both upstream and downstream regions should be more than 200 bp which required for the double crossing over event involved in the introduction of an antibiotic resistance cassette gene into a target site (Zang *et al.* 2007). After that, the mutant was constructed by natural transformation. The transformation of cyanobacteria is a first step to create of a transgenic organism which requires a protocol for transferring foreign DNA into the cell, such as natural transformation, electroporation and conjugation (Thiel 2004). The cyanobacterium *Synechocystis* sp. PCC 6803 is naturally transformable by exogenous DNA (Barten and Lill 1995). The mechanism of taking up the foreign DNA is still unclear and is not known why natural transformation is possible for only some strains. For *Synechocystis* cells, it was demonstrated that pili are essential for mobility and transformation (Yoshihara *et al.* 2001).

Cyanobacterial strains contain more than one genome copy per cell, depending on growth rate and growth stage (Vermaas 1996). In *Synechocystis* cells, they contain around 12 copies of their genome (Labarre *et al.* 1989). Therefore, mutant genome copies needs to occur before a pure mutant (lacking all copies of the wild-type gene that was deleted in this experiment) has been achieved (Vermaas 1996). The

segregation of wild type genomes into mutant copies is achieved by continuously streaking the colonies onto plates containing high level of antibiotics to which the transformant can resist. A complete segregation of colonies is verified by a colony PCR or isolated genomic DNA for PCR method (Eaton-Rye 2004).

1.5 Objective of this research

1. To examine the effect of osmotic stress on cell growth, intracellular pigment, polyamine content and agmatinase activity in *Synechocystis* sp. PCC 6803 wild type and two mutants lacking *sll1077* and *sll0228* genes, respectively.
2. To study the expression pattern of gene involve in polyamine biosynthesis under osmotic stress of *Synechocystis* sp. PCC 6803 wild type and two mutants lacking *sll1077* and *sll0228* genes, respectively.

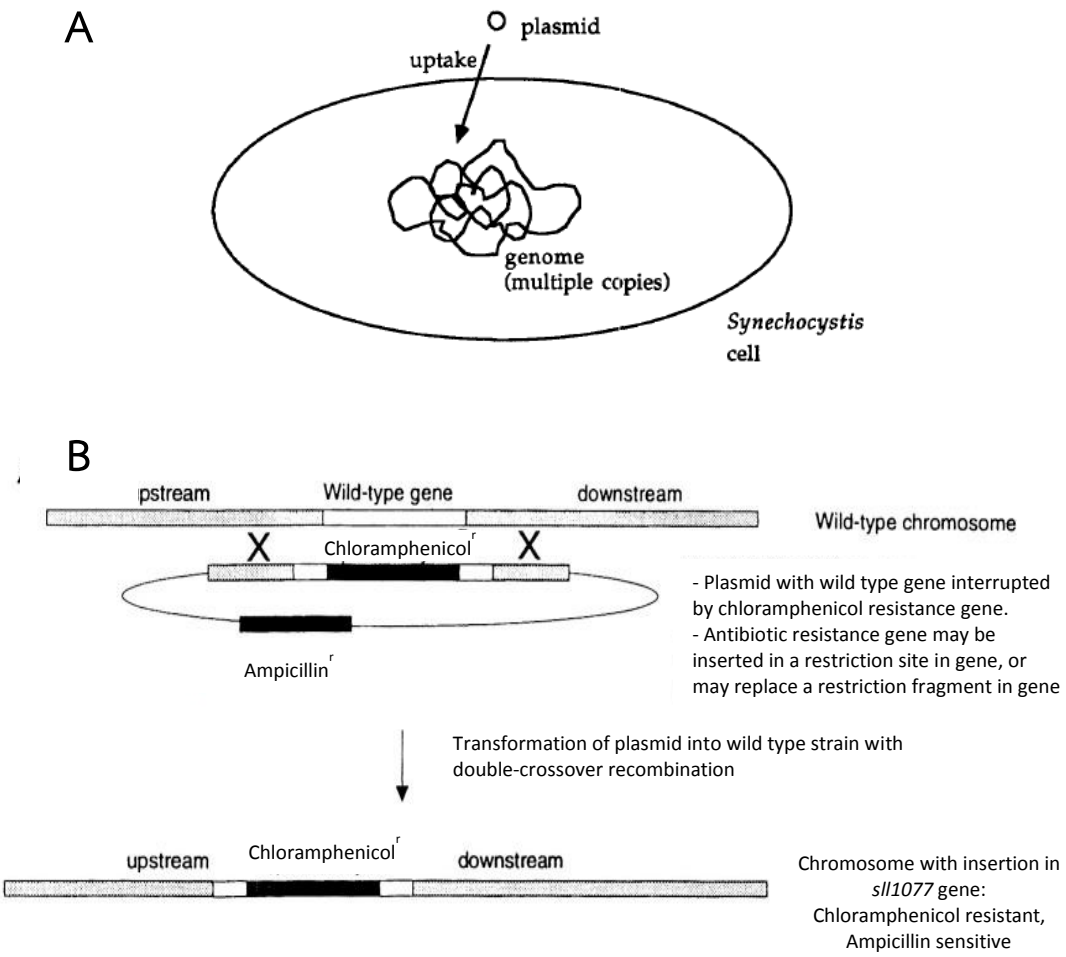

Figure 1.8 Schematic representation of the process of spontaneous transformation of *Synechocystis* sp. PCC 6803 by exogenous DNA. (A) Entry of plasmid DNA into the cell; foreign DNA also may be offered to the cell in different form (for example, as linear or single-stranded DNA (Vermaas 1996). (B) Homologous double-recombination can occur between the introduced DNA and the homologous region in one or more of the genome copies present in the cyanobacterial cell. In this way, a selectable marker gene is introduced into the genome copy. The foreign DNA will be integrated only at the one region of the cyanobacterial genome where sequence identity or homology occurs; this allows for targeted gene modification (Thiel 2004).

CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Equipments

Autoclave	Hirayama Manufacturing Cooperation, Japan
Balances	METTLER TOLEDO, USA
C-18 column	4.6 x 150 mm inertsil ODS-3 5 µm i.d.,
Centrifuge	HETTICH zentrifugen, UK
French press	THERMO ELECTRON COPORATION, USA
Gel Document	DNA cisualisation UV light, USA
HPLC	Hewlett Packard, Japan
Incubator shaker	Innova 4000 PLATFORM SHAKER, USA
Laminar flow	BVT-124 international Scientific Supply, Thailand
PCR apparatus	Thermo Cycler, Japan
pH meter	METTLER TOLEDO, USA
Power supply	BIO-RAD POWER PAC 1000, USA
UV-visible Spectrophotometer	BIOMATE 3, Thermo scientific, USA
Vortex mixer	Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.2 Chemicals

Acetic acid	Lab Scan, Poland
Agarose	Invitrogen, Spain
Agmatine	Sigma, USA
Ampicillin	Sigma, USA
Bacto-agar	Scharlau, Spain
Boric acid	Scharlau, Spain
Calcium chloride	Sigma, USA
Chloramphenicol	Sigma, USA
Chloroform	Merck, Germany
Citric acid	BDH, England
Cobalt nitrate	UNIVAR, Australia
Copper sulphate	Fisher Scientific, England
Diethyl ether	Lab Scan, Poland
Diamino octane	Sigma, USA
Ethanol	Katayama Chem, Japan
Ethylenediaminetetraacetic acid	Sigma, USA
Ethidium bromide	Sigma, USA
Ferric ammonium citrate	Sigma, USA
Glycerol	Ajax Finechem, Australia
HEPES	USB Corporation, USA
Manganese chloride	UNIVAR, Australia
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulphate	Merck, Germany
Methanol	Scharlau, Spain
Norspermidine	Sigma, USA
Perchloric acid	Merck, Germany

Phenol	Merck, Germany
Potassium acetate	Ajax Finechem, Australia
Potassium hydrogen phosphate	UNIVAR, Australia
SDS, Sodium dodecyl sulfate	Ajax Finechem, Australia
Sodium carbonate	Sigma, USA
Sodium chloride	Carlo Erba reagent, China
Sodium thiosulphate	Sigma, USA
Sodium hydroxide	Merck, Germany
Sodium molybdate	BDH, England
Sodium nitrate	BDH, England
Sodium thiosulfate	Ajax Finechem, Australia
Sorbitol	Ajax Finechem, Australia
Spermidine	Sigma, USA
Spermine	Sigma, USA
Tris (hydroxymethyl)-aminomethane	Barcelona, Spain
Tryptone	HiMedia, India
Yeast Extract	Barcelona, Spain
Zinc sulphate	UNIVAR, Australia

2.3 Kits and supplies

T4 DNA polymerase	Fermentas, Canada
T4 DNA ligase	Fermentas, Canada
Taq DNA polymerase	Invitrogen, USA
RNase A	Fermentas, Canada
*Nde*I	Fermentas, Canada
*Bam*HI	Fermentas, Canada
*Eco*RV	Fermentas, Canada
*Bsa*AI	Fermentas, Canada

*Eco*RI	Fermentas, Canada
Klenow fragmaent	Fermentas, Canada
O'gene Ruler DNA ladder mix	Fermentas, Canada
Plasmid extraction kit	Geneaid
NucleoSpin® Extract II, PCR purification kit	Machery-Nagel, USA
pGEM® -T Easy	Promega
psB1AC3	Biobrick

2.4 Primers

Table 2.1 The information of primers used in this work

Name	Primer	Sequence (5'-->3')	Product size	Annealing Temp (°C)
*sll*1077 (with restriction enzyme site of *Nde*I and *Bam*HI)	Forward	GGAATTCCATATGCTACCTATGAGCGATGCCAC	1204	49.2
	Reverse	CGGGATCCTTACTATTGCCAGGGCTCATC		
16s rRNA	Forward	AGTTCTGACGGTACCTGATGA	521	55
	Reverse	GTCAAGCCTTGGTAAGGTTCT		
RT-*sll*1077 (putative agmatinase)	Forward	GCAATTAGGCATTGGTGGTTGGCAAGTA	464	60
	Reverse	GCTCATCCACTGCCATGTTC		
RT-*sll*0228 (putative agmatinase)	Forward	TGGTTTGAAAAAGACGGCGG	404	60
	Reverse	GGCTATGCAAAGGGGGACAT		
RT-*sll*0601 (putative *N*-carbamoylputrescine amidohydrolase)	Forward	CTAGAACAGGCAACGGCGAT	358	60
	Reverse	CTTGGCGGGAAAGGTAACGA		
RT-*sll*1640 (putative *N*-carbamoylputrescine amidohydrolase)	Forward	GGCCATCGCCATAAGGAAGA	301	60
	Reverse	CATTGAGCCTGGTTTCAGCG		
RT-*sll*0873 (putative carboxynorspermidine synthase)	Forward	ACCCCGGCTCCATAATCAAC	477	60
	Reverse	TTTATGCCCCCACCTATCGC		

2.5 Organisms

Escherichia coli strain DH5α

Synechocystis sp. PCC 6803 wild type strain in Cyanobacterial Biotechnology Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

Synechocystis sp. PCC 6803 Δ*sll0228* strain, the *sll0228* gene was replaced with a chloramphenicol-resistance cassette (Dr. Wipawee Sakjirarat and Dr. Panutda Yodsang)

Methods

2.6 Bioinformatic analysis of agmatinase and arginase genes

Sequences of cyanobacteria and the others were collected from Cyanobase (Nakamura *et al.* 1998) and Genbank databases (Benson *et al.* 2000). All sequences were aligned using multiple sequence alignments with CLUSTALW2 program (Higgins *et al.* 1996) using default parameters. The phylogenic tree was created by Dendroscope program (Huson and Scornavacca 2012).

2.7 Cell culture conditions

Synechocystis sp. PCC 6803 cells were cultivated in liquid BG_{11} medium (Appendix A) at 32°C under 50 μmol photons m^{-2} s^{-1} intensity of continuous white fluorescent light. The growth of cell culture was determined at optical density (OD) of 730 nm using a spectrophotometer. In this experiment, the osmotic stress was generated by adding sorbitol in concentration of 250, 500 and 700 mM, respectively, into BG_{11} medium. The cultivated cells at mid-logarithmic phase were harvested and transferred to stress conditions with the diluted cell culture at initial OD of 0.1 for further experiments.

Escherichia coli strain DH5α cells were grown in Luria-Bertani (LB) medium (Appendix B) at 37°C with shaking at a speed of 250 rpm for liquid culture. For the agar

plate, LB medium (1 L) was added 15 g of agar and further autoclaved at 121°C for at least 15 min. After that, sterilized medium was poured onto peridish (about 15 ml/plate) and kept in 4°C refrigerator until used.

2.8 Determination of pigment contents

Chlorophyll *a* and carotenoid pigments were extracted by *N,N*-dimethylformamide. One ml of cell culture was harvested and centrifuged at 8,000xg for 5 min. The cell pellets were added 1 ml of *N,N*-dimethylformamide and vortexed vigorously. The absorbance of supernatant extracted was measured at 461, 625 and 664 nm, respectively. The pigment contents were calculated according to follow equations (Moran 1982, Chamovitz *et al.* 1993).

Chlorophyll *a* content (µg/cells) = $[(12.1 \times A_{664}) - (0.17 \times A_{625})]$ / total cells

Carotenoid content (µg/cells) = $[(A_{461} - (0.046 \times A_{664})) \times 4]$ / total cells

Total cells (cells/ml) = $(OD_{730} / 0.25) \times 10^8$ (Eaton-Rye 2004)

2.9 Oxygen-evolution measurement

The whole cells were measured for their oxygen evolution with Oxygraph plus oxygen electrode (Hansatech Instruments, U.K.). The *Synechocystis* cells were cultivated until OD 730 nm of about 0.6-0.8 and measured for chlorophyll *a* content (method 2.8), and later harvested by centrifuging at 8,000*×g* for 10 min. After that, washed cell pellets with BG_{11} medium and re-suspended in 2 ml of fresh BG_{11} medium and further incubated under darkness for at least 30 min before measuring oxygen evolution at 25°C. The fluorescent light was used as a saturating light source.

2.10 Mutant construction

2.10.1 Extraction of genomic DNA from *Synechocystis* cells

Cultivated *Synechocystis* cells with their OD at 730 nm of about 0.6-0.8 were harvested and centrifuged at 12,000*xg* for 2 min (4°C). Cell pellets was resuspended in 400 µl of TE-buffer, pH 7.5. After adding glass beads, 8 µl of 10% SDS and 400 µl of absolute phenol were added and mixed by vortexing for 1 min in 3 times. The centrifugation at 12,000*xg* for 10 min (4°C) was performed and transferred the upper part into a new tube. After that, a 1:1 ratio of phenol:chloroform mixture was added in 1 volume of sample, then mixed together. Again, centrifuged those tubes at 12,000*xg* for 10 min (4°C) and collected an upper part into a new tube. Then, the sample in new tube was added another 1 volume of chloroform. After centrifugation at 12,000*xg* for 10 min (4°C), the upper fraction was separated and added 0.1 volume of 3 M sodium acetate buffer, pH 5.2 and 2 volumes of absolute ethanol, and further incubated at -20°C for 2 h. The mixture was centrifuged at 12,000xg for 10 min (4°C). After that, collected the pellet of genomic DNA and washed with 500 µl of 70% ethanol before centrifuging at 12,000*xg* for 10 min (4°C). The genomic DNA pellet was dried at room temperature and dissolved in TE-buffer (50 µl). The content of genomic DNA was checked by electrophoresis with 0.8% agarose gel in 0.5x TAE buffer (Appendix C).

2.10.2 Polymerase chain reaction (PCR)

The genomic DNA obtained from 2.8.1 was used as template to amplify *sll1077* gene fragment. The PCR mixture and reaction were shown in following Tables 2.2 and 2.2. After PCR reaction completed, the PCR product was checked by 0.8% agarose electrophoresis.

Table 2.2 The components of PCR mixture

PCR mixture (total volume 50 µl)	Volume (µl)
Template DNA (50-500 ng)	2
10X Tag polymerase buffer	5
10 mM dNTP mixed	1
50 mM $MgCl_2$	1.5
25 mM Forward primer	2.5
25 mM Reverse primer	2.5
Tag DNA polymerase (5 Unit/µl)	0.2
autoclaved DI water	35.3

Table 2.3 PCR procedure for amplification

Step	Temperature (°C)	Time (min)
1. Initial denaturation	94	5
2. Denaturation	94	1
3. Primer annealing	50-65	0.5
4. DNA Extension	72	1.5
*repeated steps 2-4 for 25-30 cycles		
5. Final extension	72	10

2.10.3 Preparation of competent cells for calcium chloride method

E.coli strain DH5α was used as the competent cell. Cells were grown in 3 ml of LB medium on an incubation shaker at a speed of 250 rpm at 37°C for 16 h. This inoculum was transferred into 100 ml of LB medium and cultivated until their OD at 600 nm reaching 0.3-0.4. Cell harvesting was performed by centrifuging at 4,000*xg* for 10 min (4°C). The cell pellets were resuspended in 15 ml of TFBI (Appendix D) solution and centrifuged again at the same procedure. The competent cells were gently mixed with 2 ml of TFBII (Appendix D) solution and then aliquoted each 100 µl into the microcentrifuge tube for further used in transformation.

