ลักษณะสมบัติของโปรตีนที่จับกับพอลิเอมีนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาว ปณัตดา ยอดแสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

CHARACTERIZATION OF A POLYAMINE-BINDING PROTEIN IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2010

Thesis Title	CHARACTERIZATION OF A POLYAMINE-BINDING PROTEIN IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803
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Field of Study	Biochemistry
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ปณัตดา ยอดแสง: ลักษณะสมบัติของโปรตีนที่จับกับพอลิเอมีนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (CHARACTERIZATION OF A POLYAMINE-BINDING PROTEIN IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร.อรัญ อินเจริญศักดิ์ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: Assoc. Prof. Pirkko Mäenpää, Ph.D., 173 หน้า

potD ของ *Synechocystis* sp. PCC 6803 ถอดรหัสได้ periplasmic binding protein ซึ่งจัดอยู่ในระบบขนส่ง ATP-binding cassette (ABC)-type ถูกโคลนและแสดงออกใน *Escherichia coli* rPotD มีน้ำหนักโมเลกุล 43 กิโลดาลตัน ศึกษาความสามารถของ PotD ต่อการจับกับพอลิเอมีน 3 ชนิด คือ พิวเทรสซีน สเปอร์มิดีน และสเปอร์มีน มีค่าคงที่การแตกตัว (K_d) เท่ากับ 13.2, 7.8 และ 8.3 ไมโครโมลาร์ ตามลำดับ และ จำนวนการเข้าจับ (B_{max}) เท่ากับ 0.74, 1.42 และ 0.13 โมลต่อโมล PotD ตามลำดับ แสดงให้เห็นว่า PotD มีความชอบต่อการจับกับสเปอร์มิดีนมากกว่าพอลิเอมีนชนิดอื่น pH ที่เหมาะสมต่อการจับกันระหว่าง PotD และพอลิเอมีน คือ pH 8 การจับของ PotD กับพอลิเอมีนถูกกระตุ้นด้วยโซเดียมคลอไรด์และซอร์บิทอลที่ความเข้มข้น 10 มิลลิโมลาร์ แต่เมื่อเพิ่มความเข้มข้นให้สูงกว่า 50 มิลลิโมลาร์ ความสามารถในการจับจะลดลง

เซลล์สายพันธุ์กลายที่ยีน *potD* ถูกทำลายโดยแทรกชิ้นดีเอนเอยาคานามัยซินไปกลางยีน ถูกนำมาใช้ศึกษาหน้าที่ของ PotDในเซลล์ *Synechocystis* ในสภาวะแสงปกติเซลล์สายพันธุ์แท้และเซลล์สายพันธุ์กลายเจริญเติบโตได้ดีเท่าๆ กันในอาหารที่มีพิวเทรสซีนและสเปอร์มิดีน 0.1-0.3 มิลลิโมลาร์ แต่เมื่อเพิ่มความเข้มข้นของพิวเทรสซีนและสเปอร์มิดีนเป็น 0.5 มิลลิโมลาร์ พบว่าการเจริญของเซลล์สายพันธุ์กลายถูกยับยั้ง ในขณะที่เซลล์สายพันธุ์แท้มีการเจริญลดลงเพียงเล็กน้อย เมื่อนำเซลล์ทั้งสองชนิดไปศึกษาการแสดงออกของโปรตีน PotD พบว่าโปรตีนมีการแสดงออกสูงขึ้นถึง 1.6 และ 2.8 เท่า ตามลำดับ นอกจากนี้โซเดียมคลอไรด์ความเข้มข้น 550 มิลลิโมลาร์ไม่มีผลในการยับยั้งการเจริญของเซลล์สายพันธุ์แท้และเซลล์สายพันธุ์กลาย ในขณะที่ซอร์บิทอลความเข้มข้น 300 มิลลิโมลาร์ ช่วยกระตุ้นการเจริญของเซลล์สายพันธุ์แท้แต่ยับยั้งการเจริญของเซลล์สายพันธุ์กลาย การนำสเปอร์มิดีนเข้าสู่เซลล์และการขับสเปอร์มิดีนออกจากเซลล์สายพันธุ์กลายลดลง 50 และ 60 เปอร์เซ็นต์ ตามลำดับ แต่ไม่มีความแตกต่างในการขนส่งพิวเทรสซีน สภาวะที่มีโซเดียมคลอไรด์และซอร์บิทอล 10 มิลลิโมลาร์ ช่วยกระตุ้นการขนส่งพิวเทรสซีนและสเปอร์มิดีนของเซลล์สายพันธุ์แท้และเซลล์สายพันธุ์กลาย การศึกษาผลของแสงต่อการเจริญของเซลล์สายพันธุ์แท้และเซลล์สายพันธุ์กลาย พบว่าแสงยับยั้งการเจริญของเซลล์สายพันธุ์แท้เพียงเล็กน้อย แต่มีผลยับยั้งได้ชัดเจนต่อเซลล์สายพันธุ์กลายตั้งแต่ 2 วันแรก นอกจากนี้แสงยังช่วยกระตุ้นการขนส่งพิวเทรสซีนและสเปอร์มิดีนเข้าสู่เซลล์ทั้งสองชนิด มากขึ้นถึง 3 และ 2.5 เท่า ตามลำดับ เมื่อตรวจสอบการทำงานของระบบการสังเคราะห์แสงและการทำงานของระบบแสงที่ 2 ในเซลล์สายพันธุ์แท้และเซลล์สายพันธุ์กลาย พบว่าพิวเทรสซีนและสเปอร์มิดีนช่วยกระตุ้นการทำงานของระบบในช่วงแรก แต่เมื่อเวลาเพิ่มขึ้นการทำงานของระบบการสังเคราะห์แสงจะลดลง นอกจากนี้แบบจำลองโครงสร้างของ PotD ยืนยันว่า PotD มีความชอบต่อสเปอร์มิดีน สเปอร์มีน และ พิวเทรสซีน ตามลำดับ รวมทั้งสารที่มีโครงสร้างคล้ายพอลิเอมีน เช่นกรดอะมิโนต่างๆ ไม่สามารถยับยั้งการจับของ PotD ต่อพอลิเอมีน แสดงว่า PotD จำเพาะกับพอลิเอมีนเท่านั้น

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....ปณัตดา ยอดแสง.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

#5073908323: MAJOR BIOCHEMISTRY
KEYWORDS: PHOTOSYNTHESIS SYSTEM/potD KNOCKOUT MUTANT/ POLYAMINE TRANSPOR/ *Synechocystis* sp. PCC 6803

PANUTDA YODSANG: CHARACTERIZATION OF A POLYAMINE-BINDING PROTEIN IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., CO-ADVISOR : ASSOC. PROF. PIRKKO MÄENPÄÄ, Ph.D., 173 pp.

Synechocystis sp. PCC 6803 PotD protein involved in polyamine transport was overexpressed in *Escherichia coli* as His-tagged PotD (rPotD). The purified rPotD showed saturable binding kinetics with radioactively-labeled polyamines. The rPotD exhibited a similar binding characteristic for three polyamines with a preference for spermidine. The K_d and maximum binding (B_{max}) values for putrescine, spermidine and spermine were 13.2, 7.8 and 8.3 μM, respectively and 0.74, 1.42 and 0.13 mol/mol rPotD, respectively. Optimum binding of rPotD with polyamines was at pH 8.0. Although the polyamine binding was increased by NaCl and sorbitol up to 10 mM, it was reduced when the concentration was higher than 50 mM. Competition experiments of polyamines analogs, such as amino acid, showed the specific binding of rPotD with polyamines.

The *in vivo* function of *Synechocystis* PotD concerning polyamine transport was characterized by using *Synechocystis* knockout mutant disrupted *potD* gene (*potD:Km*^{r}). The mutant cells showed similar growth in BG11 to that of *Synechocystis* wild-type, although it was more sensitive to high concentration of putrescine or spermidine up to 0.5 mM than wild-type cells. Growth of *Synechocystis* cells in the medium containing either 0.5 mM putrescine or spermidine led to an increase in PotD levels, about 1.6 and 2.8 fold, respectively. The presence of 550 mM NaCl in BG11 did not significantly affect the *Synechocystis* growth in both wild-type and mutant cells, while the presence of 300 mM sorbitol in BG11 resulted in the growth stimulation in wild-type and growth inhibition in mutant cells. The putrescine and spermidine uptakes were induced by either NaCl or sorbitol up to 10 mM. Interestingly, the reduction of spermidine uptake activity was observed by 50% in mutant cells, while putrescine uptake activity was not altered. Additionally, the spermidine excretion activity was decreased by 60% in mutant cells. High light showed an inhibitory effect on *Synechocystis* growth in both wild-type and mutant cells. These mutant cells were showed growth inhibition after the first two days. Besides, the high light enhanced the putrescine and spermidine uptake activity by 3 and 2.5 fold, respectively. Furthermore, the external polyamines stimulated both PSII activity and photosynthetic capacity within 30 min but decreased sequentially after one hour in both wild-type and mutant cells. Docking of these polyamines into the homology model of *Synechocystis* PotD showed that all three polyamines are able to interact with *Synechocystis* PotD. The binding modes of the docked putrescine and spermidine in *Synechocystis* are similar to those of PotF and PotD in *E. coli*, respectively. The overall results support the role of PotD in mediating polyamine transport in *Synechocystis* sp. PCC 6803.

Department :.....Biochemistry.....Student's Signature.....Panutda Yodsang.....
Field of Study :.....Biochemistry.....Advisor's Signature.....
Academic Year :.....2010.....Co-advisor's Signature .....

ACKNOWLEDGEMENTS

This study was carried out during the past four years at the Department of Biochemistry, Faculty of Science, Chulalongkorn University and Department of Plant Physiology and Molecular Biology, University of Turku, Finland. Financial support from Office of the Higher Education Commission, Thailand for supporting by grant fund under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree for this research, the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Academy of Finland for CIMO scolarship are gratefully acknowledged.

I wish to express my deepest gratitude to my supervisors, Professor Dr. Aran Incharoensakdi and Associate Professor Dr. Pirkko Mäenpää, for their excellent guidance, enthusiasm, instruction and support from the very beginning to the very end of my Ph.D. thesis. Especially I thank Professor Dr. Aran for endless patience and advice, and Associate Professor Dr. Pirkko for introducing me to the fascinating world of molecular biology and for her warm hospitality.

My gratitude is also extended to Professor Dr. Piamsook Pongsawasdi, Associate Professor Dr. Jarunya Narangajavana, Associate Professor Dr. Teerapong Buaboocha, and Dr. Saowarath Jantaro for serving as thesis committee, for their available comments and useful suggestions.

My appreciation is also expressed to Professor Dr. Eva-Mari Aro, Professor Dr. Eevi Rintamäki, Dr. Paula Mulo, and Dr. Wuttinun Raksajit for warm support, instruction, lending helping, and interesting discussion.

Sincere thanks are also extended to all members and friends, for lending a helping hand whenever needed, sharing the great time in laboratory, their warm assistance and friendship, and at Biocity Turku Finland; Markus, Maija, Dalton, Eevi, Sumathi, Hunnu, Pengpeng, Sari, Ulla-Maija, Virpi, Minna, Nina, Saijaliisa, Kurt, Mika, Arto, Esa, Taina, Marjatta, and at Department of Biochemistry Chulalongkorn University; Pond, P'Ann, N'Ann, all members of cyanobacteria group, cyclodextrin group, P'Bum, Paman, Pui.

Last, but not least, I wish to thank my family, especially my mom, for understanding and keeping my feet tightly on the ground and my mind away from science when I was at home. It's always good to be around by family.

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ADC	arginine decarboxylase
bp	base pair
BSA	bovine serum albumin
^{o}C	degree Celsius
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
Chl *a*	Chlorophyll *a*
Ci	Curie
EDTA	ethylenediamine tetraacetic acid
HEPES	hydroxyethyl poperazineethanesulfonic acid
mM	milimolar
min	minute
µg	microgram
OD	Optical density
PCR	Polymerase Chain Reaction
PotD	Periplasmic polyamine-binding protein D
TBS	Tris Base buffer Saline
TTBS	Tris Base buffer Saline with Tween-20

CHAPTER I

INTRODUCTION

1.1 Polyamines

Polyamines are major polycationic compounds ubiquitous in all living organisms; prokaryotes, eukaryotes, parasites, plants, animals including human (Bagni and Tassoni, 2001; Berger *et al.,* 1984; Bouchereau *et al.,* 1999; Hosoya *et al.* 2005; Igarashi and Kashiwagi, 2006, Regueraa *et al.* 2005, Tabor and Tabor, 1985). The most common polyamines are triamine spermidine (1,8-diamino-4-azaoctane), tetramine spermine (1,12-diamino-4,9-diazadodecane) and diamine precursor putrescine (1,4-diaminobutane) (Figure 1). Table 1 exhibits other di- and polyamines presented in plants and microorganisms, for instance, the diamines 1,3-diaminopropane and cadaverine (1,5-diaminopentane). Unnatural polyamines have also been detected in bacteria, algae, fungi, animals and higher plants (Niitsu and Samejima, 1993). In the extreme thermophilic bacteria *Thermus thermophilus* at least 14 polyamines, among which some linear and branched pentamines, hexamines and heptamines, have been isolated (Table 1). Caldopentamine was presented in considerable amount especially in bacterial cell grown at extremely high temperatures (Oshima, 1989). Polyamines are protonated at normal cellular pH; therefore, their biological function is the binding to anionic molecules such as DNA, RNA, acidic phospholipids and various types of proteins as well as enzymes whose activity are directly regulated by polyamines (Carley *et al.,* 1983; Cohen, 1998; Feurstein and Marton, 1989; Tadolini *et al.,* 1984). Hence, polyamines have the ability to stabilize macromolecular structures, polyamines act as regulatory molecules in many fundamental cellular processes including cell division, differentiation and proliferation, cell death, DNA and protein synthesis and gene expression (Alcazar *et al.,* 2006; Childs

et al., 2003; Kusano *et al.*, 2008; Igarashi and kashiwagi, 2000; Seiler and Raul, 2005). In plants, Polyamines have been implicated in many physiological processes, for example, organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and ripening, and abiotic and biotic plant stress responses (Alcazar *et al.*, 2006; Bagni and Tassoni, 2001; Bouchereau *et al.*, 1999; Galston and Kaur-Sawhney, 1990; Kumar *et al.*, 1997; Kusano *et al.*, 2008; Malmberg *et al.*, 1998; Walden *et al.*, 1997). Polyamines are able to inhibit senescence by preventing chlorophyll, protein, RNA breakdown in leaves and by increasing macromolecular synthesis and mitotic activity in protoplasts (Kaur-Sawhney *et al.*, 1982a and 1982b). In *Escherichia coli*, polyamines play an important role to protect cells from the toxic effects of oxygen (Chattopadhyay *et al.*, 2003).

Polyamines accumulated excessively within the cells, either due to very high extracellular amount or to deregulation of the systems controlling polyamine homeostasis, they induce the toxic effects. For example, polyamine accumulation can lead to inhibition of cellular growth (He *et al.*, 1993; Pegg, 1986 and 1988; Tabor and Tabor, 1985). The addition of spermidine to cell cultures of mouse FM3A cells results in a decrease in cell growth accompanied by inhibition of protein synthesis (He *et al.*, 1993). High intracellular spermine contents increase its oxidative products inducing DNA damage or cell death (Agostinelli *et al.*, 2004; Averill-Bates *et al.*, 2005). Furthermore, the excess spermidine causes decreased protein synthesis and cell viability during the stationary phase of growth in *E. coli* (Fukuchi *et al.*, 1995). Consequently, the regulation of polyamine level must be a very fast, sensitive and precise manner. There are four different polyamines reulation strategies consisting of *de novo* biosynthesis, degradation, conjugation and transport (Bouchereau *et al.*, 1999; Urdiales *et al.*, 2001).

1.1.1 Polyamines biosynthesis

The pathways of polyamines biosynthesis have been established in many organisms (Bagni and Tassoni, 2001; Cohen, 1998; Wallace *et al.*, 2003). An overview of the general pathway is given in Figure 2. The synthesis essentially starts from two amino acid molecules, L-arginine and L-methionine. In higher plants and bacteria, the biosynthesis can be initiated directly by the decarboxylation of either ornithine or arginine, catalyzed by the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17), which gives rise to putrescine directly and arginine decarboxyase (ADC; EC 4.1.1.19) to yield agmatine, which is subsequently converted to putrescine (Smith, 1985; Slocum, 1991). In mammals and fungi, ODC reaction only leads to putrescine formation. Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group on to putrescine and spermidine, respectively. This step is catalyzed by the enzymes spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively. An aminopropyl group is transferred from the decarboxylated *S*-adenosylmethionine, which is synthesized from methionine in two sequential reactions of methionine adenosyltransferase (EC 2.5.1.6) and *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), respectively. (Bey *et al*., 1987; Kushad and Dumbroff, 1991) (Figure 2). A variety of other related compounds have been found in plants, including cadaverine (caddiamine), which derived from lysine via a reaction of lysine decarboxylase (LDC, EC 4.1.1.18). This diamine is found in *Leguminoseae* and in the flower of *Arum lilies* (Slocum, 1991). Interestingly, the model plant *Arabidopsis thaliana* had no gene coding for ornithine decarboxylase (ODC) (Hanfrey *et al.*, 2001). All other polyamine biosynthesis genes have been assigned in *A. thaliana* which consisting of two genes (*ADC1* and *ADC2*) for arginine decarboxylase (Watson and Malmberg, 1996; Watson *et al.*, 1997).

1.1.2 Polyamine conjugation

In nature, polyamines often occur as free molecular bases, but they can also be associated with small molecules like phenolic acids, called conjugated forms (Bagni and Tassoni, 2001) and also to various macromolecules like proteins, called bound forms (Martin-Tanguy, 1997). The most common amine conjugates, i.e. polyamines and aromatic amine conjugates, covalently linked to hydroxycinnamic acids have also been shown to occur at high levels in plants (Martin-Tanguy, 1985) and are thought to be correlated with developmental phenomena. They occur as water-soluble or as water-insoluble forms. In the former, the single amine group of an aliphatic amine is linked with a phenolic cinnamic acid. The water-insoluble forms can be divided into two classes. In the first, each terminal amine group of an aliphatic amine is bound to cinnamic acid, while in the second class the amine group of an aromatic amine is linked to cinnamic acid (Figure 3). These amine conjugates are found in roots but they do not normally exist in shoots. In the root of tobacco, increases in water-soluble and water-insoluble conjugates have been shown before flowering (Martin-Tanguy *et al.,* 1990) and occur in shoot apices upon floral initiation (Havelange *et al.,* 1996). The synthesis of polyamine conjugates has been elucidated in tobacco callus (Negrel, 1989). They are conjugated by the formation of an amide linkage, utilizing ester of Co-A for the provision of the activated carboxyl groups such as cinnamoylputrescine (Martin-Tanguy *et al.,* 1985), which are formed by non-specific putrescine caffeyl-CoA transferase. Therefore, the synthesis of the water-insoluble polyamine such as di-*p*-coumaroylputrescine, di-*p*-coumaroylspermidine, di-feruloylputrescine and di-feruloylspermidine has not been elucidated (Martin-Tanguy, 1997). Furthermore, posttranslational covalent linkage of polyamines to protein is catalyzed by a class of enzymes known as transglutaminases (EC 2.3.2.13) (Margosiak *et al.,* 1990).

1.1.3 Polyamine degradation

The major pathways of polyamine degradation are depicted in Figure 3. Polyamines are oxidatively deaminated by the action of amine oxidases, including copper diamine oxidases (DAO; EC 1.4.3.6) and flavoprotein polyamine oxidases (PAO; EC 1.5.3.3). PAO catalyzes oxidation of secondary amino groups of spermidine and spermine, but not other polyamines. With the formation of 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, along with the production of 1,3-diaminopropane and H_2O_2 (Cona *et al.*, 2006; Federico *et al.*, 1990; Śebela *et al.*, 2001). Spermine oxidase (SMO), a FAD-dependent amine oxidase that directs the back-conversion of spermine to spermidine with accompanying production of 3-aminopropanal and H_2O_2 (Wang *et al.*, 2001). DAO catalyzes the oxidation of primary amino groups in many biogenic amines, including mono-, di-, and polyamines. Putrescine and cadavarine is most preferable substrate of DAO producing 4-aminobutanal with concomitant production of NH_3 and H_2O_2. Diaminopropane can be converted into β-alanine, whereas pyrroline derived from 4-aminobutanal can be further catabolized to γ-aminobutyric acid (GABA) (Hausman *et al.*, 1997) in a reaction catalysed by pyrroline dehydrogenase (PDH) (Flores and Filner, 1985). The GABA is subsequently transaminated and oxidized to succinate, which is incorporated into the Krebs cycle. Thus, this pathway ensures the recycling of carbon and nitrogen from putrescine. On the other hand, the products of this catalytic process pathway including γ-aminobutyric acid (GABA), hydrogen-peroxide ammonia and aminoaldehydes produced during the degradation of polyamines also have biological significance as they trigger programmed cell death or apoptosis in certain cell types. Furthermore, decrease in polyamine levels due to catabolism may also be involved in

regulating polyamines and driving cells to the apoptotic pathway (Seiler and Raul, 2005; Thomas and Thomas, 2001).

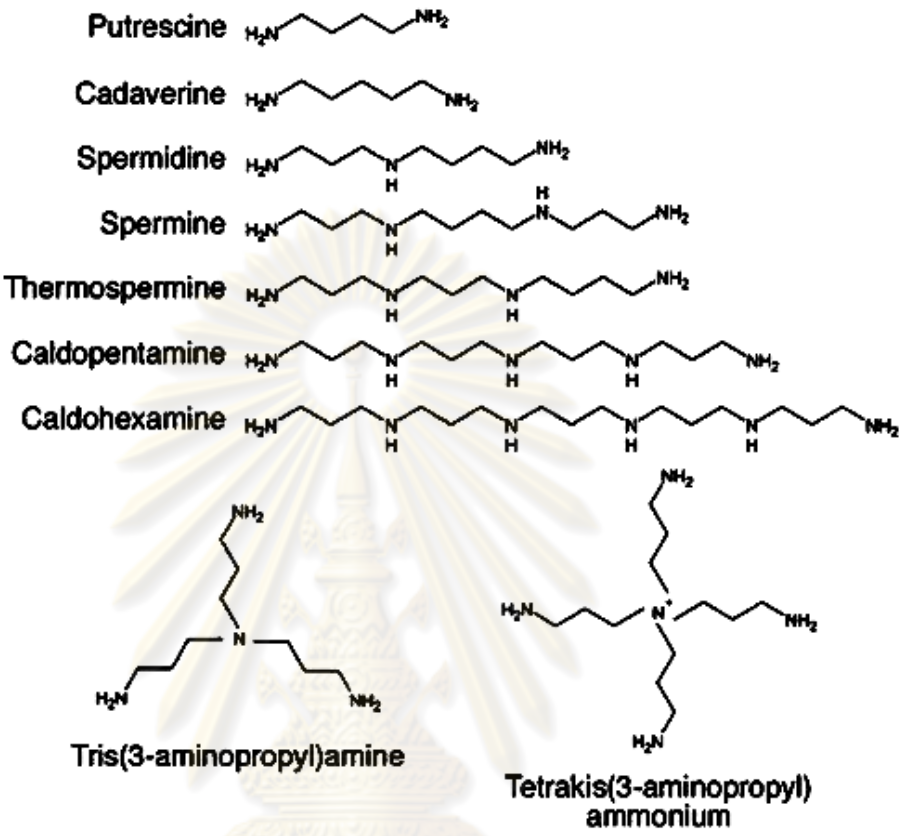


Figure 1 Structures of the common and unique polyamines. (Cohen, 1998 and Oshima, 2007)

Table 1 Common and uncommon natural occurring aliphatic polyamines*.

Trivial name	Systematic name	Chemical structure
1,3-Diaminopropane	1,3-Diaminopropane	$NH_2(CH_2)_3NH_2$
Putrescine	1,4-Diaminobutane	$NH_2(CH_2)_4NH_2$
Cadaverine	1,5-diaminopentane	$NH_2(CH_2)_5NH_2$
Norspermidine (caldine)	1,7-Diamino-4-azaheptane	$NH_2(CH_2)_3NH(CH_2)_3NH_2$
Spermidine	1,8-Diamino-4-azaoctane	$NH_2(CH_2)_3NH(CH_2)_4NH_2$
sym-Homospermidine	1,9-Diamino-5-azanonane	$NH_2(CH_2)_4NH(CH_2)_4NH_2$
Thermine	1,11-Diamino-4,8-diazaundecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Spermine	1,12-Diamino-4,9-diazadodecane	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Thermospermine	1,12-Diamino-4,8-diazadodecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
Homospermine	1,13-Diamino-4,9-diazatridecane	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_4N\ H_2$
Caldopentamine	1,15-Diamino-4,8,12-triazapentadecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH2)_3$ $NH(CH_2)_3NH_2$
Homocaldopentamine	1,16-Diamino-4,8,12-triazahexadecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3$
Homopentamine	1,19-Diamino-5,10,15-triazanonadecane	$NH(CH_2)_4NH_2$ $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_4$
Caldohexamine	1,19-Diamino-4,8,12,16-tetraazanonadecane	$NH(CH_2)_4NH_2$ $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3$
Homocaldohexamine	1,20-Diamino-4,8,12,16-tetraazaeicosane	$NH(CH_2)_3\ NH(CH_2)_3NH_2$ $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3$ $NH(CH_2)_3\ NH(CH_2)_4NH_2$
N^4-Aminopropyl-norspermidine		$[NH_2(CH_2)_3]_3N$
N^4-Aminopropyl-spermidine		$[NH_2(CH_2)_3]_2N[NH_2(CH_2)_4]$

*(Bagni and Tassoni, 2001)

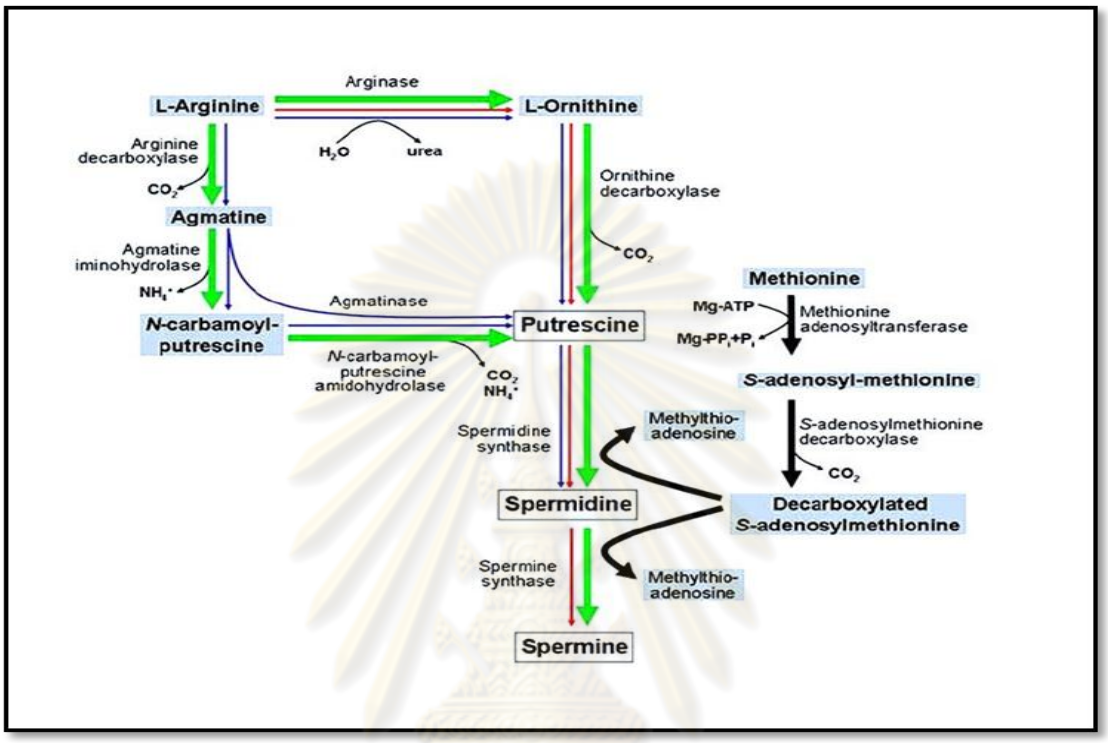


Figure 2 Pathways of polyamine metabolism (Kusano *et al.*, 2008). Plant pathways are indicated by green bold arrows. Blue and red arrows indicate bacterial and animal pathways, respectively.

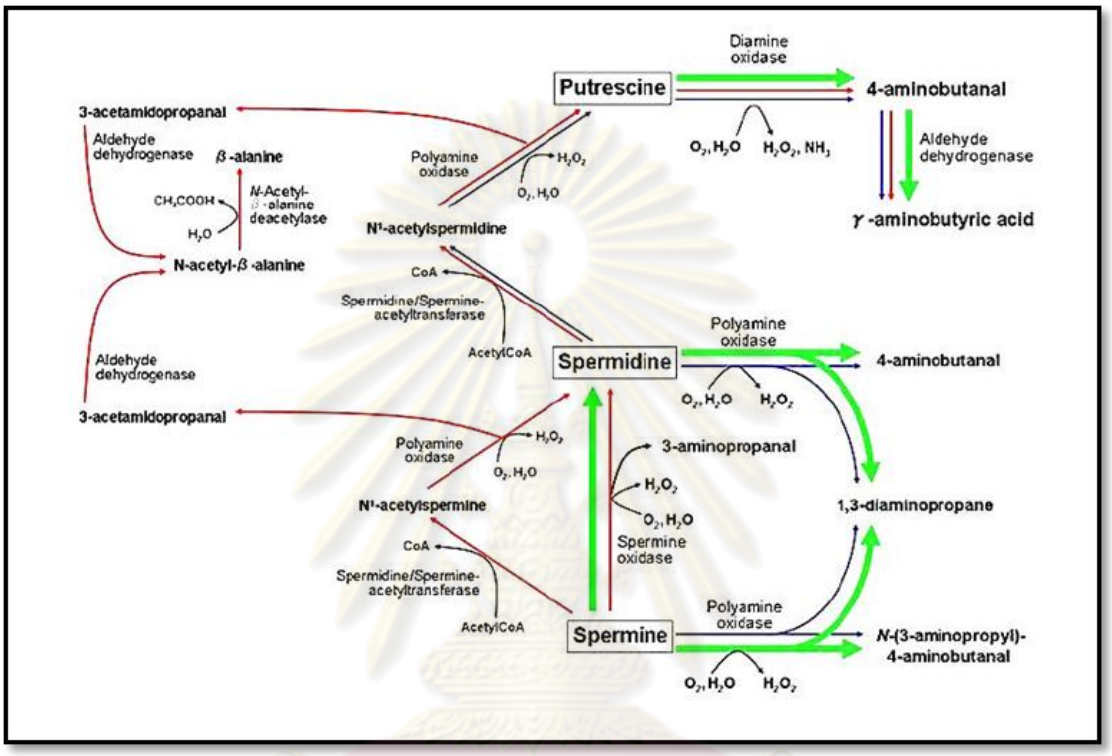


Figure 3 Pathways of polyamine degradation (Kusano *et al.*, 2008). Plant pathways are indicated by green bold arrows. Blue and red arrows indicate bacterial and animal pathways, respectively.

1.1.4 Polyamine transport

Polyamines are protonated molecules carrying positive charges on each nitrogen atom at physiological or cellular pH. Many authors have compared these molecules to Ca^{2+} and Mg^{2+}, but they differ from such cations in the homogeneous distribution of the charge along the carbon chain (Seiler *et al.,* 1996; Wallace *et al.,* 2003). For that reason, many researchers in the last decade have focused their efforts on the quest for polyamine carriers. Polyamine-specific carriers are widely distributed in prokaryotes and eukaryotes and can replenish polyamine pools upon inhibition of the biosynthetic enzymes (Seiler *et al.,* 1996). The transport of polyamines classified as ABC [ATP binding cassette]-type transporters (Higgins, 1992) have been well characterized in bacteria, yeast, parasites and animals (Igarashi and Kashiwagi, 1999; Rinehart and Chen, 1984 Tassoni *et al.,* 2002).