2.10.4 Construction of p*Sll1077-Cm*^{r} plasmid

After amplification, *sll1077* gene fragment was ligated into the pGEM T-Easy (Appendix F) vector and further transformed into *E.coli* strain DH5α cells using calcium chloride method. Competent cells were mixed with the ligation mixture and placed on ice for 30 min. After that, the sample mixture was incubated in 42°C water bath for 90 sec and immediately placed on ice for 2 min. One microliter of LB broth was added into that mixture and shaken in the incubator at 37°C for 1 h. The cells were spread on LB agar containing 100 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal and then cultivated at 37°C for 16 h. Blue-white screening method was used to select the transformants. The white colonies were grown in LB broth containing 100 µg/ml of ampicillin and incubated at 37°C for 16 h. The cell pellets were collected for further plasmid DNA extraction. Cell pellets were resuspended in 100 µl of Solution I (Appendix E), then incubated for 5 min at RT. After that, 200 µl of Solution II (Appendix E) was added into sample, shaken briefly and placed on ice for 5 min before adding solution III (Appendix E) (150 µl). The sample mixture was incubated on ice before centrifuged at 12,000*xg* for 10 min, transferred the supernatant into a new tube and later mixed with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). The sample

mixture was separated by centrifuging at 12,000*xg* for 10 min, and then transferred the upper part into a new tube. Two volumes of absolute ethanol were added in order to precipitate plasmid DNA, incubated at -20°C for 30 min and centrifuging at 12,000*xg* for 15 min (4°C). The pellet was washed with 500 µl of 70% ethanol, centrifuged again for collecting the plasmid DNA and further dissolved in 1xTE-buffer. The insertion of *sll1077* gene fragment was amplified by PCR method. The digestion with restriction enzyme *Eco*RI and DNA sequencing were carried out to confirm the correct insert. To construct plasmid for mutant creation, the chloramphenicol resistant cassette (Cm^r) was cut from the pSB1AC3 plasmid (Appendix G) by restriction enzyme digestion. The p*Sll1077* plasmid was digested with restriction enzyme *Kpn*I and further blunt end ligated with chloramphenicol resistant cassette. The transformants were selected by growing on LB agar plate containing 100 µg/ml of ampicillin and 30 µg/ml of chloramphenicol. The recombinant plasmid was amplified by PCR method, and further checked by restriction enzyme digestion and DNA sequencing. The recombinant plasmid containing chloramphenicol resistant cassette was hereafter called p*Sll1077-*Cm^r.

2.10.5 Transformation of *Synechocystis* sp. PCC 6803

This method was done according to Eaton-Rye (2004). *Synechocystis* cell culture was cultivated until its OD at 730 nm increased above 0.5, harvested by centrifuging at 5,000*xg* for 10 min (4°C) and resuspended cell pellets in fresh BG_{11} medium. *Synechocystis* cells were diluted to OD_{730} of about 2.5 into a new tube. Then, the addition of 2-10 µg of the recombinant plasmid into *Synechocystis* cell suspension, and incubated under normal growth light for 6 h at 30°C. After that, a reaction mixture was transferred into BG_{11} agar plate containing 10 µg/ml chloramphenicol. That plate was incubated under light condition at 30°C until single green colony was appeared. The appeared transformants were selected stepwise on BG_{11} solid medium containing

higher concentration of chloramphenicol up to 30 μg/ml. The *sll1077* gene disrupted mutant was verified by PCR method.

2.11 Polyamine extraction and HPLC analysis

Synechocystis cells were grown under sorbitol stress until OD at 730 nm of about 0.6 and harvested cells by centrifuging at 8,000*xg* for 10 min (4°C). In order to break cells, 5% cold $HCLO_4$ was added into the cell pellet tube. Cells were incubated with that perchloric acid (PCA) for 1 h on ice. After that, centrifuged at 8000*xg* for 10 min (4°C) and collected both supernatant (PCA-soluble) and pellet (PCA-insoluble) fractions, served as free form and bound form of polyamines, respectively. Both fractions were derivatized with benzoyl chloride. To 500 μl aliquot of sample in glass tube, 1 ml of 2 M NaOH solution was added, following by 25 μl of internal standard (100 mM of 1,8-diamino octane) solution and 10 μl of benzoyl chloride. This mixture was shaken by vortexing and allowed to stand for 20 min at room temperature. Following the addition of saturated NaCl (2 ml), polyamine derivatives were separated by 2 ml of diethyl ether. The upper fraction was collected into a new tube and dried in the fume hood. After that, the dried sample was dissolved with 1 ml of methanol. Before using in next step, filtrated the sample through a 0.45 μm cellulose acetate membrane. Standard polyamines were prepared following the same procedure. High performance liquid chromatography (HPLC) instrument with inertsil® ODS-3 C-18 reverse phase column (5 μm; 4.6 x 150 mm) was applied to analyze polyamine derivatives using UV-Vis detector at 254 nm. The 60% methanol was used as isocratic mobile phase using flow rate 1 ml/min.

2.12 Agmatinase activity assay

Cell culture (200-1000 ml) was harvested by centrifuging at 4000*xg* for 10 min (4°C), and washed with 50 mM Tricine-NaOH buffer (pH 8.5). In order to break cells, French press apparatus was performed at 16000 lb in^{-2}. Both the concentration of that protein extract and incubation time were optimized firstly for enzyme activity assay. The crude extract was incubated with a substrate of 8 mM agmatine sulphate at 30°C in appropriate time and stopped reaction by 250 µl of 25% perchloric acid. The supernatant was obtained after centrifuging at 12,000*xg* for 10 min (4°C) and subsequently continued the derivatization of the product of putrescine. After that, benzoylation and HPLC determination were performed (method 2.11). The protein concentration was determined by Bradford method (Bradford 1976). Unit of enzyme specific activity was calculated from nmol putrescine product per min per mg protein.

2.13 Determination of transcript level of polyamine biosynthetic gene

2.13.1 RNA extraction

The cell culture (50 ml) was collected at OD 730 nm of about 0.6 by centrifuging at 4000*xg* for 10 min (4°C) and immediately frozen cell pellets in liquid nitrogen. TRIzol® reagent was used for total RNA extraction. After homogenizing sample by glass bead vortexing, supernatant sample was transferred into screw cap tube. Then, 0.75 ml of TRIzol® reagent was added, and further incubated at room temperature for 5 min. After centrifuging at 12000*xg* for 10 min (4°C), aqueous phase was transferred into a new tube and added 0.2 ml of chloroform. Mixing sample was done by hand and incubated that tube for 2 min at room temperature, and then centrifuged the sample tube at 12000*xg* for 10 min (4°C). The upper phase was collected into a new tube, and added 0.5 ml of isopropanol, later incubated at room temperature for 10 min before centrifuging at 12000*xg* for 10 min (4°C). The obtained RNA pellet was

washed with 1 ml of 75% ethanol and centrifuged again at 12000*xg* for 15 min. Finally, RNA pellet was dried for 5-10 min, and further dissolved with 30 µl of DEPC–treated water. The extracted RNA quality was determined by 0.8% agarose gel electrophoresis. In order to remove the contaminated DNA, total RNA solution was treated with DNase I as described in following Table 2.4. The reaction was incubated at 37°C for 20 min, and then stopped reaction immediately by adding 2 µl of 50 mM EDTA following by incubated at 65°C for 10 min. The total RNA was verified by agarose gel electrophoresis. Moreover, the RNA concentration was measured by UV spectrophotometer. The absorbance of a diluted RNA solution at 260 nm was calculated using this equation.

$$\text{RNA concentration (ng/µl)} = A_{260} \times \text{Dilution factor} \times 40$$

Table 2.4 The reaction mixture of DN*ase* treatment

RNA/Primer mixture (total volume 30 µl)	Volume (µl)
10X reaction buffer with $MgCl_2$	3
Total RNA	25
Dn*ase* I (1U/µl)	2

2.13.2 **Reverse transcription-polymerase chain reaction (RT-PCR)**

One microgram of total RNA was added into cDNA synthesis mixture. The reaction was performed by SuperScript™ III First-Strand Synthesis System kit for RT-PCR according to the following the procedure. The reaction mixture was prepared as in Table 2.5. After that, the mixture was incubated at 65°C for 5 min, then chilled on ice for at least 1 min. Next step, the mixture was added into cDNA synthesis mixture as described in following this Table 2.6.

Table 2.5 The RNA/Primer mixture

RNA/Primer mixture	Volume (µl)
Total RNA 1 µg	x
50 ng/µl random hexamer primer	1
10 mM dNTP mixed	1
DEPC –treated water	to 10

Table 2.6 The cDNA synthesis mixture

cDNA synthesis mixture	Volume (µl)
10X RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNaseOUT™ (40 U/µl)	1
SuperScript™ III RT (200 U/µl)	1

After mixing gently, the reaction was incubated at 25°C for 10 min and following 50°C for 50 min for cDNA synthesis. To stop the reaction, the heat inactivation was allowed at 85°C for 5 min, then placed on ice before adding 1 µl of RNase H and further incubated at 37°C for 20 min to remove RNA. Finally, the reaction was kept in -20°C before use in further experiment. The first-strand cDNA was amplified using PCR method. The PCR product was analyzed by 1.5% agarose gel electrophoresis in 1X TBE buffer (Appendix C).

CHAPTER III

RESULTS

3.1 Phylogenetic tree of agmatinase and arginase related genes

The collection of 42 agmatinase or arginase genes from *Synechocystis* sp. PCC 6803 and other species were aligned from amino acid sequences using CLUSTALW2 and further constructed the phylogenetic tree which was shown in Figure 3.1. The amino acid encoded by *sll1077* and *sll10228* genes were predicted in the same group with putative agmatinase enzyme. The amino acid sequences encoded by *sll1077* of *Synechocystis* sp. PCC 6803 belonged to the same clade with unicellular cyanobacteria, such as, *Cyanothece* sp. PCC 7424 (AGM), *Synechococcus* sp. PCC 7002 (AA), *Acaryochloris marina* MBIC11017 (AGM1), and *Synechococcus* sp. RCC307 (AGM2). Likewise, the amino acid sequence encoded by *sll0228* of *Synechocystis* sp. PCC 6803 belonged to the same clade with AGM of both filamentous cyanobacteria, *Trichodesmium erythraeum* IMS101 and *Arthrospira platensis* NIES-39, and unicellular cyanobacteria of *Cyanothece* sp. PCC 7424 and *Synechococcus* sp. PCC 7002. However, sll0228 showed the correlation in the same clade of ARG1 and ARG2 with unicellular cyanobacteria *Microcystis aeruginosa* NIES-843. On the other hand, the result of pairwise alignment of *sll1077* and *sll10228* amino acid sequences showed 35.3% of similarity and 20.7% of identity (Figure 3.2).

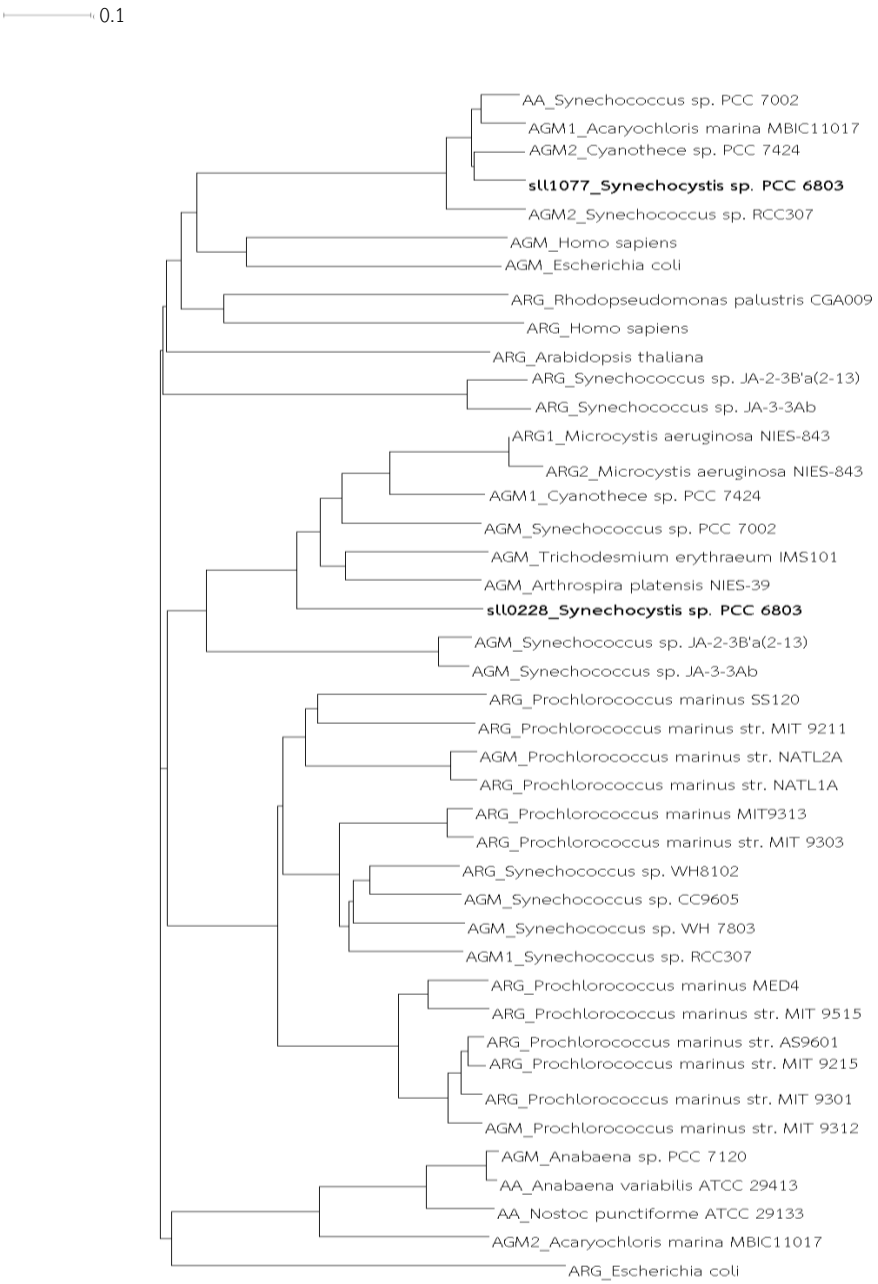

Figure 3.1 Unrooted phylogenetic analysis based on the multiple alignment of the amino acid sequences. The amino acid sequence of arginase and/or agmatinase from 42 strains of cyanobacteria and other species were constructed for phylogenetic tree. Symbols: AGM; agmatinase, ARG; arginase, and AA; agamatinase/arginase.

Table 3.1 Gene index of agmatinase and arginase genes in cyanobacteria and other species

No.	Gene code	Species	Predicted as
1	sll1077	*Synechocystis* sp. PCC 6803	Agmatinase
2	sll0228	*Synechocystis* sp. PCC 6803	Arginase
3	alr2310	*Anabaena* sp. PCC 7120	Agmatinase
4	MAE47100	*Microcystis aeruginosa* NIES-843	Arginase1
5	MAE47180	*Microcystis aeruginosa* NIES-843	Arginase2
6	Pro1849	*Prochlorococcus marinus* SS120	Arginase
7	PMM1686	*Prochlorococcus marinus* MED4	Arginase
8	PMT2214	*Prochlorococcus marinus* MIT9313	Arginase
9	SYNW2422	*Synechococcus* sp. WH8102	Arginase
10	SYNPCC7002_A1751	*Synechococcus* sp. PCC 7002	Arginase/Agmatinase
11	SYNPCC7002_A1109	*Synechococcus* sp. PCC 7002	agmatinase
12	AM1_2833	*Acaryochloris marina* MBIC11017	Agmatinase
13	AM1_3528	*Acaryochloris marina* MBIC11017	Agmatinase
14	PMN2A_1287	*Prochlorococcus marinus* str. NATL2A	Agmatinase
15	Ava_0127	*Anabaena variabilis* ATCC 29413	Arginase/Agmatinase
16	Syncc9605_2591	*Synechococcus* sp. CC9605	Agmatinase
17	PMT9312_1779	*Prochlorococcus marinus* str. MIT 9312	Agmatinase
18	CYB_2252	*Synechococcus* sp. JA-2-3B'a(2-13)	Arginase
19	CYB_1744	*Synechococcus* sp. JA-2-3B'a(2-13)	Agmatinase
20	CYA_0535	*Synechococcus* sp. JA-3-3Ab	Agmatinase
21	CYA_0859	*Synechococcus* sp. JA-3-3Ab	Agmatinase
22	A9601_18961	*Prochlorococcus marinus* str. AS9601	Arginase
23	P9515_18771	*Prochlorococcus marinus* str. MIT 9515	Arginase
24	P9303_29511	*Prochlorococcus marinus* str. MIT 9303	Arginase
25	NATL1_21591	*Prochlorococcus marinus* str. NATL1A	Arginase
26	P9301_18771	*Prochlorococcus marinus* str. MIT 9301	Arginase
27	SynWH7803_2454	*Synechococcus* sp. WH 7803	Agmatinase
28	P9215_19591	*Prochlorococcus* marinus str. MIT 9215	Arginase
29	SynRCC307_2435	*Synechococcus* sp. RCC307	Agmatinase1
30	SynRCC307_2478	*Synechococcus* sp. RCC307	Agmatinase2
31	P9211_18171	*Prochlorococcus marinus* str. MIT 9211	Arginase
32	Npun_R6035	*Nostoc punctiforme* ATCC 29133	Arginase/Agmatinase
33	RPA4729	*Rhodopseudomonas palustris* CGA009	Arginase
34	Tery_3780	*Trichodesmium erythraeum* IMS101	Agmatinase
35	NIES39_C03400	*Arthrospira platensis* NIES-39	Agmatinase
36	PCC7424_2696	*Cyanothece* sp. PCC 7424	Agmatinase1
37	PCC7424_5245	*Cyanothece* sp. PCC 7424	Agmatinase2

No.	Gene code	Species	Predicted as
38	AAL24446	*Homo sapiens*	Agmatinase
39	CAA31188	*Homo sapiens*	Arginase
40	KGM85012	*Escherichia coli*	Agmatinase
41	WP_042093148	*Escherichia coli*	Arginase
42	AEE82694	*Arabidopsis thaliana*	Arginase

```
sll1077           1 MSDATPFRPPSEAEEALIKETRLPLTGWQQEVDQGLTYGLEAAASIKDRS     50

sll0228           1 --------------------------------------------------       0

sll1077          51 IPTFSRGELPHYAGINTFMKAP--YLED--VREVGKYDVAIVGVPHDSGT     96
                          ....|.|...|  :||.  :.......|.:|.:|:::.|
sll0228           1 -----------MHSPNKFTSGPKQFLESEAITSYADAAVVVVPIPYEATT     39

sll1077          97 TYRPGTRFGPQGIRRISALYTPYNFEMGVDLREQISLC-DVGDIFTI---    142
                    :||.|...||:.:...|.....|:.|:|.      |.| |:| |:|.
sll0228          40 SYRKGCEHGPEAVLEASDQLEAYDEELGT------SPCHDLG-IYTCAPL     82

sll1077         143 ------PA-NNEKSFDQISKGIAHIFSSGAFPIILGGDHSIGFPTVRGIC    185
                         || ..:....::..|||.....|.|.:.:||:|:|....||.:.
sll0228          83 ADSNKHPALAGDAMVTEVCDGIAPFVEDGKFVVAIGGEHAITTGVVRAMQ    132

sll1077         186 RHLGDKKVGIIHFDRHVDTQETDLDERMHTCPWFHATNMANAPAKNLVQL    235
                    |...: ...::..|.|.|.:: ..:...|.    ||..|     :.:::|
sll0228         133 RGTSE-PFTVVQIDAHGDMRD-KFEGSCHN----HACVM-----RRVLEL    171

sll1077         236 GIGGWQVPRQGVKVCRERATNILTVTDITEMSLDAAAD------FAIARA    279
                    |:.  .:|.....:|:|.| :::...:|........||      .|||..
sll0228         172 GLP--TLPIAIRAICQEEA-DLIREKNIPVFWAREMADNPNWINEAIASI    218

sll1077         280 TDGTDCVWISFDIDCIDAGFVPGIGWPEPGGLLPREALYLLKRIIRETNV    329
                    |  |..|:::.|:|..|.||:||.|.|||||...|.|...:|:.:...|
sll0228         219 T--TQKVFLTIDMDGFDPGFMPGVGTPEPGGLGWYEGLNFFRRLFQTKQV    266

sll1077         330 CGMEVVEVSPPYDISDMTSLMATRVICDTMAHLVVSGQLPRTEKPAYIHA    379
                    .|.:::|::|... |.::.....::....|.:.   |:..|.:.
sll0228         267 IGCDLMELAPVRG-SVVSEFSTAKLAYKLMGYW---GESQRKKL------    306

sll1077         380 EANMAVDEPWQ    390

sll0228         307 -----------    306
```


Figure 3.2 ClustalW2 amino acid alignment of the putative agmatinase *sll1077* and putative arginase *sll0228* in *Synechocystis* sp. PCC 6803 from Cyanobase (http://genome. microbedb.jp/cyanobase/).

3.2 PCR condition of *sll1077* amplification

After the extraction of the genomic DNA from *Synechocystis* cells, the *sll1077* gene fragment was amplified by PCR method. The annealing temperature was varied between 45-60°C for optimizing condition. The suitable annealing temperature was 49.2°C as shown in Figure 3.3. The PCR product of *sll1077* fragment was separated in 0.8% agarose gel which a size of 1204 bp (Figure 3.4).

3.3 Construction of the recombinant plasmid containing disrupted *sll1077* gene

The PCR product of *sll1077* gene fragment was ligated into pGem T-easy vector using DNA ligase. After that, the reaction mixture was transformed into to *E.coli* DH5**α** strain. After selecting the white colony on LB plate containing ampicillin, IPTG and X-Gal, PCR method was used for verifying the *sll1077* gene fragment on plasmid. Only one colony (number 1) had its expected size corresponding to *sll1077* gene (Figure 3.5A). Then, this colony was taken and grown in LB medium. Next, the plasmid DNA was extracted and confirmed by restriction enzyme digestion namely *Eco*RI enzyme. The result showed the correct size of *sll1077* gene in pGem T-easy vector, hereafter named p*Sll1077*, which had its size of 1204 bp (Figure 3.5B).

The p*Sll1077* was inserted by chloramphenicol resistant cassette gene (*Cm*^{r}) which obtained from pSB1AC3 vector (Appendix G). This cassette gene fragment was obtained by *Bsa*AI and *Eco*RV digestion which had its product size of 759 bp. After insertion of *Cm*^{r} gene fragment to disrupt *sll1077* gene, two colonies were obtained on LB plate containing chloramphenicol and checked the recombinants with colony PCR method. The result showed the correct size of 1963 bp (Figure 3.6A). Plasmid DNA was extracted and digested with restriction enzymes *Nde*I and *Bam*HI. After checking by electrophoresis, plasmid DNA digested by two enzyme showed correct sizes of pGem T-easy vector fragment (3015 bp) and *sll1077* gene fragment inserted with chloramphenicol resistance cassette (1963 bp), so called p*Sll1077-Cm*^{r} (Figure 3.6B).

3.4 *Synechocystis* transformants lacking *sll1077* gene

The recombinant plasmid p*Sll1077-Cm*^{r} was transformed into *Synechocystis* cells which segregated into genome by double homologous recombination. The transformants were selected in BG_{11} medium contain antibiotic chloramphenicol. The complete segregation was confirmed by PCR analysis (Figure 3.7) which had its correct size of 1963 bp.

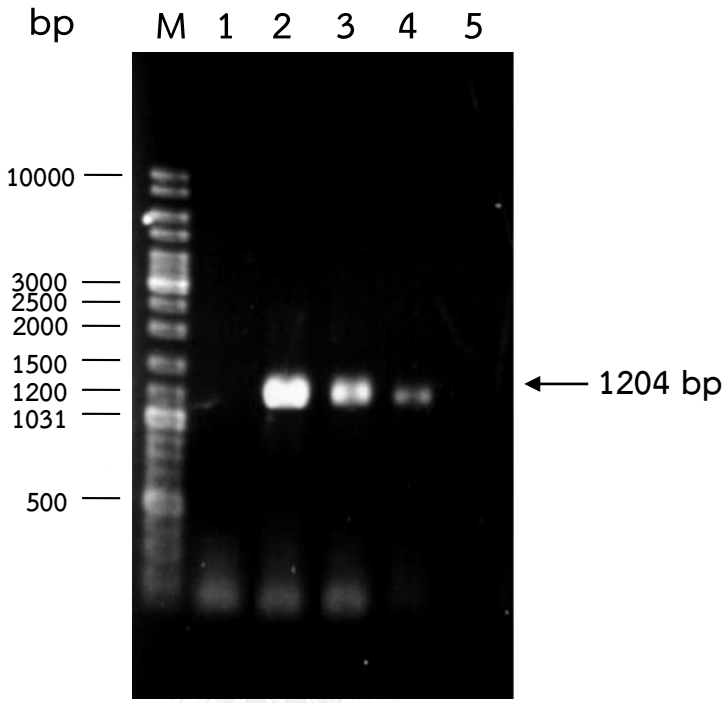

Figure 3.3 Amplification of *sll1077* gene fragment of gradient annealing temperatures. The 0.8% agarose gel electrophoresis in 0.5xTAE buffer of PCR products was performed. Lane M; DNA marker, lanes 1– 5; PCR products at 46.5, 49.2, 55.7, 60.1 and 64.0°C respectively.

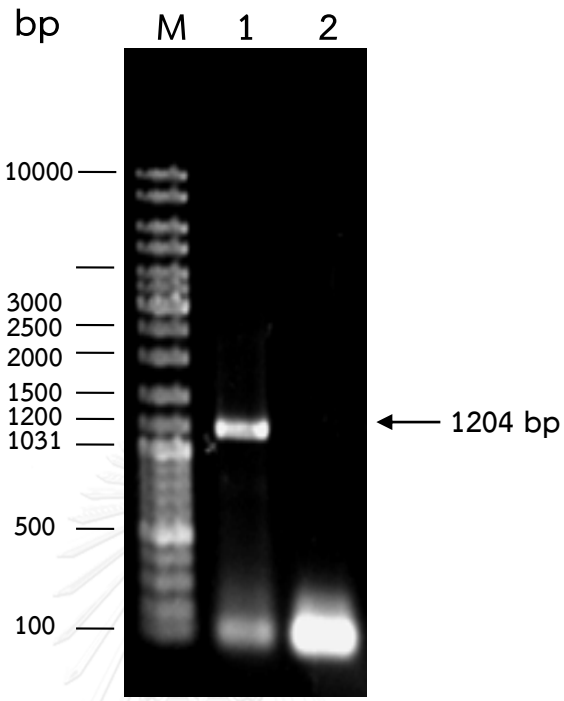

Figure 3.4 Gel electrophoresis (0.8%) of PCR product of *sll1077* gene fragment lane M; DNA marker, Lane 1; PCR product of *sll1077* gene fragment and Lane 2; negative control.

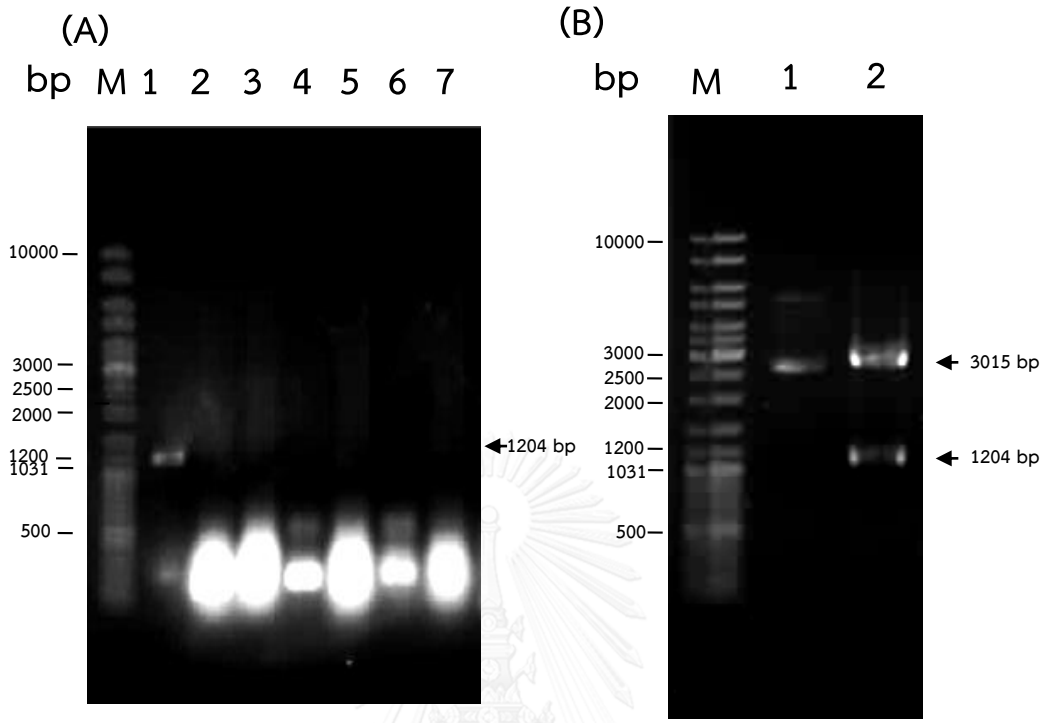

Figure 3.5 Agarose gel (0.8%) electrophoresis of *sll1077* gene in pGem T-easy vector (p*Sll1077*). (A) The PCR amplification of selected clone. Lane M; DNA marker, and lanes 1-7, colony PCR of *sll1077* gene fragment. (B) The restriction enzyme digestion of p*Sll1077*. Lane 1; p*Sll1077* and lane 2; p*Sll1077* digested with *Eco*RI enzyme.

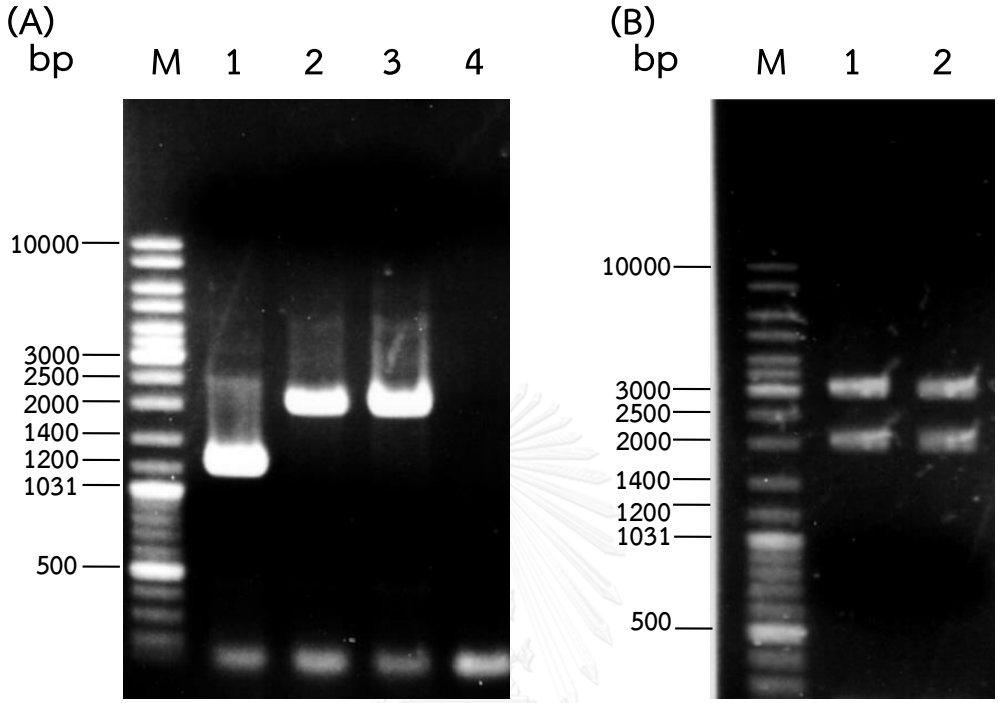

Figure 3.6 Agarose gel (0.8%) electrophoresis of *sll1077* gene inserted with chloramphenicol resistant cassette in pGem T-easy vector (p*Sll1077-Cm*^{r}). (A) The PCR amplification of selected clone. Lane M; DNA marker, lane 1; PCR of *sll*1077 gene, lanes 2-3; colony PCR of *sll*1077 gene with *Cm*^{r} and lane 4; negative control. (B) The restriction enzyme digestion of p*Sll1077- Cm*^{r}. Lanes 1-2; p*Sll1077- Cm*^{r}1 and p*Sll1077-Cm*^{r}2 digested with *Nde*I and *BamH*I enzyme.

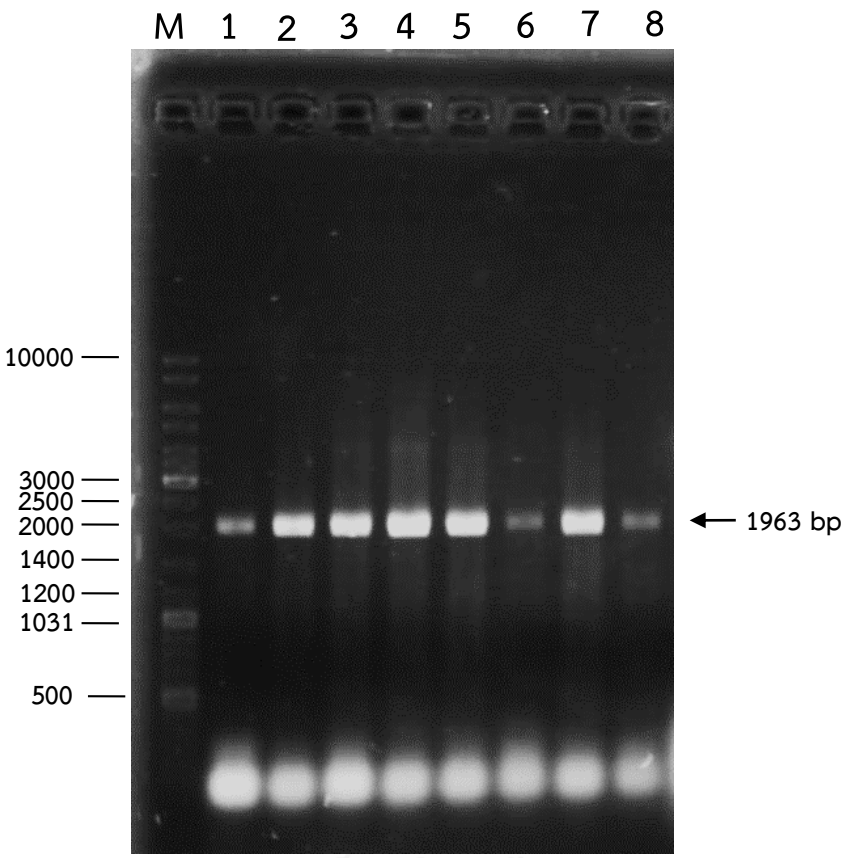

Figure 3.7 Confirmation of complete segregated Δ*sll1077* by PCR method and agarose gel (0.8%) electrophoresis. Lane M; DNA marker and lanes 1-8; colony PCR products of *sll*1077 gene with *Cm*^{r}.