1.1.4.1 Polyamine transport in bacteria

The genes encoding polyamine transporter subunits have been identified in *E. coli*. This transporter is a saturable, carrier-mediated and energy-dependent process. The genes encoded the transport proteins for putrescine, spermidine and cadaverine were identified and clarified the properties (Kashiwagi *et al.,* 1990) (Figure 4). There are two polyamine uptake systems which belong to ATP binding cassette (ABC) transporters. Those are the spermidine-preferential uptake system consisting of PotA (ATPase), PotB and PotC (channel-forming proteins), and PotD (a substrate binding protein), and putrescine-specific uptake system consisting of PotF (a substrate binding protein), PotG (ATPase), and PotH and PotI (channel-forming proteins). Spermidine uptake was not observed in *E. coli* in which any one of the four proteins was disrupted. However, transformation of an intact gene corresponding to the one disrupted restored spermidine uptake activity. Thus, all four proteins are in dispensable for spermidine uptake (Furuchi

et al., 1991). Two other polyamine transporters belong to non-ABC type transporter, comprising single proteins each with twelve transmembrane segments, are PotE and CadB, which function as antiporters of putrescine/ornithine and cadaverine/lysine (Kashiwagi *et al.,* 1991; Kashiwagi *et al.,* 1997; Kashiwagi *et al.,* 2000; Meng and Bennett, 1992; Soksawatmaekhin *et al.,* 2004 and 2006; Watson *et al.,* 1992). PotE and CadB catalyzed both the uptake at neutral and excretion at acidic pH of putrescine and cadaverine, respectively. These two proteins are important for cell growth at acidic pH because they can help neutralize the pH in the medium and make membrane potential for nutrient uptake (Neely and Olson, 1996; Soksawatmaekhin *et al.,* 2004). The spermidine excretion protein (MdtJI) consisted of four transmembrane segments was recently identified (Higashi *et al.,* 2008). The level of *mdtJI* mRNA was increased by spermidine stimulation/induction. Polyamine content in *E. coli* is regulated by feedback inhibition of biosynthetic enzymes and polyamine uptake systems by polyamines themselves, and there is no antizyme or equivalent system known existing in *E. coli* (Kashiwagi *et al.,* 2002a; Tabor and Tabor, 1969). To date, a new putrescine transporter PuuP was identified as one of the proteins which are involved in the putrescine utilization pathway in *E. coli* K12 (Kurihara *et al.,* 2005 and 2009).

1.1.4.2 Polyamine transport in yeast

Polyamine uptake in *Saccharomyces cerevisiae* is energy-dependent (Figure 5). Two polyamine-preferential uptake proteins, DUR3 and SAM3, consist of 12 or 16 transmembrane segments. DUR3 catalyzed the uptake of polyamine together with urea, whereas, SAM3 was found to catalyze the uptake of polyamines together with *S*-adenosylmethionine, glutamic acid, and lysine (Uemura *et al.,* 2007). The K_m values for putrescine and spermidine of DUR3 were 479 and 21.2 μM, respectively, and those of

SAM3 were 433 and 20.7 μM, respectively (Uemura *et al.*, 2007). Cell growth of a polyamine-requiring mutant was completely inhibited by disrupting both DUR3 and SAM3. The results indicated that DUR3 and SAM3 are major polyamine uptake proteins in yeast (Uemura *et al.*, 2007). Moreover, exogenous polyamines were also taken up by amino acids transporter, GAP1 and APG2 (Aouida *et al.*, 2005; Uemura *et al.*, 2005) and transporter of 4-aminobutyric acid on the vacuolar membrane, UGA4 (Uemura *et al.*, 2004). Besides, four genes encoding polyamine transport proteins, TPO1 through TPO4, were found in *S. cerevisiae* (Tomitori *et al.*, 1999 and 2001). TPO1 and TPO4 showed their recognition on putrescine, spermidine and spermine whereas TPO2 and TPO3 recognized specifically to spermine only. Moreover, TPO1 function served as a pH dependent-polyamine transporter. It is interesting that polyamines uptake showed appropriate condition at pH 8.0 whereas, polyamines excretion effectively occurred at pH 5.0. These might be dued to yeast cells usually grown at acidic pH, which suitable for TPO1-4 function on polyamines excretion as similar as PotE and CadB. Additionally, TPO5 which is a polyamine preferential excretion protein was recently found in *S. cerevisiae* (Tachihara *et al.*, 2005). The addition of external putrescine increased the level of *TPO5* mRNA in *S. cerevisiae*, in agree with that of *MdtJI* in *E. coli*.

1.1.4.3 Polyamine transport in parasite

The scientific evidence of an influx system for polyamines in parasites has been reported, for instant on *leishmanial* cells, *L. infantum* promastigotes (Balaña-Fouce *et al.*, 1989; Kaur *et al.*, 1986; Reguera *et al.*, 2005). This organism is able to uptake putrescine by a saturable, concentration- and energy-dependent, specific carrier but not for higher polyamines, spermidine and spermine, other analogous polyamine such as MGBG, or basic amino acids. These results were conclusively confirmed in *Leishmania donovani*, the etiological agent of visceral leishmaniasis, in which a second polyamine

carrier was described, in this case for spermidine (Kandpal and Tekwani, 1997). The influx of this latter polyamine was found to be over seven times more effective than the uptake of putrescine, a diamine found not to share this mechanism, unlike spermine, which was observed to significantly inhibit spermidine uptake. Spermine, in turn, was completely not taken up by the putrescine transport system, but only moderately by the spermidine carrier. These findings were also reported in *Trypanosoma cruzi* (González *et al.,* 1992), *Leishmania mexicana* promastigotes (González and Algranati, 1994) and *Crithidia fasciculata* parasites (Calonge *et al.,* 1996a). In these all cases, putrescine uptake recognition was specific and only slightly inhibited by spermidine, spermine and a number of variable length diamines. A common feature in all polyamine transporters of parasites is their proton motive force dependence to take up the substrate. The fact that sodium ionophores, uncoupling agents and sulfhydryl group reagents, were found to inhibit polyamine influx significantly in all parasites studied suggesting that the mechanism involved is coupled to a sodium electrochemical gradient (Balaña-Fouce *et al.,* 1989; Kandpal and Tekwani, 1997). Recently, the molecular characterization of polyamine transporters in protozoa is also a field open to research. None of genes with this putative function has been identified before in any of the major genome databases. When functionally expressed in *Xenopus laevis* oocytes, the polyamine transporter protein showed a high affinity for putrescine and spermidine (Enkvetchakul *et al.,* 2003).

1.1.4.4 Polyamine transport in mammalian cells

Polyamine transport mechanisms have been recently studied in mammalian cells (Hoshino *et al.,* 2005). The mammalian polyamine transport activity is also acutely controlled by cell cycle events and hormonal stimulation (Lessard *et al.,* 1995). Two polyamine transport systems have been proposed in mammalian cells. The first model

proposed a role for the heparin sulphate side chains of recycling glypican 1 (GPC1) in the transport of spermine, and assumed that GPC1 recycling was a basis of polyamine transport (Belting *et al.*, 2003). In the second model mechanism, polyamines were transported into cells through unidentified membrane transporters/carriers driven by a membrane potential. The polyamines then were sequestered into vesicles by proton exchange over a pH gradient built by a vacuolar ATPase (Casero and Marton, 2007; Soulet *et al.*, 2004). In addition to these suggested transport mechanisms that also act as control points for the adjustment of intracellular polyamine levels, two additional mammalian mechanisms have also been proposed (Wallace *et al.*, 2003). The polyamine uptake into rat liver mitochondria is mediated by specific polyamine uniporter which is dependent on membrane potential and insensitive to medium pH (Tominello *et al.*, 1992). Uptake and excretion are catalyzed by one and the same polyamine transporter (Sakata *et al.*, 2000). A similar reversal of roles has also been observed in *E. coli* PotE, which catalyses both the uptake and excretion of putrescine (Kashiwagi *et al.*, 1997). In animal cells, excess polyamines are metabolized to acetyl derivatives, which are better substrates for excretion than the polyamines themselves, although polyamines are better substrates for uptake than acetylpolyamines (Seiler, 1987).

1.1.4.5 Polyamine transport in cyanobacteria

Polyamine transport in cyanobacteria has been studied in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). *Synechocystis* is able to regulate the polyamine uptake, putrescine and spermidine into its cells (Raksajit *et al.*, 2006 and 2009). The optimum activity of putrescine uptake was occured at pH 7.0 whereas that of the spermidine uptake was at pH 8.0. The increase of the external osmolality generated by either NaCl or sorbitol at 10 - 20 mOsmol/kg upshifts caused an increase of putrescine

and spermidine uptake with 1.5 - 2.0 fold (Raksajit *et al.,* 2006 and 2009). This suggests that cells required polyamines to better thrive against osmotic upshift. The genome of *Synechocystis* contains *potD* gene (*slr0401*) that encodes a periplasmic substrate-binding protein (PotD-like protein) of the polyamine transport system (Kaneko *et al.,* 1996; Igarashi and Kashiwaki, 1999). The sequence identity between *E. coli* PotD and *Synechocystis* PotD is 24%, but the residues from active site are more conserved and share a sequence identity of 38% (Brandt *et al.,* 2010). The sequence identity of *Synechocystis* PotD to *E. coli* PotF (19%) and to TpPotD (18%) is considerably lower than to *E. coli* PotD (Brandt *et al.,* 2010). However, the function of PotD on polyamine transport remains unclear.

Altogether, the importance of polyamine uptake in living cells is far from clear, since all organisms have enzymes able to synthesize polyamines and maintain optimum levels for their metabolism. Most of recent studies on the polyamine uptake were carried out deeply in *E. coli* and yeast (Igarashi and Kashiwaki, 1999) and some from our research group focusing on cyanobacteria. Moreover, one of the useful tools on Molecular Biology study is the genome sequence database. Accordingly, genes for several kinds of putative polyamine transports have been described mostly in living organism as show in Table 2.

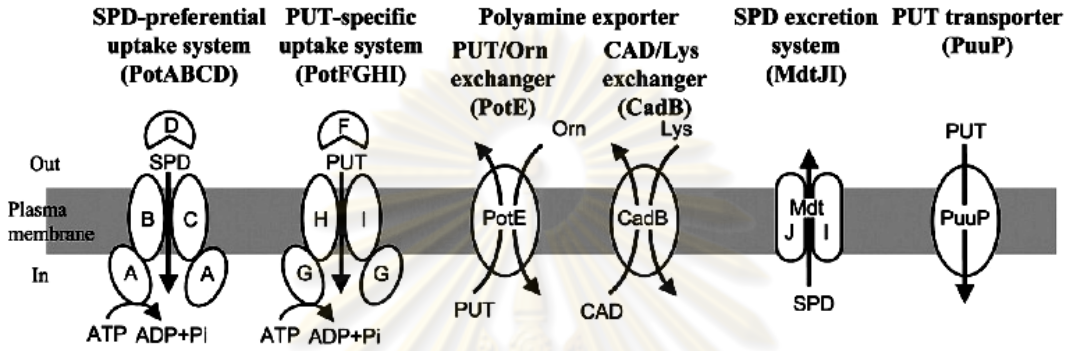


Figure 4 Polyamine transport systems in *Escherichia coli*. ATPase (A, PotA and G, PotG), the substrate-binding proteins (D, PotD and F, PotF), channel-forming protein (B, PotB; C, PotC; H, PotH and I, PotI) SPD, spermidine; PUT, putrescine; CAD, cadaverine (Igarashi and Kashiwagi, 2010).

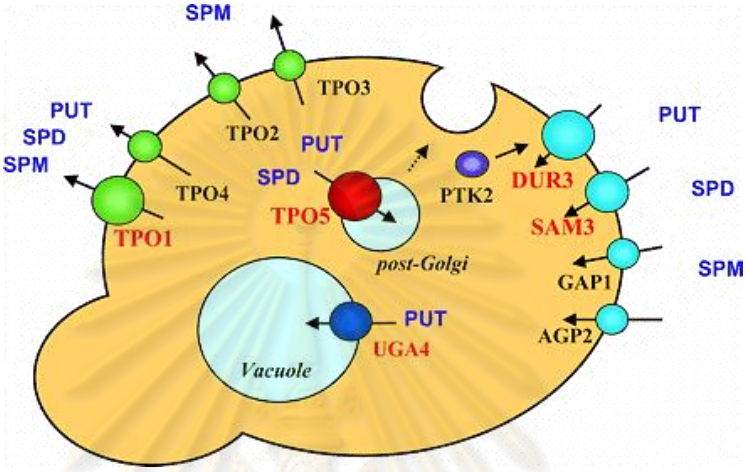


Figure 5 Polyamine transport in *Saccharomyces cerevisiae*. Polyamine uptake is mainly catalyzed by DUR3 and SAM3. Excretion of polyamines is mainly catalyzed by TPO1 and TPO5, located on either the plasma membrane or post-Golgi secretory vesicles. Putrescine uptake into vacuole is catalyzed by UGA4 (Igarashi and Kashiwagi, 2010).

Table 2 Genes related to and involved in polyamine transport*****

Bacterium or archaeon	Size of chromosome (kbp)	Number of genes annotated	Genes suggested to be linked to polyamine transport
Archaea			
Archaeoglobus fulgidus	2178	2407	*potABC*
Bacteria			
Bacillus subtilis	4215	4100	*blt*
Borrelia burgdorferi	911	850	*potABCD*
Escherichia coli	4639	4289	*potABCD, potFGHI, potE*
Haemophilus influenza	1830	1709	*potABCD, potD, potE*
Mycoplasma genitalium	580	467	*potABC*
Mycoplasma pneumoniae	816	677	*potABC*
Rickettsia prowazekii	1112	834	*potE*
Synechocystis sp. PCC 6803	3573	3169	*potD*
Treponema pallidum	1138	1031	*potABC*

*Igarashi and Kashiwagi, 1999

1.2 Photosynthesis system in cyanobacteria

Cyanobacteria perform oxygenic photosynthesis. Light reactions of photosynthesis convert solar energy into chemical energy in the form of ATP and NADPH, and release oxygen as a by-product. The light reactions are catalyzed by four multiprotein complexes, namely photosystems I and II (PSI and PSII), cytochrome b_6f complex (Cyt b_6f) and ATP synthase, all of which are embedded in photosynthetic thylakoid membrane (Figure 6). In cyanobacteria, PSII is served mainly by a large extramembraneous phycobilisome antenna. The phycobilisome are mainly composed of water-soluble phycobiliproteins carrying linear tetrapyrrole chromophores, which absorb visible light in 450 - 660 nm range (Grossman *et al.*, 1993). PSI, in turn, captures light through its own membrane-intrinsic core antenna consisting mainly of chlorophyll *a* (chl *a*), which absorbs strongly in the blue-violet and red regions of the visible spectrum. Under certain conditions, however, PSI can obtain part of its excitation energy from the phycobilisome antenna through a process called state transitions (Mullineaux and Emlyn-Jones, 2005).

1.2.1 Polyamine and photosynthesis system

Generally, polyamines are known as stabilizing factors of biomembranes and macromolecules (Watanabe *et al.*, 1991; Igarashi and Kashiwagi, 2000). Exogenous di- and polyamines had the ability to retard protein degradation, inhibit chlorophyll loss, stabilize chloroplast thylakoid membranes, regulate the photosynthetic rate and minimize the rise in ribonuclease activity normally accompanying senescence of detached leaves maintained in darkness (Beigbeder *et al.*, 1995; Besford *et al.*, 1991 and 1993; Duan, 2000; Shih *et al.*, 1982).

Polyamines could act as regulators in adaptation of the photosynthetic apparatus and consequently protect photosynthetic machinery in an ozone-polluted environment

(Navakoudis *et al.,* 2003). During loss of viability either of cereal protoplasts due to osmotic shock or during dark-induced senescence in leaves, there is a rapid increase in arginine decarboxylase and a massive accumulation of putrescine (Flores and Galston, 1984). This is in sharp contrast to dicotyledons where osmotic treatments lead to increases in spermidine and spermine with a decline in putrescine and in the activities of ornithine decarboxylase and arginine decarboxylase (Tiburcio *et al.,* 1986). When oat leaves exposed to osmotic stress, there is an accompanying rise in the activities of RNase and protease, and a loss of chlorophyll (Altman *et al.*, 1977; Kaur-Sawhney and Galston, 1979). Treatment with the spermidine or spermine inhibited or retarded these processes (Kaur-Sawhney and Galston, 1979; Galston *et al.,* 1978) and induced DNA synthesis and limited protoplast mitosis (Kaur-Sawhney *et al.,* 1980). Furthermore, the three main polyamines were found to be associated with thylakoid membranes and with photosynthetic subcomplexes (Kotzabasis *et al.,* 1993). Dornemann *et al.,* (1996) noted changes in thylakoid-bound polyamines during chloroplast development. Correlations between polyamines and photosynthesis are in line with observations in synchronized cultures of *Scenedesmus obliquus*, in that the maxima for the membrane bound polyamines and the photosynthetic rate appear at the same point of the cell cycle (Kotzabasis and Senger, 1994).

The involvement of polyamines in stress resistance and their association with thylakoid membranes and the light reactions of photosynthesis indicate that polyamines are likely to interact directly with photosystems. Spermine at relatively low concentration was reported to enhance or restore the loss of photochemical activity both *in vivo* and *in vitro* (Ioannidis and Kotzabasis, 2007; Subhan and Murthy, 2001). However, some other studies have shown that exogenous polyamines added to isolated PSII submembrane

fractions were able to interact with the luminal side of PSII, which led to the loss of photosynthetic activity at high concentration of polyamines (>1 mM) (Beauchemin *et al.,* 2007a and 2007b). It was reported that exogenous polyamines can penetrate to the luminal side of thylakoid membranes (Ioannidis *et al.,* 2006). Thus, polyamine can interact with hydrophilic portions of proteins and the extrinsic polypeptides of the oxygen evolving complex. This interaction released the extrinsic proteins of PSII leading to the loss of PSII activity (Beauchemin *et al.,* 2007b; Boisvert *et al.,* 2007).

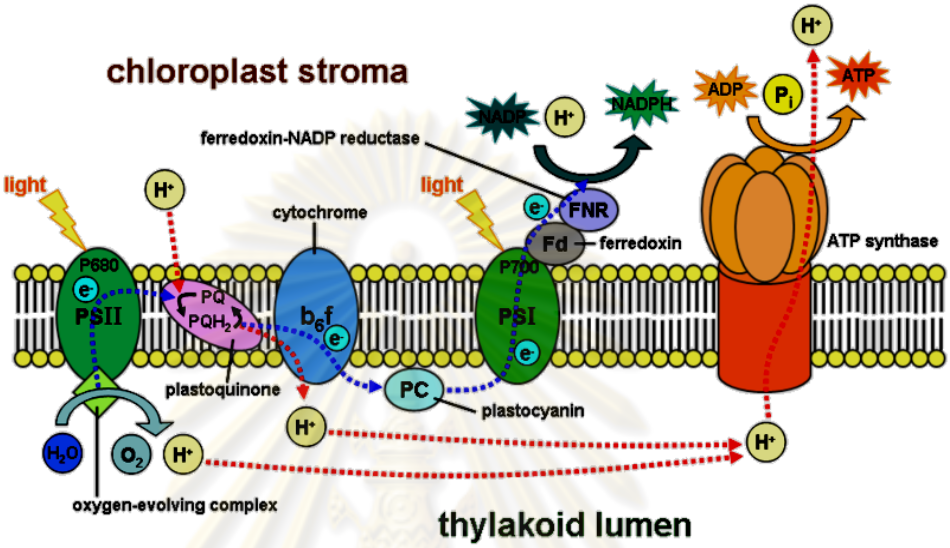


Figure 6 Simplified scheme showing the structure and function of the photosynthetic light reactions and eletron transfer routes in the thylakoid membrane. Light energy is converted into chemical energy, in the form of the energy-carriers ATP and NADPH by four major protein complexes in the thylakoid membrane, namely photosystem I (PSI), photosystem II (PSII), cytochrome b6f complex and ATP synthase.

(http://upload.wikimedia.org/wikipedia/commons/1/18/Thylakoid_membrane.png)

1.3 Cyanobacteria

Cyanobacteria can be classified as the Procaryota, Division of Cyanophyta and Cyanophyseae class. They are also diverse group of oxygen-evoluting photosynthetic bacteria and also major contributors to global primary production (Bryant, 2003; Ting *et al.*, 2002). Cyanobacteria can be found in terrestrial, freshwater and marine environments ranging from dim caves to hot springs and hyper-saline lakes (Rappka, 1988). Their phylogenetic position in the bacterial kingdom is still obscure, although recent analysis of ancient genes has indicated a genetic relationship with Gram-positive bacteria (Xiong *et al.*, 2000). Morphologically, cyanobacteria range from unicellular to filamentous forms. Some species of filamentous cyanobacteria have specialized cell type called heterocysts, akinetes or hormogonia (Adams and Duggan, 1999; Rippka, 1988; Tomitani *et al.*, 2006). Cyanobacteria have a cell wall consisting of plasma membrane, a peptidoglycan layer and outer membrane, which are often surrounded by external surface layer, the glycoclayx layer (Figure 7A and B) (Gantt, 1994; Hoiczyk and Hansel, 2000). The glycoclayx protects the cell from dehydration. The peptidoglycan layer is responsible for maintaining their cell shape and ability of the cell to withstand highly internal osmotic pressure. The outer membrane consists of a lipid layer and a lipopolysaccharide layer and porins, which function as a diffusion channels. The plasma membrane is a lipid bilayer which serves as a selectively permeable barrier. In addition, cyanobacteria have internal membranes called thylakoids in which the photosynthetic and respiratory electron transfer reactions located in.

1.3.1 *Synechocystis* sp. PCC 6803

Synechocystis is an unicellular, non-nitrogen (N_2)-fixing cyanobacterium and a ubiquitous inhabitant in fresh water (Figure 7C). They divide by binary fission at two or three successive planes. Based on their GC contents, many cultured strains of

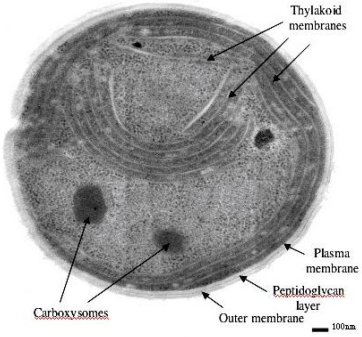
Synechocystis can be classified into three groups; the marine group, the low GC group and the high GC group (Holt *et al.*, 1994). Strain PCC 6803 belongs to the latter group, whose members, including PCC 6714, have been mostly isolated from freshwater. They also have the propensity to utilize glucose (Rippka *et al.*, 1979). A phylogenetic tree based on 16S rRNA sequences suggests that high GC content species are more closely related to *Microcystis aeruginosa*, which is a unicellular spherical cyanobacterium with gas vesicles, than to other *Synechocystis* groups (Honda *et al.*, 1999). There are four substrain cultures of *Synechocystis* ('PCC', 'ATCC', 'GT' (glucose-tolerant) and 'Kazusa'), all of which were derived from the Berkeley strain 6803, which was isolated from freshwater in California by R. Kunisawa (Stanier *et al.*, 1971) shown in Figure 8.

The complete nucleotide sequence of *Synechocystis* was announced in 1996. This was the first photoautotrophic organism to be fully sequenced. Sequencing was carried out using a clone-by-clone strategy based on the physical map of the genome, resulting in a highly accurate sequence. The circular genome was originally deduced to be 3,573,470 bp long (Figure 9). The average GC content is 47.7% (Kaneko *et al.*, 1996). Consequently, *Synechocystis* has been one of the most popular organisms for genetic and physiological studies of photosynthesis for two major reasons; it is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982) and grows heterotrophically at the expense of glucose (Rippka *et al.*, 1979). Moreover, *Synechocystis* cells has several features that make this strain particularly suitable for studying stress response at the molecular level (Glatz *et al.*, 1999). There is unequivocal evidence that in higher plant cells exposed to heat stress, the photosynthetic apparatus is irreversibly damaged prior to impairment of other cellular functions (Berry and Bjorkman, 1980). The general assembly of photosynthetic membranes in cyanobacteria is similar to that of higher plant, therefore *Synechocystis* might serve as a powerful model

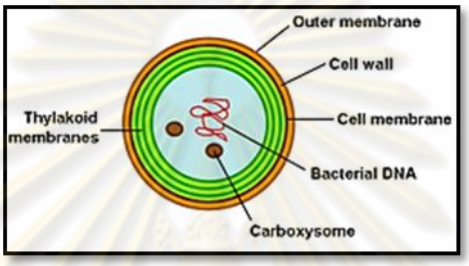
for studying the molecular mechanisms of stress response and long-term adaptation (Jantaro *et al.*, 2003; Lehel *et al.*, 1993).

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

A



B



C

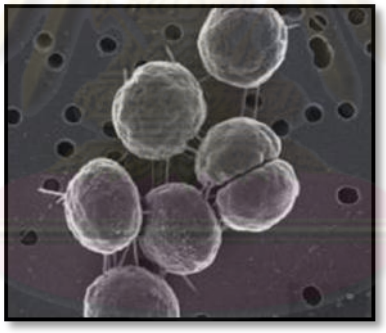


Figure 7 Ultra structure of a cyanobacterial cell. (A) Illustrates the compartmentalization of the cyanobacterial cell. (B) Schematic representation of a thin section of A. (C) Scanning Electron Microscope Image of *Synechocystis* cultivated with CO_2 limitation and excess light.

(www.cyanosite.bio.purdue.edu/images/images.html),
(http://www.flickr.com/photos/blueridgekitties/4619195795/),
(http://www.pnas.org/content/103/30/11358.full)

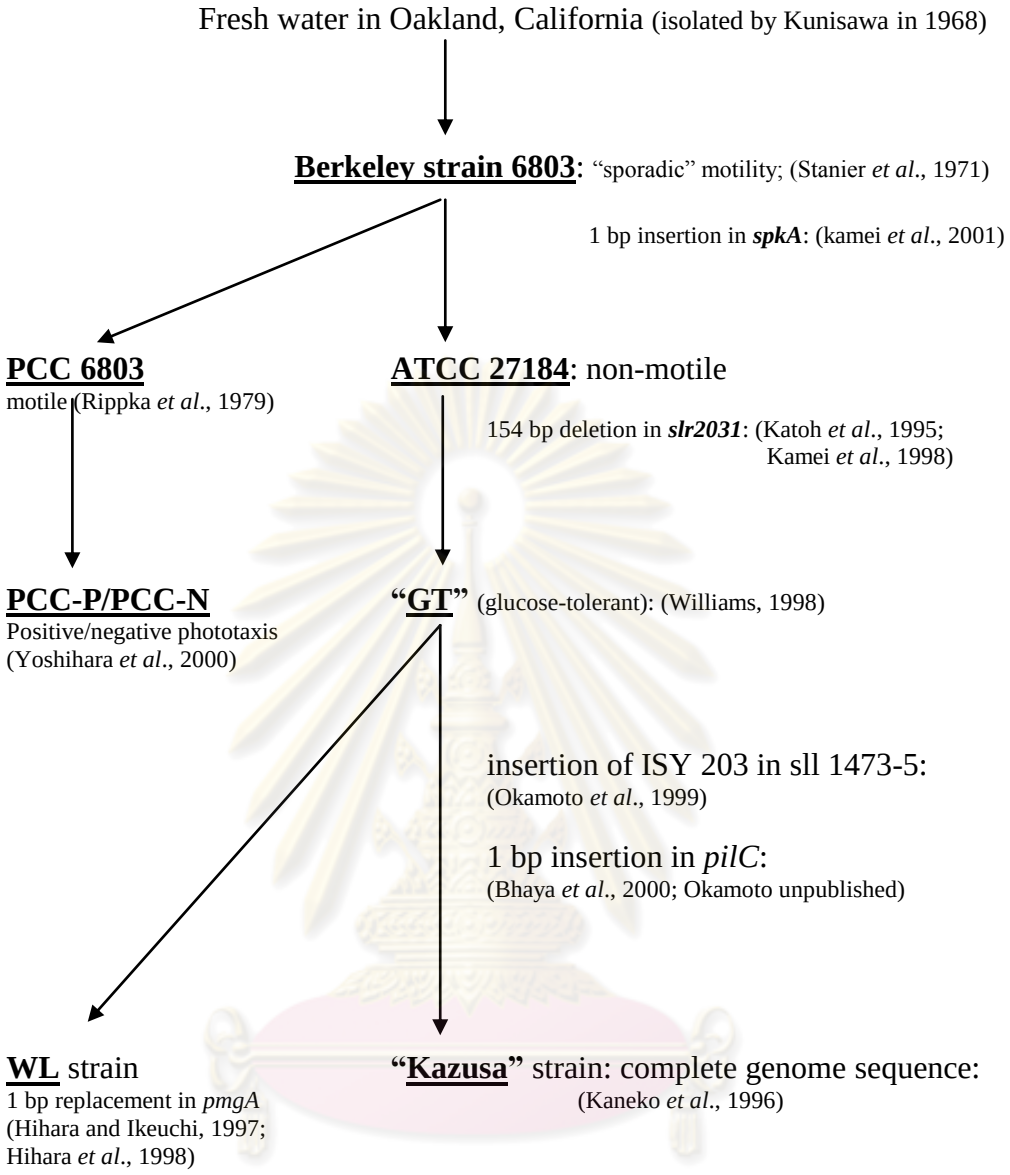


Figure 8 Strain histories of *Synechocystis* (Ikeuchi and Tabata, 2001).

1.4 Acclimation to changing environmental conditions

All organisms have some capacity to adapt to environmental stress, but the extent of this adaptive capacity varies widely. Heat, cold, high pressure, and acid or alkaline conditions can all produce stress. There are 2 routes to adapt easily to environmental stress. First, environmental conditions induce regulation of metabolic pathways employing existing cellular components. Gradual exposure to the stress, for example, may enable bacteria to synthesize new enzymes that allow them to continue functioning under the stressing conditions or that enhance their capacity to deal with the stressing agent. These adaptations enable bacteria to grow in a variety of conditions. Second, to resist environmental stress in other ways for instant, bacteria that live in extremely acidic conditions can pump out acid from their cell. Bacteria are able to function in these extreme conditions generally cannot function under conditions we consider normal. Most of earlier studies relating to stress responses have been done as the external symptoms of abiotic challenge become acute (Bouchereau *et al.,* 1999) but their precise molecular mechanism of action still remains unclear (Bagni and Tassoni, 2001).

Cyanobacteria must acclimate to changing light intensity, osmolarity, and temperature in their environments by strictly regulating the expression of distinct sets of genes, as shown in *Synechocystis* (Murata and Suzuki, 2005). Light is not only energy source, but also a signal in regulation of cyanobacterium gene expression (Gill *et al.,* 2002; Hübschmann *et al.,* 2005; Hihara *et al.,* 2001). Acclimation to changing light conditions involves several mechanisms such as state transitions, phototaxis, changes in PSII/PSI ratio and chromatin adaptation (reviewed by Mullineaux, 2001). The responsiveness of photosynthesis genes to white light has recently become obvious in studies of *Synechocystis* global gene expression profile (Huang *et al.,* 2002).

Transcriptional levels of a polyamine biosynthesis gene (ADC) were observed to remain unchanged under light-to-dark transition in *Synechocystis* (Jantaro *et al.,* 2005). However, the up-regulation of carotenoid biosynthesis has been detected as a respond to light stress in *Synechococcus* 7942 as well (Schafer *et al.,* 2006). Although, the osmotic stress generated by NaCl or by sorbitol induces similar changes in the expression of some genes, other genes are specifically regulated only by NaCl or by sorbitol (Kanesaki *et al.,* 2002; Marin *et al.,* 2006). Furthermore, NaCl and sorbitol have different effects on cytoplasmic volume (Kanesaki *et al.,* 2002; Marin *et al.,* 2006). These regulations were perceived as distinct signals through the Hik-Rre systems (Shoumskaya *et al.,* 2005) which may respond by direct osmolarity-induced activation of the transport. Correspondingly the accumulation of cellular polyamine levels was increased after exposure to long-term salt and osmotic stress in *Synechocystis* (Jantaro *et al.,* 2003). Non-optimal temperature, either low or high temperature, is an effective abiotic factor, which is known to influence polyamine metabolism in plants (Bouchereau *et al.,* 1999). The expression of certain cold-inducible genes in *Synechocystis* was perceived as distinct signals through the histidine kinase system (Suzuki *et al.,* 2001).

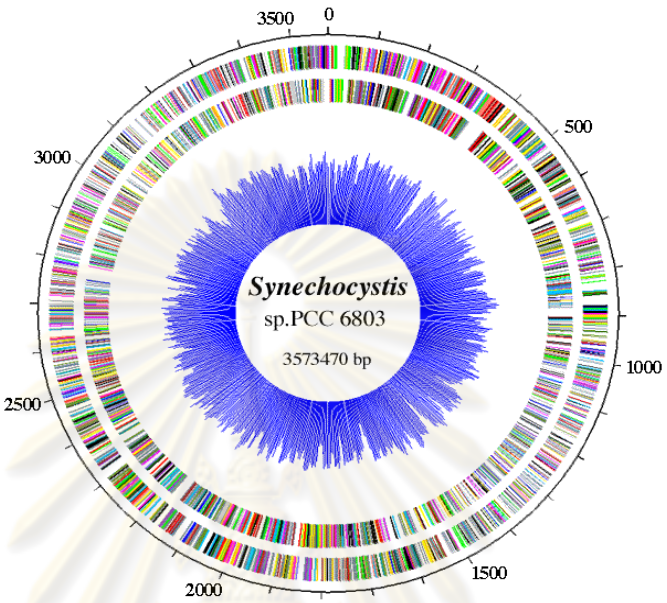


Figure 9 The cellular genome of *Synechocystis* according to Cyanobase (www.kazusa.or.jp/cyano/Synechocystis).