3.5 Growth rate and pigment contents of wild type and mutant strains

In this experiment, the growth rate and pigment contents of wild type and two mutant strains, lacking of *sll1077* gene (Δ*sll1077*) and *sll0228* (Δ*sll0228*) gene, respectively, were measured. Growth rate of wild type under control condition without sorbitol addition showed no different change with other two mutant strains (Figure 3.8A). Also, in 250 mM (Figure 3.8B) and 500 mM sorbitol condition (Figure 3.8C), all three strains showed similar patterns of growth rate. The highest stress condition of 700 mM sorbitol obviously affected the growth rate of all strains (Figure 3.8D). Surprisingly, mutant strain Δ*sll1077* slightly grown in this condition. When comparing wild type strain at different sorbitol conditions (Figure 3.9A), the highest growth rate was occurred under in control condition. Although 250 mM and 500 mM sorbitol conditions affected the growth rate, cells still could grow under these conditions whereas 700 mM sorbitol condition completely inhibited growth rate. In Δ*sll1077* strain, the growth rate under control condition was as similar as that under 250 mM sorbitol concentration (Figure 3.9B). The 500 mM and 700 mM sorbitol conditions also decreased growth of Δ*sll1077*. The growth rate of Δ*sll0228* were not different significantly under both control condition and 250 mM sorbitol condition (Figure 3.9C). In 500 mM sorbitol condition, cells were grown slowly and was inhibited significantly under 700 mM sorbitol condition.

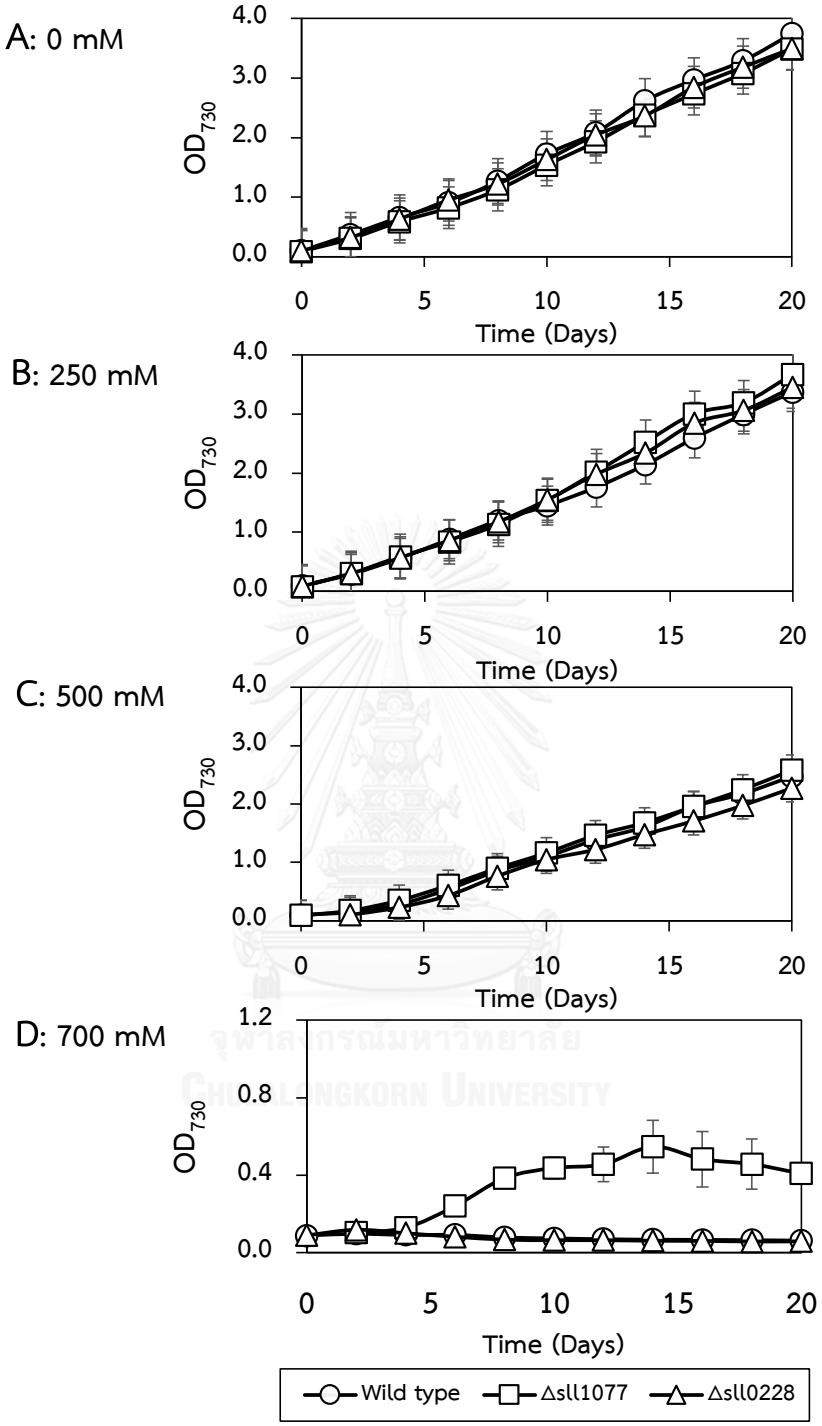

Figure 3.8 The growth rates of *Synechocystis* wild type (○), Δ*sll1077* (□) and Δ*sll0228* (△) mutant strains under various sorbitol conditions of (A) 0 mM (B) 250 mM (C) 500 mM and (D) 700 mM concentrations respectively. Data represent Mean ± S.D., n = 3.

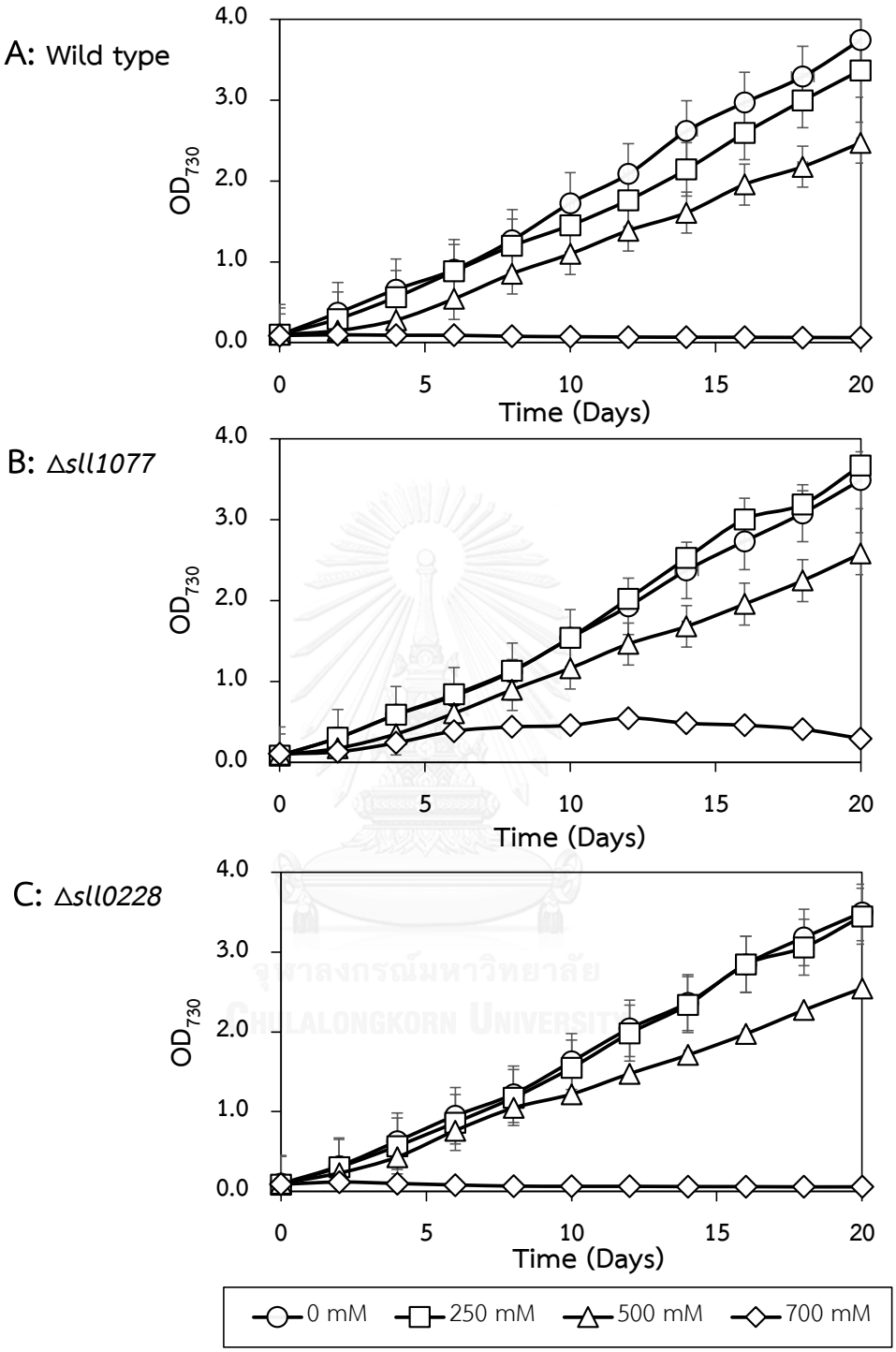

Figure 3.9 The growth rates of *Synechocystis* wild type (A), Δ*sll1077* (B), Δ*sll0228* (C) strains. Symbols: conditions of 0 mM (○), 250 mM (□), 500 mM (△) and 700 mM (◇) sorbitol concentrations. Data represent Mean ± S.D., n=3.

The chlorophyll *a* content of cells at control condition showed similar amounts along 20 days and remained steady all wild type and mutant strains (Figure 3.10A). In 250 mM sorbitol condition, the chlorophyll *a* content also showed no apparent change (Figure 3.10B). While in 500 mM sorbitol condition (Figure 3.10C), all three strains were shapely increased within day 2 and further dropped in day 6, and the amounts were stable after that up to day 20. The chlorophyll *a* content of wild type had similar accumulation as in Δ*sll1077* whereas Δ*sll0228* accumulated it less amount than under 700 mM sorbitol condition. The chlorophyll *a* content in wild type and Δ*sll0228* were decreased significantly. In contrast, the chlorophyll *a* content in Δ*sll1077* was increased and fluctuated along 14 days and later decreased at day 16 (Figure 3.10D). Moreover, the 250 mM and 500 mM sorbitol conditions did not decrease the chlorophyll *a* content compared to that under control condition (Figure 3.11A). Under 700 mM sorbitol condition, the chlorophyll *a* content was significantly decreased. In Δ*sll1077*, the chlorophyll *a* content under all conditions were not changed significantly, except the decrease after day 12 under 700 mM sorbitol condition (Figure 3.11B). For Δ*sll0228*, the sorbitol conditions at 250 and 500 mM concentrations did not harm chlorophyll *a* accumulation whereas 700 mM sorbitol condition significantly inhibited the chlorophyll *a* accumulation (Figure 3.11C).

The carotenoid contents of wild type and mutant strains were shown in Figure 3.12 and 3.13. All wild type and mutant strains had carotenoid accumulation along 20 day-culture under control (Figure 3.12A), as well as under 250 mM sorbitol condition (Figure 3.12B). The 500 mM sorbitol condition significantly decreased the carotenoid contents in all strains at day 6 and later showed constant amounts up to day 20 (Figure 3.12C). In 700 mM sorbitol condition, Δ*sll1077* showed gradually a decrease on the carotenoid content (Figure 3.12D). For wild type strain (Figure 3.13A), the highest amount of carotenoid content was observed under control condition whereas the 250 and 500 mM sorbitol conditions decreased slightly carotenoid contents. The lowest

content of carotenoid was observed under 700 mM sorbitol condition, which significantly declined at day 4. For Δ*sll1077* strain (Figure 3.13B), all sorbitol conditions significantly decreased the carotenoid accumulations, except under 700 mM sorbitol condition after day 12 which obviously reduced the carotenoid contents. The carotenoid content at high concentration of 700 mM sorbitol was decreased gradually. On the other hand, the carotenoid content of Δ*sll0228* (Figure 3.13C) showed a slight decrease under 250 mM sorbitol condition whereas 500 mM sorbitol condition significantly decreased the carotenoid content. Strikingly, the carotenoid content was dramatically decreased under 700 mM sorbitol condition after day 2 of treatment.

3.6 Oxygen evolution of *Synechocystis* cells under sorbitol stress

Synechocystis wild type and mutant strains were cultivated in BG_{11} medium supplemented with various concentrations of sorbitol until OD 730 nm of around 0.6-0.8 (6 days) before performing O_2 evolution measurement. The O_2 evolution rates of *Synechocystis* wild type and mutant strains were shown in Figure 3.14 which represented to their photosynthetic efficiency. Wild type strain showed the similar O_2 evolution rate as other two mutants under control condition. The sorbitol conditions at 250 mM did not harm photosynthetic efficiencies of wild type and both two mutant strains. However, 500 mM sorbitol concentration decreased the O_2 evolution of wild type strain whereas showed no reduction in mutants. The highest sorbitol stress at 700 mM dramatically decreased the oxygen evolution in all strains compared to control and lower sorbitol concentrations. Interestingly, The O_2 evolution rates of Δ*sll1077* and Δ*sll0228* mutants were not changed by sorbitol stress conditions at 0 mM, 250 mM and 500 mM, concentration.

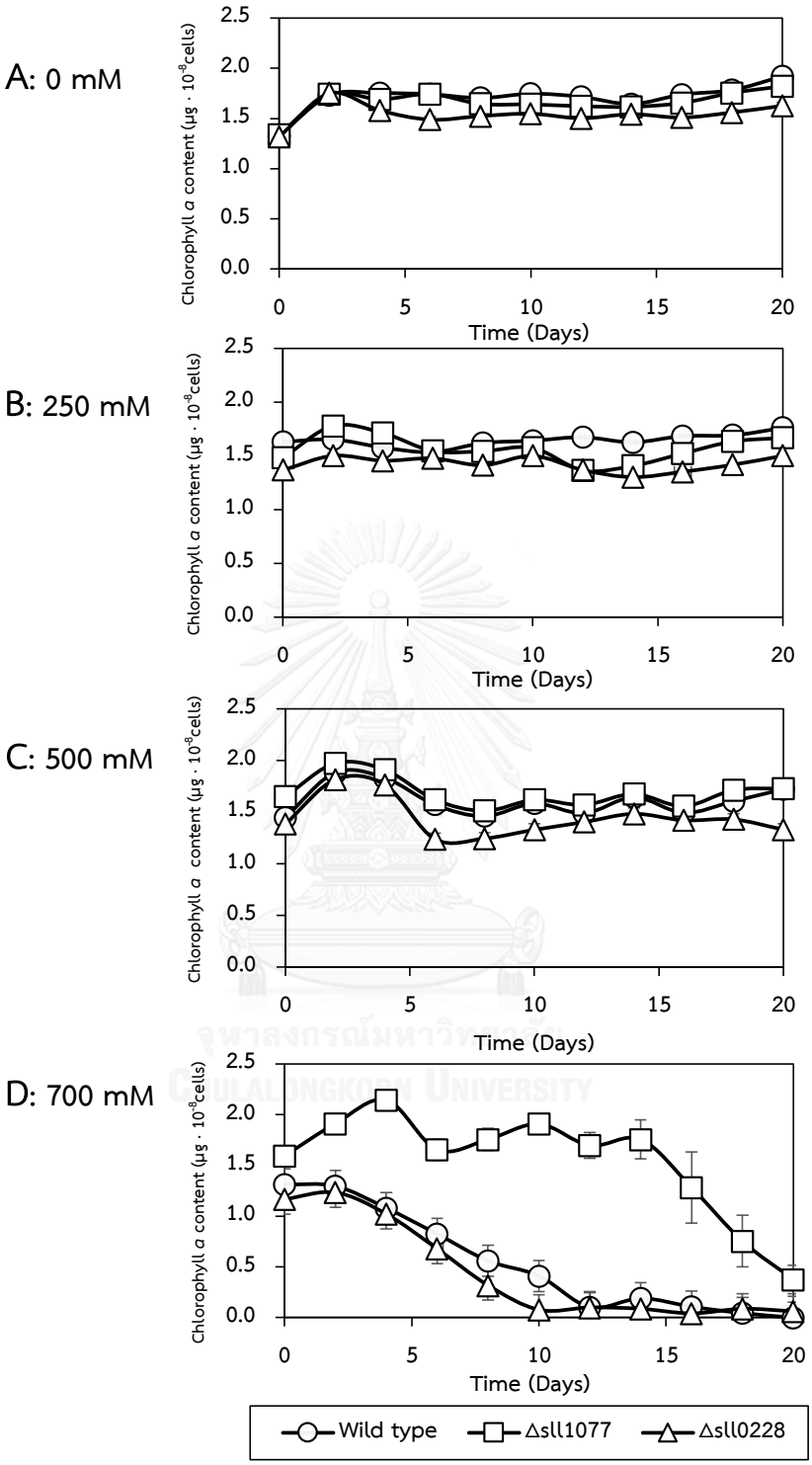

Figure 3.10 The chlorophyll *a* content of *Synechocystis* wild type (○), Δ*sll1077* (□) and Δ*sll0228* (△) mutant strains under various sorbitol concentrations of (A) 0 mM (B) 250 mM (C) 500 mM and (D) 700 mM, respectively. Data represent Mean ± S.D., n = 3.

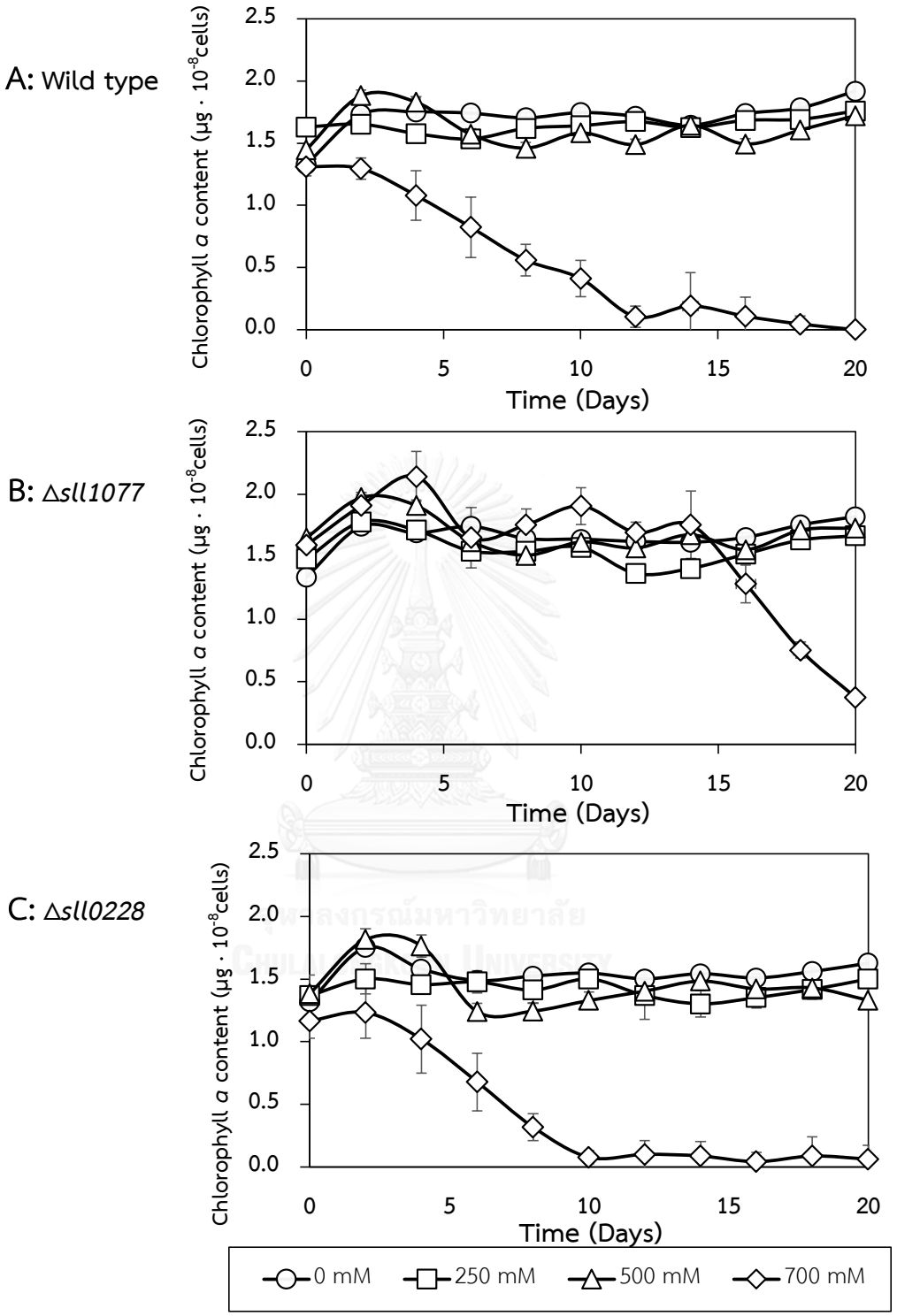

Figure 3.11 The chlorophyll *a* content of *Synechocystis* wild type (A), Δ*sll1077* (B), Δ*sll0228* (C) strains. Symbols: 0 mM (○), 250 mM (□), 500 mM (△) and 700 mM (◇) sorbitol concentrations. Data represent Mean ± S.D., n=3.

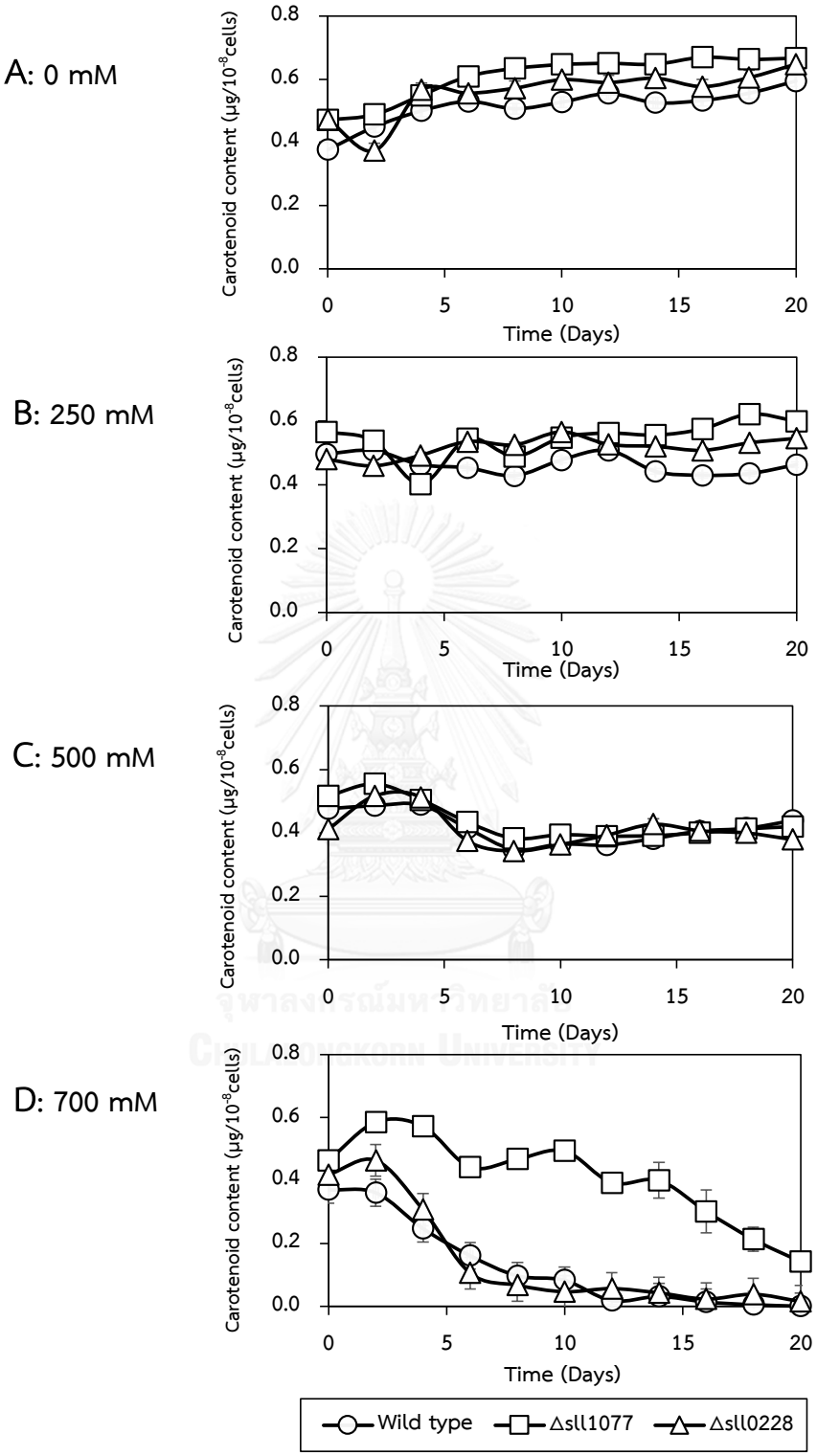

Figure 3.12 The carotenoid content of *Synechocystis* wild type (○), Δ*sll1077* (□) and Δ*sll0228* (△) mutant strains under various sorbitol concentrations of (A) 0 mM (B) 250 mM (C) 500 mM and (D) 700 mM, respectively. Data represent Mean ± S.D., n = 3.

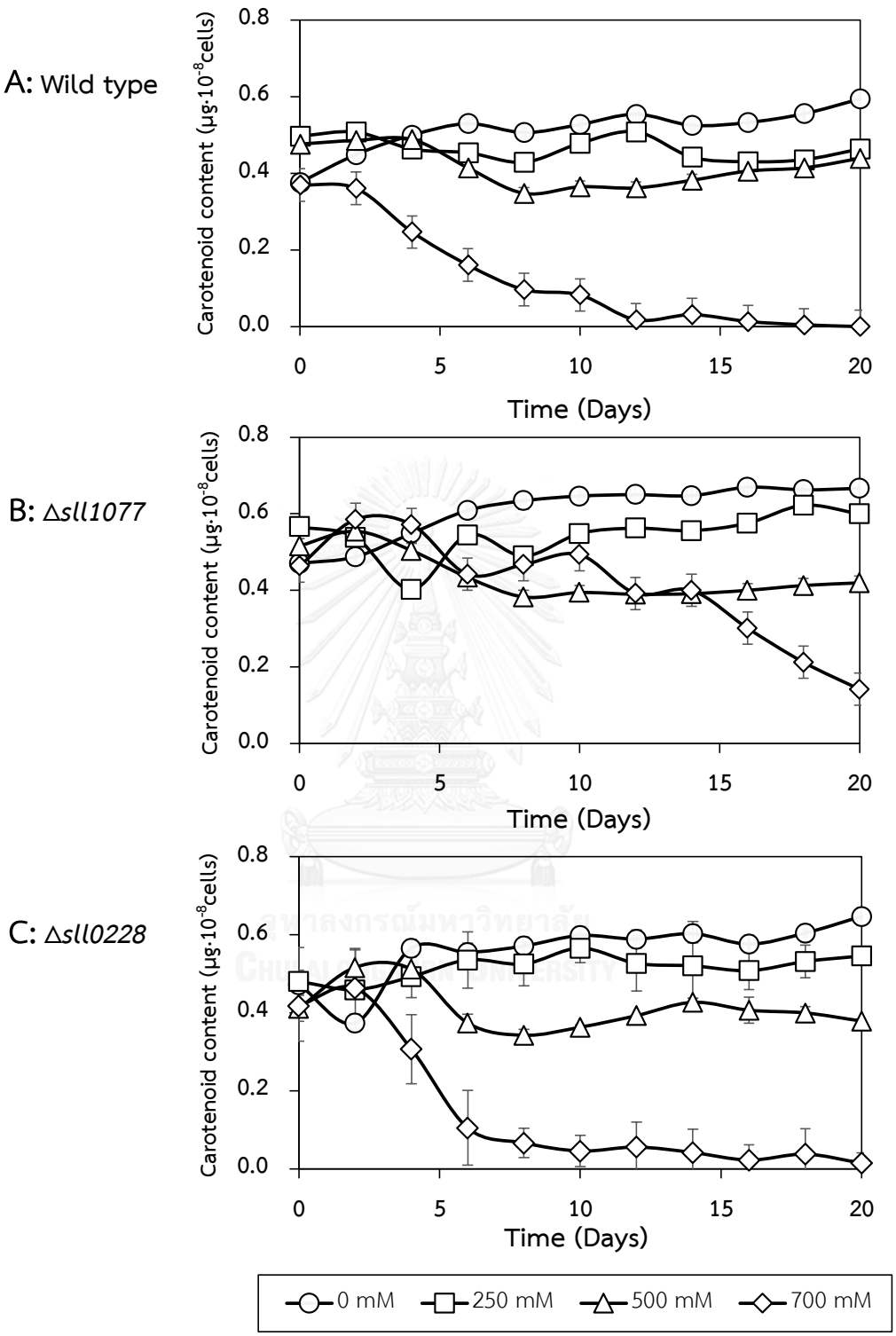

Figure 3.13 The carotenoid content of *Synechocystis* wild type (A), Δ*sll1077* (B), Δ*sll0228* (C) strains. Symbols: 0 mM (○), 250 mM (□), 500 mM (△) and 700 mM (◇) sorbitol concentrations. Data represent Mean ± S.D., n=3.

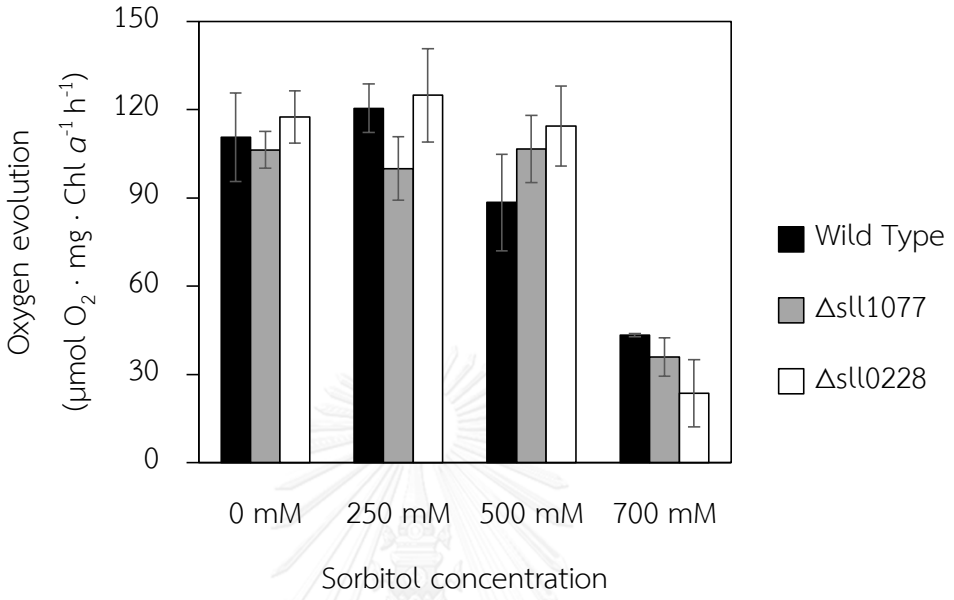

Figure 3.14 The oxygen evolution of wild type (■) and mutant strains of Δ*sll1077* (▩) and Δ*sll0228* (□), respectively, under various sorbitol concentrations. The photosynthetic efficiency represents by oxygen evolution was measured using Oxygraph plus oxygen electrode (Hansatech Instruments, U.K.) under saturated light intensity at 25°C. Data represent Mean ± S.D., n = 3.

3.7 Polyamine contents under sorbitol stress

The polyamine contents were measured under various sorbitol conditions in Figure 3.15. In wild type strain (Figure 3.15A), putrescine and spermidine contents of control condition (at 0 mM), 250 mM and 500 mM sorbitol concentrations were slightly increased and futher reached their highest level at 700 mM sorbitol condition around 3.7 nmol · 10^{-8} cells. In Δ*sll1077* strain (Figure 3.15B), spermidine content was slightly increased in 250 mM sorbitol concentration and then decreased slowly in 500 mM and 700 mM sorbitol conditions. On the other hand, putrescine content was induced gradually in 250 mM and 500 mM sorbitol concentrations and dropped in 700 mM sorbitol concentration. In Δ*sll0228*, spermidine was significantly decreased when compared to other two strains (Figure 3.15C). The sorbitol concentrations of 250 mM and 500 mM slightly induced few spermidine content and showed no spermidine detected under 700 mM sorbitol condition. Interestingly, there were no putrescine and spermine detected in Δ*sll0228*.