OBJECTIVES OF THIS RESEARCH

1. To determine the binding ability of *Synechocystis* PotD encoded by *potD (slr0401*) to polyamines: putrescine, spermidine and spermine.
2. To examine the function of PotD in polyamine transport in detail through *Synechocystis potD* mutant.
3. To study the effect of external polyamines on cell growth and photosynthesis system.
4. To study the effect of osmotic and light stress on cell growth, polyamine uptake and photosynthesis system.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipments

Autoclave	Model HA-30, Hirayama Manufactering Corporation, Japan
Balances	METTLER PJ360 DeltaRange® GWB, USA IKAMAG® REO DREHZAHL ELECTRONIC Precisa 800M SWISS QUALITY, USA
Centrifuge	HERMLE Z233 MK, USA
C-18 column	4.6 x 150 mm inertsil®ODS-3 5 µm i.d., Japan
Electrophoresis	Unit BIO-RAD PROTEIN® II xi Cell, USA
French press cell disrupter	THERMO ELECTRON COPORATION, USA
	Geiger counter RADOS RDS-120 Universal Survey Meter, USA
Geldoc® enabled	DNA visualisation UV light, USA
GENE pulser apparatus	BIO-RAD, USA
HPLC	Hewlett Packard series 1050, Japan
Laminar flow	BVT-124 International Scientific Supply, Thailand
Light source unit	Prekeo S250 Zeiss IKON, Japan

PCR apparatus	PERKIN ELMER DNA Thermal Cycler, USA
pH meter	ORION model 420A, USA
Power supply	BIO-RAD POWER PAC 1000, USA
Scintillation counters LS6500	Multi-Purpose Scintillation Counter, BECKMAN COULTER, USA
Shaker	Innova™ 2100 PLATFORM SHAKER, USA
Spectrophotometer	SPECTRONIC® GENESYS™2, USA Jenway UV/VIS 6400, USA
Ultracentrifuge	BECKMAN COULTER OPTIMA™ L-100XP, USA
Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.1.2 Chemicals

2,4-Dinitrophenol(DNP)	Merck, Germany
3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)	Sigma, USA
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma, USA
Acetic acid	BDH, England
Acetone	Merck, Germany
Acrylamide	Merck, Germany

Agarose	Invitrogen, USA
Agmatine	Sigma, USA
Amiloride	Sigma, USA
Ammonium persulfate	Merck, Germany
Brilliant blue	Sigma, USA
Bromophenol blue	Sigma, USA
BSA (Fraction V)	Sigma, USA
Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)	Sigma, USA
Chloramphenicol	Sigma, USA
Chloroform	Merck, Germany
Dithiothreitol	Sigma, USA
Dimethylformamide (DMF)	Sigma, USA
EDTA	Sigma, USA
Ethanol	Merck, Germany
Ethidium bromide	Sigma, USA
Glucose	Sigma, USA
Glycerol	Ajax Finechem, Australia
Glycine	Sigma, USA
Glyoxal	Sigma, USA
Gramicidine D	Sigma, USA

Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Mercaptoethanol	Sigma, USA
Methanol	Lab Scan, Poland
Metylene blue	Sigma, USA
N,*N*-dicyclohexylcarbodiimide (DCCD)	Sigma, USA
N-2-hydroxyethylpiperazine- *N*′-2-ethanesulfonic acid (HEPES)	Sigma, USA
N-ethylmaleimide	Sigma, USA
Nigericin	Sigma, USA
Ouabain	Sigma, USA
p-Chloromercurisulfonic acid	Sigma, USA
p-Nitro blue tetrazolium chloride (NBT)	Sigma, USA
Phenol	Merck, Germany
Potassium cyanide	Sigma, USA
Putrescine dihydrochloride	Sigma, USA
Putrescine-1,4-^{14}C dihydrochloride	Sigma, USA
Pyridoxal-5-phosphate	Sigma, USA
Silver nitrate	Sigma, USA
Sodium arsenate	Sigma, USA

Sodium bicarbonate	BDH, England
Sodium chloride	APS, Australia
Sodium citrate	Sigma, USA
Sodium dodecyl sulfate	Sigma, USA
Sodium fluoride	Sigma, USA
Sodium thiosulfate	Sigma, USA
Sorbitol	Sigma, USA
Spermidine trihydrochloride	Sigma, USA
Spermidine-8-^{14}C trihydrochloride	Sigma, USA
Spermine tetrahydrochloride	Sigma, USA
Sucrose	Sigma, USA
TEMED	BIO-RAD, USA
Toluene	BDH, England
Tris base	USB Corporation, USA
Triton X-100	Packard, USA
Tween-20	BIO-RAD, USA
Urea	Sigma, USA
Valinomycin	Sigma, USA
Xylene cyanol FF	Sigma, USA

2.1.3 Kits and suppliers

1 kb DNA Ladder	BioLabs, New England, England
DyNAzymeTM DNA Polymerase kit	FINNZYMES, Finland
ECL Western blotting detection	GE Healthcare, USA
Horseradish peroxidase conjugated	Promega Corporation, USA
Milipore Ultrafree-DA	Millipore Coorperation, USA
Ni Sepharose column kit	Amersham bioscience, USA
Nylon membrane filters 0.45 and 0.22 μm	Sartorius, Germany
Parablot NCL membrane	Machery-Nagel, USA
PCR amplification kit	MBI Fermentas, Germany
Prestained Protein Ladder, 10 - 160 kDa	MBI Fermentas, Germany
Whatman 3MM paper	Whatman International, England

2.1.4 Organisms

The two organisms, namely *Escherichia coli* and *Synechocystis* sp. PCC 6803 were used in this study.

2.1.4.1 *Escherichia coli* strains were obtained from the Laboratory of Biochemistry, Department of Biochemistry, Chulalongkorn University, Thailand.

2.1.4.1.1 Strain Top10 (F- *mcr*A, Δ*(mrr-hsdRMS-mcrBC,* φ*80lacZ*Δ*M15* Δ*lacX74 deoR rec1 araD139* Δ*(ara-leu)7696galU galK rpsL (Str*^{R}*) endA1 nupG)* was used for DNA manipulation.

2.1.4.1.2 Strain BL21 (DE3) (F- *ompT hsdS(rBmB) gal dcm*(DE3)) was used for *PotD* expression.

2.1.4.2 *Synechocystis* sp. PCC 6803 wild-type strain (Williams, 1988), is glucose tolerant, and was obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biochemistry and Food Chemistry, University of Turku, Finland.

2.1.5 Plasmids

The three plasmids, namely pGEM-T Easy, pET-19b and pUC4K Vector were used in this study. Circle maps are shown in APPENDIX N, O and P.

2.1.6 Oligonucleotides

Target gene	Name	primers	Amplified fragment length (bp)
potD *(slr0401)*	forward-potD	5'-AGTTCTGACGGTACCGTGATGA-3'	1,150
	reverse-potD	5'-GTCAAGCCTTGGTAAGGTTCT-3'	

2.2 Methods: polyamine binding study

2.2.1 Expression of recombinant *Synechocystis potD*

The recombinant plasmid potD was constructed as described by Raksajit (2007), briefly, *potD* gene was amplified by using the pair of specific primer as shown in 2.1.6, and then ligated into pET-19b. After that, the recombinant plasmid, namely pPETPOTD, obtained was transformed into the expression host. The expression hosts used in this experiment were *E. coli* strains BL21 (DE3). A single colony was picked from the spread plate recombinant plasmid and then inoculated in 10 ml of LB containing the 100 μg/ml

of ampicillin in 125 ml Erlenmeyer flask. This innoculated culture was incubated at 37ºC with shaking overnight. Consequently, 1 ml of cell culture was inoculated in the 500 ml of LB medium containing 100 μg/ml of ampicillin. When the optical density at 600 nm (OD_{600}) reached 0.6 - 0.8 (approximately in 2 - 3 hours), isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added into the culture flask with the final concentration of 0.1 mM. One milliliter culture medium was collected after induction at 5 hours-induction. The pellet was then collected by centrifuging at 5,000 x g at 4ºC for 10 min, and subsequently analyzed by SDS-PAGE. The cell pellets were washed twice with 50 mM Tris-HCl buffer, pH 8.0 and resuspended in the same buffer. After that, sonication in three short pulses of 30 s with 2 min in ice between each pulse was done in order to disrupt cells. To obtain sample fraction, centrifugation at 13,000 x g, 4°C for 20 min was performed. The centrifuged soluble and insoluble fractions were then analyzed by 15% (w/v) SDS-PAGE.

2.2.2 Purification of the recombinant *Synechocystis* PotD (rPotD)

As recombinant *Synechocystis* PotD protein was found in the pellet fraction as inclusion bodies, the pellet was then subjected forward to purification by a prepared Ni^{2+}-affinity chromatography column according to the manufacturer suggestions for insoluble proteins (His GraviTrap™ kit, GE Healthcare, England). Briefly, inclusion bodies were washed twice in 50 mM Tris-HCl pH 8.0 and dissolved in washing buffer (APPENDIX G). The insoluble proteins were removed by centrifugation. Subsequently, the clear solution was applied onto a Ni^{2+}- column equilibrated with three volumes of washing buffer. The rPotD protein was eluted by elution buffer containing 500 mM instead of 20 mM imidazole (APPENDIX G). The purified rPotD was analyzed by 15% (w/v) SDS-PAGE.

2.2.3 Protein refolding and immunological analysis of rPotD

The rPotD was refolded by dialysis overnight at 4°C against dialysis buffer (APPENDIX G). The dialyzed protein sample was then centrifuged at 13,000 x g, 4°C for 20 min to remove unfolded or aggregated proteins and analyzed by 15% (w/v) SDS-PAGE. The concentration of rPotD protein was determined by Bradford's method (Bradford, 1976). The molecular mass of rPotD protein was estimated using standard markers and its specificity was confirmed by immunoblotting analysis. Briefly, the protein from SDS-PAGE was transferred onto polyvinylidene fluoride membranes (Immobilon-P; pore size, 0.45 μm; Millipore, USA) by electroblotting. Immunoblotting was performed by using a 1:3,000 dilution of an anti-His tag monoclonal antibody (Amersham Biosciences, USA) and a 1:5,000 dilution of a polyclonal rabbit immune PotD serum. The immunoblots were developed with ECL^{TM} anti-rabbit immunoglobulin G, horseradish peroxidase conjugate of goat antibodies against rabbit immunoglobulin in a 1:10,000 dilution (GE Healthcare, England), as instructed by the manufacturer.

2.2.4 Affinities for polyamine binding

Binding assay was carried out as described previously (Kashiwagi *et al.*, 1993). One milliliter of the reaction mixtures containing 100 μg of purified rPotD protein, 50 mM HEPES-KOH pH 7.5, 30 mM KCl and 50 μM each radioactively labeled polyamine (^{14}C putrescine, ^{14}C spermidine and ^{14}C spermine) with a specific activity of 2 mCi/μmol were incubated at 37^{o}C for 5 min. The reaction was stopped by rapid filtration through a filter (cellulose nitrate membrane, pore size: 0.22 μm, Millipore, USA). The filter was rinsed with 5 ml of cold buffer (50 mM HEPES-KOH pH 7.5, 30 mM KCl). The radioactivity was counted with a liquid scintillation counter (Beckman Coulter Multipurpose Scintillation Counter LS6500, USA). All binding experiments were

repeated at least twice with triplicate samples. The assay of competition experiments by substrate analogs was done by adding 20 fold excess of unlabeled analogs to the assay mixture. The apparent dissociation constants (K_d) and the maximum binding (B_{max}) parameters were calculated from Scatchard plots as described previously (Kashiwagi *et al.,* 1993). The effect of external pH on polyamine binding was assayed at different pHs where 100 mM potassium acetate was used for buffer pH 4.0 - 6.0, 100 mM Tris-HCl for buffer pH 7.0 - 8.0, and 100 mM glycine-KOH for buffer pH 9.0 - 11.0.

2.2.5 Docking of polyamines into the homology model of rPotD

Spermine, spermidine and putrescine were docked into the homology model of *Synechocystis* PotD (Brandt *et al.,* 2010) using the program GOLD 3.2 (Jones *et al.,* 1995 and 1997). Prior to the docking experiments, spermidine in the *Synechocystis* PotD model was removed and hydrogens were added to the *Synechocystis* PotD model in Sybyl 8.0 (Tripos International, St Louis, MO, USA). In the putrescine docking experiment, the conserved water molecule involved in putrescine binding in the *E. coli* PotF (PDB code 1A99) crystal structure, was added to the *Synechocystis* PotD model prior to adding hydrogens. Ten independent genetic algorithm runs with the default docking parameters were made in GOLD. The binding site in the *Synechocystis* PotD model was restricted within 15 Å radius from the OD2 atom of Asp206 in the spermidine and spermine docking experiments, while the putrescine docking experiment was centered at the OD2 atom of Asp295. The docking was stopped if three docking solutions were within 1.5 Å rmsd. The docking results were visualized and examined with Bodil modeling and visualization environment (Lehtonen *et al.,* 2004) and figures were prepared with PyMOL (DeLano, 2003).

2.3 Methods: characterization of *Synechocystis potD* and *potD::Km*^{r} mutant

2.3.1 Mutant construction

The *potD* gene of the wild-type was interrupted with a kanamycin resistance (*Km*^{r}) gene cassette to construct the knockout mutant recombinant and transformed it forward to *Synechocystis* segregation.

2.3.1.1 Culture condition

Axenic cells of the cyanobacterium *Synechocystis* were grown photoautotrophically at 30^{o}C in BG-11 medium containing 17.6 mM $NaNO_3$ as nitrogen source buffered with 20 mM HEPES-NaOH (pH 7.5) (Rippka *et al.,* 1979) under continuous illumination provided by white light fluorescent tubes. The incident light intensity in the growth chamber was 40 - 50 μmol photons/m^2/s. Growth of liquid cultures was monitored by measuring the optical density at 750 nm (OD_{750}) with a Spectronic® Genesys^{TM}2 spectrophotometer.

2.3.1.2 Genomic DNA extraction

Two milliliter of exponentially growing cultures of *Synechocystis* was extracted genomic DNA. Cells were collected by centrifuging at 4,000 x g for 10 min and resuspended in 400 μl of TE-Buffer pH 7.5. Half volume of glass bead, 8 μl of 10% SDS and 400 μl of absolute phenol were added into the mixture. Cells were broken by vortexing 1 min for 3 times, cool on ice between intervals. The lysate was centrifuged (10,000 x g, 10 min, room temperature) to remove major amount of polysaccharides, and the clear supernatants (containing total DNA) were transferred to a new microcentrifuge tube. After that, one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed and centrifuged at 12,000 x g, room temperature for 10 min. Kept viscous

supernatant in new microcentrifuge tube, added 1:1 volume of chloroform:isoamyl alcohol (24:1) and then centrifuged at 12,000 x g, room temperature for 10 min. Transferred supernatant in new microcentrifuge tube, added 2 volume of absolute ethanol and incubated on ice for 30 min, then centrifuged at 12,000 x g, 4^{o}C for 10 min. Pellets were kept and added 70% ethanol (v/v). After gentle mixing by inversion, the mixture was centrifuged at 12,000 x g, 4^{o}C for 10 min. Washed pellets were air dried at room temperature. One hundred µl of TE-Buffer pH 8.0 was suspended and then checked by monitoring concentration at absorbance 260 nm (OD 1.0 = 50 µg/ml) while the purity was checked by the absorbance ratio A260/A280 (Sambrook and Russell, 2001). DNA sample was run in 0.8% agarose gel electrophoresis with 1x TAE buffer pH 8.0.

2.3.1.3 Amplification of *Synechocystis potD*

The *slr0401* sequence encoding the putative periplasmic binding protein D of *Synechocystis* (PotD) (Kaneko *et al.*, 1996) was amplified from the genomic DNA using *Pfu* DNA polymerase (Promega, USA) and gene specific primers. For the convenience of cloning, an *Nde*I site was designed to the 5′-end of the forward-potD primer and a *Bam*HI site was added to 5′-end of the reverse-potD primer after the stop codon. *Pfu*-based PCR amplification was performed under the conditions as shown in APPENDIX D. The PCR products were then fractionated on a 0.8% (w/v) agarose gel.

2.3.1.4 Plasmid construction of pGEMPOTD

A *potD* gene was purified by plasmid purification kit. The gene was tailed with an adenine nucleotide and ligated to the pGEM-T Easy vector, called pGEMPOTD. The pGEMPOTD plasmid was transformed into *E. coli* Top10 using electroporation. The transformants were selected using the blue white screening on the ampicillin agar plates. A single white colony of *E. coli* harboring a pGEMPOTD was grown in 1.5 ml LB broth

containing 100 μg/ml of ampicillin and incubated at 37ºC overnight. The pGEMPOTD was prepared by a minipreparation method as described by Sambrook and Russell (2001). Briefly, a single-colony of *E. coli* TOP 10, containing the pGEMPOTD, was inoculated into 2 ml of LB medium supplemented with 100 μg/ml ampicillin. The culture was incubated overnight at 37ºC, transferred into a 1.5 ml tube and harvested by centrifugation at 5,000 x g, 4ºC for 5 min. The pellet was resuspended in 100 μl of Solution I to which 20 μg/ml RNase was added and incubated at room temperature for 5 min. The sample was placed on ice for 5 min, 200 μl of Solution II was added, shaken vigorously and further incubated on ice for 5 min. This was followed by the addition of 150 μl of Solution III and 5 min incubation on ice. After centrifuging at 12,000 x g for 10 min, the supernatant was transferred to a fresh microcentrifuge tube and extracted with one volume phenol:chloroform:isoamyl alcohol (25:24:1), and mixed by vortexing. The sample was then centrifuged at 12,000 x g for 10 min; the upper phase was transferred to a new microcentrifuge tube. After that, two volume of absolute ethanol was added to the sample and incubated at -20^{o}C for 30 min. The sample was centrifuged at 12,000 x g for 10 min, the supernatant was discarded whereas the pellet was washed with 800 μl of cold 70% ethanol. The plasmid was collected by centrifugation at 12,000 x g, 4ºC for 15 min, and left to dryness by air flow. The plasmid was resuspended in TE-buffer and digested with *Nde*I and *Bam*HI at 37ºC an overnight. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. After that, the selected clones were sequenced with an automated sequencer by a commercial service (Macrogen Inc., Korea).

2.3.1.5 Plasmid construction of pPotD::KM^{r}

A 1.15 kb DNA fragment containing *potD* gene was amplified by PCR with specific primers and subsequently inserted into the pGEM T-easy vector as described

previously in 2.3.1.4. The pGEMPOTD was digested with *Bst*EII getting the 5′ sticky end and made the blunt end fragment by adding DNA Polymerase I Large (Klenow) Fragment. Briefly, 50 µl of reaction was composed of 10 µg of *Bst*EII digested pGEMPOTD, 1x of Klenow buffer, and 10 unit of the enzyme. The reaction was incubated at 37ºC for 20 min. After incubation, the reaction mixture was loaded to 0.8% agarose gel electrophoresis and purified the blunt-end fragment by using DNA elution and purification kit. In addition, pUC4 K (Amersham-Bioscience, USA) carrying Km^r cassette was digested with *Bam*HI and made the blunt end fragment as describe above. This plasmid was called pPotD::KM^r.

2.3.1.6 Ligation reaction

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The 10 µl ligation reaction was composed of 1 µl of 10×T4 DNA ligase buffer, 1 unit of T4 DNA ligase, 10 ng of *Bst*EII digested pGEMPOTD and 7 ng of *Bam*HI digested Km^r cassette. The reaction was incubated at 16ºC overnight. One microlitre of the ligation mixture was transformed into the competent *E. coli* Top10.

2.3.1.7 Electrotransformation

The competent cells were gently thawed on ice. Forty microlitre of cell suspension were mixed well with 1 µl of the ligation reaction, and placed on ice for 1 min. The cells were transformed by setting the GENE pulser apparatus (Bio-RAD, USA)

as follows: 25 μF, 200 Ω of the pulse controller unit, and 2.50 kV. After one pulse was applied, the cells were immediately resuspended with 1 ml of LB broth. The cell suspension was incubated at 37ºC with shaking at 250 rpm for 45 min. The cell suspension was spread on the LB agar plate containing 100 μg/ml ampicillin and 100 μg/ml kanamycin, then incubated at 37ºC for overnight. After incubation, colonies were randomly selected for plasmid isolation.

2.3.1.8 Isolation of the pPotD::KM^r

The pPotD::KM^r construction was selected by restriction enzyme digestion using *Eco*RI. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. The clone containing the correct DNA fragment of approximately 2.41 kb for *potD::Km^r* mutation was selected. DNA sequencing was performed to confirm the correct frame of the vector and the inserted DNA as well as the sequence of the *potD*.

2.3.1.9 Natural transformation into *Synechocystis*

The mutant plasmid was then transformed into *Synechocystis* cells according to Williams (1988). Briefly, 40 ml of the culture (OD_{730} ~ 0.5) were centrifuged (5,000 x g, 5 min, 30^{o}C). The cell pellets were resuspended in 1 ml fresh BG11. Forty microliter of cell suspensions was aliquot into new steriled eppendrof tubes. The 20 μg of potD::Km^r plasmid was added into the cell suspensions. The mixture of cells and mutant plasmid was incubated for 6 hours under darkness at 30ºC, and shaken tube once after 3 hours. Consequently, 1 ml of fresh BG11 containing 0.4% glucose was added and incubated with shaking at 30ºC for overnight under light illumination at 30 μmol photons/m^2/s. Cells were spread on BG11 containing 0.4% glucose and grown in chamber at 30ºC, light for 18 hours. After 18 hours, the transformants were plated onto medium agar plates containing 20 μg/ml of kanamycin. After 3 days, transformants were

transferred on fresh agar plates containing kanamycin at the higher concentration, 30 - 100 μg/ml. PCR amplification technique was used to confirm the complete segregation of mutant by using the same primers that were used to amplify *potD* from *Synechocystis* wild-type.

2.3.2 Growth study

The growth rates of *Synechocystis* in liquid cultures were monitored under various conditions as follow; BG11 medium, the presence of 0.1 - 0.5 mM putrescine, spermidine and spermine; the presence of 250 and 550 mM NaCl; the presence of 300 and 700 mM sorbitol. The *Synechocystis* was grown photoautotrophically in BG11 under various conditions. In addition, the mutant strain, *potD::Km*^{r} was grown in BG11 medium supplemented with 25 μg/ml kanamycin under various conditions. The initial of cell concentration was adjusted to an OD_{730} of 0.1 and cultures of 100 ml volume were grown in 250 ml Erlenmeyer flasks on a rotatory shaker at 160 rpm, 30°C under continuous illumination of 50 μmol photons/m^2/s. These conditions were used as the standard growth conditions. Cells were also grown under continuous illumination of 250 μmol photons/m^2/s for studying in the effect of high light intensity on growth. Two-ml samples were taken from a culture flask every day to determine growth rate by measuring the optical density at 730 nm with a Spectronic® Genesys™ 2 spectrophotometer.

2.3.3 Extraction and determination of cellular polyamine content

Synechocystis wild-type and mutant cells under various conditions were harvested and broken the cells by 5% cold $HClO_4$. After the extraction by 5% perchloric acid for 1 hour on an ice bath, the samples were centrifuged at 8,000 x g, 4^{o}C for 10 min. The supernatant and pellet fractions (represented as free and bound forms of polyamines,

respectively) were derivatized by benzoylation reaction. One ml of 2 M NaOH was added into 500 µl of 5% $HClO_4$ extract firstly following by mixing with 10 µl of benzoyl chloride. The mixture was vigorously vortexed and incubated for 20 min at room temperature. The reaction was stopped by 2 ml of saturated NaCl. The benzoyl-polyamines were separated by solvent fractionation with 2 ml of cold diethyl ether. Taken 2 ml of the ether phase with benzoyl-polyamines to a new tube and evaporated to dryness, then redissolving in 1 ml of methanol. Authentic polyamine standards were prepared similarly as the stressed cells. The derivatized-polyamines were then analyzed by high performance liquid chromatography (HPLC) (Flores and Galston, 1982), using 1,6-hexanediamine as an internal standard. Samples were filtered through a 0.45 µm cellulose acetate membrane filter before injecting to HPLC. Derivatized polyamines were analyzed by high performance liquid chromatography (HPLC) with inertsil®ODS-3C-18 reverse phase column (5 µm; 4.6 x 150 mm) using UV-Vis detector at 254 nm. The mobile phase was a gradient of 60 - 100% methanol:water. The flow rate was 0.5 ml/min. Chromatogram and standard curve of polyamines are showed in the APPENDIX K.

2.3.4 Polyamine transport assay

2.3.4.1 Polyamine uptake

Exponential cells were harvested by centrifugation (8,000 x g, 10 min, 4°C), washed twice with 50 mM HEPES-KOH buffer pH 7.5 containing 0.4% glucose and suspended in the same buffer. The cell suspension containing 0.1 mg cell protein was preincubated at 37°C for 5 min. The addition of 5 µl ^{14}C polyamine (Radiochemical Centre, Amersham, England) with a specific activity of 2 mCi/mmol at a final concentration of 50 µM was added to start the uptake experiment. The reaction was incubated, then, the cells were rapidly collected on membrane filters (cellulose acetate,

0.45 μl pore size; Millipore USA). The filters were washed twice with 1 ml of cold buffer containing 1 mM specific polyamine to remove the adsorbed ^{14}C polyamine on the membrane. The amount of amine adsorbed to the cell surface and the filter was less than 0.1% of the added amine under these experimental conditions. The radioactivity on the filter was determined with a liquid scintillation counter. Initial polyamine uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of polyamine taken up per min per milligram protein.For the ionic and osmotic stress assay, cells were preincubated with the NaCl or sorbitol for 30 min at 37ºC before the addition of ^{14}C polyamine. For the assay of inhibition by substrate analogues, cells were incubated with a mixture of labelled substrate and 20 fold excess of unlabeled analogues for 30 min at 37ºC before the addition of ^{14}C polyamine. For the light intensity assay, cells were stood under darkness or 110 μmol photons/m^2/s for 30 min before adding ^{14}C polyamine.

2.3.4.2 Polyamine excretion by intact cells

Exponential cells were preloaded with a specific activity of 2 mCi/mmol at a final concentration of 1 mM for 90 min. After incubation, cells were washed twice as described in uptake assay. ^{14}C polyamine-loaded cells were incubated in the buffer containing either 50 mM HEPES-KOH buffer pH 7.5 containing 0.4% glucose or 50 mM sodium acetate buffer pH 6.0 containing 0.4% glucose. Cells were collected by membrane filters every 15 min. The radioactivity was determined with a liquid scintillation counter.

2.3.5 Western blot analysis

2.3.5.1 Isolation of total membrane and soluble protein fraction

Soluble protein was isolated essentially as described by Gombos *et al.* (1994). Forty milliliter of the exponential phase of wild-type and mutant *Synechocystis*

cells under various conditions were harvested by centrifuged at 4,000 x g, 4°C for 8 min and washed twice with washing buffer (50 mM HEPES-NaOH pH 7.5 and 30 mM $CaCl_2$). The pellet was resuspended with 200 µl resuspention buffer (50 mM HEPES-NaOH pH 7.5, 30 mM $CaCl_2$, 800 mM sorbitol and 1 mM *ε*-amino-*n*-caproic acid). The cell pellets were broken by vortexing them in the presence of glass beads (150 - 212 microns; Sigma, USA) 1 min for 6 times, cool on ice between intervals. Unbroken cells and glass beads was removed by centrifugation at 1,000 x g for 5 min. Centrifuged again, then the membranes was pelleted from the supernatant by centrifugation at 18,000 x g for 30 min, resuspended in storage buffer (50 mM Tricine-NaOH pH 7.5, 600 mM sucrose, 30 mM $CaCl_2$ and 1 M glycinebetaine), and stored at -80°C. All steps were performed in dim light at 4°C. Isolated membranes were highly enriched with thylakoids but apparently also contained fragments originating from plasma membrane.

2.3.5.2 Immunoblotting

Fifty microgram of total membrane and soluble protein fractions were loaded into a 15% (w/v) SDS-PAGE. Consequence, proteins were transferred from the gel onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, USA). After the transfer, the gel can be stained with Coomassie blue stain to ensure that proteins have been transferred to the membrane completely. Membrane was immediately placed into the blocking buffer (Tris base buffered saline, and 5% skim milk) with shaking at 4^oC for 1 hour. Subsequently, the blotted membrane was washed twice with TTBS buffer, each wash for 5 min (Tris base buffered saline, 0.05% Tween-20) and incubated with 1:5,000 of primary antibody (polyclonal anti-PotD, Biomedical Technology Research Center, Chiangmai University, Chiangmai, Thailand) in the Antibody Binding Buffer (Tris base buffered saline, 0.05% Tween-20 and 1% skim milk) with shaking at 4°C for

overnight. After incubation, the blot was washed three times 5 min each. The blot was incubated with a secondary antibody conjugated horseradish peroxidase (1:10,000) for one hour with shaking at 4°C. After incubation of the secondary antibody, the blot was washed three times in TTBS, each wash for 5 min. The blot was developed by addition of substrate for chemiluminescence horseradish peroxidase. The signal was detected with X-ray film (Fuji, Japan) and quantified by densitometric evaluation by using the Geliance 1000 Imaging System (Perkin Elmer, USA).

2.3.6 Photosynthesis capacity and PSII activity determination

Cell was cultivated in BG-11 medium supplemented with each polyamine, NaCl or sorbitol. The cell culture at 2, 24, 72 hours was harvested and resuspended with fresh BG11 medium to the final concentration of 10 μg of chlorophyll *a* per milliliter prior to the determination of oxygen evolution .

PSII activity was measured as oxygen evolution under saturating light intensity with Clark-type oxygen electrode (Hansatech, King's Lynn, GB) at 32^{o}C. The 0.5 mM DCBQ (2,6-dichloro-*p*-benzoquinone was used as electron acceptor and 0.5 mM ferricyanide added to keep the acceptor in oxidized form. Photosynthetic capacity was measured similarly as PSII activity except that 0.6 mM bicarbonate was supplemented instead.

CHAPTER III

RESULTS

3.1 Polyamine binding activity

3.1.1Expression of recombinant *Synechocystis* PotD (rPotD)

The recombinant *Escherichia coli* BL21(DE3) cells harboring pETpotD were grown at 37 ^{o}C in LB medium containing 100 μg/ml ampicillin. Protein production was induced by isopropyl-*β*-D-thiogalactopyranoside (1 mM). After induction, one millilitre of culture medium was collected at 0, 1, 2, 3 and 5 hours. Whole cells and a crude extract from sonicated cells were analyzed subsequently by SDS-PAGE (Figure 10). The induction of an approximate 43 kDa recombinant protein occurred after 1 hour of induction by IPTG and gradually increased over the 5 hour of induction. The result shown in Figure 10 illustrated that recombinant *Synecocystis* PotD (rPotD) protein was expressed in *Escherichia coli* strain BL21 (DE3) and formed itself as an inclusion body.

3.1.2 Purification, refolding and imunoblotting of rPotD

The rPotD-inclusion body was dissolved by washing buffer. The insoluble proteins were removed by centrifugation. The clear solution was then applied onto a Ni^{2+}-column equilibrated with 3 volumes of washing buffer. The elution fraction was collected and dialyzed extensively against dialysis buffer to remove the imidazole and NaCl. The purified rPotD was analyzed by 15% SDS-PAGE. Recombinant purified PotD was analyzed by immunoblotting analysis using a monoclonal antibody raised against His-tag at a dilution of 1:3,000 and a secondary antibody conjugated anti-rabbit immunoglobulin G at a dilution of 1:5,000 to confirm the molecular weight and purity. After incubation of

antibody, immunoblotting membrane was developed by addition of substrate for horseradish peroxidase. During exposure for 20 min, the signal corresponding to the specific antibody-antigen reaction was apparently visualized. An analysis by immunoblotting with anti-His monoclonal antibody and polyclonal PotD antiserum indicated that the rPotD had an apparent molecular mass of 43 kDa corresponding to the size of the predicted rPotD (Figure 11).

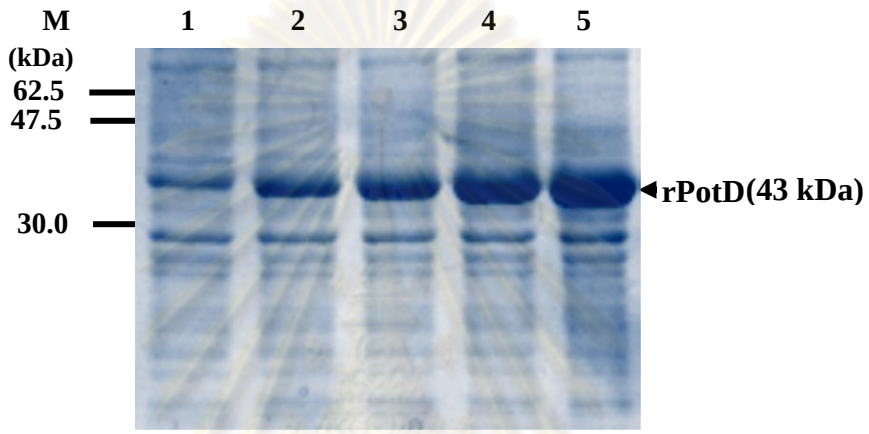


Figure 10 Coomassie-stained gel analysis of His-tagged *Synechocystis* PotD (rPotD) expression in *E. coli*. Whole-cell extracts were loaded on 15% SDS-PAGE. Lane M, molecular weight markers (in kilodaltons); lane 1, whole-cell extracts of non-induced cultures after growing overnight; lanes 2 - 5, whole-cell extracts of cultures kept 1, 2, 3 and 5 hours, respectively after IPTG induction in LB medium at 37^{o}C.

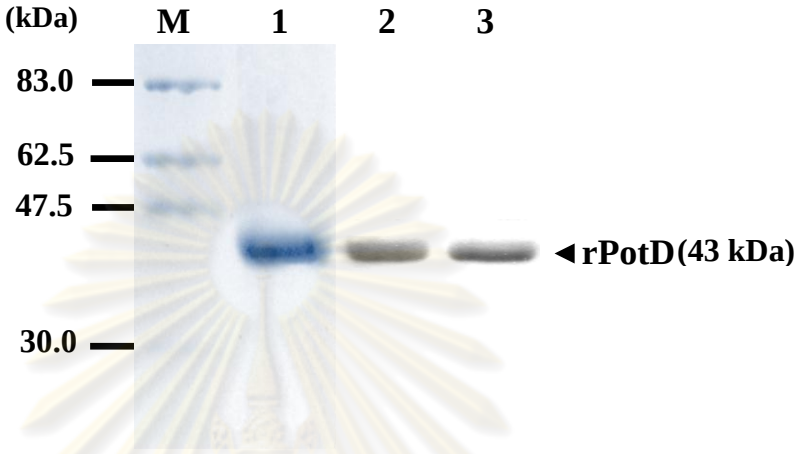


Figure 11 Coomassie-stained gel analysis of rPotD eluted fraction from Ni^{+} chromatography purification and immunoblotting analysis. Lane M, molecular weight markers (in kilodaltons); lane 1, fraction eluted with buffer containing 500 mM imidazole; lane 2, Ni^{2+}-purified rPotD probed with monoclonal anti-His antibody; lane 3, Ni^{2+}-purified rPotD probed with polyclonal rabbit immune serum against the purified rPotD.

3.1.3 Affinities for polyamine binding

The binding of varying putrescine, spermidine or spermine concentration to rPotD was saturable (Figure 12A). Scatchard plots showed linear transformation of the binding of all three polyamines to rPotD demonstrated in Figure 12B. The values of the dissociation constant (K_d), calculated from the reciprocal of the slope for putrescine, spermidine and spermine *in vitro* were 13.2, 7.8, and 8.3 μM, respectively. The maximum binding (B_{max}) values, the X-intercepts of the Scatchard plot, of putrescine, spermidine and spermine were 0.74, 1.42 and 0.13 mol/mol rPotD, respectively (Table 3). The data indicate that the three polyamines bind to rPotD with a 1:1 binding stoichiometry based on the molecular mass for rPotD of 43 kDa.

3.1.4 The effect of pH on polyamine binding

Polyamine carries a net positive charge; hence, we further tested whether pH could affect its binding. The binding assay was done by using 50 mM potassium acetate buffer pH 4.0 - 6.0, 50 mM HEPES-KOH buffer pH 7.0 - 8.0 and 50 mM Tris-HCl buffer pH 9.0 - 11.0. The result showed that the changes in the polyamine binding capacity as a function of external pH were observed (Figure 13). The specific binding of all types of polyamines to rPotD displayed an optimum at pH 8.0. However, there was the reduction of binding capacity which occurred under the decrease and increase of pH. It is noted that the rPotD had the highest binding capacity for spermidine at all pH values analysed.

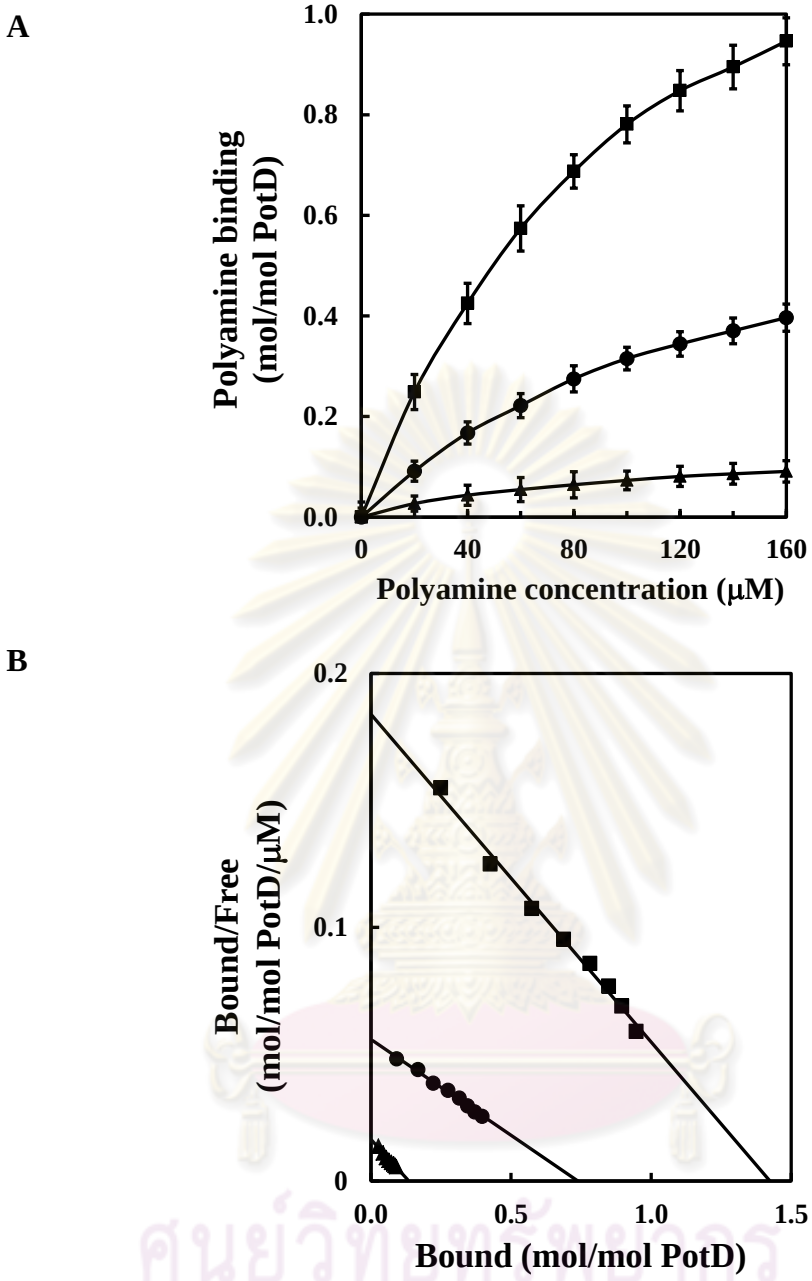


Figure 12 Concentration dependence of putrescine (●), spermidine (■), and spermine (▲) binding. (A) Different amounts of each polyamine were added to give different external polyamine concentrations. The data represented three independent biological replicates. (B) Scatchard analysis of the data. The line drawn is derived from regression analysis of the data. K_d and B_{max} were obtained from the reciprocal of the slope and the intercept on the X-axis, respectively.

Table 3 Parameters for rPotD binding to different polyamines

Polyamine	K_d (μM)	B_{max} (mol/mol)
Putrescine	13.2 ± 0.1	0.74 ± 0.05
Spermidine	7.8 ± 0.1	1.42 ± 0.40
Spermine	8.3 ± 0.1	0.13 ± 0.03

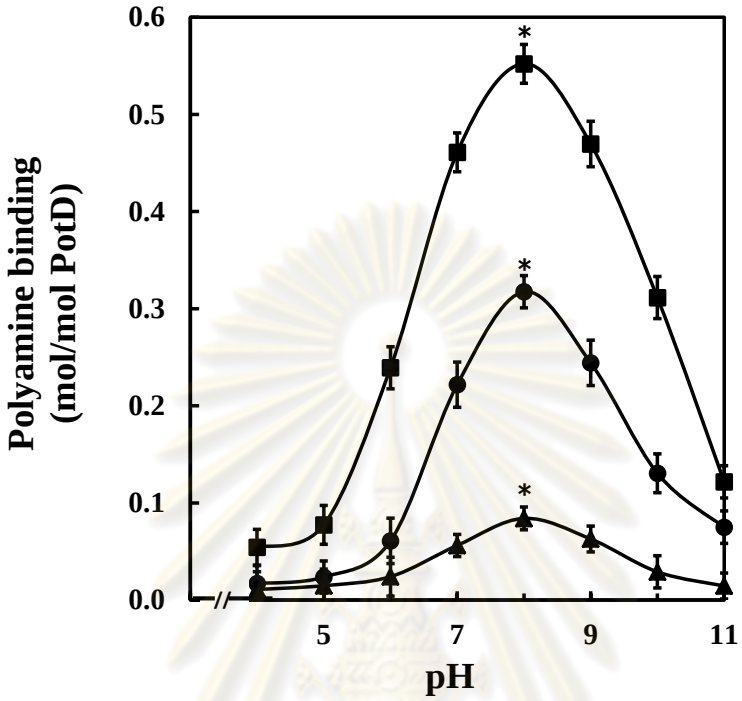


Figure 13 Effect of external pH on putrescine (●), spermidine (■), and spermine (▲) binding. The binding assays were performed with different pHs. Asterisk indicates statistically significant differences (Student's *t*-test, $P < 0.05$, n =3).

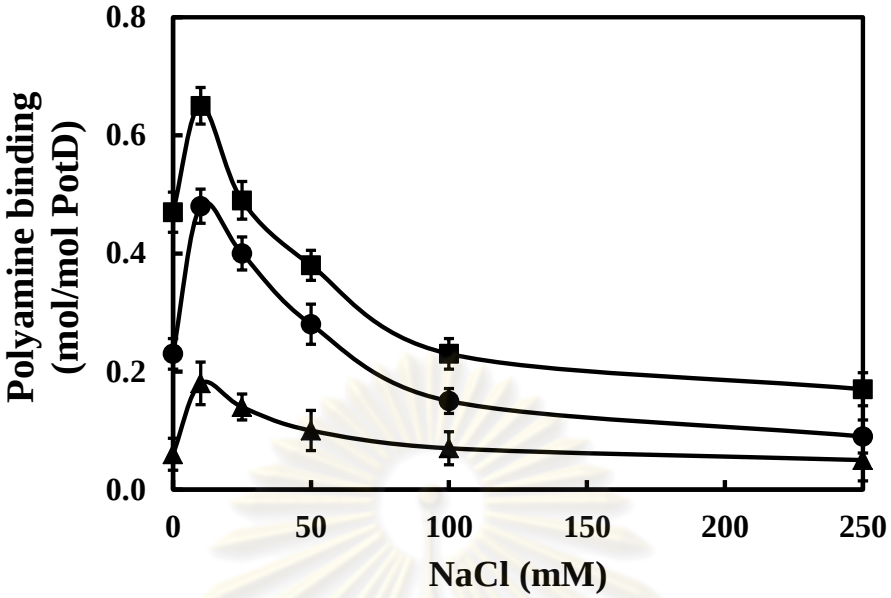
3.1.5 Effect of NaCl and sorbitol on polyamine binding

Since polyamines had been reported that have protective mechanism inside the cells against environmental stresses. Therefore, we further assessed whether osmotic stresses generated by NaCl and sorbitol affect polyamine binding. The presence of 10 mM NaCl showed the highest capacity binding for the three polyamines. However, increasing of NaCl concentration from 50 to 250 mM decreased the binding capacity (Figure 14A). Similarly, the highest binding capacity of rPotD to polyamines was at 10 mM sorbitol. Increasing of sorbitol concentration from 50 to 200 mM resulted in decrease of polyamine binding capacity (Figure 14B). Overall, this suggests that slight osmotic stress generated by either sorbitol or NaCl enhanced the polyamine binding capacity of rPotD.

3.1.6 Specificity of polyamine binding

Experiments were carried out to determine if there are specific interactions between the rPotD and polyamines. As shown in Table4, the binding of each radioactively labeled polyamine to rPotD was not inhibited by compounds structurally related to polyamines, including L-arginine, L-asparagine, L-glutamic acid, L-lysine, and L-ornithine. Binding of ^{14}C spermidine to rPotD was inhibited by non-labeled spermine and putrescine 35 and 42%, respectively. Binding of ^{14}C spermine to rPotD was inhibited by non-labeled putrescine and spermidine 39% and 58%, respectively. Similarly, binding of ^{14}C putrescine to rPotD was inhibited by non-labeled spermine and spermidine 38% and 58%, respectively. These results proved that rPotD is able to bind specifically to the three polyamines putrescine, spermidine and spermine.

A



B

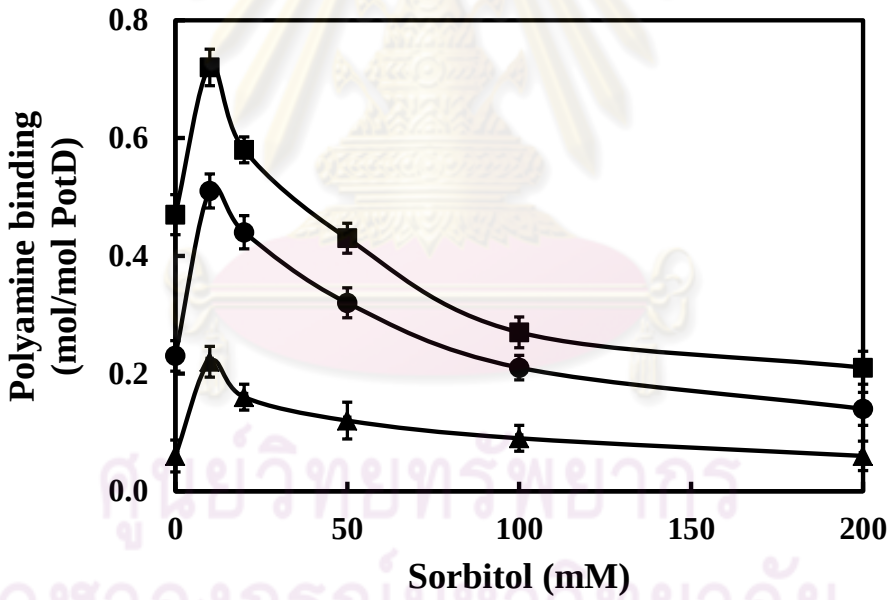


Figure 14 Effect of external NaCl at various concentrations of 0 - 250 mM (A) and sorbitol at various concentrations of 0 - 200 mM (B) on putrescine (●), spermidine (■), and spermine (▲) binding. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3

Table 4 Effect of polyamine analogs on specific binding activity of *Synechocystis* rPotD**.**

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

[a] statistically significant differences (Student's *t*-test, $P < 0.05$, n = 3) with respect to the control. The data shown are the means of three independent experiments, representing the percent binding activity relative to the control (100%) for putrescine, spermidine and spermine, which were 0.33 ± 0.03, 0.55 ± 0.18, and 0.05 ± 0.01 mol/mol, respectively.

3.1.7 Docking of polyamines into the homology model of *Synechocystis* PotD.

Putrescine, spermidine and spermine were docked into the *Synechocystis* PotD model to examine differences in the binding of the three polyamines. The binding modes of spermidine and putrescine were also compared with their binding to the preferred periplasmic substrate-binding proteins in *E. coli*, PotD and PotF, respectively (Pistocchi *et al.*, 1993). The binding mode of the docked spermidine in *Synechocystis* PotD was very similar to the binding mode of spermidine in the X-ray structure of *E. coli* PotD as well as to the binding mode of spermidine in the previously modeled complex of *Synechocystis* PotD and spermidine. *E. coli* and *Synechocystis* PotDs share the conserved residues (Asp206, Glu209, Trp267, Trp293 and Asp295; *Synechocystis* numbering), which are involved in the binding of spermidine (Figure 15A). Additionally, Leu40 binds spermidine through hydrophobic interactions while Asn269 and Gln270 bind the N1 nitrogen of spermidine. The volume of the active site cavity in *Synechocystis* PotD is large enough to accommodate spermine, which is largest of the docked polyamines. Based on the docking results, spermine binds similarly as spermidine does (Figure 15B). An additional residue, Gln71, was found to interact with the N14 nitrogen of spermine. A conserved water molecule in *E. coli* PotF (water number 471 in PDB structure 1A99) has been shown to be important for putrescine binding (Vassylyev *et al.*, 1998) and, thus, it was added to the active site of the *Synechocystis* PotD model prior to putrescine docking. Putrescine was docked to the model in a similar binding mode as in the crystal structure of *E. coli* PotF (Figure 15C). *Synechocystis* PotD residues Leu40, Glu209, Trp267, Gln270, Trp293 and Asp295 are involved in binding putrescine, while Asp206 and Asn269 are too far away to be able to bind. Glu209 interacts with N2 atom of putrescine via water molecule.

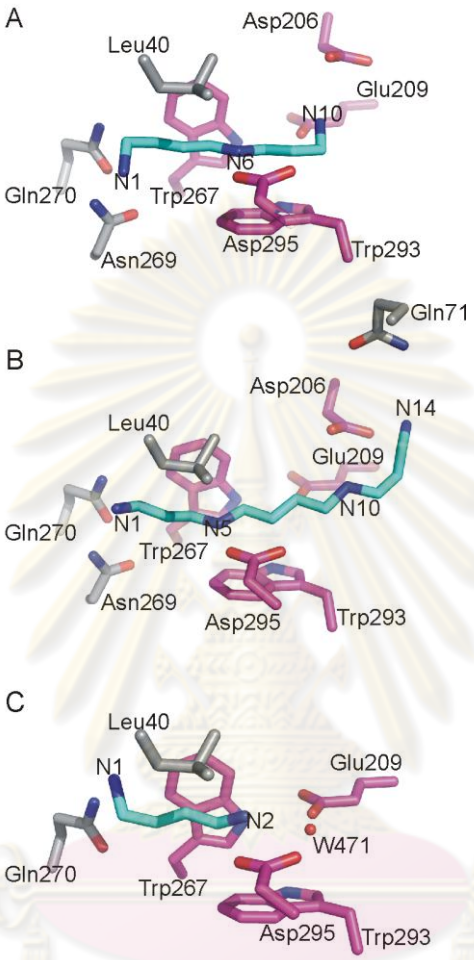


Figure 15 The docked binding mode of spermidine (A), spermine (B) and putrescine (C) in the *Synechocystis* PotD model. The amino acid residues involved in the polyamine interactions are shown as sticks and the conserved residues colored magenta. The polyamines are colored cyan.

3.2 Characterization of *Synechocystis potD* and PotD::Km^r*Synechocystis* knockout

3.2.1 The *potD* gene amplification

The *potD* gene of *Synechocystis* was amplified from genomic DNA using specific primers, designed from the complete sequence data in cyanobase (Kaneko *et al.*, 1986). PCR amplification was performed by pre-denaturation at 95ºC for 5 min, 29 cycles of denaturation at 95ºC for 30 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min, and the final extension at 72ºC for 10 min. The PCR product was electrophoretically separated on a 0.8% agarose gel. A prominent PCR product of *potD* gene with the approximated size of 1.15 kb was observed as shown in Figure 16.

3.2.2 Construction of a pGEM-T easy recombinant plasmid containing the *potD*

The PCR product from the previous step was purified using PCR purification kit (Qiagen, USA). The purified PCR product and pGEM-T easy vector were ligated together by ligase for overnight at 4^{o}C. The ligation reaction was transformed into the *E. coli* Top10 cells. The recombinant clone was selected on LB agar plate containing ampicillin and X-Gal. The white colonies were randomly selected and cultured in LB broth containing ampicillin overnight. The cultures were subjected to plasmid extraction and digestion with *Nde*I and *Bam*HI restriction enzymes to prove the correction of recombinant plasmid. Subsequently, these reactions were analyzed by 0.8% agarose gel electrophoresis. The recombinant plasmid harboring *potD* gene was confirmed by double digestion using *Nde*I and *Bam*HI enzymes. Two bands of pGEM-T easy vector and *potD* gene were observed at 3.0 and 1.15 kb, respectively (Figure 17).

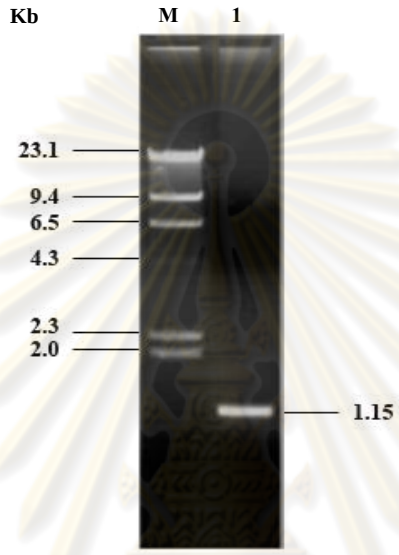


Figure 16 Agarose gel electrophoresis of the amplified *potD* gene. The PCR product was separated on a 0.8% agarose gel and visualized by ethidium bromide staining. M is a λ/*Hind*III DNA marker and lane 1 is the PCR product of *potD*.

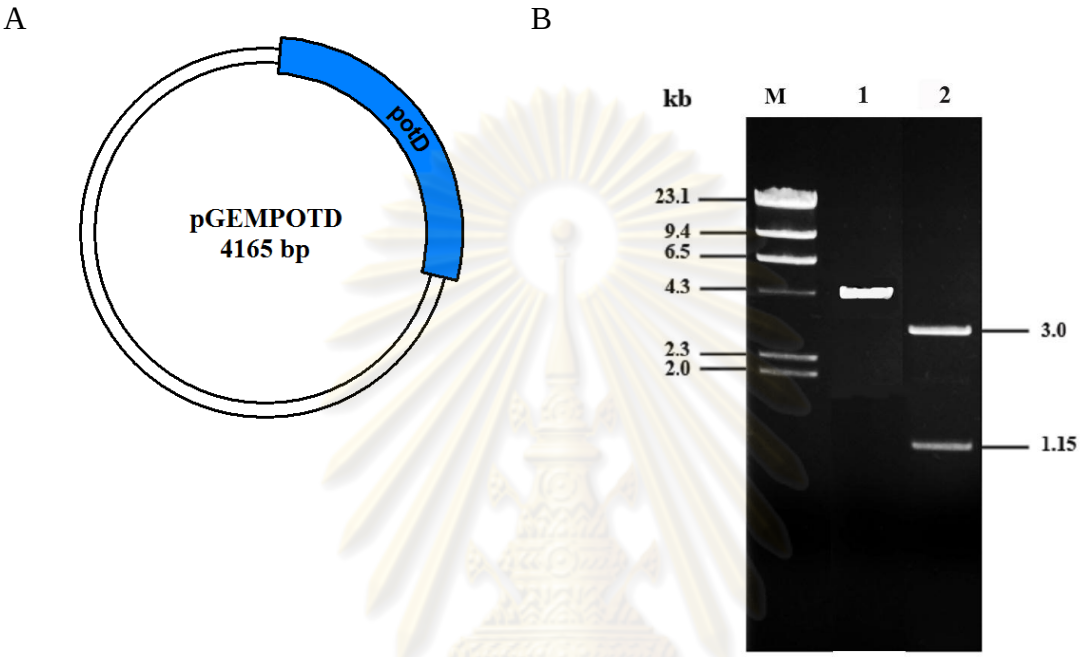


Figure 17 Agarose gel electrophoresis of the recombinant *potD* gene in pGEM-T easy vector (pGEMPOTD). (A) The construction of pGEMPOTD (B) The analysis of pGEMPOTD. Lane M is a λ/*Hind*III DNA marker, lane 1 is pGEMPOTD and lane 2 is the pGEMPOTD digested with *Nde*I and *Bam*HI. The digested fragments were separated on a 0.8% agarose gel and then visualized by ethidium bromide staining.

3.2.3 Construction of a *potD* inactivation strain

The chromosomal *potD* gene of *Synechocystis* was disrupted by inserting a kanamycin resistance (*Km*^{r}) gene cassette of pUC4K (Amersham-Bioscience, USA) into the *potD* gene. Briefly, pGEMPOTD was digested with *Bst*EI as well as pUC4K was digested with *Bam*HI for overnight. These fragments then were made blunt ends by using Klenow (Promega, USA). The restriction enzyme activity was inactivated by heat at 65^{o}C for 20 min. The reaction was added 2 µl of Klenow (NEB: 3' - 5' exo-) and 1 µl of 2.5 mM dNTPs, incubated at 37^{o}C for 30 min. Subsequently, these reactions were loaded to 0.8% agarose gel electrophoresis. The target band was eluted from gel and purified by using NucleoSpin® Extract II. The ligation mixture was performed, transformed into the *E. coli* Top10 cells. The recombinant clone was selected on LB agar plate containing ampicillin and kanamycin. The colonies were randomly selected and cultured in LB broth containing ampicillin and kanamycin overnight. The cultures were subjected to plasmid extraction and digestion with *Nde*I and *Bam*HI restriction enzymes to prove the correction of recombinant plasmid. Subsequently, these reactions were analyzed by 0.8% agarose gel electrophoresis to confirm the presence of inserted fragment. The analysis revealed that the recombinant plasmid contained a kanamycin resistance (*Km*^{r}) gene in pPOTD::Km^r. Two bands of pGEM-T easy vector and *potD::Kmr* gene were represented at 3.0 and 2.41 kb, respectively (Figure 18). The knockout plasmid was transformed to *Synechocystis*. To ensure gene knockout is completely segregation, colony PCR was performed by using specific primers. PCR products were analyzed by 0.8% agarose gel electrophoresis as shown in Figure 19. The PCR result revealed that the DNA fragment isolated from *Synechocystis potD* mutant was larger than *Synechocystis* wild-type strain.

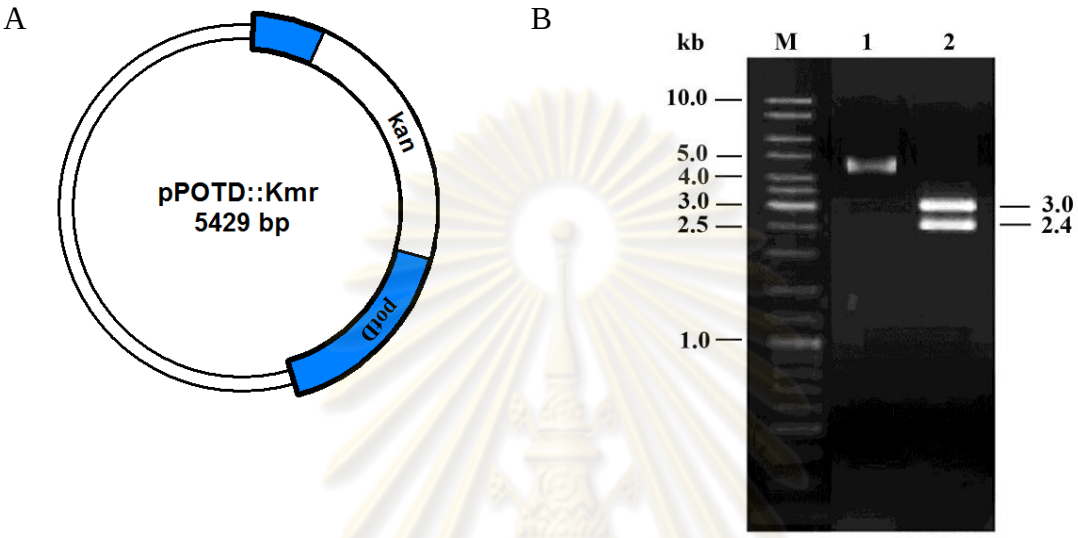


Figure 18 Agarose gel electrophoresis of pPOTD::Km^r. The DNA was separated on a 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is a 1 kb DNA marker, lane 1 is the undigested pPOTD::Km^r, and lane 2 is the pPOTD::Km^r digested with *Eco*RI.

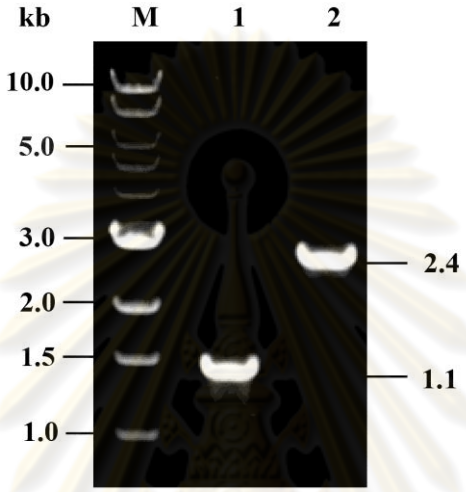


Figure 19 Agarose gel electrophoresis of *potD* amplified using chromosomal DNA of the *Synechocystis* wild-type and *potD::km*^{r} knockout. The PCR product was analyzed on a 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is a 1 kb DNA marker, lane 1 is the wild-type *potD*, 1.15 kb and lane 2 is *potD::km*^{r}, 2.41 kb.

3.3 Effect of external polyamines and environmental stresses on *Synechocystis* growth comparing wild-type and *potD* mutant

Synechocystis wild-type strain and *Synechocystis potD* mutant (hereafter wild-type and mutant cells, respectively) were grown in BG11 medium supplemented with various concentrations of each polyamine; putrescine, spermidine or spermine. In BG11 medium without supplementation, the mutant had the same growth rate as wild-type strain during 10 days as shown in Figure 20. To investigate the role of PotD to the three major forms of polyamines consisting of putrescine, spermidine and spermine on growth, polyamines were added to the medium. In the presence of 0.1 - 0.3 mM putrescine, both the mutant and wild-type had increased growth rate during 10 days. The higher putrescine concentration resulted in the less growth rate. In addition, the mutant strain showed strong negative sensitivity to 0.4 and 0.5 mM putrescine compared with wild-type strain (Figure 21). Similar results were observed in the presence of spermidine (Figure 22). Additionally, increasing the external concentration of spermine from 0.1 to 0.5 mM resulted in decreasing of wild-type strain growth rate. Interestingly, the mutant strain could not tolerate the external spermine (Figure 23). These results suggest that the mutant strain is more sensitive to all external polyamines than wild-type strain.

Subsequently, cells were grown in the medium containing 250 and 550 mM NaCl. It was observed that the growth rate of wild-type cells was not affected by external NaCl, whilst the mutant cell was slightly affected (Figure 24). The presence of sorbitol at 300 mM in BG11 medium strongly promoted the growth of wild-type whereas only growth inhibition was observed in the mutant cell. Sorbitol at 700 mM significantly reduced the growth of wild-type and mutant cells (Figure 25).