3.8 Norspermidine contents under sorbitol stress

In this experiment, norspermidine content was detectable only under sorbitol stressed condition. The result was shown in Figure 3.16. Under control condition, all these strains showed no any detectable norspermidine. Under 250 mM condition, wild type cells gave the highest level of norspermidine among three strains of about 1.6 nmol · 10^{-8} cells. The Δ*sll1077* and Δ*sll0228* strains had lower amounts of about 3 fold and 1.33 fold, respectively, compared with wild type strain. Under 500 mM condition, norspermidine amount of wild type strain showed as similar as that in Δ*sll0228* while Δ*sll1077* had lowest content among two other strains of about 1.28 fold of wild type. Under 700 mM sorbitol, Δ*sll0228* showed the highest norspermidine content of about 2.7 nmol · 10^{-8} cells. Interestingly, PCA-insoluble form of norspermidine was observed apparently under 700 mM sorbiltol condition. The Δ*sll1077* gave the lowest content

among others. Interestingly, the higher concentration of sorbitol, the higher amounts of norspermidine in all strains were clearly observed.

3.9 The expression of polyamine biosynthetic genes

Synechocystis cells were grown under 700 mM sorbitol concentration until the optical density at 730 nm of about 0.6-0.8 and then collected cells for RNA extraction and cDNA synthesis. The amplification technique PCR and gel electrophoresis were used for verifying the expression of polyamine biosynthetic genes. From the result in Figure 3.17, *sll1077* and *sll0228* mRNAs of wild type (encoding agmatinase and arginase enzyme, respectively, in Cyanobase) showed higher level under sorbitol stress than control at 0 mM. The *sll1077* transcript was up-regulated by 700 mM sorbitol condition in both wild type and Δ*sll1077* strain whereas the reduction of *sll1077* transcript was significantly decreased in Δ*sll0228* strain. Moreover, the *sll0228* transcript showed the same pattern as the *sll1077* transcript which up-regulated by 700 mM sorbitol condition in wild type and Δ*sll1077* strain and down-regulated in Δ*sll0228* strain.

On the other hand, the expression levels of s*ll0601* and *sll1640* (predicted by Schriek *et al*. 2007 as both encode *N*-carbamoylputrescine amidohydrolase enzyme) were observed (Figure 3.18). Interestingly, the gene expressions of these two genes were found in all strains. In wild type, there was no significant change on *sll0601* transcript under control (0 mM) and 700 mM sorbitol conditions. The increased level of *sll0601* transcript was observed apparently in Δ*sll1077* strain. In contrast, the transcript level of *sll0601* was decreased significantly by 700 mM sorbitol condition in Δ*sll0228* strain. On the other hand, the gene transcript of *sll1640* was increased by 700 mM sorbitol in wild type strain, as well as in Δ*sll1077* strain whereas the significant decrease of *sll1640* transcript was observed in Δ*sll0228* strain.

Nevertheless, the *sll0873* transcript encoding carboxynorspermidine synthase, in Cyanobase, was shown in Figure 3.19. The up-regulation of *sll0873* mRNA was

obviously seen under 700 mM sorbitol condition compared to control in all wild type, Δ*sll1077* and Δ*sll0228* strains.

3.10 Agmatinase activity

The effect of sorbitol on agmatinase activity in *Synechocystis* wild type and mutant strains was shown in Figure 3.20. At control condition, wild type strain showed the agmatinase activity around 1.63 nmol · min^{-1} mg · $protein^{-1}$ whereas Δ*sll1077* strain had less activity than wild type strain of about 30%. Interestingly, the strain lacking *sll0228* gene gave lower activity around 0.1 nmol · min^{-1} mg · $protein^{-1}$ than wild type of about 94%. The higher sorbitol concentrations increased the agmatinase activity in wild type. On the other hand, the sorbitol concentrations insignificantly increased the activity in Δ*sll1077* strain whereas the activity was decreased significantly under 700 mM sorbitol condition compared with control condition. Strikingly, the Δ*sll0228* strain gave lowest or trace level of activity in all sorbitol stresses.

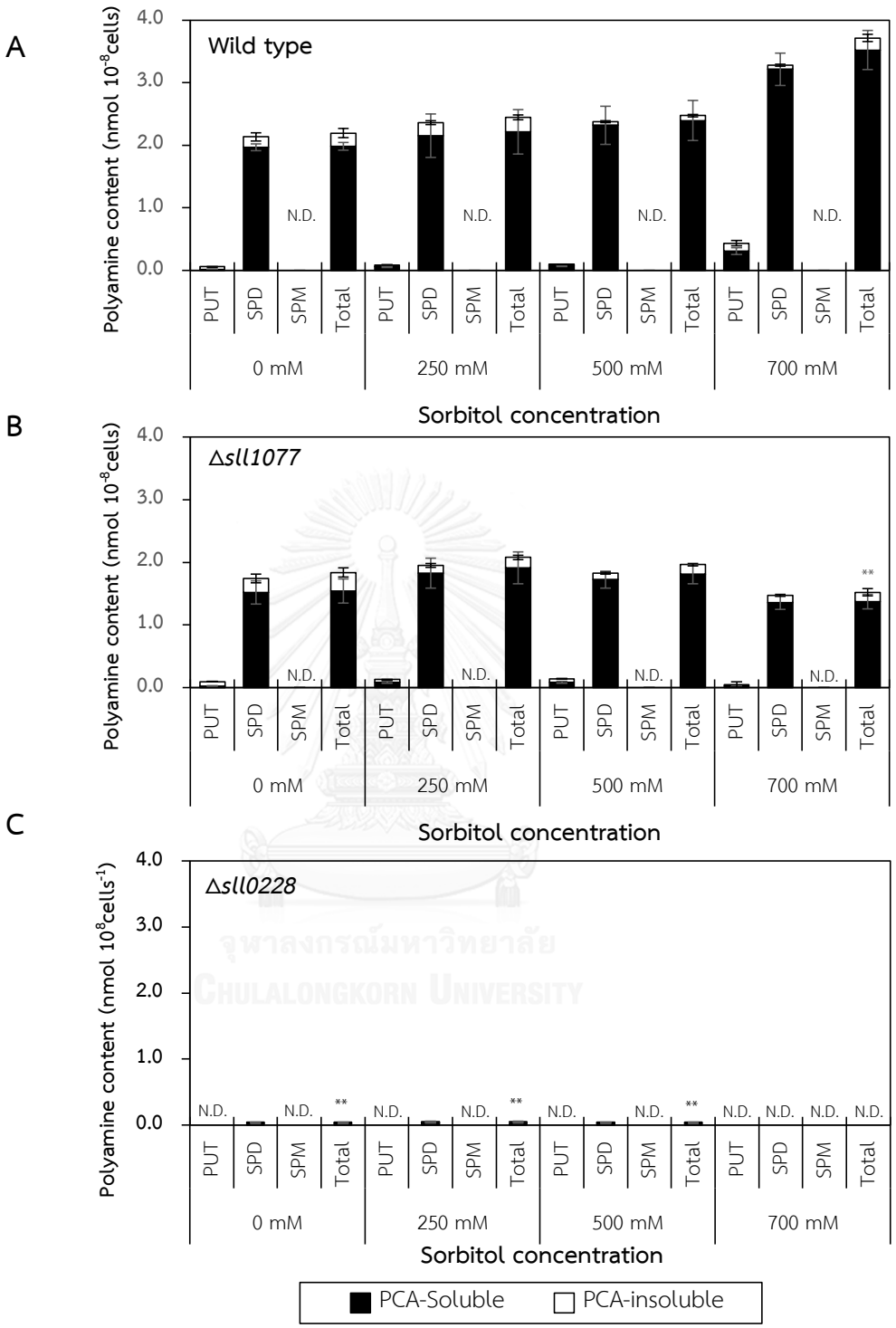

Figure 3.15 Polyamine contents, PCA-soluble (■) and PCA-insoluble (□) polyamines, in wild type (A) and mutant strains of Δ*sll1077* (B) and Δ*sll0228* (C) under various sorbitol concentrations. Data represent Mean ± S.D., n = 3. N.D. = undetectable level while asterisks represent the bar with significantly difference with ** $P < 0.01$ compared to wild type strain in each sorbitol concentration.

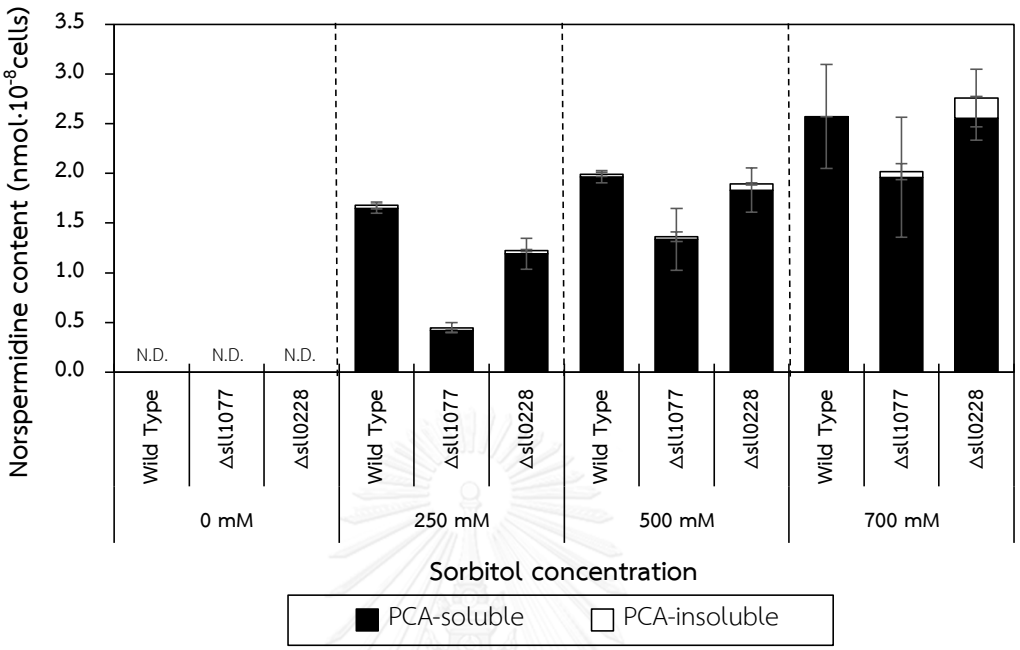

Figure 3.16 Norspermidine contents, PCA-soluble (■) and PCA-insoluble (□) polyamines, in wild type (A) and mutant strains of Δ*sll1077* (B) and Δ*sll0228* (C) under various sorbitol concentrations. Cells grown with mid-log phase were harvested and further measured for norspermidine content by polyamine derivatization and HPLC detection. Data represent Mean ± S.D., n = 3. N.D. = undetectable level.

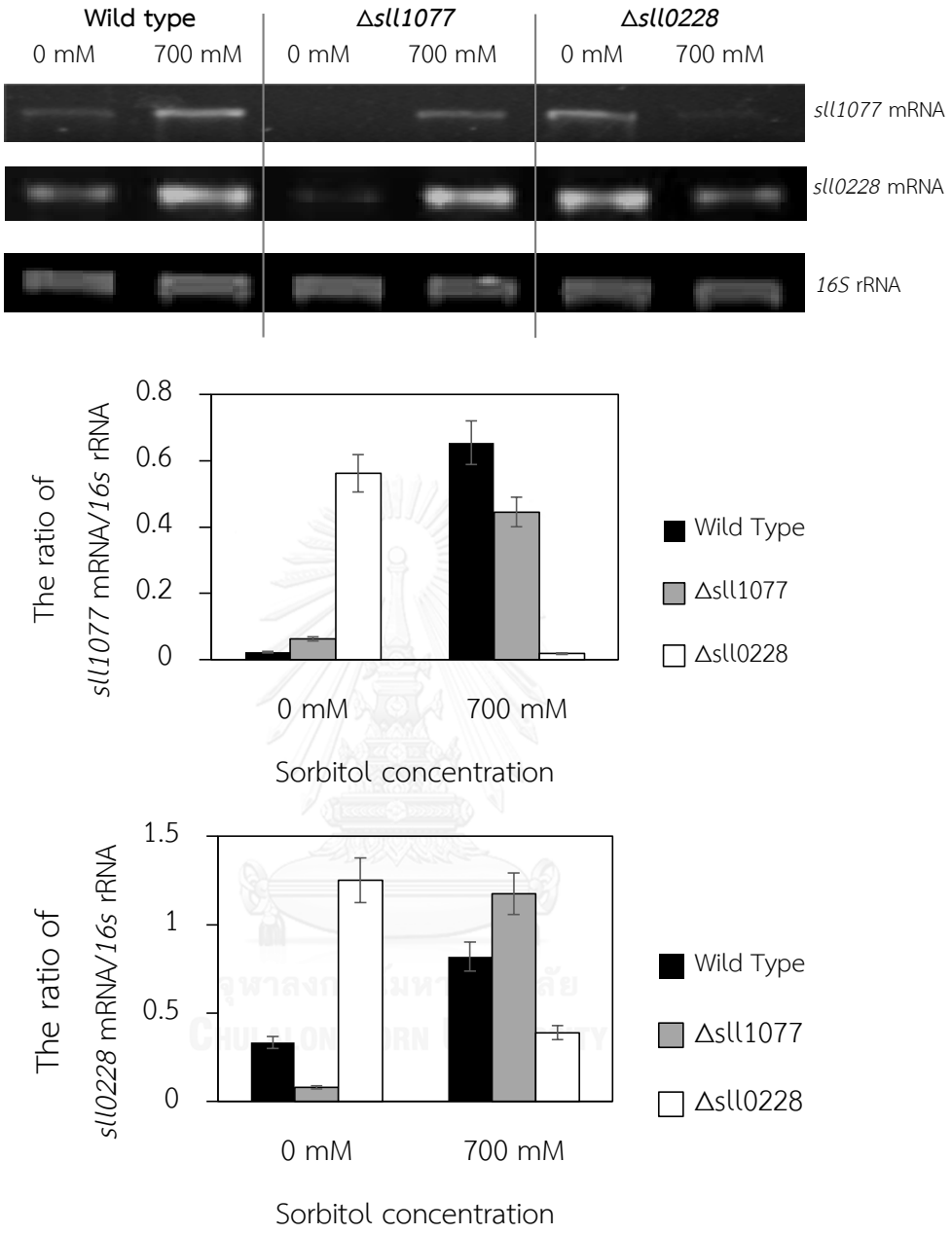

Figure 3.17 The gene expressions of *sll1077* and *sll0228* (putative agmatinase))transcripts in wild type (■) and mutant strains of Δ*sll1077* (▩) and Δ*sll0228* (□). The relative abundance of *16s* rRNA was shown in lower row.

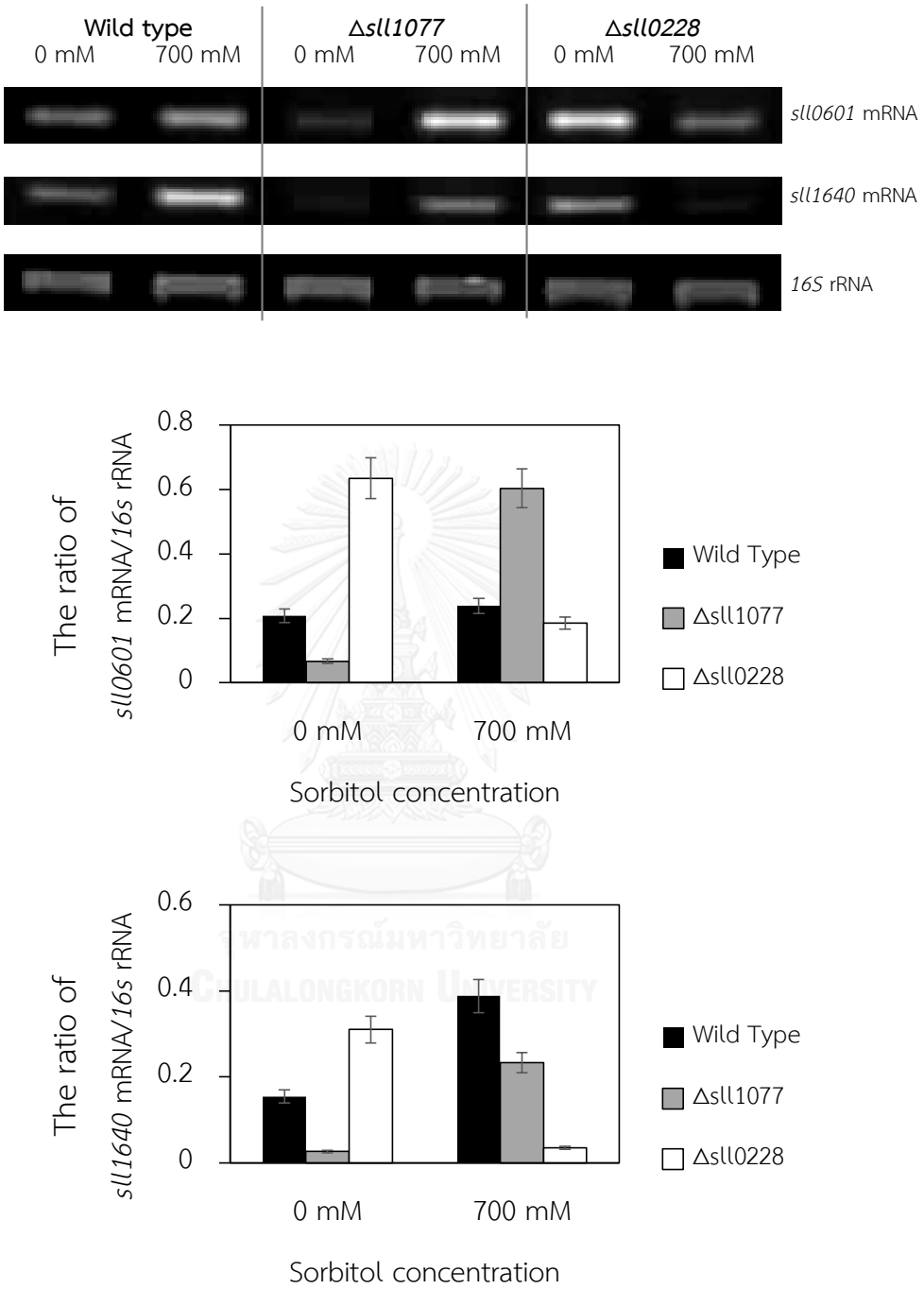

Figure 3.18 The gene expressions of *sll0601* and *sll1640* (putative *N*-carbamoylputrescine amidohydrolase) transcripts in wild type (■) and mutant strains of Δ*sll1077* (▩) and Δ*sll0228* (□). The relative abundance of *16s* rRNA was shown in lower row.