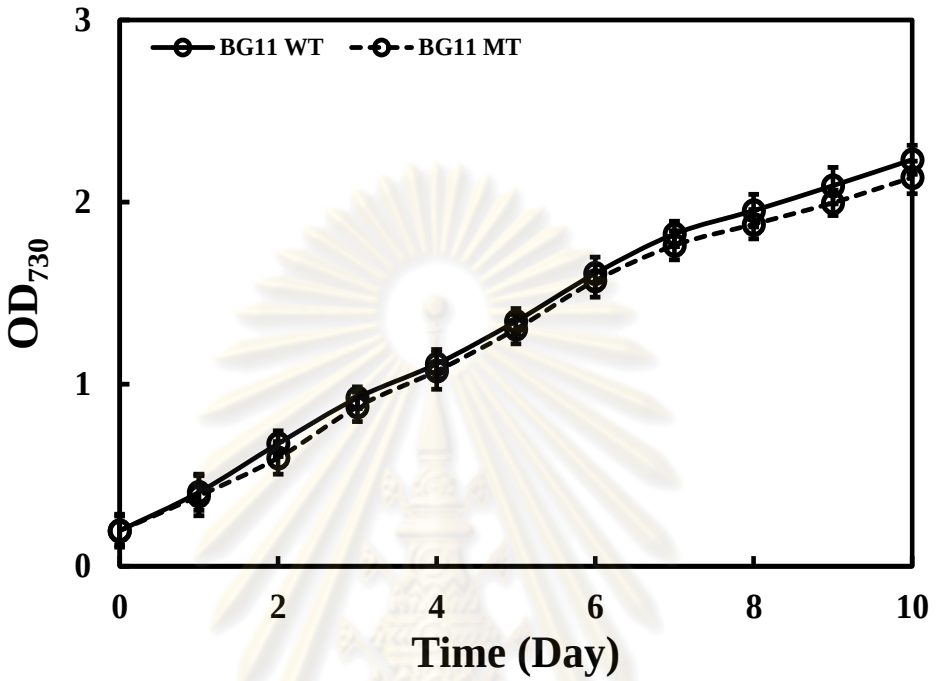


Figure 20 Growth of *Synechocystis* wild-type and mutant cells in the BG11 medium. Solid line represents wild-type growth and square dot represents mutant growth. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

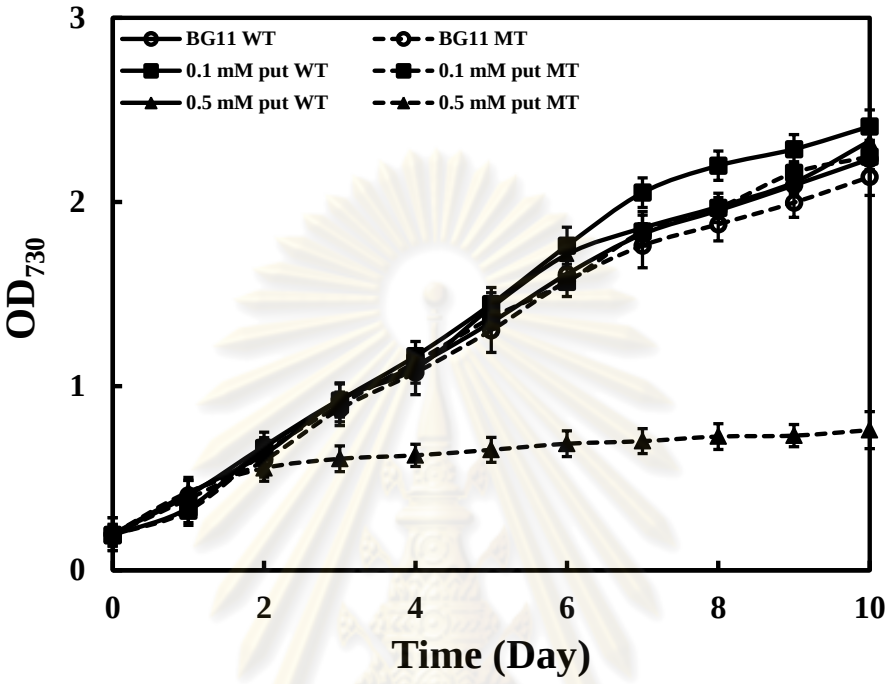


Figure 21 Effect of external putrescine on the cell growth of *Synechocystis* wild-type and mutant cells. Different amounts of putrescine were added to BG11 medium giving different external putrescine concentrations (0.1 - 0.5 mM). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

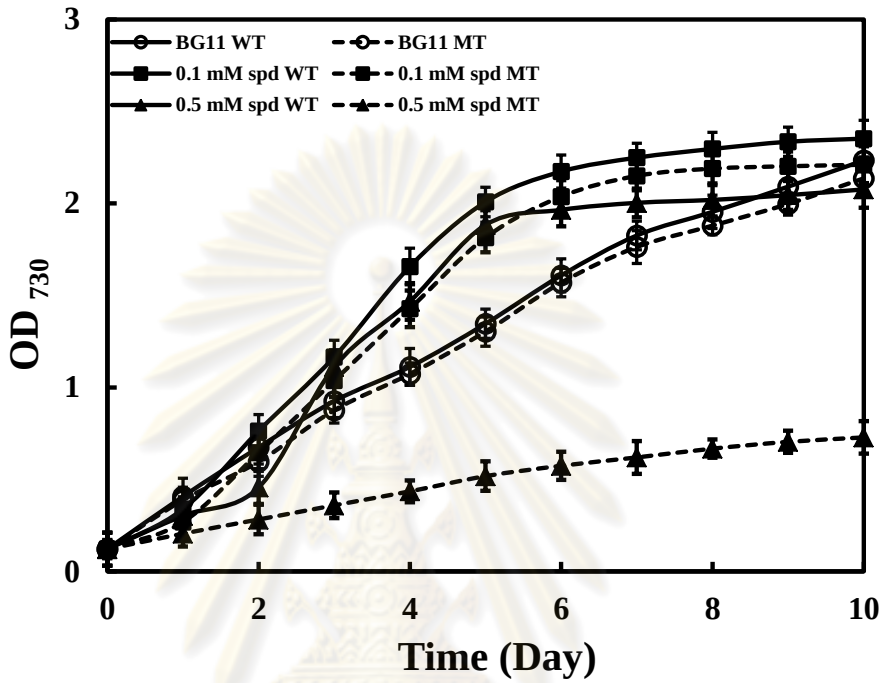


Figure 22 Effect of external spermidine on the cell growth of *Synechocystis* wild-type and mutant cells. Different amounts of spermidine were added to give different external spermidine concentrations (0.1 - 0.5 mM). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

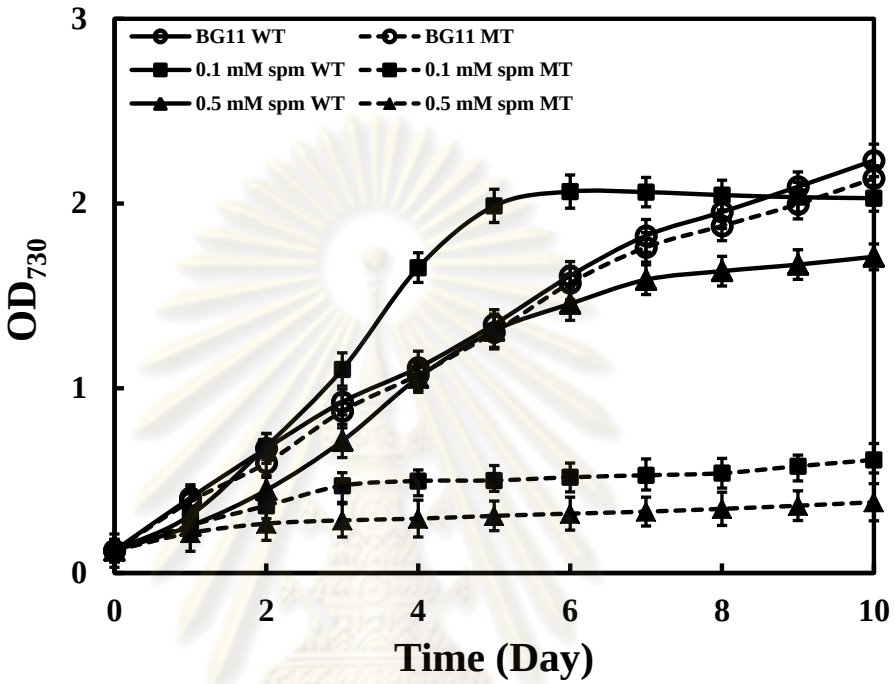


Figure 23 Effect of external spermine on the cell growth of *Synechocystis* wild-type and mutant cells. Different amounts of spermine were added to give different external spermine concentrations (0.1 - 0.5 mM). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

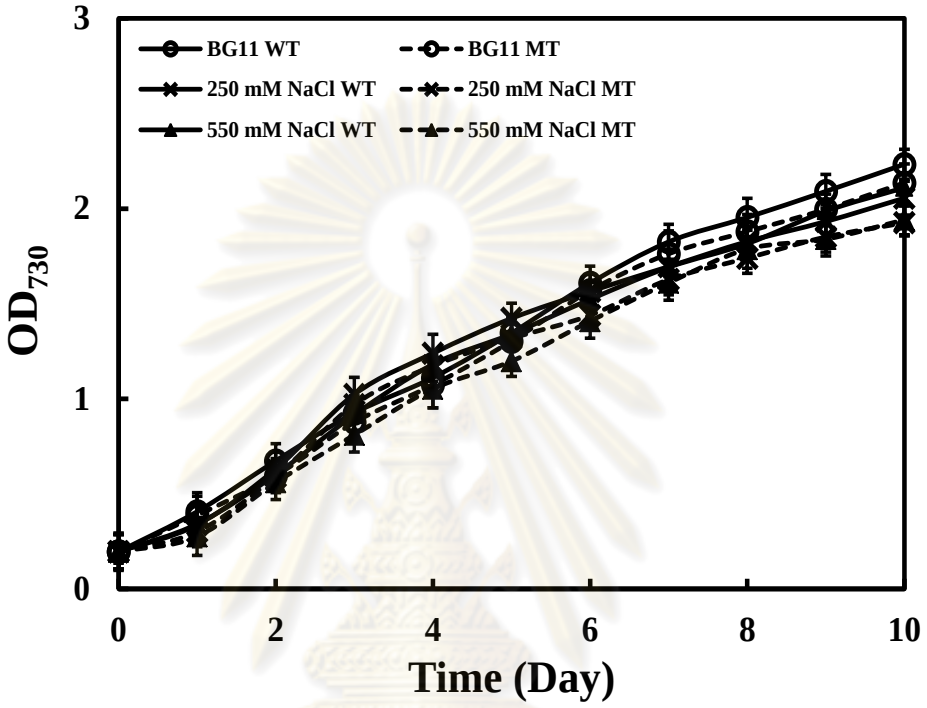


Figure 24 Effect of NaCl on the cell growth of *Synechocystis* wild-type and mutant cells. Different amounts of NaCl were added to give different external NaCl concentrations (250 and 550 mM). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

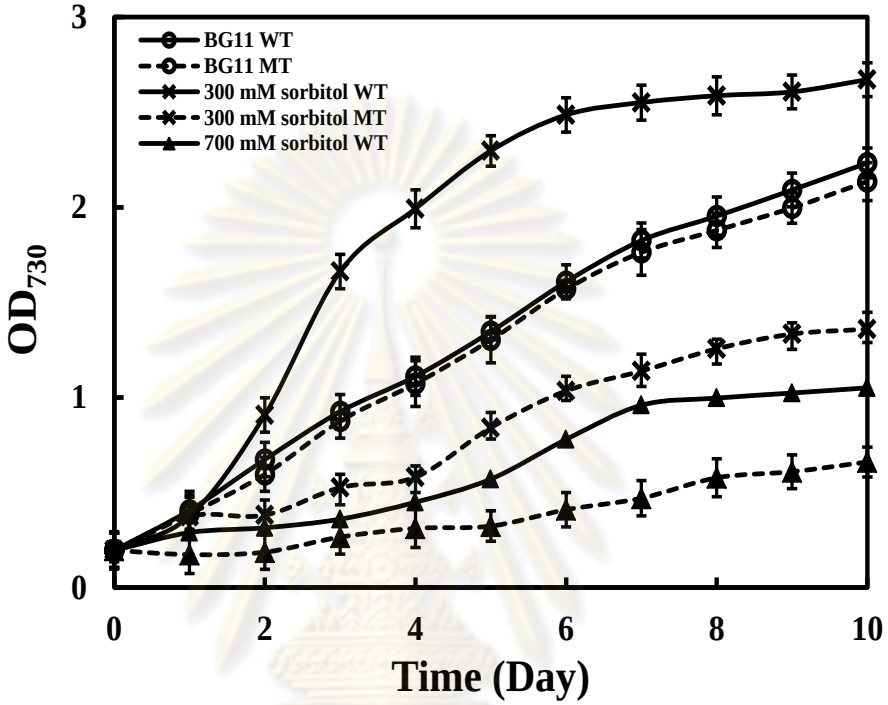


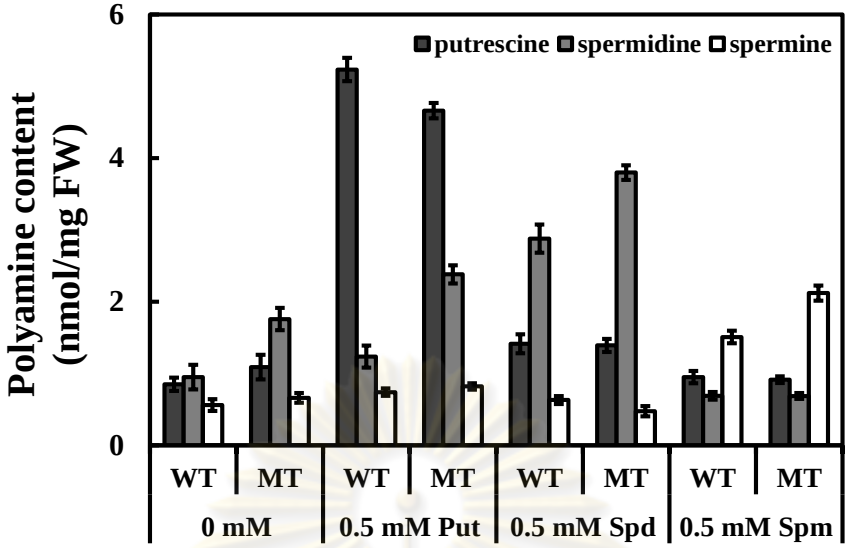
Figure 25 Effect of sorbitol on the cell growth of *Synechocystis* wild-type and mutant cells. Different amounts of sorbitol were added to give different external sorbitol concentrations (300 and 700 mM). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

3.4 Effect of external polyamines on cellular polyamine contents

Total polyamine contents in the cells of *Synechocystis* wild-type and mutant grown in BG11 containing 0.5 mM of each polyamine for 5 days are shown in Figure 26A. For total polyamine contents, they are included both free- and bound-forms of polyamines. The results revealed that mutant cell accumulated putrescine and spermidine with 1.2 and 3.1 fold compared to that of the wild-type, respectively. Additionally, the level of spermine showed no difference between wild-type and mutant. The cells cultured in BG11 containing 0.5 mM putrescine showed higher amount of putrescine than that without the polyamines addition. It should be noted that *Synechocystis* wild-type and mutant can uptake regularly putrescine into the cell. Intriguingly, the increase of spermidine was sharp increased about 2 fold in mutant cell compared to wild-type. Likewise, 0.5 mM spermidine was added to the medium, the putrescine and spermidine contents inside the cells were higher in *potD* mutant than that in wild-type. On the other hand, the level of spermine was not increased in cells cultured in the presence of putrescine and spermine. It was found the overaccumulation of spermine occurred in cells grown with 0.5 mM spermine presence. These findings suggested that mutant cell where *potD* gene was disturbed had the high intracellular spermidine and spermine compare to wild-type cell. Thus, PotD should be involved in the transport of spermidine and spermine, but not putrescine.

Since, the addition of sorbitol had affected cell growth, the accumulated polyamine amount was then determined. The enhancement of polyamine content was established in mutant cultured in 300 mM sorbitol condition. Also, when the sorbitol concentration was rose up to 700 mM, the apparent increase of polyamine was markedly observed (Figure 26B).

A



B

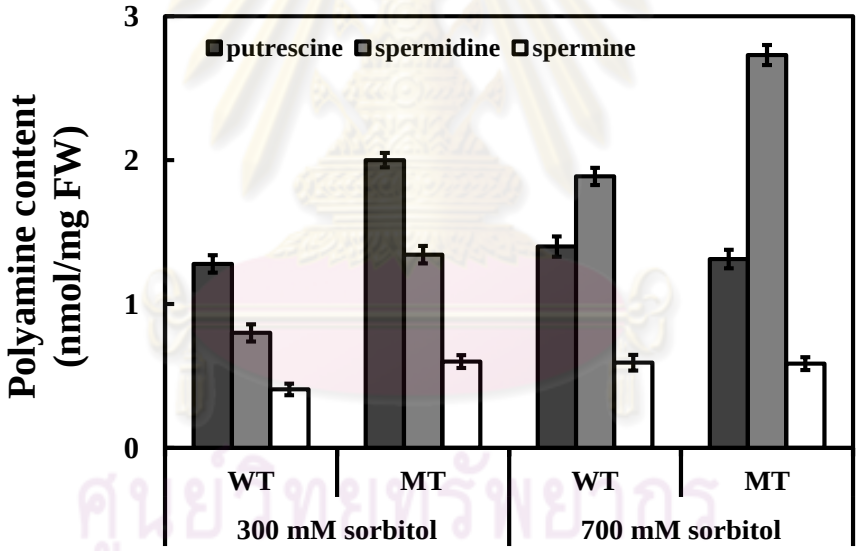


Figure 26 Total polyamine contents in *Synechocystis* wild-type and *potD* mutant cells. Cells were grown in BG11 medium containing 0.5 mM of each polyamine (A) and 300 mM or 700 mM sorbitol (B) for 5 days. The polyamines were extracted and analyzed by HPLC. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

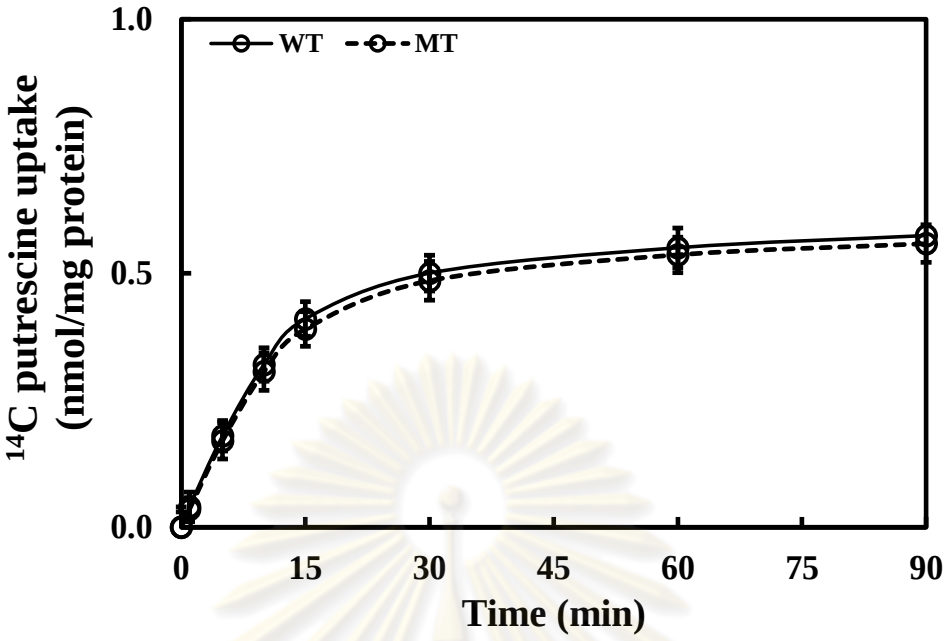
3.4 Polyamine uptake

In order to elucidate whether *potD* is involved in polyamine transport, the uptake and excretion of polyamine were determined in *Synechocystis* wild-type and mutant at interval time for 90 min. *Synechocystis* could immediately uptake ^{14}C polyamine into cells gradually and showed constant uptake after 30 min. The uptake activity of putrescine in both strains was not different overtime observed at start until going to saturation (Figure 27A). On the other hand, *Synechocystis* mutant had about 50% lower uptake activity of spermidine than that in wild-type strain (Figure 27B). Additionally, the polyamine uptake activity of wild-type strain was highest for spermidine with 1.7 and 1.3 fold greater activity than putrescine and spermine, respectively. Then these data demonstrated a significant role of *Synechocystis* PotD in spermidine uptake.

3.4.1 Effect of external NaCl and sorbitol on polyamine uptake

Changes in polyamine uptake activity could be observed when changing NaCl or sorbitol concentration. Figure 28 and 29 revealed that the uptake of polyamines, including putrescine and spermidine, was sensitive to NaCl and sorbitol in both *Synechocystis* wild-type and mutant cells. Increasing NaCl concentration to 10 mM, cells showed the highest polyamine uptake activity. It probably supported that NaCl stimulated the uptake activity. However, the higher concentrations of 50 mM NaCl resulted in dramatic decrease of their uptake. The same tendency results were also shown responded to changing concentration of sorbitol. The elevation of sorbitol concentration up to 20 mM promoted the polyamine uptake. Nevertheless, the diminishing of polyamine uptake was detected when sorbitol concentration was higher than 50 mM. These observations indicated that high concentration of NaCl and sorbitol perturbed the uptake of polyamines in *Synechocystis* cells.

A



B

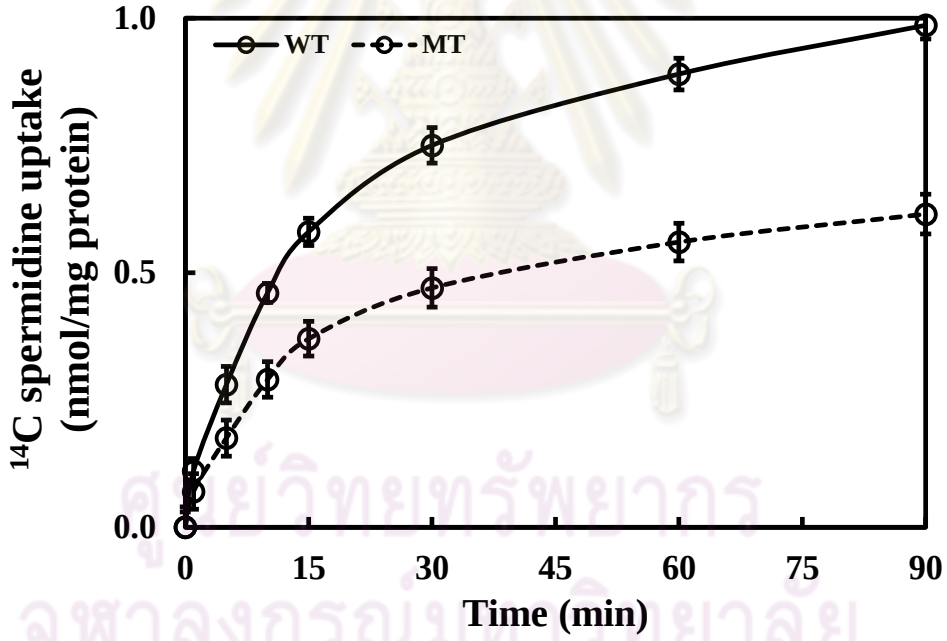
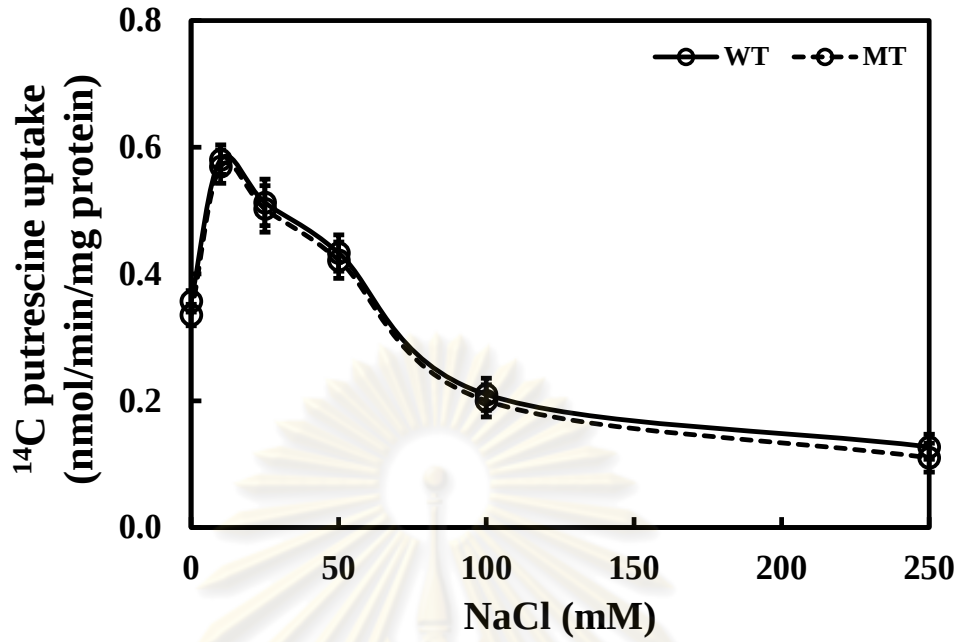


Figure 27 Time intervals of putrescine uptake (A) and spermidine uptake (B) by *Synechocystis* wild-type and mutant cells. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

A



B

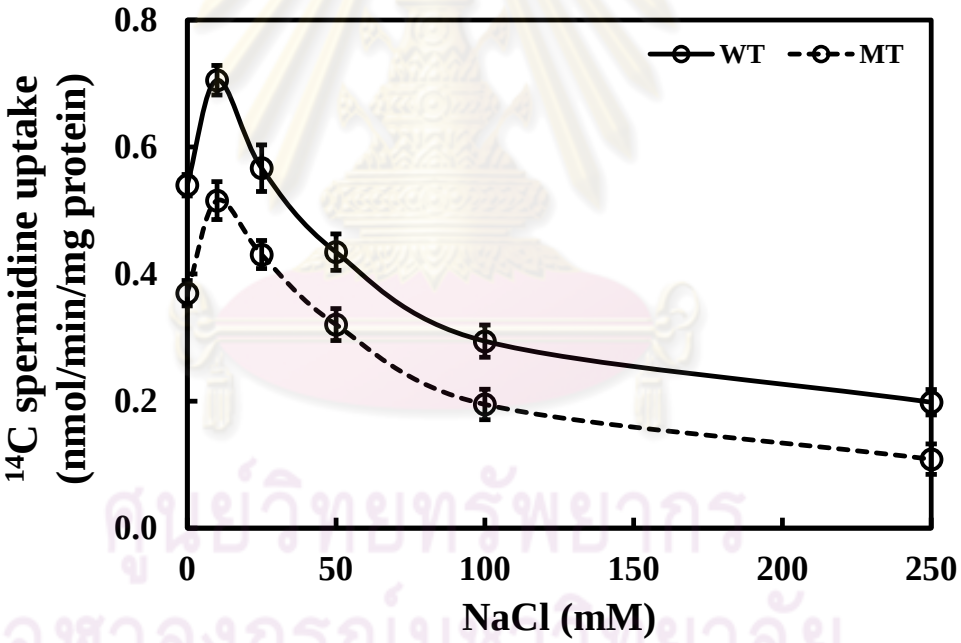
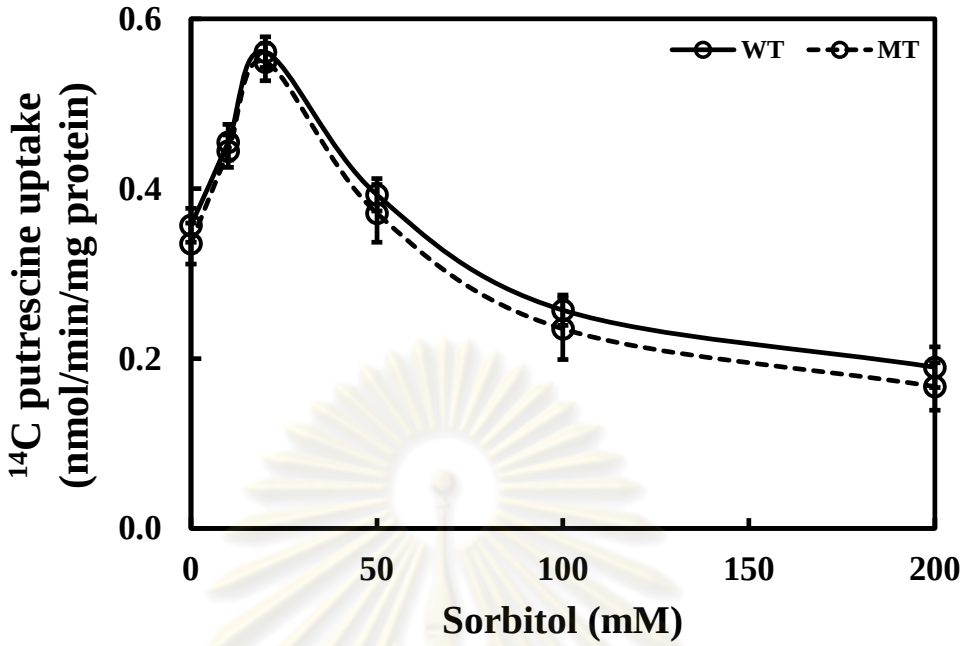


Figure 28 Effect of NaCl on putrescine uptake (A) and spermidine uptake (B) by *Synechocystis* wild-type and mutant cells. Initial uptake rates were determined in the presence of increasing NaCl concentration varied from 0 - 250 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

A



B

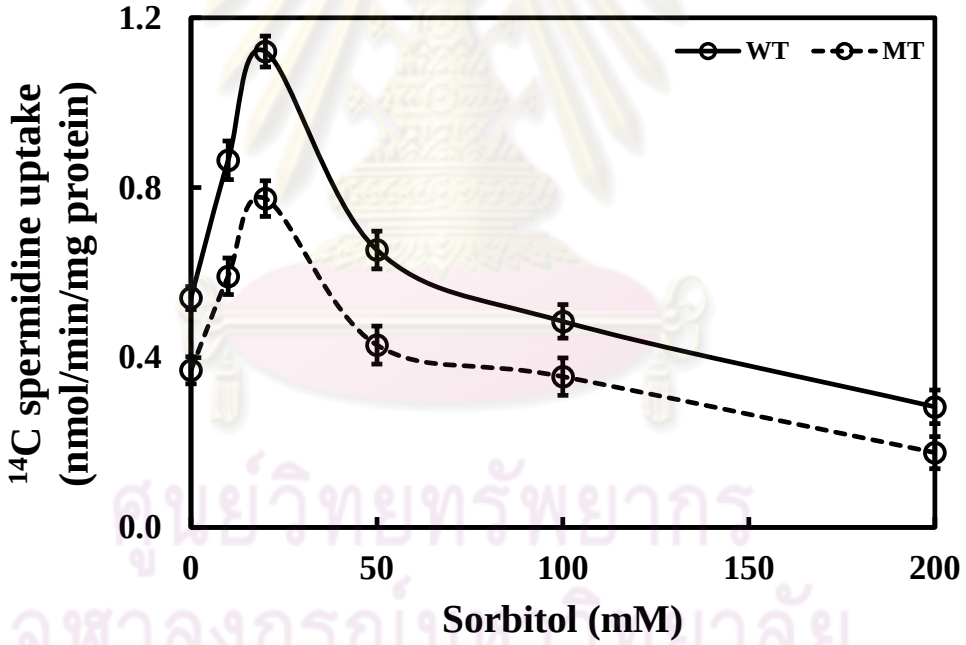


Figure 29 Effect of sorbitol on putrescine uptake (A) and spermidine (B) by *Synechocystis* wild-type and mutant cells. Initial uptake rates were determined in the presence of increasing sorbitol concentration varied from 0 - 200 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

3.4.2 Effect of amino acids on polyamine uptake

In order to investigate whether *Synechocystis* PotD binds specifically to polyamine, the competitive substrates, such as amino acids, were added into the reaction and measured for the uptake rate. The results showed that lysine and asparagine had no effect on the putrescine, spermidine and spermine uptake. The presence of alanine, serine including cadaverine, one of polyamines found in plant, slightly inhibited all putrescine and spermidine uptake rates. On the contrary, putrescine uptake rate was strongly inhibited by 40 and 55% in the presence of spermidine and spermine, which are structurally similar to putrescine but had more amino groups, respectively (Table 5). In the same way, putrescine and spermine showed 40 and 50% inhibitions of spermidine, respectively (Table 6). These results suggested that PotD of *Synechocystis* cell has a high specificity for polyamine uptake.

Table 5 Effect of polyamine analogues on the putrescine uptake of wild-type and *potD* mutant of *Synechocystis.*

Analogs	Putrescine uptake[a] (%)	
	wild-type	mutant
None	100 ± 2	100 ± 3
Lysine	98 ± 4	95 ± 3
Alanine	88 ± 4	84 ± 4
Serine	86 ± 2	83 ± 3
Asparagine	94 ± 5	92 ± 3
Cadaverine	87 ± 2	83 ± 3
Spermidine	63 ± 3	58 ± 2
Spermine	45 ± 3	43 ± 2

[a] Cells were incubated in the mixture containing 1 mM unlabeled analogue and 50μM ^{14}C putrescine. The data shown are the means of three independent experiments representing the percent of putrescine uptake rate relative to the control rate which was 0.14 ± 0.02 and 0.13 ± 0.03 nmol/mg protein for wild-type and mutant, respectively.

Table 6 Effect of polyamine analogues on the spermidine uptake of wild-type and *potD* mutant of *Synechocystis.*

Analogs	Spermindine uptake[a] (%)	
	wild-type	mutant
None	100 ± 3	100 ± 4
Lysine	95 ± 2	94 ± 4
Alanine	85 ± 2	83 ± 3
Serine	83 ± 3	82 ± 3
Asparagine	93 ± 4	91 ± 4
Cadaverine	86 ± 2	85 ± 3
Putrescine	58 ± 3	56 ± 4
Spermine	48 ± 2	46 ± 3

[a] Cells were incubated in the mixture containing 1 mM unlabeled analogue and 50 μM ^{14}C spermidine. The data shown are the means of three independent experiments representing the percent of spermidine uptake rate relative to the control rate which was 0.28 ± 0.04 and 0.17 ± 0.02 nmol/mg protein for wild-type and mutant, respectively.

3.4.3 Effect of metabolic inhibitors on polyamine uptake

We next determined the energy source for polyamine uptake by inhibitor application. The results are shown in Tables 7 and 8 for putrescine and spermidine uptake, respectively. The pattern of inhibitors tested had similar effects on both putrescine uptake and spermidine uptake. The *p*-chloromercurisulfonic acid (PCMS), which is the protein structure modifier, strongly reduced putrescine uptake whereas moderately inhibited spermidine and spermine uptakes. The inhibitors for ATP formation, sodium arsenate and sodium fluoride clearly decreased uptake activity. This suggests directly the requirement of ATP for polyamine transport. The transmembrane potential was disrupted in order to assess the role of electrochemical gradient on polyamine transport. Potassium cyanide, an inhibitor on the electron transport chain, caused effective inhibition on polyamine uptake. Transport uncouplers, gramicidin D, which dissipate proton motive force, could significantly inhibit putrescine, spermidine and spermine uptakes. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nigericin, an ionophore which abolishes transmembrane proton gradient (ΔpH) and the proton motive force (Δp), respectively, showed strongly cease of polyamine uptake. Moreover, the inhibition of uptake rates occurred in the presence of amiloride, a potent inhibitor of many Na^+-coupled transport systems including Na^+/H^+ antiporter. Reagents, which interfere the transmembrane sodium ion gradient such as monensin for a sodium ionophore and ouabain for an inhibitor of the plasma membrane Na^+/K^+-ATPase, caused an effective reduction onto polyamine uptake. The sensitivity of polyamine transport to various types of inhibitors suggests that these uptake systems were ATP-dependent requiring proton motive force.

Table 7 Effect of metabolic inhibitors on the putrescine transport of wild-type and *potD* mutant of *Synechocystis.*

Inhibitors	concentration	putrescine uptake[a]	
		wild-type	mutant
None	-	100 ± 3	100 ± 2
Sodium fluoride	1 mM	26 ± 2	23 ± 4
Ouabain	1 mM	20 ± 3	17 ± 3
DNP	1 mM	32 ± 5	30 ± 4
Sodium arsenate	1 mM	44 ± 5	41 ± 4
PCMS	1 mM	11 ± 2	10 ± 2
KCN	1 mM	42 ± 3	40 ± 3
Nigericin	10 μM	13 ± 2	11 ± 2
Amiloride	100 μM	37 ± 3	35 ± 3
Gramicidin D	100 μM	72 ± 2	70 ± 4
Monensin	100 μM	45 ± 4	42 ± 5
CCCP	100 μM	20 ± 5	19 ± 4
Sodium ionophore	100 μM	63 ± 5	61 ± 2
Chloramphenicol	1 μg/ml	39 ± 3	36 ± 3

[a] Cells were pre-incubated with inhibitors for 30 min before the addition of 50 μM ^{14}C putrescine to initiate the uptake as described in Materials and methods. The data shown are the means of three independent experiments representing the percent of putrescine uptake rate relative to the control rate which was 0.14 ± 0.02 and 0.13 ± 0.03 nmol/mg protein for wild-type and mutant, respectively.

Table 8 Effect of metabolic inhibitors on the spermidine transport of wild-type and *potD* mutant of *Synechocystis.*

Inhibitors	concentration	spermidine uptake[a]	
		wild-type	mutant
None	-	100 ± 3	100 ± 4
Sodium fluoride	1 mM	66 ± 3	65 ± 2
Ouabain	1 mM	58 ± 2	57 ± 3
DNP	1 mM	58 ± 3	54 ± 2
Sodium arsenate	1 mM	64 ± 4	63 ± 4
PCMS	1 mM	68 ± 5	65 ± 2
KCN	1 mM	61 ± 4	57 ± 3
Nigericin	10 μM	57 ± 2	52 ± 4
Amiloride	100 μM	52 ± 2	47 ± 3
Gramicidin D	100 μM	83 ± 3	81 ± 3
Monensin	100 μM	56 ± 4	49 ± 2
CCCP	100 μM	55 ± 3	55 ± 2
Sodium ionophore	100 μM	70 ± 4	67 ± 3
Chloramphenicol	1 μg/ml	68 ± 3	64 ± 2

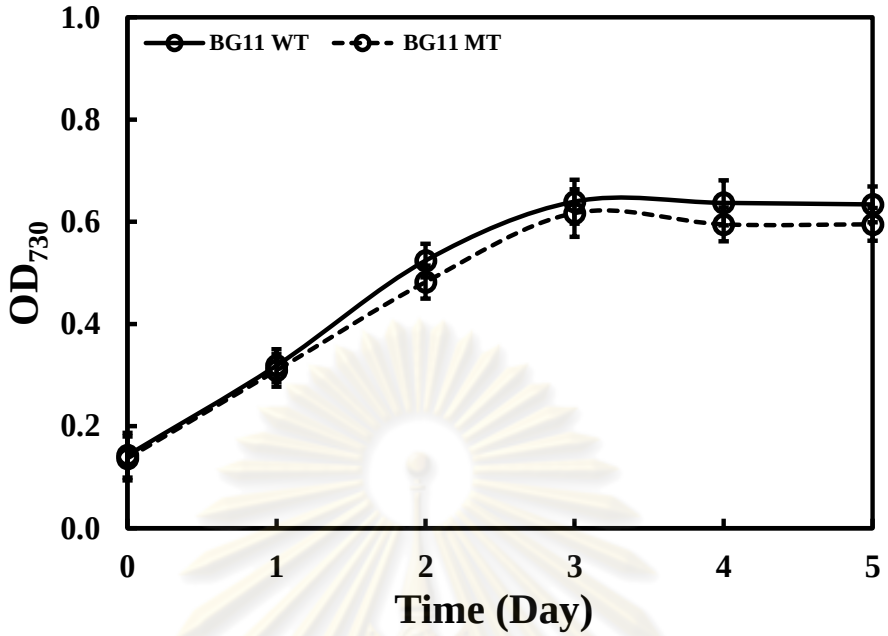
[a] Cells were pre-incubated with inhibitors for 30 min before the addition of 50 μM ^{14}C spermidine to initiate the uptake as described in Materials and methods. The data shown are the means of three independent experiments representing the percent of spermidine uptake rate relative to the control rate which was 0.28 ± 0.04 and 0.17 ± 0.02 nmol/mg protein for wild-type and mutant, respectively.

3.5 Effect of light illumination on *Synechocystis* wild-type and mutant

3.5.1 Effect of light illumination on cell growth

Since light is a major cause of stress for photosynthetic organisms, we further tested whether light had the effect on cell growth or polyamine uptake of cells. Cells were grown in BG11 medium without or with 0.2 mM of putrescine or spermidine. Both of wild-type and mutant grew normally. Cell cultures were started with OD 730 at 0.15 under light intensity which changed from 50 to 250 μmol photons/m^2/s for 5 days. The seemingly slow growth in both of wild-type and mutant was observed in all conditions compared to normal light intensity. The growth of both cells cultured in BG11 medium is shown in Figure 30A and B. There were no major differences in the growth rate of wild-type and mutant, but differences of chlorophyll *a* (chl *a*) content were detected. Three-day culturing showed normal chl *a* content of wild-type, thereafter, chl *a* was reduced. The chl *a* content of mutant was lower than that of wild-type. Similar results were noticed in cells cultured in the presence of 0.2 mM putrescine. Growth rates in this condition were not different between wild-type and mutant. Moreover, the amount of chl *a* was significantly different after 2 days (Figure 31A and B). Interestingly, mutants which grewn normally in BG11 containing 0.2 mM spermidine under light intensity of 50 μmol photons/m^2/s could not grow under 250 μmol photons/m^2/s, while wild-type showed slow growth rate. The amount of chl *a* was consistent with the cells growth rate (Figure 32A and B). From all results subport the fact that, both *Synechocystis* wild-type and mutant were sensitive to light intensity.

A



B

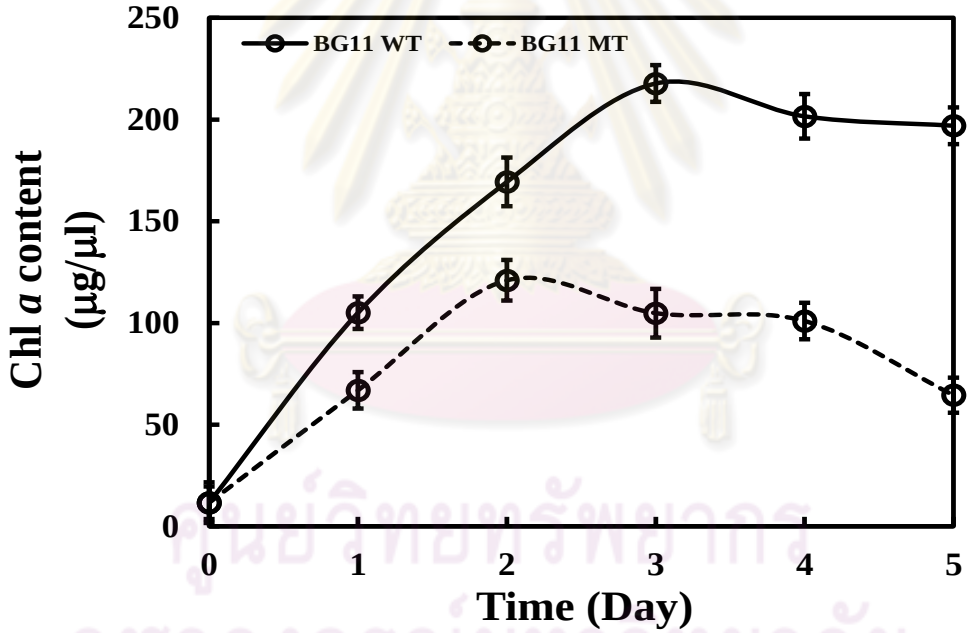
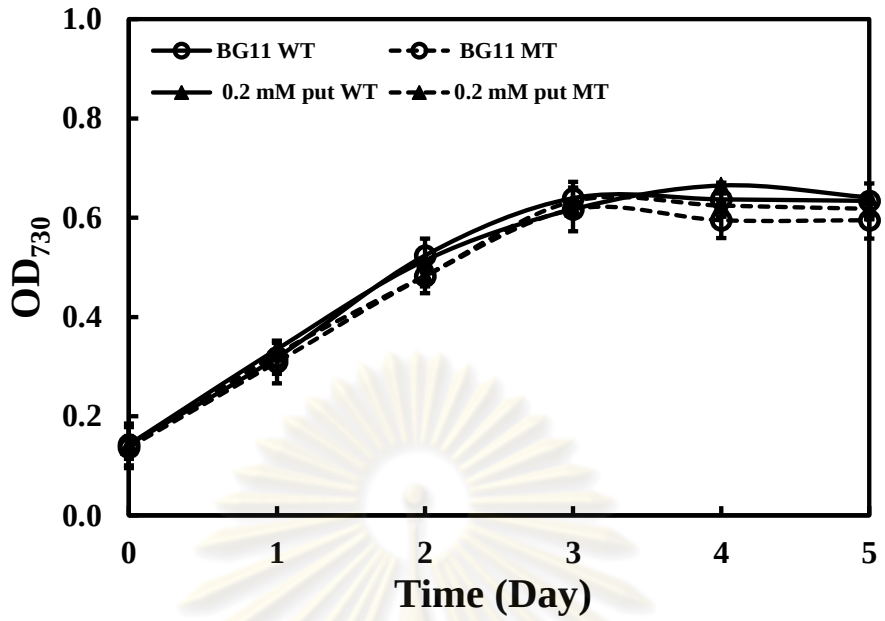


Figure 30 Growth (A) and chlorophyll *a* content (B) of *Synechocystis* wild-type and *potD* mutant grown in BG11 medium under continuous light intensity of 250 µmol photons/m^2/s. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

A



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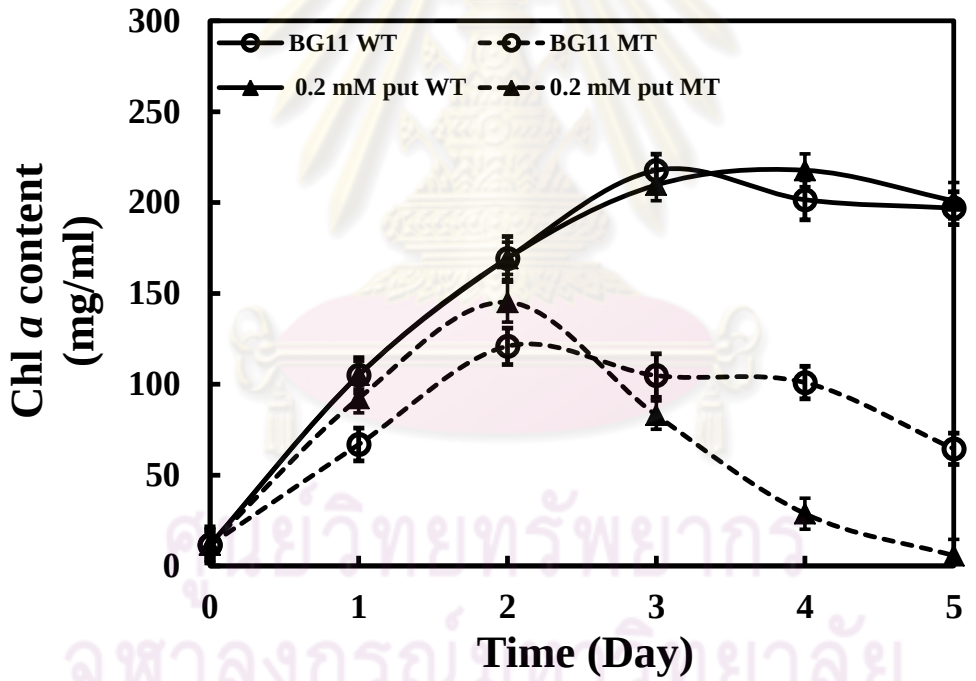
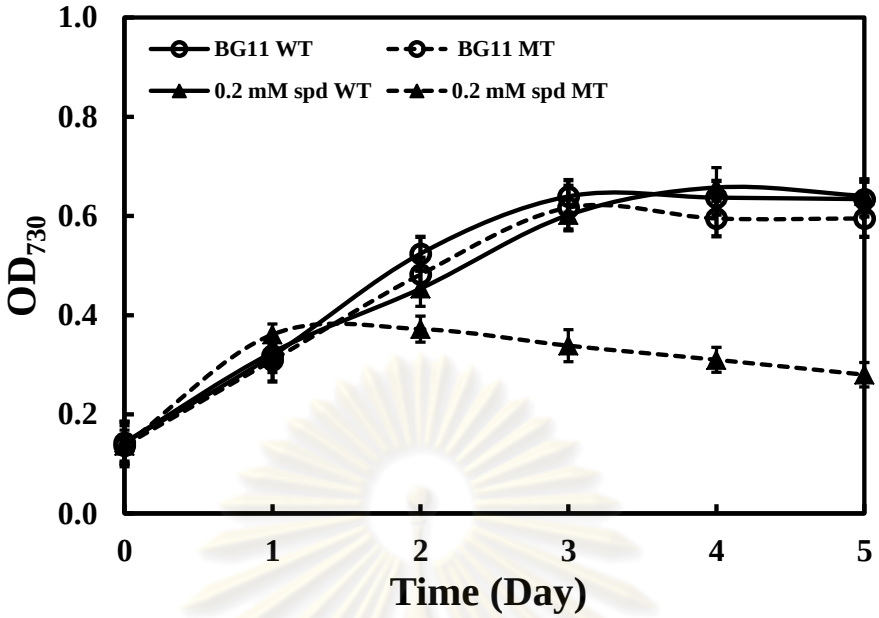


Figure 31 Growth (A) and chlorophyll a content (B) of *Synechocystis* wild-type and *potD* mutant grown in BG11 medium supplemented with 0.2 mM putrescine under continuous light intensity of 250 μmol photons/m^2/s.The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

A



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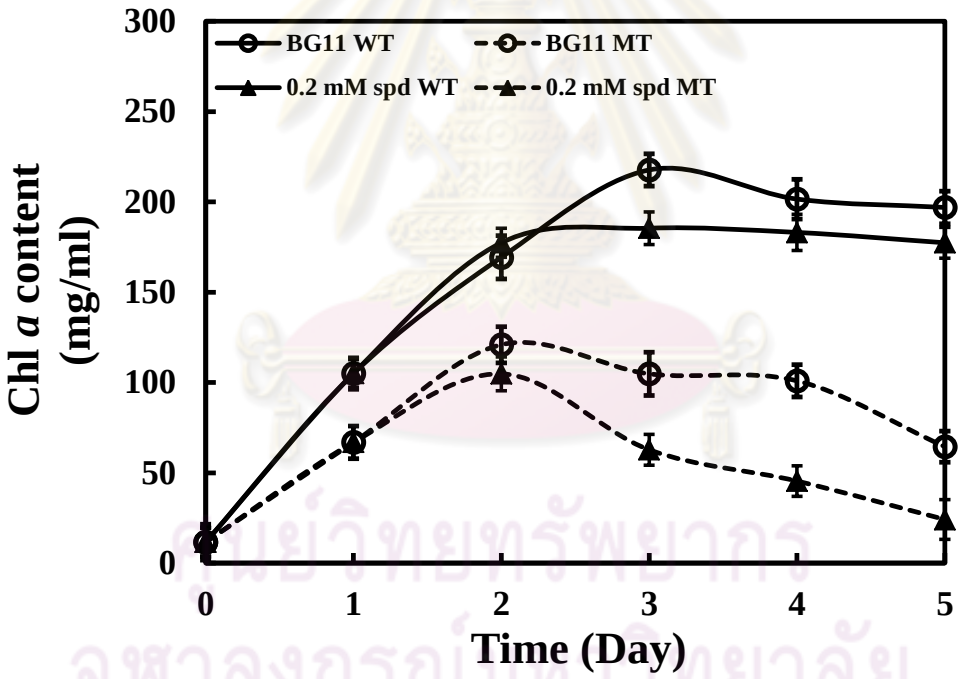
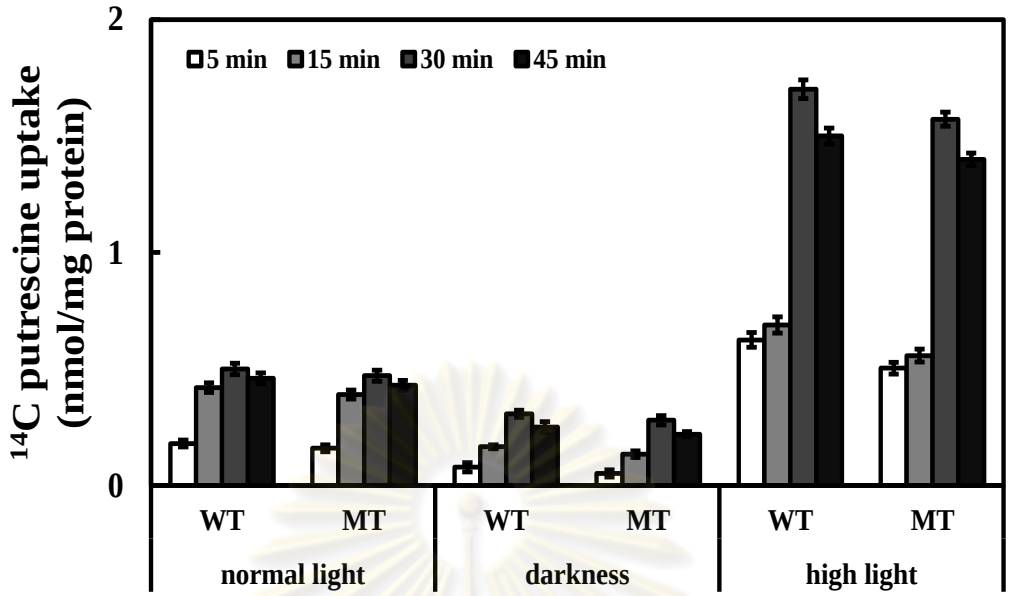


Figure 32 Growth (A) and chlorophyll a content (B) of *Synechocystis* wild-type and *potD* mutant grown in BG11 medium supplemented with 0.2 mM spermidine under continuous light intensity of 250 μmol photons/m^2/s. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3

3.5.2 Effect of light illumination on polyamine uptake

Cells were pre-incubated under darkness and light at either 50 μmol photons/m^2/s (control) or 110 μmol photons/m^2/s (high light) before determing polyamine uptake. The results showed similar uptake rates for the two polyamines in both wild-type and mutant cells. The uptake activity of pre-incubated cells up to 30 min under high light was increased; however, the reduced activity was detected at 45 min. The uptake activity of darkness-exposed cells was slightly declined comparing to the control (Figure 33A). The results showed that cells pre-incubated under high light were able to uptake polyamine better than cells pre-incubated under darkness (Figure 33B). It seems that light energy during uptake was an important contributing factor for polyamine transport system.

A



B

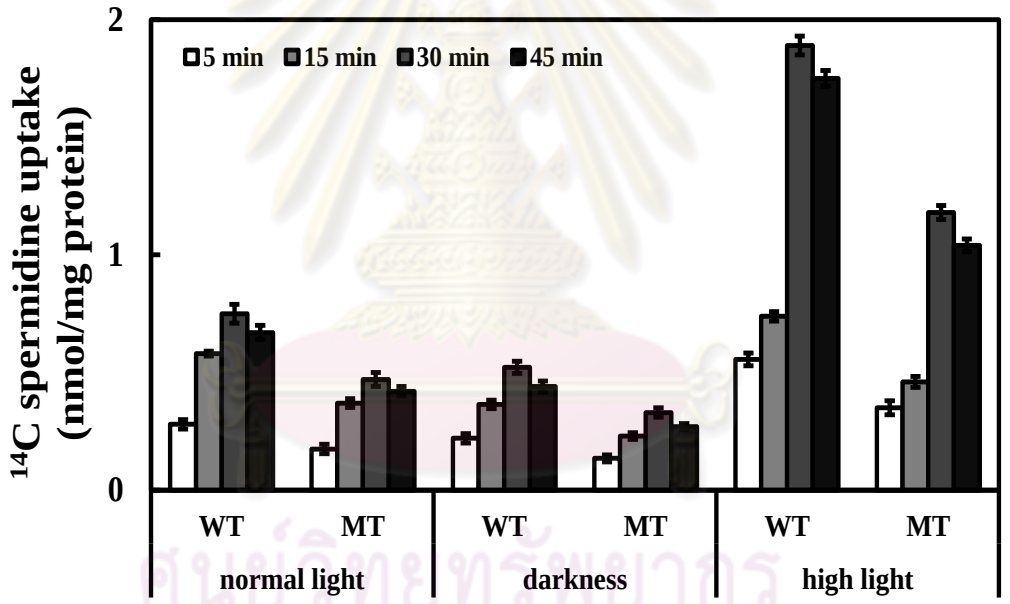


Figure 33 Effect of light stress on putrescine uptake (A) and spermidine uptake (B) by wild-type and *potD* mutant of *Synechocystis*. Initial uptake rates were determined under normal light (50 μmol photons/m^2/s), high light (110 μmol photons/m^2/s) and darkness. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

3.6 Study of photosynthesis system

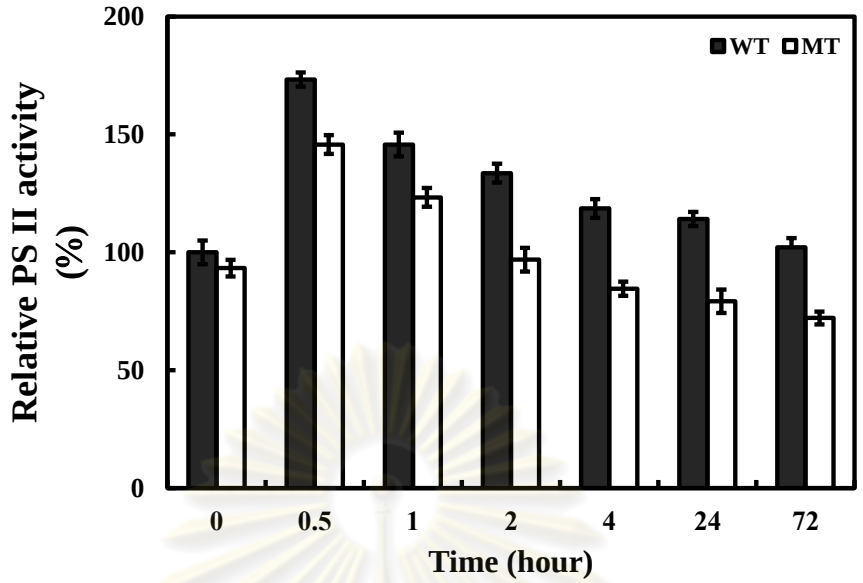
3.6.1 Effect of external polyamine on photosynthesis system

Synechocystis wild-type and mutant cells were grown in BG11 medium for 3 days, then, adapted cells to BG11 medium supplemented with 0.5 mM putrescine or spermidine. Consequently, cells were continuously cultured for 0.5 - 72 hours, harvested cells to further measure both of the photosystem II (PSII) activity and photosynthetic capacity. The results showed that the activity of PSII of wild-type strain was higher than that of mutant strain in all conditions as demonstrated in Figures 34 to 36. Rapidly increasing activity of PSII occurred when culturing time was extended up to 30 min. On the other hand, the activity was slightly reduced after 30 min. In the presence of either putrescine or spermidine, although the PSII activity of wild-type strain was increased, the PSII activity of mutant was declined. Additionally, the photosynthetic capacity seems to be increased in the presence of either putrescine or spermidine. The results suggested that external polyamines are a factor supporting photosynthetic machinery.

3.6.2 Effect of external NaCl and sorbitol on photosynthesis system

Synechocystis wild-type and mutant cells were adapted to BG11 medium supplemented with 550 mM NaCl or 300 mM sorbitol for 0.5 - 72 hours. Both conditions exhibited that PSII activity and photosynthetic capacity was speedily altered during 30 min. Additionally, its PSII activity and photosynthetic capacity was slightly decreased after 30 min (Figures 37 and 38). It seems that either NaCl or sorbitol had the ability to induce the PSII activity and photosynthetic capacity at short time, whereas, slightly inhibited at a longer time.

A



B

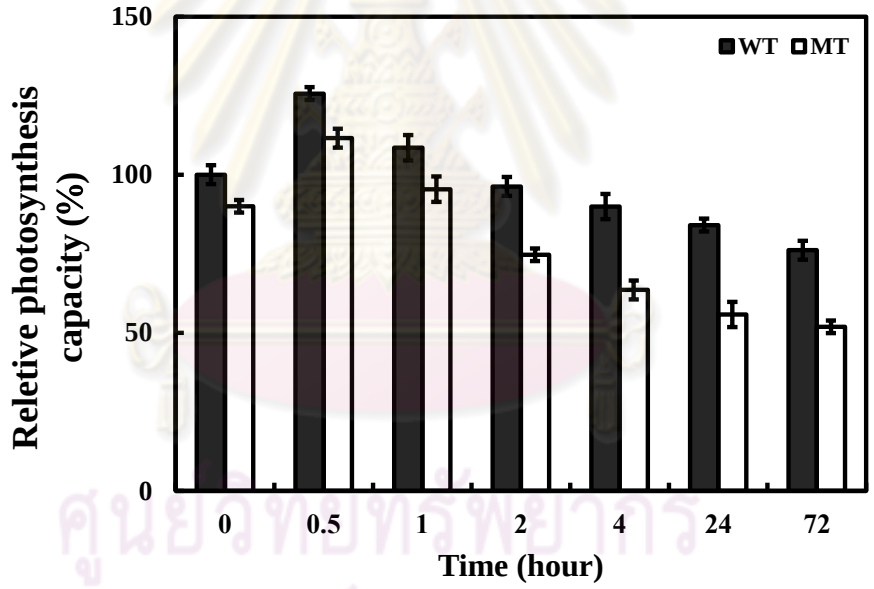
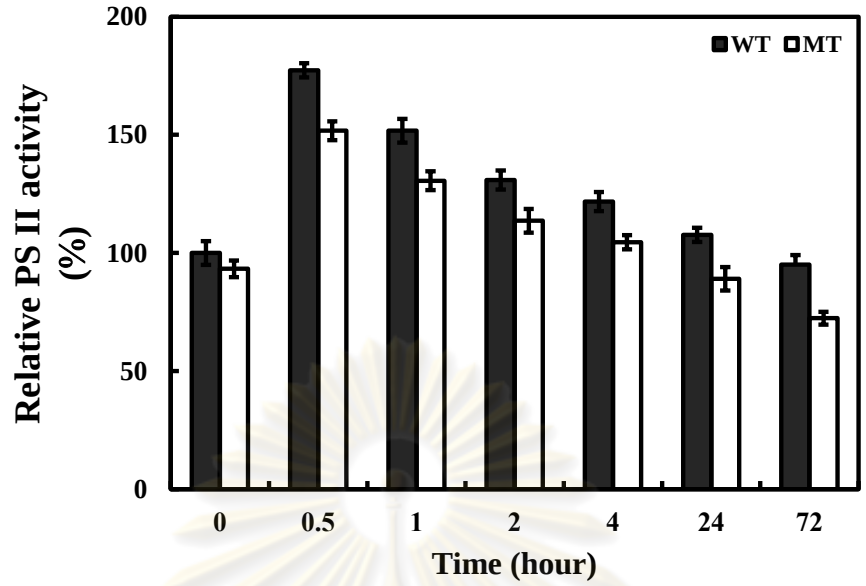


Figure 34 Photosynthetic oxygen evolution of *Synechocystis* wild-type (solid bars) and mutant cells (open bars) measured in a Clark type oxygen under saturating light intensity at 32^{o}C. The relative PSII activity (A) measured with 0.5 mM DCBQ as electron acceptor in the presences of 0.5mM ferricyanide. The PSII activity of wild-type and mutant that corresponded to 100% was 178 ± 3.5 and 166 ± 4 μmol O_2 mg/Chl *a*/hour, respectively. Photosynthetic capacity (B) was determined using 0.6 mM bicarbonate. The Photosynthetic capacity of wild-type and mutant that corresponded to 100% was 125 ± 3 and 113 ± 4 μmol O_2/mgChl *a*/hour. The data represent means ± SE, n=3.

A



B

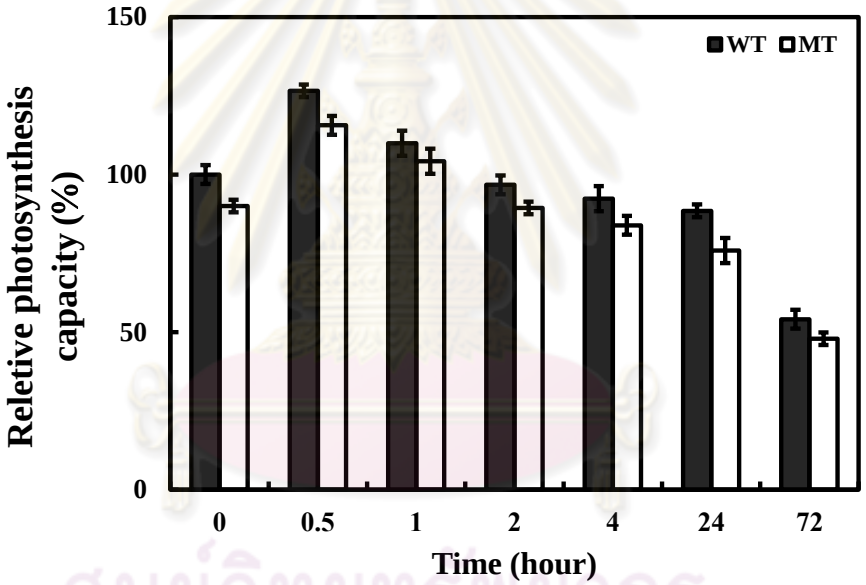
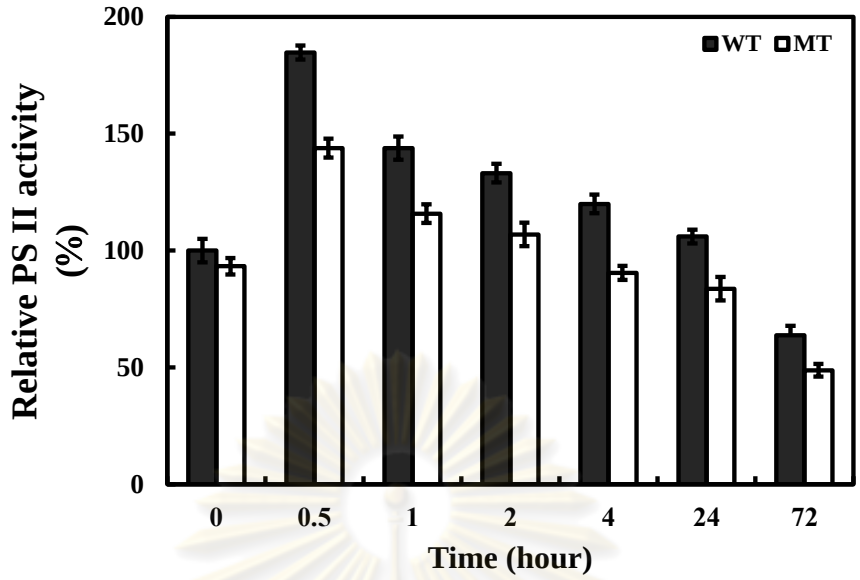


Figure 35 Effect of external putrescine on photosynthetic oxygen evolution of *Synechocystis* wild-type (solid bars) and mutant cells (open bars) measured in a Clark type oxygen under saturating light intensity at 32°C. The relative PSII activity (A) measured with 0.5mM DCBQ as electron acceptor in the presences of 0.5 mM ferricyanide. The PSII activity of wild-type and mutant that corresponded to 100% was 178 ± 3.5 and 166 ± 4 μmol O_2/mgChl *a*/hour, respectively. Photosynthetic capacity (B) was determined using 0.6 mM bicarbonate. The Photosynthetic capacity of wild-type and knockout that corresponded to 100% was 125 ± 3 and 113 ± 4 μmol O_2/mgChl *a*/hour. The data represent means ± SE, n=3.