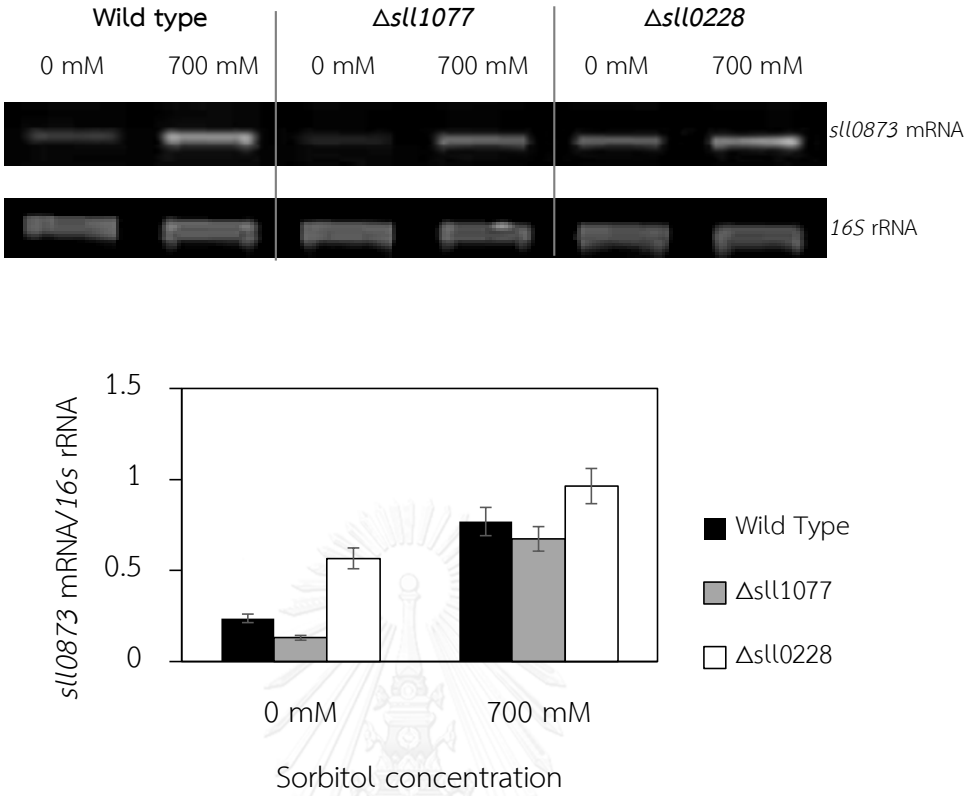

Figure 3.19 The gene expression of *sll0873* (putative carboxynorspermidine synthase) transcript in wild type (■) and mutant strains of Δ*sll1077* (▒) and Δ*sll0228* (□). The relative abundance of *16s* rRNA was shown in lower row.

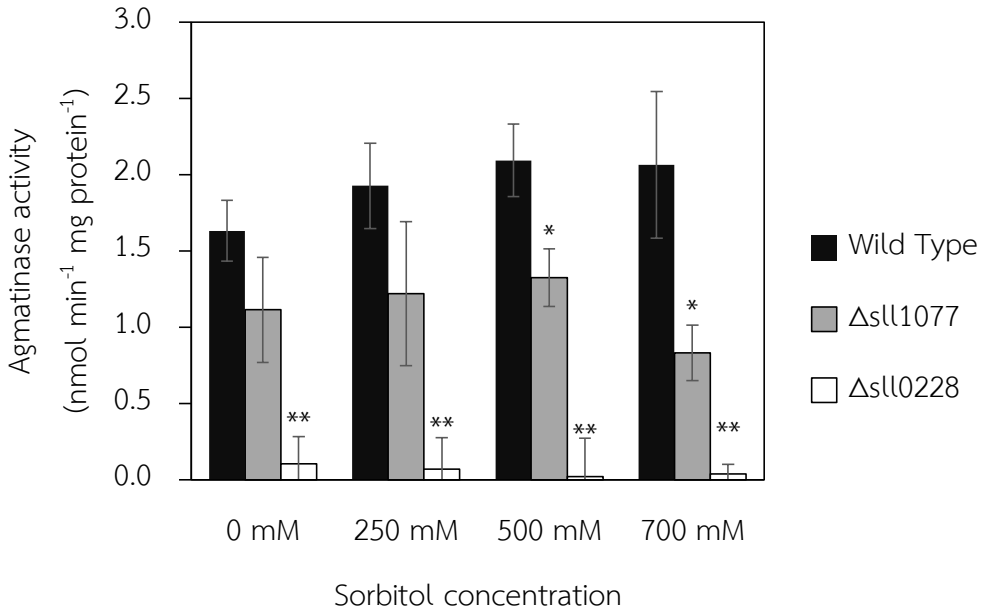

Figure 3.20 Agmatinase activities under various sorbitol conditions in wild type (■), Δ*sll1077* (▩) and Δ*sll0228* (□) strains, respectively. The control condition was represented by 0 mM sorbitol condition. Data represent Mean ± S.D., n = 3 while asterisks represent the bar with significantly difference with * $P < 0.05$ and ** $P < 0.05$ compared to wild type strain in each sorbitol concentration.

CHAPTER IV
DISCUSSION

Effect of sorbitol stress on growth rate, chlorophyll *a* and carotenoid contents and oxygen evolution

The results of growth rates in wild type and mutant strains which cultivated along 20 days under sorbitol-induced osmotic stress were shown in Figures 3.8 and 3.9. The growth rate of wild type and mutant strains were not significantly different under control and 250 sorbitol conditions whereas 500 mM sorbitol condition decreased growth rates of all strains and inhibited completely under 700 mM sorbitol condition, except in Δ*sll1077*. It was found that the highest sorbitol stress at 700 mM concentration did not completely harm cell growth of Δ*sll1077* cells. Cells lacking *sll1077* gene seemed to tolerate to sorbitol stress rather than wild type. In the previous studies, the sorbitol effect at 300 mM concentration significantly decreased growth rate of *Synechocystis* cells and completely inhibited growth rate at 700 mM sorbitol condition (Jantaro *et al.* 2003). It was also consistent to maize that the 200 mM sorbitol condition decreased germination rate and length of root and shoot while the 600 mM sorbitol condition highly inhibited them (Jain *et al.* 2010).

The pigment contents of chlorophyll *a* and carotenoids were measured and shown in Figures 3.10-3.13. It was indicated that the concentrations at 250 and 500 mM sorbitol conditions did not decrease the pigment contents in wild type and mutant strains whereas the 700 mM sorbitol concentration significantly reduced pigment contents in all strains. These results were coincident with previous findings that the reduction of pigment content including chlorophyll *a*, chlorophyll *b* and carotenoid were observed in soybean under osmotic stress (Radhakrishnan and Lee 2013). In

maize leaf, the higher concentration of sorbitol-induced osmotic stress significantly lowered pigment contents (Jain *et al.* 2010). The function of chlorophyll *a* in the photosynthetic apparatus of oxygen-producing organisms was accompanied by auxiliary light-harvesting pigments (Katz *et al.* 1978). In addition, carotenoids in cyanobacteria were also light-harvesting pigments in photosynthesis, and could protect against photooxidative damage (Hirschberg and Chamovitz 1994). By their functions against sorbitol stress, we found that chlorophyll *a* and carotenoid pigments were increased under 500 mM sorbitol concentration at day 2 of treatment in all strains (Figure 3.10C and 3.12C). However, the decrease of chlorophyll content in osmotically stressed plants might be the result of photooxidation and chlorophyll degradation (Anjum *et al.* 2011). Interestingly, Δ*sll1077* strain was not only able to grow under 700 mM sorbitol condition but also efficiently maintained intracellular pigment contents along 14 days of treatment.

The O_2 evolution rates in wild type and mutant strains were shown in Figure 3.14. The 500 mM sorbitol condition did not effect on the O_2 evolution rate in mutant strains whereas decreased it in wild type strain. Moreover, 700 mM sorbitol condition dramatically decreased the phosynthetic efficiencies of all strains. The effect of osmotic stress on photosystem was observed in *Synechococcus* sp. PCC 7942 (Allakhverdiev et al. 2000). Under ionic and osmotic stress at 0.5 M NaCl and 1 M sorbitol conditions, respectively, the declined oxygen-evolving activity of photosystem was observed by decreasing the amount of water in the cytosol, rapidly increasing the intracellular concentration of salts and influxing of Na^+ ions through potassium/Na^+ channels to inactivated PSI and PSII (Allakhverdiev et al. 2000). In green alga *Dunaliella tertiolecta*, the process of photosynthesis was inhibited by the addition of NaCl, KCl, sucrose or ethylene glycol that induced osmotic and ionic stresses (Gilmour et al. 1984). It might suggest that osmotic stress decreased the water level in the cytoplasm

through the efflux of water through water channels (aquaporins), with resultant increases in intracellular concentrations of ions and a decrease in photosynthetic activity (Allakhverdiev et al. 2000).

Effect of sorbitol stress on polyamine biosynthesis

Polyamine contents shown in Figure 3.15 was found in PCA-soluble form or free form as major form in *Synechocystis* cells. Moreover, spermidine content was a dominant component in all strains. The previous studies reported that free form of polyamines was normally detected in high amount, such as in cereal leaves (Flores and Galston 1984), chestnut and walnut (Rugini *et al.* 1993), and *Oryza sativa* (Roy and Ghosh 1996). Moreover, spermidine in many organisms showed highest amount among three types of polyamines, such as in tabacco (Slocum and Galston 1985) and *Lotus glaber* (Sanchez *et al.* 2005). In wild type strain, we found that the higher amount of polyamines was observed under higher sorbitol concentration, especially spermidine content. In the previous research, polyamines in oat leaves were increased under 400 mM sorbitol condition and 10% PEG 6000 condition (Flores and Galston 1984). The spermidine contents in *Lupinus luteus* hypocotyls and leaves were increased up to 50% and 70%, respectively, under sorbitol condition as compared with control. In addition, the polyamine supplementation to osmotically stressed oat leaves enhanced the protection to thylakoid membranes from degradation (Besford *et al.* 1993). In Δ*sll1077* strain, the tendency of polyamine accumulation was higher induced under 250 mM sorbitol condition whereas reduced under 500 and 700 mM sorbitol conditions. In our experiment, *Synechocystis* cells lacking of *sll1077* significantly decreased polyamine accumulation under osmotic stress. Moreover, it was interesting that Δ*sll0228* mutant completely inhibited polyamine accumulation in all conditions. It was consistent with the previous study in *Escherichia coli,* polyamine gene deficient strain (lacking of arginine decarboxylase, agmatinase and ornithine decarboxylase) in

Escherichia coli showed undetectable level of putrescine and spermidine (no spermine) in cells (Jung *et al.* 2003). The study of *Arabidopsis thaliana*, double mutated gene of spermine synthase gave no spermine content but did not affect to putrescine and spermidine contents (Imai *et al.* 2004). These results were indicated that *sll1077* and *sll0228* genes might involve in polyamine biosynthesis, as correspondingly predicted in Cyanobase.

Interestingly, norspermidine, the uncommon polyamine, which found in some organisms, was also observed in *Synechocystis* cells only under sorbitol stress. The previous studies showed that norspermine was widely observed in various species of algae included *Rhodophyta, Pyrrophyta, Chrysophyta, Phaeophyta, Euglenophyta, Chlorophyta*, and *Charophyta* (Hamana and Matsuzaki 1982). In this study, norspermidine was mostly presented in free form (PCA-soluble fraction). It was only detected under osmotic stress condition and increased its accumulation when increased the sorbitol concentrations in all strains (Figure 3.16). The recent studies showed the evidence suggesting that norspermidine was up-accumulated under sorbitol stress condition. In *Thermus thermophilus* was only found norspermidine under high temperature (Oshima 1983). In addition, norspermidine was detected in rice (*Oryza sativa*) during heat stress (Roy and Ghosh 1996). Moreover, we also noticed that nospermidine and polyamine contents accumulated in the similar levels about 2-4 nmol $\cdot 10^{-8}$ cells depending on conditions and strains. Therefore, it is possible whether the decrease of polyamine contents did not effect to cells growth because there was norspermidine existing in cells which may help cells to survive under sorbitol stress. Moreover, the polyamine degradation (Figure 1.6) which might be induced by sorbitol stress studied possibly give a precursor 1,3-diaminopropane which enables to produce norspermidine

Effect of sorbitol stress on the expression of gene involved in polyamine biosynthesis

The *sll1077* and *sll0228* gene expressions (Figure 3.17) showed the similar transcription patterns. Under 700 mM sorbiotol condition, there were induced expressions of these genes, except in Δ*sll0228* strain. The environmental stress affected on transcript levels of polyamine biosynthetic genes. It was previously reported that *Arabidopsis* gene expression of arginine decarboxylase and S-adenosyl-L-methionine decarboxylase was up-regulated under salt stress (Bagni *et al.* 2006). In tomato *Lycopersicon esculentum,* up-regulations of arginine decarboxylase and ornithine decarboxylase genes were observed under cold acclimation (Song *et al.* 2015), which in the similar pattern with the increase of rice S-adenosyl-L-methionine decarboxylase gene under chilling stress which up-accumulated spermidine levels to cold-resistance (Pillai and Akiyama 2004). These suggested that up-regulation of polyamine biosynthetic genes were involved in stress response and polyamine contents. Interestingly, the *Arabidopsis* mutant lacking of *ACL5* gene (spermine synthase) showed increased levels of *ACL5* transcripts compared with wild type which suggested as negative feedback mechanism for *ACL5* expression (Hanzawa *et al.* 2000).

The *sll0601* and *sll1640* genes were bioinformatically predicted as *N*-carbamoylputrescine amidohydrolase by Schriek *et al*. 2007 (Figure 3.18). Putrescine could be synthesized from agmatine via agmatine deiminase and *N*-carbamoylputrescine amidohydrolas. (Figure 1.5A). This pathway mostly found in higher plants and some bacteria, such as *Pseudomonas aeruginosa* (Nakada and Itoh 2003). Transcriptional level of genes demonstrated the similar pattern as those of both *sll1077* and *sll0228* genes which up-regulated under sorbitol condition in both wild type and Δ*sll1077* strains whereas down-regulated in Δ*sll0228* strain. In *Synechocystis* cells, it showed only bioinformatics information of gene related to *N*-carbamoylputrescine amidohydrolase but not to agmatine deiminase (Schriek *et al.*

2007). The expressions of these genes might suggest whether *Synechocystis* cells had another polyamines biosynthesis pathway via agmatinase and/or *N*-carbamoylputrescine amidohydrolase.

The transcript level of *sll0873* encoding carboxynorspermidine decarboxylase which producing norspermidine (Figure 3.19). It was up-regulated in all strains under sorbitol condition. The gene expression tendency was tightly related to the norspermidine content which also increased its accumulation under sorbitol condition (Figure 3.16). Up to date, there was few research studying the abiotic stress on norspermidine response. However, our result might offer the critical point on norspermidine function that associated with stress response in *Synechocystis*.

Effect of sorbitol stress on agmatinase activity

The agmatinase activity in all strains was shown in Figure 3.20. It was significantly increased under 700 mM sorbitol condition. In recent studies, enzymes related to polyamine biosynthesis were reported their relation to stress response. In tomato *Lycopersicon esculentum*, arginine decarboxylase activity was induced by cold stress and also found an increase of polyamine accumulation (Song *et al.* 2015). Moreover, sorbitol and NaCl stresses induced arginine decarboxylase activity in *Lupinus luteus* roots (Legocka and Kluk 2005). These results suggested that polyamine accumulation in *Synechocystis* cells was induced highly by sorbitol stress. In Δ*sll0228*, the agmatinase activity was undetectable in all condition. In addition, *Arabidopsis* mutant lacking of both arginine decarboxylase (*adc*1, *adc*2) still had its activity of about 18% compared with wild type strain (Watson *et al.* 1998). In this study, the loss of agmatinase activity in Δ*sll0228* strain was tightly correlated with loss of polyamine contents. Therefore, *sll0228* gene might potentially encode agmatinase enzyme in *Synechocystis* cells and also confirm that *Synechocystis* cells possesses main polyamine biosynthesis via arginine decarboxylase and agmatinase pathway.