A



B

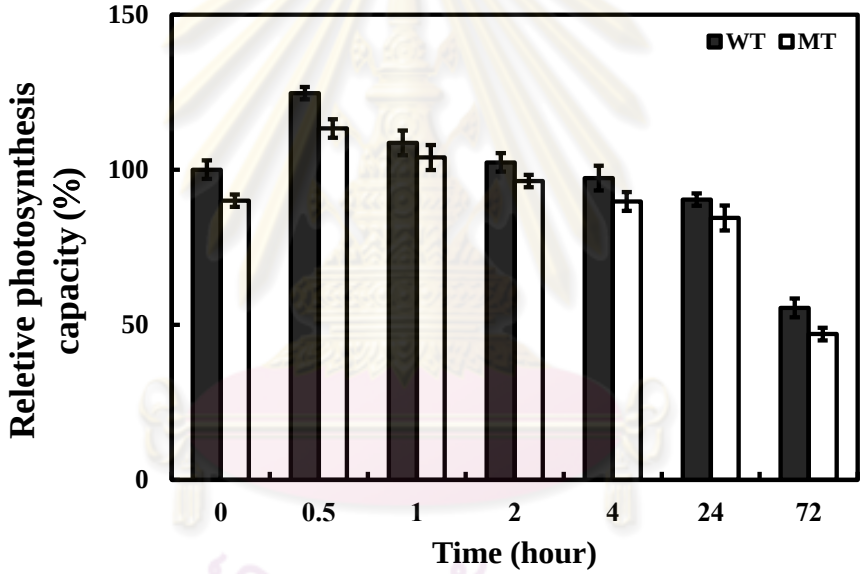
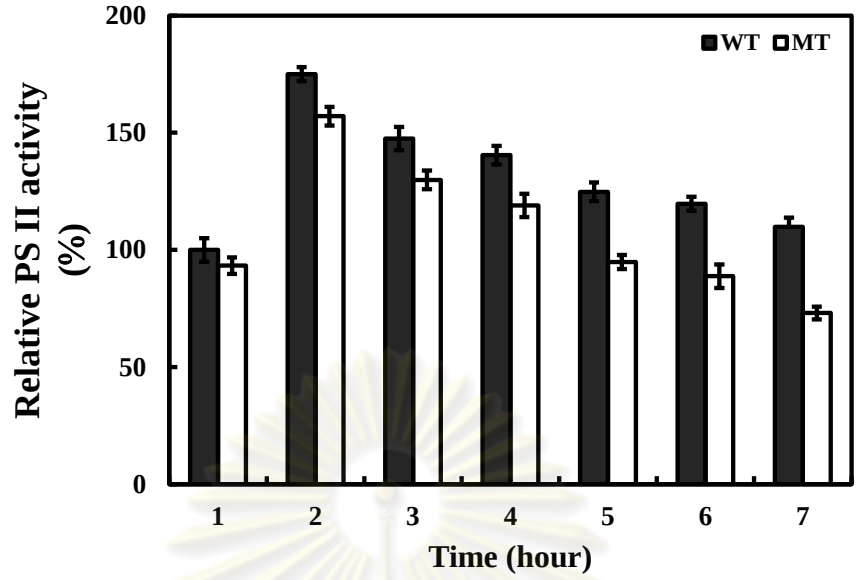


Figure 36 Effect of external spermidine on photosynthetic oxygen evolution of *Synechocystis* wild-type (solid bars) and mutant cells (open bars) measured in a Clark type oxygen under saturating light intensity at 32°C. The relative PSII activity (A) measured with 0.5mM DCBQ as electron acceptor in the presences of 0.5 mM ferricyanide. The PSII activity of wild-type and knockout that corresponded to 100% was 178 ± 3.5 and 166 ± 4 μmol O_2/mgChl *a*/hour, respectively. Photosynthetic capacity (B) was determined using 0.6 mM bicarbonate. The Photosynthetic capacity of wild-type and mutant that corresponded to 100% was 125 ± 3 and 113 ± 4 μmol O_2/mgChl *a*/hour. The data represent means ± SE, n=3.

A



B

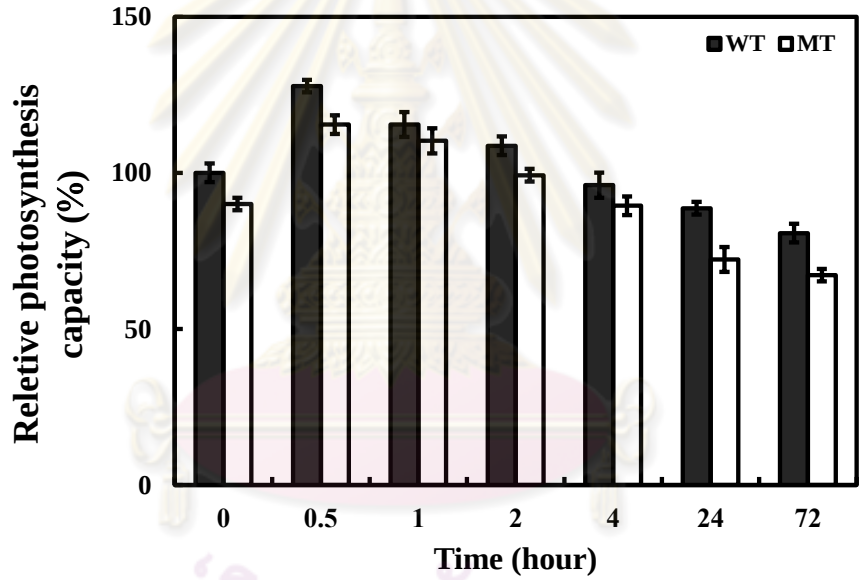
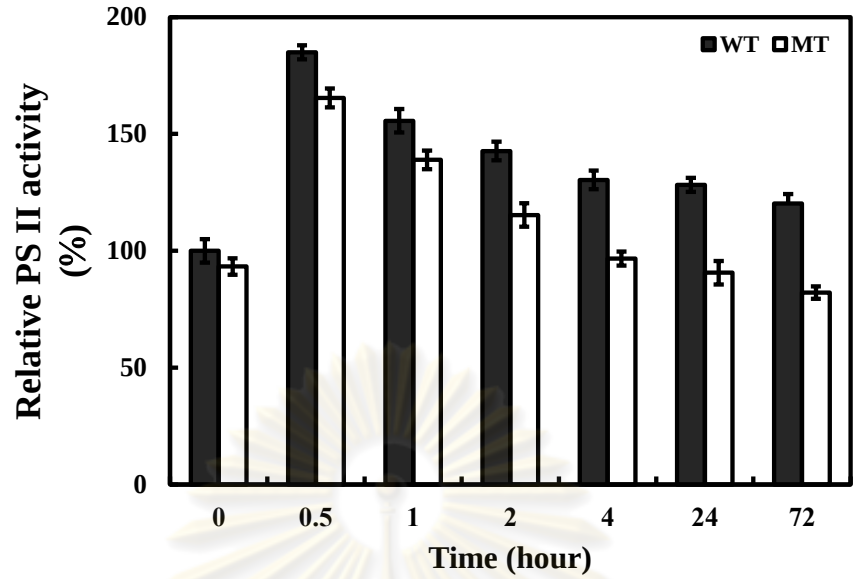


Figure 37 Effect of NaCl on photosynthetic oxygen evolution of *Synechocystis* wild-type (solid bars) and mutant cells (open bars) measured in a Clark type oxygen under saturating light intensity at 32°C. The relative PSII activity (A) measured with 0.5mM DCBQ as electron acceptor in the presences of 0.5 mM ferricyanide. The PSII activity of wild-type and mutant that corresponded to 100% was 178 ± 3.5 and 166 ± 4 μmol O_2/mgChl *a*/hour, respectively. Photosynthetic capacity (B) was determined using 0.6 mM bicarbonate. The Photosynthetic capacity of wild-type and mutant that corresponded to 100% was 125 ± 3 and 113 ± 4 μmol O_2/mgChl *a*/hour. The data represent means ± SE, n=3.

A



B

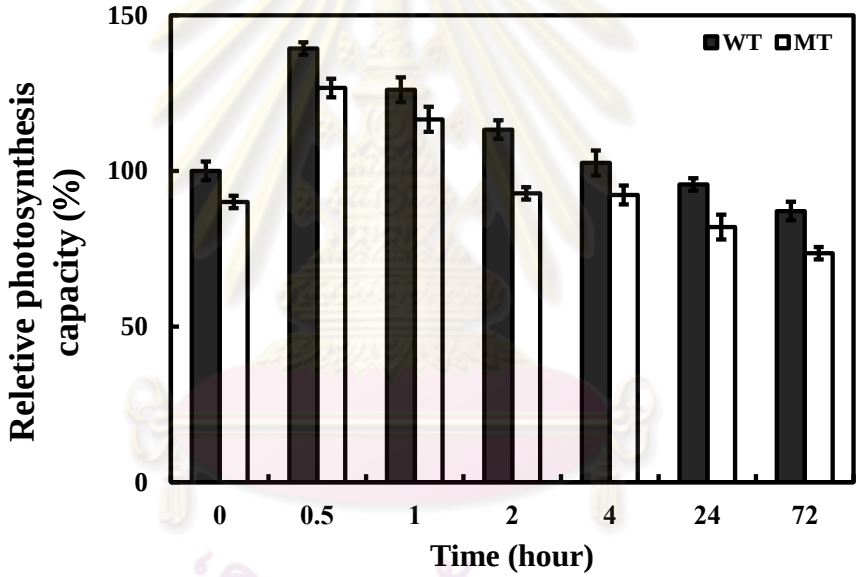


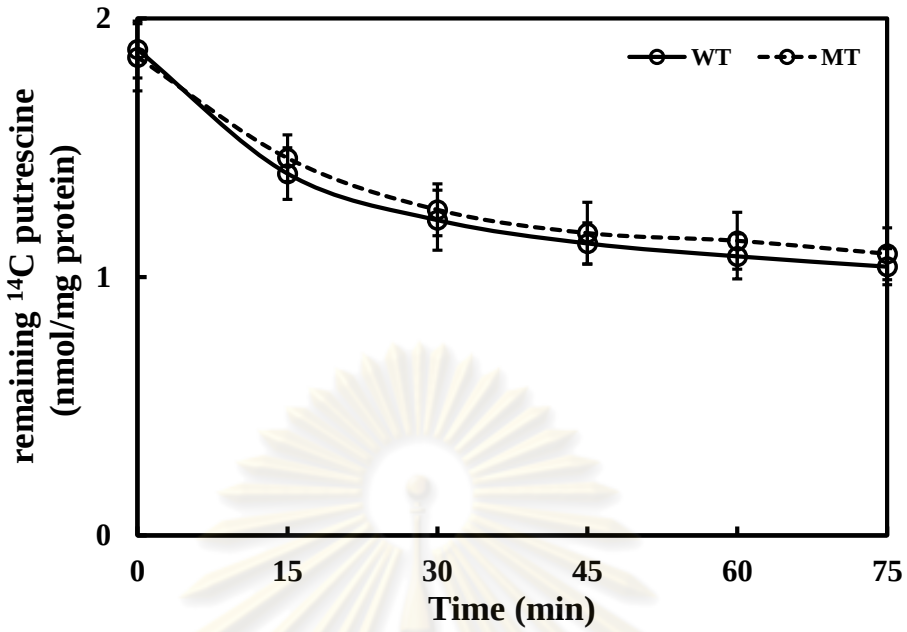
Figure 38 Effect of sorbitol on photosynthetic oxygen evolution of *Synechocystis* wild-type (solid bars) and mutant cells (open bars) measured in a Clark type oxygen under saturating light intensity at 32°C. The relative PSII activity (A) measured with 0.5mM DCBQ as electron acceptor in the presences of 0.5 mM ferricyanide. The PSII activity of wild-type and mutant that corresponded to 100% was 178 ± 3.5 and 166 ± 4 μmol O_2/mgChl *a*/hour, respectively. Photosynthetic capacity (B) was determined using 0.6 mM bicarbonate. The Photosynthetic capacity of wild-type and mutant that corresponded to 100% was 125 ± 3 and 113 ± 4 μmol O_2/mgChl *a*/hour. The data represent means ± SE, n=3.

3.7 Excretion of polyamine by *Synechocystis* wild-type and mutant strains

When the polyamine contents were measured in cells cultured with or without 0.5 mM of each polyamine, overaccumulation of polyamines was observed (especially spermidine). It was then tested whether *Synechocystis* can excrete polyamine using PotD protein. The experiment was examined by using cell preloaded with each ^{14}C polyamine, then, measured the remaining of ^{14}C polyamine inside the cells. No differences in putrescine excretion between wild-type and mutant cells were observed. The excretion of spermidine in wild-type strain was significantly 40% higher than mutant strain (Figure 39A and B). The results indicated that PotD plays the role of spermidine excretion from *Synechocystis* cell.

In addition to the increased polyamine transport under acidic pH, we furthermore studied whether external pH influenced polyamine excretion. The reaction mixture pH was adjusted from pH 7.5 to 6.0. The major difference of polyamine excretion rates was observed between wild-type and mutant cells. The putrescine excretion rate at pH 6.0 in wild-type cell had the 20% higher activity than the mutant cell, nevertheless no difference rate was observed in both strains under pH 7.5 (Figure 40A). The excretion of spermidine in mutant cell had the 60% excretion activity compared to wild-type cell (Figure 40B). The results revealed that acidic pH affected excretion ability of polyamines in *Synechocystis* cell.

A



B

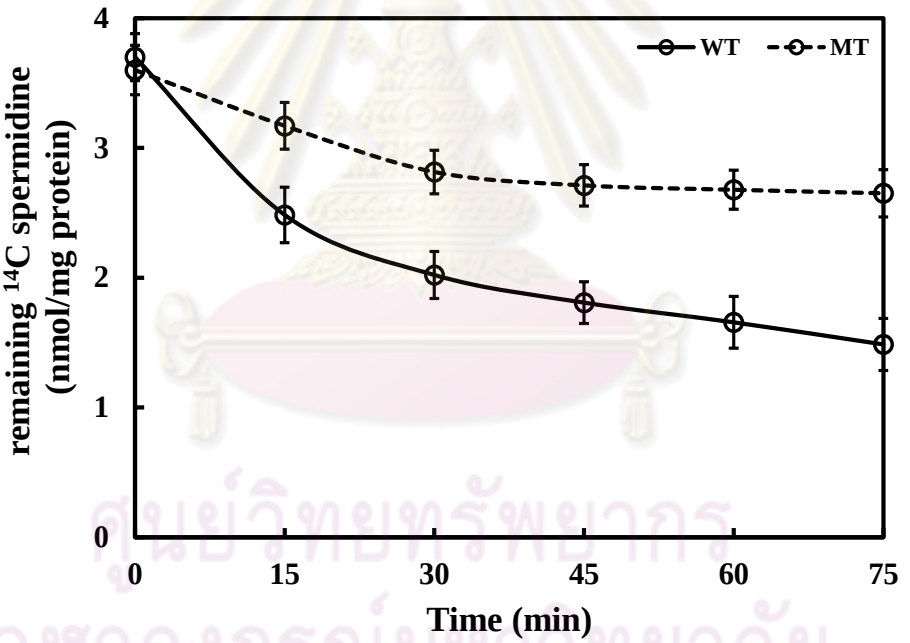
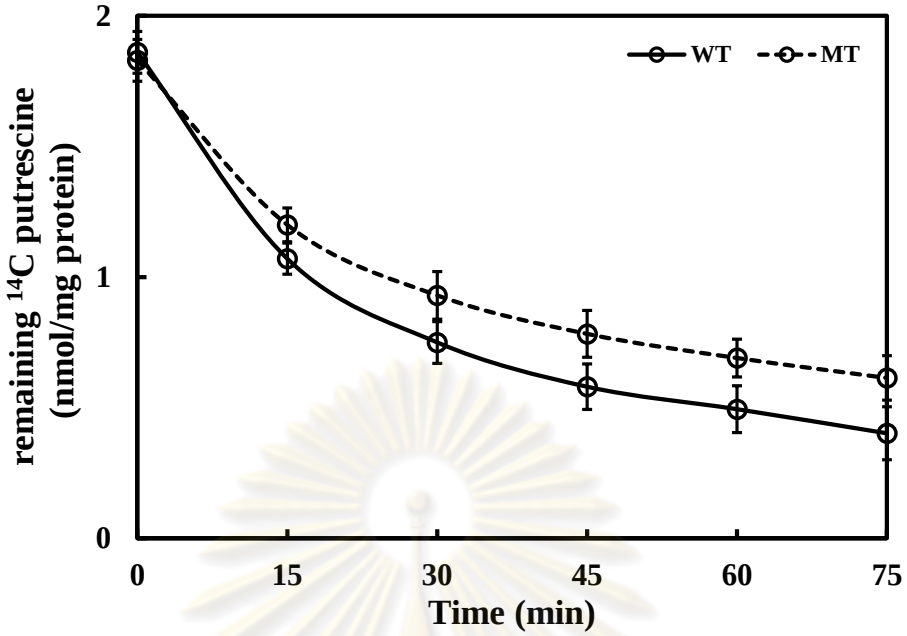


Figure 39 Remaining of preloaded ^{14}C putrescine (A) and ^{14}C spermidine (B) in *Synechocystis* wild-type and mutant cells. The reaction of excretion was done at pH 7.5. The data represent means ± SE, n=3.

A



B

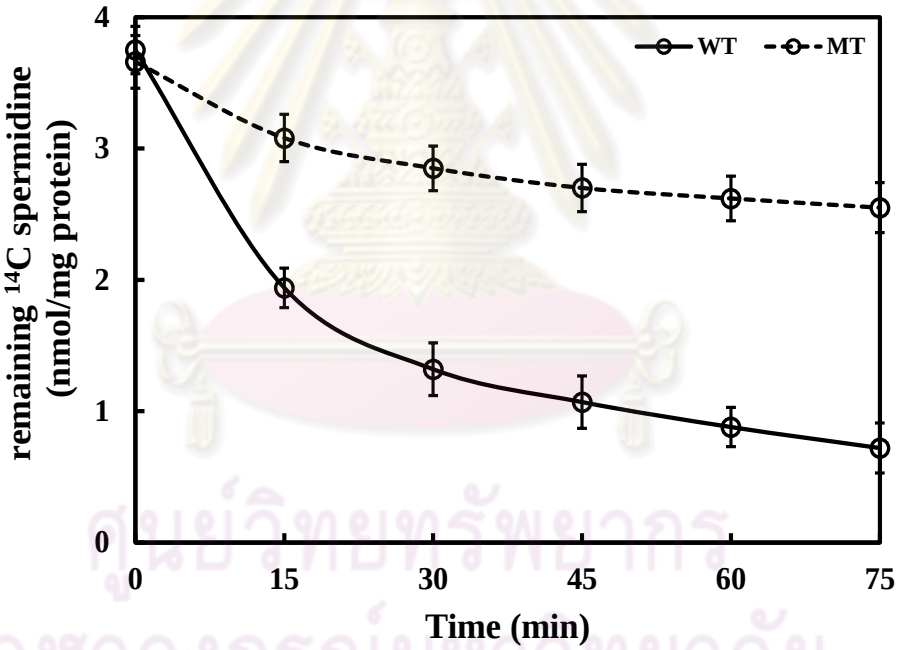


Figure 40 Remaining of preloaded ^{14}C putrescine (A) and ^{14}C spermidine (B) in *Synechocystis* wild-type and mutant cells. The reaction of excretion was done at pH 6.0. The data represent means ± SE, n=3.

3.8 Immonoblotting assay on PotD protein

Synechocystis wild-type was grown in BG11 medium for 5 days. Consequently, soluble and membrane proteins were isolated and subjected to immunoblot analysis. Forty-three kDa of band corresponding to the predicted molecular weight of PotD protein was detected in the soluble protein fraction but it was absent from the membrane protein fraction (Figure 41).

3.8.1 Effect of external polyamine on PotD protein

We further studied influence of external polyamines on PotD expression by using immunoblot analysis with specific PotD antiserum. *Synechocystis* wild-type was grown in BG11 medium containing either 0.1 mM or 0.5 mM of each polyamine. The PotD level was examined at 2, 24 and 120 hours. For first 2 and 24 hours of exposed cells, it was found that the presence of 0.1 mM putrescine in the medium did not affect the *Synechocystis* PotD level. However, the presence of 0.5 mM putrescine resulted in a slight increase of PotD level. Besides, the presence of 0.1 and 0.5 mM spermine caused the slight increase of PotD level. Five days exposure of cells showed the significantly changed PotD level. PotD was moderately increased about 1.3 fold by putrescine (Figure 42). Surprisingly, exposure of spermidine resulted in enormously elevated amount of PotD about 2.7 fold (Figure 43). On the other hand, the results showed a clear decrease in the amount of the PotD protein about 1.5 fold due to spermine (Figure 44). The observations confirmed that PotD was involved in polyamines transport with preference towards spermidine.

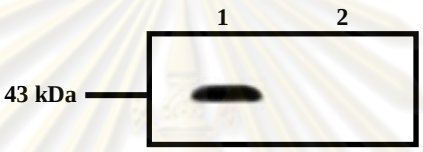


Figure 41 Western blot analysis of PotD in soluble protein fraction and membrane protein fraction probed with polyclonal rabbit immune serum against purified rPotD. Lane 1 is PotD protein in soluble fraction; Lane 2 is PotD protein in membrane fraction.

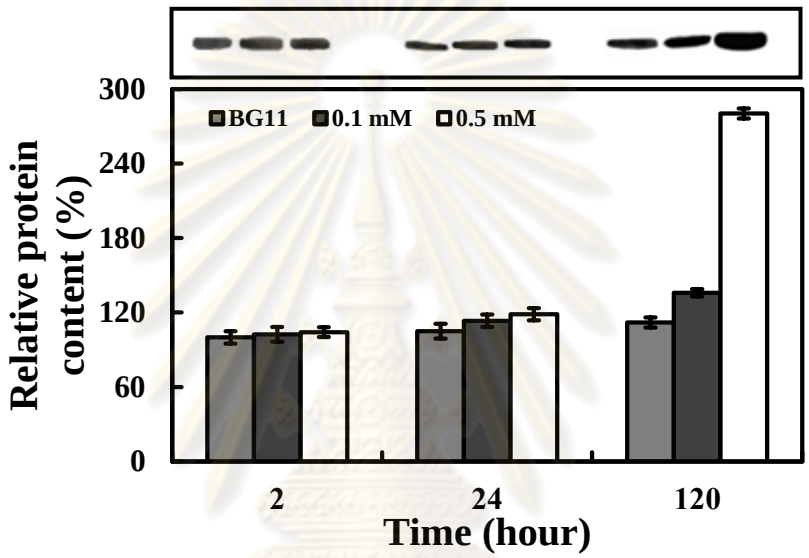


Figure 42 Western blot analysis of PotD induction by 0.1 or 0.5 mM putrescine. *Synechocystis* cells grown for 2, 24 and 120 hours were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

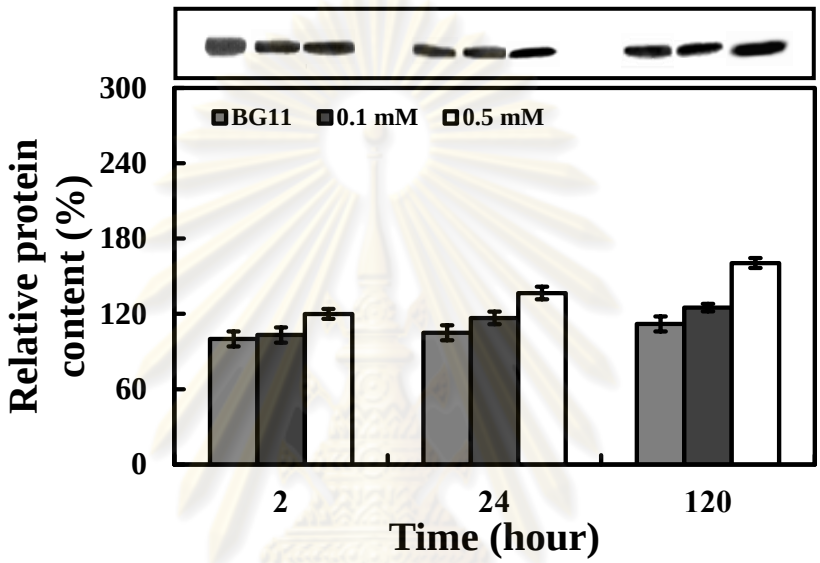


Figure 43 Western blot analysis of PotD induction by 0.1 or 0.5 mM spermidine. *Synechocystis* cells grown for 2, 24 and 120 hours were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

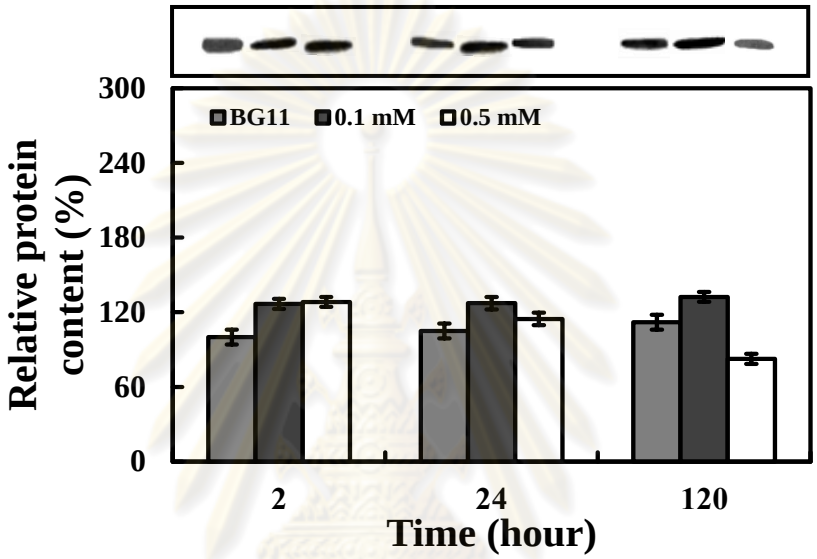


Figure 44 Western blot analysis of PotD induction by 0.1 or 0.5 mM spermine. *Synechocystis* cells grown for 2, 24 and 120 hours were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

3.8.2 Effect of external NaCl and sorbitol on *Synechocystis* PotD protein

Afterwards, we further estimated whether *Synechocystis* PotD levels were changed under osmotic stress generated by NaCl and sorbitol. The amount of *Synechocystis* PotD dramatically decreased at 2 hours when cells were incubated with 250 mM and 550 mM NaCl compared to control. Nevertheless, it was interesting that the amount of PotD remained relatively unchanged at 24 hours. Furthermore, the small increase of PotD level was observed after 120 hours in the presence of 550 mM NaCl (Figure 45).

Likewise, two-hour exposure to 150 mM and 300 mM sorbitol resulted in declined amount of PotD protein. At the long-exposure, sorbitol generated the higher amount of PotD than control. Cells exposed to 150 mM sorbitol had significant increase of PotD level. The small amount of PotD was increased by 300 mM sorbitol (Figure 46). The changes of PotD level might be due to the influence of NaCl and sorbitol.

3.8.3 The combination effect of external polyamine and environmental stresses on PotD protein

We thereafter estimated the induction of PotD in the presence of 0.5 mM of each polyamine including either 550 mM NaCl or 300 mM sorbitol. *Synechocystis* cells grown for 5 days under various conditions were analyzed. The results showed no synergistic effect on the increase of PotD by the simultaneous presence of polyamine and either NaCl or sorbitol (Figure 47).

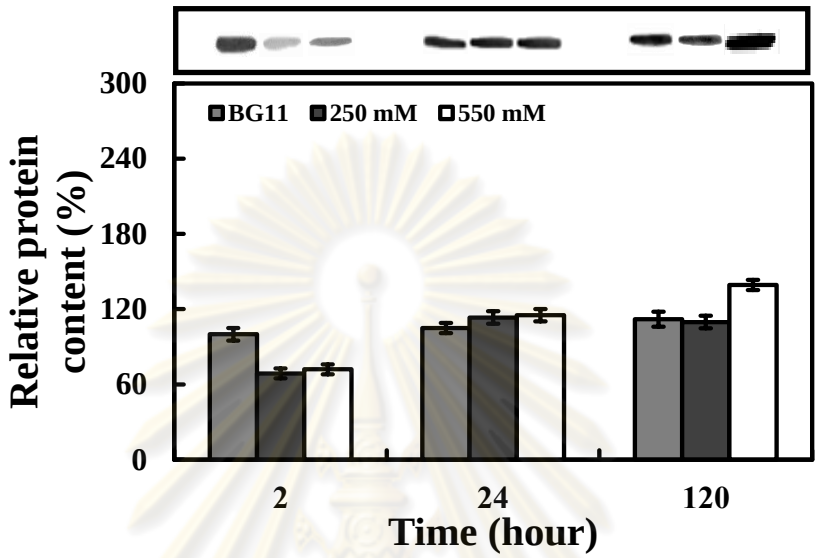


Figure 45 Western blot analysis of PotD induction by either 250 or 550 mM NaCl. *Synechocystis* cells grown for 2, 24 and 120 hours were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

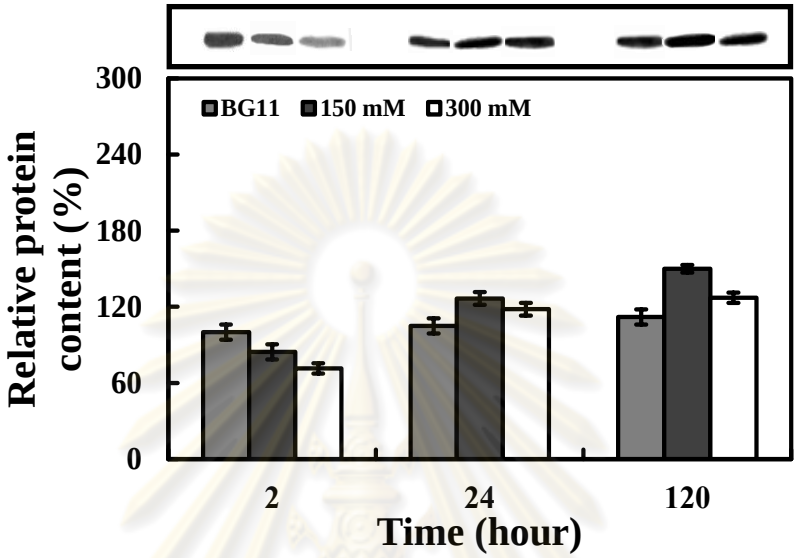


Figure 46 Western blot analysis of PotD induction by either 150 or 300 mM sorbitol. *Synechocystis* cells grown for 2, 24 and 120 hours were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

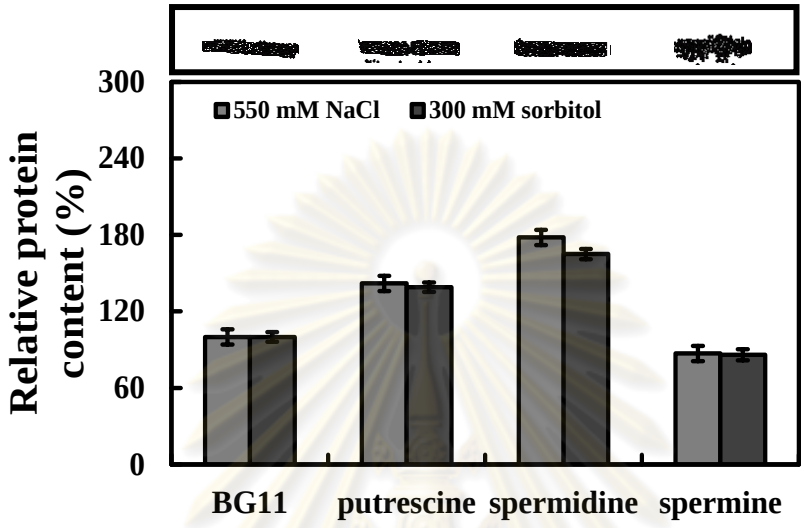


Figure 47 Western blot analysis of PotD induction through the combination of 0.5 mM polyamine and osmotic stress imposed by 550 mM NaCl or 300 mM sorbitol. The mean values (± SE) are calculated from the results representing three independent biological replicates. The top panel shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

CHAPTER IV

DISCUSSION

We have demonstrated that *Synechocystis* sp. PCC 6803 is capable of taking up exogenous polyamines passing through ATP-binding cassette (ABC)-type transporter although the mechanism of their interactions at the molecular level remains unclear (Raksajit *et al.*, 2006 and 2009). Therefore, we have studied the *in vitro* binding capacity of polyamines to a binding protein of *Synechocystis* in order to clarify the characteristics of polyamine transport. Sets of genes involved in polyamine uptake have been identified in the annotated genome of *Escherichia coli* so far (Igarashi and Kashiwagi, 1999). Unlike *E. coli* and other bacterial species genes, the gene encoding the binding component subunit of *Synechocystis* (PotD) are not physically linked to those encoding the hydrophobic components, suggesting alternative regulatory pathways controlling the balanced expression of the transport system components (Igarashi and Kashiwagi, 1999). The transporters in cyanobacteria consist of several polypeptide subunits but their structure is not much related to the corresponding *E. coli* ones. Moreover, the low homology between the PotD in *E. coli* and *Synechocystis* subunits does not allow identification of the *Synechocystis* ones using homology searches. Hence, it is necessary to clarify the details of the transport mechanism in *Synechocystis* since it is not obvious that the functions of PotD are identical in *E. coli* and *Synechocystis*. For attempts to express the *Synechocystis* PotD, then the *potD* gene (*slr0401*) was amplified by using pair of specific primers. The blastn sequence-alignment result showed 100% homology to *potD* gene which was published in cyanobase (http://genome.kazusa.or.jp/cyanobase). Moreover, this sequence also showed 5e-93 (85%) homology to periplasmic polyamine binding protein of *Cyanothece* sp. PCC 7474 (ACC. No. PC001291). Consequently, *potD*

gene was cloned and overexpressed in *E. coli* BL21(DE3) as a recombinant His-tagged protein (rPotD). The SDS-PAGE gel showed an overexpression of the His-tagged PotD, with an apparent molecular mass of 43 kDa, which is slightly higher than the theoretical full-length native form of PotD (41 kDa) which is previously reported in other bacteria (Shah and Swiatlo, 2006; Sugiyama *et al.*, 1996a and b; Svensson *et al.*, 2006). Because most of the expressed recombinant PotD protein is accumulated intracellularly as inclusion bodies, the high recovery yields of soluble rPotD are routinely attained to allow purification of the recombinant protein at near homogeneity (Balan *et al.*, 2005). To evaluate the integrity and biological activity of the purified protein, the polyamine-binding property of the recombinant protein is then evaluated in binding assays. It is worth noting that the additional histidine residues of rPotD do not perturb the biological activity of polyamine transport (Svensson *et al.*, 2006).