The function of *sll1077* and *sll0228* genes in *Synechocystis* sp. PCC 6803

In this experiments, *Synechocystis* cells lacking of *sll0228* gene clearly showed the loss of agmatinase activity and polyamine content in Δ*sll0228* under all conditions. The *Synechocystis* cells lacking of *sll1077* gene showed a partial decrease of agmatinase activity and polyamine content. However, the lack of either *sll0228* or *sll1077* gene did not harm cell growth. This result would hint us that these two genes were not encode the key enzyme in polyamine biosynthesis. The alignment of *sll1077* and *sll0228* protein sequences showed 35.3% similarity and 20.7% identity (Figure 3.2). Based on our findings, we proposed that the *sll1077* possibly encoded another enzyme which could partial decrease the agmatinase activity when compared with Δ*sll0228* which complete loss of agmatinase activity. Both arginase and agmatinase belong to ureohydrolase-type enzymes which released urea as by-product. According to the same type of enzyme, it hardly identified the real function by only enzyme assay and bioinformatics approaches. Moreover, the previous research of *Lycopersicon esculentum* (tomato) showed that an arginase activity also had a very low agmatinase activity (Chen *et al.* 2004).

The phylogenetic tree was performed in Figure 3.1. Interestingly, both *sll1077* and *sll0228* was shown in the same clade with related to agmatinase. The *sll0228* showed similar to agmatinase in its cluster while *sll1077* had many arginase proteins in cluster, such as *Arabidopsis thaliana*, *Homo sapiens*, *Synechococcus* sp. JA-3-3Ab, *Synechococcus* sp. JA-2-3B'a(2-13) and *Rhodopseudomonas palustris* CGA009. *Schriek* and colleagues (2007) suggested that *sll1077* gene might be 4-guanidino butyrase based on the alignment of *sll1077* with 4-guanidino butyrase (GbuA) from *Pseudomonas putida* F1 which showed 25% identical, 20% similar, and 15% weakly similar amino acid residues. Although the gene encoding arginase was unknown and the recent studies showed neither lack of *sll0228* nor *sll1077* have arginase activity

(Quintero *et al.* 2000, Sekowska *et al.* 2000), it could be concluded in our study that *sll0228* has complete agmatinase activity while *sll1077* has partial agmatinase activity in *Synechocystis* cells.

CHAPTER V

CONCLUSION

The two mutant strains, both lacking of *sll1077* and *sll0228* genes, were used in this study to investigate the functional role of agmatinase, an enzyme in polyamine biosynthetic pathway, under sorbitol-induced osmotic stress. The growth and pigment contents showed no significance between wild type and mutant strains under osmotic stress, except in Δ*sll1077* strain which could maintain its growth and pigment contents along 14 days. The spermidine content was the dominant type of common polyamines among three strains. The spermidine content of Δ*sll1077* was less than that of wild type strain while Δ*sll0228* spermidine content was significantly decreased in all sorbitol conditions corresponding to the tendency results of agmatinase activity. The lacking of *sll1077* gene partially effected on both polyamine contents and agmatinase activity while a lacking of *sll0228* gene extremely decreased both polyamine contents and agmatinase activity in all conditions studied. It was reasonably indicated that *sll0228* gene had a tight correlation with agmatinase gene in *Synechocystis* sp. PCC 6803 rather than *sll1077* gene which was earlier predicted as a putative agmatinase in Cyanobase. Moreover, the transcript levels of both *sll1077* and *sll0228* were up-regulated under 700 mM sorbitol condition in both wild type and Δ*sll1077* whereas those levels in Δ*sll0228* were down-regulated. On the other hand, we also presented the new finding of norspermidine, an uncommon polyamines in *Synechocystis* sp. PCC 6803, which was induced significantly only by sorbitol-induced osmotic stress. Altogether, this study not only specified the agmatinase gene of *Synechocystis* sp. PCC 6803, but also discovered the sorbitol-induced norspermidine which might be involved to stress adaptation and tolerance.

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APPENDIX

APPENDIX A

- **BG_{11} medium** (Rippka *et al.* 1979)

Nutrients	Stock solution (1 L)	BG11 medium (1 L)	
		Liquid medium	Solid medium
KH_2PO_4	30 g	1 ml	1 ml
$MgSO_4 \cdot 7H_2O$	75 g	1 ml	1 ml
$CaCl_2 \cdot 2H_2O$	36 g	1 ml	1 ml
Na_2CO_3	20 g	1 ml	1 ml
Citric acid	6 g	1 ml	1 ml
Ferric ammonium citrate	6 g	1 ml	1 ml
EDTA	1 g	1 ml	1 ml
Trace element Solution	See below	1 ml	1 ml
$NaNO_3$	150 g	10 ml	10 ml
HEPES	238.3 g	10 ml	10 ml
30%$Na_2S_2O_3 \cdot 5H_2O$	300 g	-	10 ml
Bacto-agar	-	-	15 g
H_2O added up to	-	1000 ml	1000 ml

Adjust pH to approximately 7.5 (Initial pH is approximately 8.5)

***Trace element Solution (1 L)

H_3BO_3	2.86 g	$CuSO_4 \cdot 5H_2O$	0.079 g
$ZnSO_4 \cdot 7H_2O$	0.222 g	$Co(NO_3)_2 \cdot 6H_2O$	0.0494 g
$Na_2MoO_4 \cdot 2H_2O$	0.390 g		
$MnCl_2 \cdot 4H_2O$	1.81 g		

APPENDIX B

- **LB medium (Bertani 1951)**

Nutrients composition	Liquid medium	Solid medium
Bacto tryptone	10 g	10 g
NaCl	10 g	10 g
Yeast extract	5 g	5 g
Agar	-	15 g
dH_2O	1 L	1 L

All composition were dissolved together and bring volume up to 1 liter. Autoclave on liquid cycle for 20 min at 15 psi. Store at room temperature or +4°C.

APPENDIX C

- **TAE buffer**

  Stock solution (1 L)

50X:	Tris-base	240.0 g
	Glacial acetic acid	51.1 ml
	0.5 M EDTA, pH 8.0	100 ml
	Added distilled water to make 1 liter.	

- **TBE buffer**

  Stock solution (1 L)

5X:	Tris-base	54.0 g
	Boric acid	27.5 g
	0.5 M EDTA (pH 8.0)	20 ml
	Distilled water	800 ml

APPENDIX D

- **TfBI solution (500 ml)**

Potassium acetate	1.47 g
$MnCl_2$	4.95 g
$CaCl_2$	0.74 g
Glycerol	75 ml

Adjust pH 5.8 with 0.2 M acetic acid. Autoclave and store at 4°C

- **TfBII solution (100 ml)**

100 mM MOPS, pH 7.0	10 ml
$CaCl_2$	1.10 g
RbCl	0.12 g
Glycerol	15 ml

Autoclave and store at 4°C in the dark (i.e. wrap bottle in tinfoil)

APPENDIX E

Alkaline lysis reagents for plasmid extraction

- **Solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose and 10 mM EDTA)**

100 mM glucose	5 ml
1M Tris-HCl, pH 8.0	0.25 ml
0.5 M EDTA, pH 8.0	0.2 ml

  Added distilled water to make 10 ml.

- **Solution II (0.2 N NaOH and 1% SDS)**

0.4 N NaOH	12.5 ml
25% SDS	1 ml

  Added distilled water to make 25 ml.

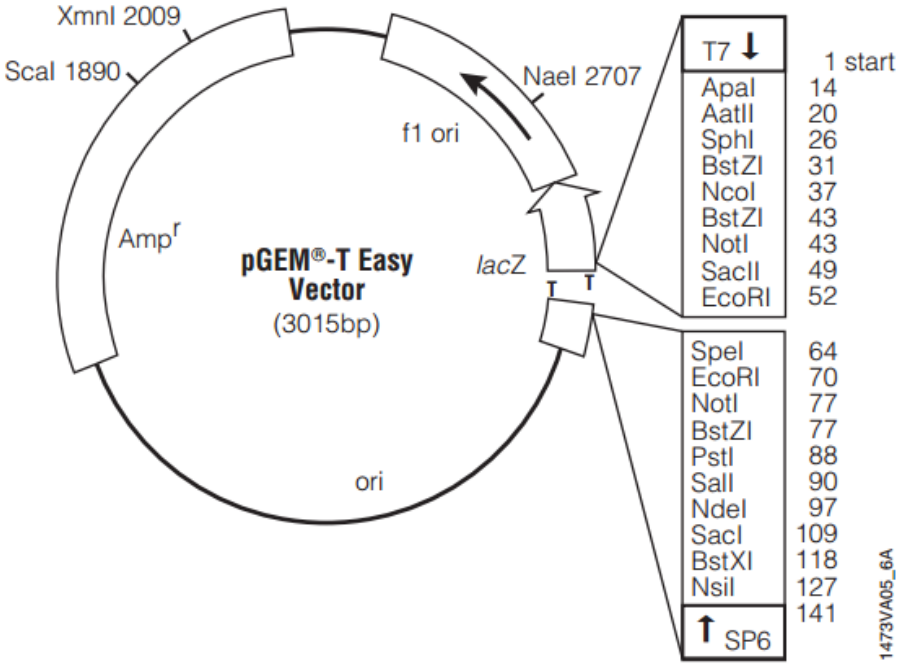
- **Solution III (3 M potassium acetate, pH 4.8)**

Potassium acetate	2.94 g in
Added glacial acetic acid	1.15 ml

  Added distilled water to make 10 ml.

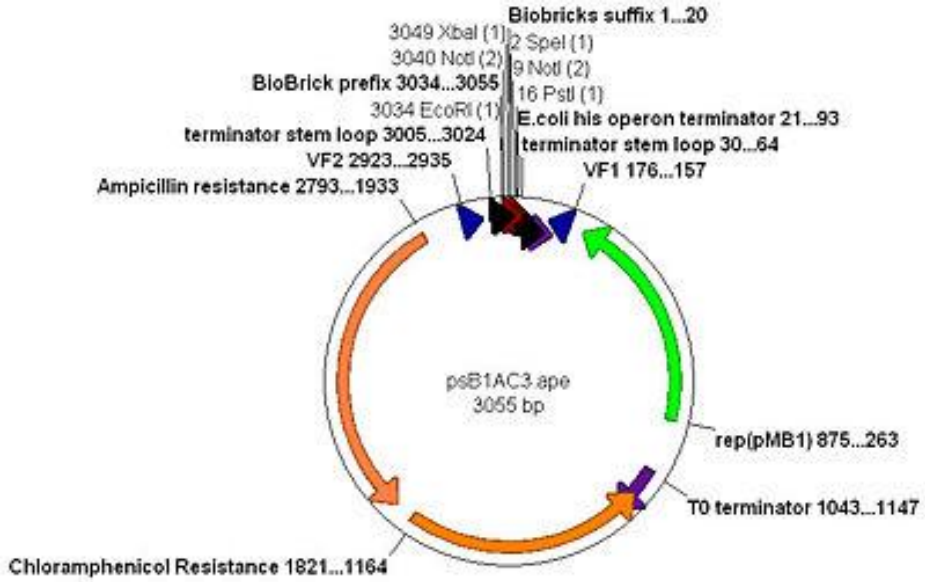
APPENDIX F

- pGEM® T-Easy vector



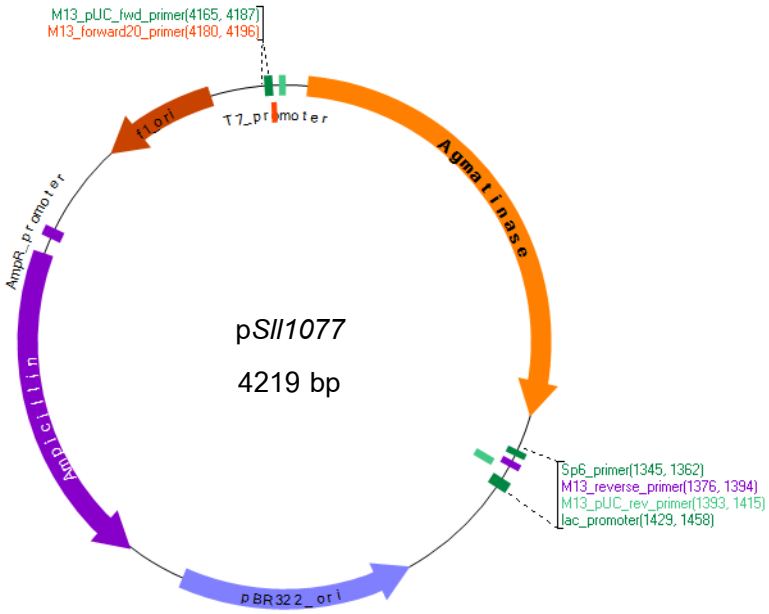
APPENDIX G

- psB1AC3



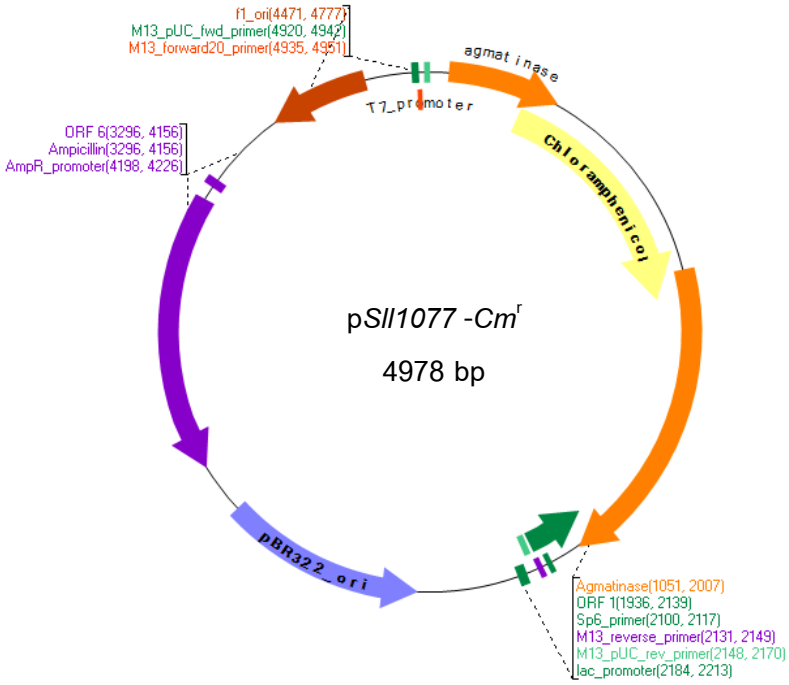
APPENDIX H

- p*Sll1077*



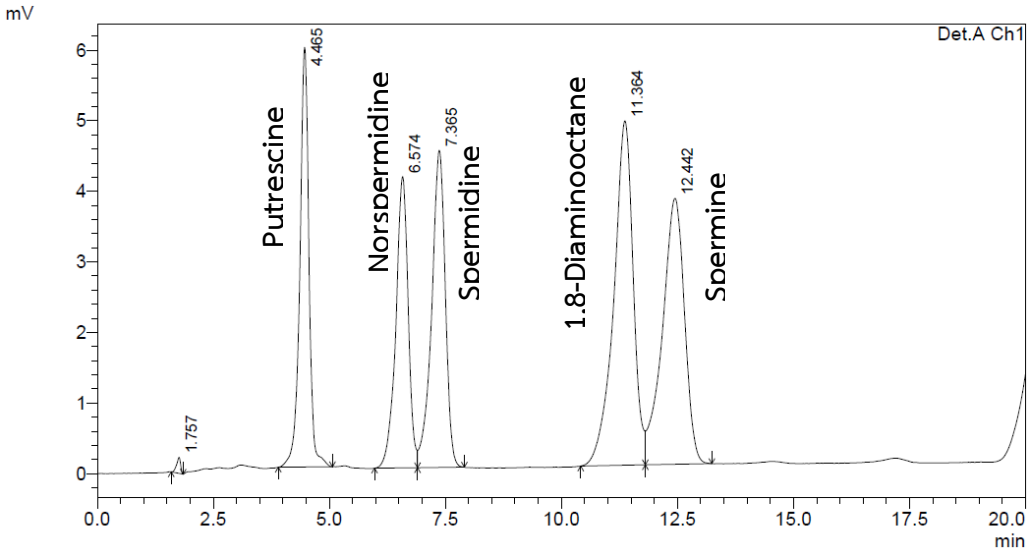
APPENDIX I

- p*Sll1077-Cm*^{r}



APPENDIX J

- Chromatogram of standard polyamines



VITA

Miss Supapid Eknikom was born in 1990 in Bangkok, Thailand. She was graduated with a Bachelor of Science degree in Biochemistry, Faculty of Science, Chulalongkorn University in 2012. She has studied for the Master degree of Science at the program in Biotechnology, Faculty of Science, Chulalongkorn University since 2012.

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