The rPotD is capable of binding putrescine, spermidine as well as spermine although the affinity for putrescine is about 2 fold lower than that for spermidine (Figure 12). The maximum binding (B_{max}) values of putrescine, spermidine and spermine were 0.74, 1.42 and 0.13 mol/mol rPotD, respectively. The data indicate that the three polyamines bind to rPotD with a 1:1 binding stoichiometry based on the molecular mass for rPotD of 43000. The seemingly low values of less than 1 for putrescine and spermine might be due to the dissociation of the complex occurring during the filtration/washing of the membrane before counting the radioactivity remaining on the membrane. Another possibility is that during a refolding process a portion of the improper refolded rPotD was incompetent to bind polyamines. Moreover, the K_d values for polyamine binding in this report are different from those previously reported by Brandt *et al.* (2010) using Surface Plasmon Resonance (SPR) method for binding studies. SPR is an optical method that

measures differences in refractive index close to the surface of modified gold (Oskarsson and Holmberg, 2006). The rPotD must be immobilized on the surface of modified gold and as a result, the protein could not maintain its native form. In contrast, the binding studies using radioactive polyamines are quite sensitive and the native form of rPotD was maintained during the experiment. Polyamines can effectively bind to the binding site of rPotD giving rise to reliable K_d values for the binding of polyamines.

Comparison of the affinity of rPotD for different polyamines reveals that both spermidine and spermine, which similarly bound to rPotD according to the results of docking experiments than putrescine. Our results were in agreement with those in *E. coli* PotD which favors the binding of spermidine (K_d = 3.2 μM) rather than putrescine (K_d = 100 μM) (Kashiwagi *et al.*, 1993). On the contrary, *E. coli* PotF shows a high binding affinity to only putrescine (K_d = 2.0 μM) and does not bind other polyamines (Vassylyev *et al.*, 1998). Likewise, TpPotD of *Treponema pallidum* (PotD homologue) also exhibits a higher binding affinity for putrescine (K_d = 10 nM) than spermidine (K_d = 430 nM) (Machius *et al.*, 2007). These results may indicate the different characteristics of polyamine binding sites among different organisms.

Worth noticing is that, at alkaline pH 8.0, *Synechocystis* cells are more proficient to take up spermidine than putrescine (Raksajit *et al.*, 2009). Similarly in the current study, rPotD also had the highest binding capacity for spermidine at pH 8.0 compared to those for spermine and putrescine (Figure 13). The effect of pH on the binding activity of rPotD indicates that the protonation state of the target amino acids involved in polyamines binding of rPotD is optimal at pH 8.0. The specific spermidine binding to plasmalemma vesicles isolated from Zucchini (*Cucurbita pepo* L.) shows pH-dependence with a maximum at pH 8.0 (Tassoni *et al.*, 1996). Furthermore, the dependence of

polyamines uptake on the extracellular pH was previously reported in *Leishmania* (Basselin *et al.,* 2000), sea water red alga *Ulva rigida* (Badini *et al.,* 1994) as well as in Wistar rats (Kobayashi *et al.,* 1992).

Specific binding interaction of polyamines to rPotD performed using a series of compounds structurally related to polyamines indicates the characteristics of polyamine binding sites which bind specifically polyamines. Amino acids such as L-arginine, L-asparagine, L-glutamic acid or L-lysine which possesses one positively charged nitrogen atoms and one negatively charged carboxyl group, hardly competitively inhibited spermidine binding. Spermidine had higher inhibition (58%) of spermine binding activity than did spermine on inhibition of spermidine binding activity (35%) (Table 4). This lent further support for the preference of rPotD to bind spermidine rather than spermine. The less favorable binding of spermine to rPotD might be related to the four positively charged nitrogen atoms leading to the hindrance structure of spermine. Previously, Igarashi (1999) demonstrated that PotD protein had a stronger binding and uptake activity toward spermidine than toward putrescine due to the fact that spermidine interacted with Glu171, Trp255, and Asp257 on the PotD protein, whereas putrescine interacted with PotD at the position occupied by the diaminobutane moiety of the spermidine molecule.

The unique network of hydrogen bonds presumably limits the binding cavity of putrescine-preferential *E. coli* PotF, making it too small to bind the larger polyamines. The conserved water molecule plays a special role in preventing putrescine from protruding too deep into the binding cavity (Vassylyev *et al.,* 1998). *E. coli* PotD seems to lack the hydrogen bond network, resulting in more flexible binding cavity and, thus, being able to bind both spermidine and putrescine, the latter with a lower affinity

(Vassylyev *et al.*, 1998). Regarding the binding preference, the binding cavity of *Synechocystis* PotD is more similar to the binding cavity of *E. coli* PotD. The docking experiments conducted with putrescine, spermidine and spermine showed that the binding site of *Synechocystis* PotD is capable of accommodating all three polyamines (Figure 15). The fact that *Synechocystis* PotD had more favorable interactions with spermidine than with putrescine provides an explanation for the higher affinity binding of spermidine among different organisms. Taken together, the results from the present study reveal the capacity of the rPotD of *Synechocystis* to bind specifically to polyamine and the docking results provide an explanation for the preferred spermidine binding.

The *in vitro* results revealed that PotD had the ability of polyamine binding, especially spermidine. In this study, we tried to characterize the function of PotD on polyamine transport in *Synechocystis*. The *potD* gene was inactivated by homologous recombination to the target gene using insertion mutagenesis method with kanamycin resistance cassette. After *potD* was inactivated, the complete segregation of *potD* mutant was used in the further experiments. The growth rate of both wild-type and mutant indicated that *potD* gene can be inactivated simultaneously without any effect on growth under control growth conditions. This was similar to the finding of gene response to temperature in *Synechocystis*, ΔsigBD mutant did not affect the growth under normal conditions at 32^{o}C (Tuominen *et al.*, 2005). The presence of putrescine and spermidine at 0.1 - 0.3 mM resulted in slightly enhanced growth in both wild-type and mutant. Such a growth difference of wild-type and mutant cells was observed in the presence of 0.4 - 0.5 mM putrescine and spermidine. Greatly, the sensitivity to high polyamine concentration was detected in mutant. The wild-type cells in the presence of 0.1 - 0.5 mM spermine also showed faster initial growth than that lacking spermine. It should be noted that the growth

of cells cultured with spermine can reach to early stationary phase at day 5^{th} whereas growth of cells cultured without spermine normally can reach to this phase at day 9^{th}. Our results are in accordance with the finding that *S. cerevisiae* with disrupted *TPO5* gene involving in putrescine excretion was sensitive to high concentration of putrescine. It was also observed that polyamine content in mutant is higher than that in wild-type (Tachihara *et al.*, 2005). The addition of spermidine to *E. coli* lacking spermidine acetyltransferase (*speG*) at 7^{o}C inhibited cellular growth due to the spermidine accumulation (Limsuwun and Jones, 2000). In parallel, the presence of spermidine or spermine in growth medium greatly inhibited cell growth of mouse FM3A cells caused by polyamine accumulation (He *et al.*, 1993). The toxicity of polyamines has been reported in *E. coli* and mammalian cells that the overaccumulation of spermidine and/or spermine inhibited the growth (Fukuchi *et al.*, 1995; He *et al.*, 1993). Polyamines were able to disrupt several metabolic functions within the cells by inhibition of protein biosynthesis accompanied by an irreversible dissociation of ribosomes (Ramakrishna *et al.*, 1978). Thus, there are two mechanisms to maintain the intracellular polyamine levels. Firstly, when enzymes involved in polyamine degradation are induced, spermidine/spermine will be acetylated. That N′-acetylspermidine and N′-acetylspermine cannot interact to ribosome. Therefore, the inhibition of protein biosynthesis due to overacumulation of spermidine/spermine is relieved (Kakegawa *et al.*, 1991). The second mechanism is to excrete polyamine out of the cells. For instance *S. cerevisiae* which do not exist spermidine acetyltransferase has 5 excretion proteins (Tachihara *et al.*, 2005). Besides, *E. coli* containing PotE, CadB or MdtJI is able to excrete putrescine, cadaverine and spermidine, respectively (Igarashi and Kashiwagi, 2010). However, it has not been reported the excretion protein in *Synechocystis* so far. It is worth noting that *Synechocystis* was capable to uptake putrescine, spermidine into itself (Raksajit *et al.*, 2006 and 2009).

From all reasons, it is possible that reduction of growth in wild-type and *potD* mutant cell is due to the accumulation of intracellular polyamines. The absence of external polyamines in the medium, the accumulation of internal polyamines is increased by mutant cells indicating that its accumulation was induced via polyamine biosynthesis. Additionally, spermidine accumulated was higher than putrescine, indicating that putrescine might be used as a substrate in spermidine biosynthesis (Figure 26). The spermidine overaccumulation released by either degradation or excretion. Our results revealed that the growth of mutant cells accumulated polyamine was inhibited after 2 days, suggesting that cells could not maintain the internal polyamine or were not able to excrete polyamine. This observation indicates the function of PotD in spermidine transport.

Growth rate of *Synechocystis* both wild type and mutant cells is also affected by the osmotic stress, generating by NaCl and sorbitol. The slow growth was detected in both NaCl-treated cells and sorbitol treated cells compared to non-treated cells. This was similarly reported by Jantaro *et al.* (2003). The finding showed *Synechocystis* was normally grown in 550 mM NaCl. The growth rate was decreased in the presence of 300 mM sorbitol and completely inhibited at concentrations of 700 mM sorbitol.

The levels of putrescine and spermidine accumulated in *Synechocystis* cells after salt and osmotic stress treatments. Nevertheless, some studies on plant showed that cereal cells and protoplasts exposed to sorbitol not only accumulate putrescine and spermidine but also activate biosynthetic pathway mediated by arginine decarboxylase (*ADC*) (Flores *et al.*, 1984). In *Arabidopsis thaliana* which has *ADC1* and *ADC2*, some studies demonstrated that *ADC2* is responsible for induction of the polyamine biosynthetic pathway by osmotic stress. No induction of *ADC* activity by the osmolyte sorbitol could

be observed in the *ADC* knockout mutant, indicating a predominant role of *ADC2* in stress response (Soyka and Heyer, 1999). It seems that the accumulation of polyamine in the presence of sorbitol enhancing the growth reduction in both wild-type and mutant cells may result from the activation of polyamine biosynthesis.

Comparison of putrescine uptake between wild-type and mutant strain noticed similar uptake pattern. Interestingly, the mutant cell showed a 50% lower spermidine uptake than the wild-type cell. While, reduction of spermine uptake in mutant cell occurred as well, but 20% of activity lower than in wild-type was detected (data not show). It is likely that *potD* highly involved in spermidine uptake. This finding is in accordance with the previous results of *E. coli* which reported that spermidine uptake activity is decreased greatly with mutated PotD protein (Kashiwagi *et al.,* 1993 and 1996). Although, the spermidine uptake activity of mutant cells is decreased, it still had the ability to uptake spermidine. This finding suggested that *Synechocystis* could have other channels to participate in polyamine transport. As reported, there are six and ten polyamine transporters in *E. coli* and *S. cerevisiae,* respectively (Igarashi and Kashiwagi, 2010).

The induction of the polyamines uptake, including putrescine, spermidine and spermine was detected at low concentration of either NaCl or sorbitol. Increasing concentration caused a gradually declined uptake activity. Noticeably, cells required polyamine to protect cells against osmotic stress. Mutant cells also were able to uptake polyamines with lower activity compared to wild-type. This seems that PotD protein may involve somewhat against osmotic stresses. The uptake activity of both wild-type and mutant was reduced during darkness, while it was induced under light. Correspondingly, *Synechocystis* incubated for 3 days in darkness showed marked decrease in the amount of

the PotD protein. However, when cell was transferred from darkness to 30 min light treatment, there was the up-shift of PotD level (Brandt *et al.,* 2010). The observation seems support an involvement of PotD under light treatment.

Light plays an essential role for photosynthetic organisms, nevertheless change in light intensity affect somehow to them. The photo-oxidative damage directly affect to PSII reaction center through protein D1 (Mattoo *et al.,* 1981; Ohad *et al.,* 1984). However, this activity can be restored by a complex process known as the PSII repair cycle (Aro *et al.,* 1993 and 2005). Our results showed that the high intensity light affected on *Synechocystis* wild-type and mutant cells enhancing the decrease in chlorophyll *a* content by accumulation of polyamines. Previous study has reported that high polyamines content are capable to promote the disappearance of Chl *a*. Polyamines act as a chelator, therefore, it is possible that polyamine induced chlorophyll bleaching in light is the same phenomenon as chelator-induced chorophyll bleaching (Kotaka and Krueger, 1969; Pjon, 1982).

The low molecular weight amines including polyamine have ability to stimulate ATP synthesis during light periods (Giersch, 1981; Pick and Weiss, 1988; Sigalat *et al.,* 1988). Due to the function of polyamines, their proton sequestering capacity might accept protons from PSII, Cyt b_6f, PSI and deliver protons to ATPase. It has only recently been demonstrated that this kind of stimulation is feasible by the biogenic putrescine (Ioannidis *et al.,* 2006). The low amount of polyamines improves the photosynthetic functions under stress. Besides the conjugation of polyamines with LHC polypeptides, polyamines are likely to interact with extrinsic proteins and the hydrophilic part of intrinsic proteins of PSII by electrostatic interaction. This could stabilize the conformation of proteins under various stresses (Hamdani *et al.,* 2011). The binding mode of amines with PSII proteins

showed that spermine and putrescine form H-bonding with protein through polypeptide C=O, C-N and N-H groups with major perturbations of protein secondary structure (Hamdani *et al.,* 2011). The high concentration of polyamines added to PSII-enriched submembrane fractions presented a significant loss of PSII activity. Polyamines can induce major protein secondary structural changes and inhibit photosynthetic oxygen evolution (Bograh *et al.,* 1997). In parallel with our results, addition of external polyamine induced the photosynthesis system at short time, thereafter, inhibition of photosynthesis occurred, mostly by spermine (data not shown). From all results, effect of light on polyamine uptake, effect of light on growth and effect of polyamine on photosynthesis, we concluded that *potD* gene would be necessary for *Synechocystis* cell under light stress and for photosynthesis system.

Salt stress is considered to be one major environmental factor that limits the efficiency of photosynthesis. However, the effects of light and salt stress on photosystem II (PSII) in the *Synechocystis* are completely different. Strong light induces photodamage to PSII, whereas salt stress inhibited the repair of the photodamaged PSII and did not accelerate damage to PSII directly (Allakhverdiev *et al.,* 2002). As reported that NaCl-exposed *Spirulina platensis* decreased the oxygen evolution activity which correlated with the decrease in the quantum yield of PSII electron transport (ΦPSII). Phycocyanin content also decreased significantly while chlorophyll content remained unchanged in salt-stressed cells (Lu and Vonshak, 2002). In contrast, both of photosynthetic capacity and PSII activity of *Synechocystis* tolerated NaCl (Jantaro *et al.,* 2005). Corresponding to our present results, the external NaCl hardly affected on photosynthetic capacity and PSII activity of *Synechocystis* wild-type, but it markedly affected on those activities in mutant cells.

Moreover, Jantaro *et al.* (2005) reported that high concentration of sorbitol completely inhibited photosynthesis system. The amount of the D1 protein of PSII, *psbA* (encoding D1) and *psaA* (encoding PsaA) transcripts were decreased under severe osmotic stress. Similar to present findings, the photosynthesis system of *Synechocystis* wild-type was unaffected by sorbitol stress. Conversely, those activities were moderately decreased by mutant cells. This suggests the function of PotD might involve in photosynthesis system under osmotic stress.

Polyamine analogs, for instance, amino acids inhibited neither putrescine nor spermidine uptake in *Synechoscystis* (Raksajit *et al.*, 2006 and 2009). Therefore, we further tested that whether polyamine analogs inhibited the polyamine uptake in *Synechocystis* lacking *potD* gene. Our results indicated that the uptake of polyamine by mutant cells was not inhibited by amino acids. Moreover, in the presence of inhibitors which inhibited the energy-producing system, both wild-type and mutant cells were similarly affected by these inhibitors.

The results of induction of PotD protein indicated that spermidine was more effective than putrescine in the induction of PotD in *Synechocystis*. In *E. coli*, spermidine was also shown to induce the transcription of *TPO5* gene higher than putrescine (Tachihara *et al.*, 2005). The TPO5 protein had a role in the excretion of polyamine with higher capacity for putrescine than spermidine. For *Synechocystis*, the increase in PotD content by spermidine is necessary for the acclimation of cells to external spermidine during which the uptake and accumulation of spermidine can be detected, since high intracellular spermidine is inhibitory to cell growth (Tachihara *et al.*, 2005). We found that the increase of PotD is beneficial to the cells due to the ability of PotD to excrete

spermidine to the medium. This would enable the cells to maintain a low level of intracellular spermidine when exposed to high external concentration of spermidine.

The *potD* gene encodes periplasmic binding protein involved in preferential spermidine transport which has 24% homology to *potD* gene in *E. coli*. The role of *E. coli potD* is not related to spermidine excretion (Kashiwagi *et al.,* 1996). However, *Synechocystis* with perturbed *potD* gene seems to accumulate polyamine inside its cells. It is then possible that *Synechocystis potD* might be involved in polyamine excretion. As reported in *E. coli*, when excess amounts of putrescine accumulate in cells, they are excreted to medium by PotE protein (Kashiwagi *et al.,* 1992). Beside, spermidine content in *E. coli* cultured in the presence of 2 mM spermidine was decreased and excretion of spermidine from cells was enhanced by MdtJI (Higashi *et al.,* 2008). Therefore, excretion of polyamine in *Synechocystis* was investigated. Both wild-type and mutant did not participate in putrescine excretion. Interestingly, spermidine excretion activity was decreased by 40% in mutant cell. Furthermore, acidic pH was more efficient to polyamine excretion. A small increase of putrescine excretion activity was observed in wild-type cell, but not in mutant cell. There was a reduction of spermidine excretion activity ranging from 40 to 70 % between wild-type and mutant cells. Even the mutant did not excrete spermidine at higher rate at pH 6. At this pH, wild-type exhibited excretion at higher rate when compared to excretion at pH 7.6. Moreover, we also found that mutant cell had spermine excretion activity lower than that of wild-type. *S. cerevisiae*, *TPO5* excretes putrescine effectively and less in spermidine. The excretion of ^{14}C putrescine was faster in wild-type cell than disturbed *TPO5* mutant cell but no difference in spermidine (Tachihara *et al.,* 2005). Our results are reasonable to support that *Synechocystis* PotD plays a role in spermidine excretion. However, although PotD in

E. coli is related to polyamine uptake, there is no report about the role of *E. coli* PotD for polyamine excretion. In our studies, this response did not agree with the result demonstrated by Kashiwagi *et al.* (1996) in *E. coli*. We suggested that differences in the organism species might be responsible for this discrepancy. *Synechocystis* PotD belongs to ABC type transporter and has only 24% identity to *E. coli* PotD (Brandt *et al.*, 2010). The type of PotD was confirmed by using bioinformatics program, indicating that PotD is periplasmic binding protein. Hence, it was not able to transport polyamine by itself, suggesting that the other protein should interact with PotD. We have tried to identify the candidate proteins for PotA, PotB and PotC in *Synechocystis* genome, but the similarity of about only 11% was obtained. As a consequence, the existence of Pot ABC in *Synechocystis* needs further investigation. It is possible that this polyamine transport system in *Synechocystis* might have its own transmembrane (Figure 47A) and ATPase subunits or it might share the channel-forming proteins (PotB and PotC) and ATPase (PotA) with another ABC transport system (Figure 47B) (Incharoensakdi *et al.*, 2010).

Future perspectives: The results I have obtained in this thesis indicated the role of PotD for polyamine transport with high affinity for spermidine. However, the interaction of PotD with other proteins is still unclear. Further work on identification of those proteins involved in spermidine transport should be continued.

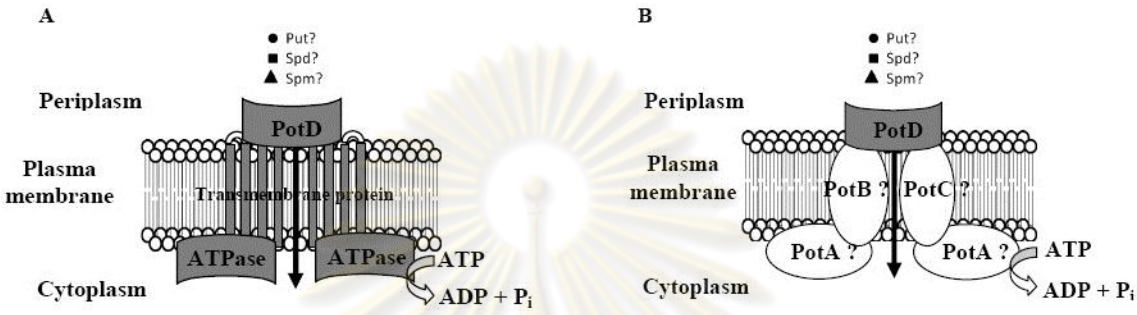


Figure 47 The proposed polyamine transport system in *Synechocystis* sp. PCC 6803. (A) PotD is substrate-binding protein which might have its own transmembrane and ATPase subunits, (B) PotD is substrate-binding protein which might share the channel-forming proteins (PotB and PotC) and ATPase (PotA) with another ABC transport system.

CHAPTER V

CONCLUSION

The characterization of a polyamine-binding protein in cyanobacterium *Synechocystis* sp. PCC 6803 has been investigated by inactivation of *potD* gene by insertional mutagenesis. Interestingly, the reduction of spermidine uptake and excretion was observed by 50% and 60% in mutant cells, respectively. However, putrescine uptake activity was not altered. Furthermore, the effect of either putrescine or spermidine on growth of both wild-type and mutant cells were determined. Although, wild-type and mutant cells showed similar growth in BG11, mutant cell was more sensitive to high concentration of putrescine or spermidine up to 0.5 mM. Next, the effect of osmotic stresses generated by NaCl or sorbitol on polyamine uptake and growth were examined. The putrescine and spermidine uptake were induced by either NaCl or sorbitol up to 10 mM. Furthermore, the presence of 300 mM sorbitol in BG11 resulted in the growth stimulation in wild-type and growth inhibition in mutant cells. The effect of light intensity on polyamine uptake and growth were further studied. The high light intensity stimulated the putrescine and spermidine uptake activity by 3- and 2.5-fold, respectively. Furthermore, inhibition of growth by high light was observed in *Synechocystis*, both wild-type and mutant cells. The growth of mutant cells was completely inhibited by combination effect of polyamine and high light during the first two days. Furthermore, the effect of polyamine on photosynthetic system was also considered. The finding can be concluded that the external polyamines induced both PSII activity and photosynthetic capacity within 30 min but decreased sequentially after one hour in both wild-type and mutant cells. Additionally, the effect of either polyamines or NaCl or sorbitol on the PotD protein level was examined. The PotD levels of *Synechocystis* cells grown in the medium

containing either 0.5 mM putrescine or spermidine led to an increase about 1.6 and 2.8 fold, respectively. The purified rPotD demonstrated a similar binding characteristic for putrescine, spermidine and spermine with a preference for spermidine. The polyamine binding capacity on rPotD revealed that the discosiation consant (K_d) and maximum binding (B_{max}) values for putrescine, spermidine and spermine were 13.2, 7.8 and 8.3 μM, respectively and 0.74, 1.42 and 0.13 mol/mol rPotD, respectively. The optimum binding between PotD and three polyamines was at pH 8.0. The presence of NaCl and sorbitol up to 10 mM induced the polyamine binding, whereas the concentration of both higher than 50 mM reduced the binding activity. Furthermore, the competition experiments provided evidence that exhibited specific binding of rPotD occurred with polyamines. Morover, docking of these polyamines into the homology model of *Synechocystis* PotD showed that all three polyamines are able to interact with *Synechocystis* PotD. The overall results support the role of PotD in mediating polyamine transport in *Synechocystis* sp. PCC 6803.

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APPENDICES

APPENDIX A

BG-11 medium

BG-11 medium (1 liter)

	Solid medium	Liquid medium
H_2O	947 ml	967 ml
Becto-agar	15 g	-
100 x BG-FPC*	10 ml	10 ml
189 mM Na_2CO_3	1 ml	1 ml
175 mM K_2HPO_4	1 ml	1 ml
6 mg/ml Feric ammonium citrate	1 ml	1 ml
1 M TES	10 ml	-
30% $Na_2S_2O_3.5H2O$	10 ml	-
1 M HEPES-NaOH, pH 7.5	20 ml	20 ml

100xBG-FPC*

	100 ml
$NaNO_3$	14.96 g
$MgSO_4.7H_2O$	0.75 g
$CaCl_2.2H_2O$	0.36 g
Citric acid	0.065 g
0.5M Na-EDTA	55.4 ml

*After autoclaved, add 10 ml of 1000xTrace metal

1000x Trace metal mix (1,000 ml) **

	1000 ml
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.221 g
$Na_2MoO_4.2H_2O$	0.390 g
$CuSO_4.5H_2O$	0.080 g
$Co(NO_3)_2.6H_2O$	0.049 g

** Sterile filtrate, store at 4 ^oC

APPENDIX B

LB medium

LB medium (1 liter)

	Solid medium	Liquid medium
Bacto tryptone	10 g	10 g
NaCl	5 g	5 g
Yeast extract	5 g	5 g
Agar	15 g	-

All compositions were dissolved together with 800 ml of distilled water; and then the mixture was adjusted to pH of 7.0 with 10 M NaOH. The total volume of solution was then adjusted to 1 liter with deionized water. The medium was sterilized by autoclaving at 15 Ib/in^2 for 15 min.

APPENDIX C

Bradford protein determination

Bradford stock solution	
95 % ethanol	100 ml
88 % phosphoric acid	200 ml
Serva Blue G	350 mg
Stable indefinitely at room temperature	

Bradford working buffer	
distilled water	425 ml
95 % ethanol	15 ml
88 % phosphoric acid	30 ml
Bradford stock solution	30 ml
Filter through Whatman No. 1 paper, Store at room temperature. Usable for several week, but may need to be refiltered.	

Assay

1. Prepare protein solution 20 µl into tube.
2. Add 1 ml Bradford working buffer and vortex.
3. Read OD_{595} within 10 min - 1 hour**.**

APPENDIX D

PCR amplification protocol

PCR amplification mixture

10x PCR buffer*	5.0	µl
5 mM dNTP mix	2.5	µl
Primer 5' (5 pmol/µl)	5.0	µl
Primer 3' (5 pmol/µl)	5.0	µl
DyNAzyme DNA polymerase** (2unit/µl)	0.5	µl
DNA	X	µl
MQ-water	32-X	µl
Total	50.0	µl

** DyNAzyme™II is a thermostable DNA polymerase, has a half-life of 2.5 hour at 96 ^{o}C.

Program running PCR

95^{o}C	5.00	min (Pre denaturation time)	
95 ^{o}C	1.00	min (Denaturation time)	34 cycles
55 ^{o}C	1.00	min (Annealing time)	
72 ^{o}C	2.30	min (Extension time)	
72 ^{o}C	10.00	min (Final Extension cycle)	
16 ^{o}C	for ever		

*** 10x PCR buffer**

100 mM Tris-HCl buffer pH 8.8 at 25 ^{o}C

15 mM $MgCl_2$, 500 mM KCl

1 % Triton X-100

APPENDIX E

Alkaline lysis reagents for plasmid extraction

Solution I (100 ml)

1.0 M Glucose	5.0 ml
1.0 M Tris-HCl, pH 8.0	2.5 ml
0.5 M EDTA, pH 8.0	2.0 ml

After autoclave 20 μg/ml of RNase was added and stored at 4 ^{o}C

Solution II (25 ml)

10 M NaOH	0.5 ml
20% SDS	1.25 ml

Solution III (500 ml)

potassium acetate	147 g
glacial acetate	57.5 ml

Autoclave and store at 4 ^{o}C

TE buffer (500 ml)

5 ml 1 M Tris-HCl, pH 8.0

1 ml 0.5 M EDTA

Autoclave and store at room temperature

APPENDIX F

Polyacrylamide gel electrophoresis

Stock solutions

50% (w/v) Acrylamide, 1.33% (w/v) bis-acrylamide, 100 ml

Acrylamide	50 g
N,N′-methylene-bis-acrylamide	1.33 g

Distilled water was added to make a total volume of 100 ml and stirred until completely dissolved.

1.5 M Tris-HCl buffer pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane	18.15 g

Adjusted pH to 8.8 with concentrated HCl slowly. Added distilled water to the total volume of 100 ml.

0.5 M Tris-HCl buffer pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane	6.05 g

Adjusted pH to 6.8 with concentrated HCl slowly. Added distilled water to the total volume of 100 ml.

20% w/v SDS, 100 ml (stored at room temperature)

SDS	20 g

Distilled water was added to make a total volume of 100 ml.

10% Ammoniumpersulfate (APS), 1 ml

APS	0.1 g

Distilled water was added to make a total volume of 100 ml.

0.5% Bromophenol blue

Bromophenol blue	0.05 g

Distilled water was added to make a total volume of 100 ml.

20% v/v Glycerol

Glycerol	20 ml

Distilled water was added to make a total volume of 100 ml.

15% Separating gel (2 Gel)

1.5 M Tris-HCl pH8.8	11.5 ml
50% Acrylamide Solution	15.0 ml
Distilled water	26.8 ml
20% SDS	1.0 ml
10% APS	300 µl
TEMED	30 µl

6% Stacking gel (2 Gel)

0.5 M Tris-HCl pH6.8	5.0 ml
50% Acrylamide Solution	2.4 ml
Distilled water	14.1 ml
20% SDS	400 µl
10% APS	200 µl
TEMED	20 µl

Laminii solution 50 ml

0.5 M Tris-HCl pH6.8	13.8 ml
50% glycerol	20.0 ml
20% SDS	10.8 ml

Distilled water was added to make a total volume of 50 ml.

Solubilizing buffer

Laminii solution	900 μl
2-mercaptoethanol	100 μl
0.5% Bromophenol blue	5 μl

* The ratio of sample and sample buffer is 1:1. Mixture was incubated at 65 ^{o}C for 10 min. Then, centrifuged at 10,000 g for 1 min, samples were loaded into the gel.

Electrophoresis buffer, 1 liter (25 mM Tris, 192 mM glycine)

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

Distilled water was added to make a total volume of 1 liter.

APPENDIX G

Protein purification buffer

washing buffer	elution buffer	dialysis buffer (refold)
50 mM Tris-HCl pH 8.0	50 mM Tris-HCl pH 8.0	50 mM Tris-HCl pH 8.0
500 mM NaCl	500 mM NaCl	0.1% Triton X-100
20 mM imidazole	500 mM imidazole	0.5 mM PMSF
0.1% Triton X-100	0.1% Triton X-100	
0.5 mM PMSF	0.5 mM PMSF	
8 M urea	8 M urea	

APPENDIX H

Chlorophyll *a* content determination

1. The 900 µl of 90 % methanol was added into the microcentrifuge tube.
2. 100 µl of cell suspensions was added.
3. The mixture was then vortexed for 1 - 2 min
4. Incubate the mixture for 1 hour in the darkness
5. Centrifugation at 10,000xg for 5 min at 4 ^{o}C
6. Measurement the optical density at 665 nm
7. Calculation of Chlorophyll content by

   OD665 x 12.7 x 10 =…….. µg Chl *a*/ ml

   Coefficient constant is 12.7

APPENDIX I

Thylakoid isolation reagents

Stock solution

1 M HEPES-NaOH, pH 7.5, 100 ml

HEPES 23.83 g

Adjusted pH to 7.5 with concentrated NaOH slowly. Added distilled water to the total volume of 100 ml.

0.5 M Tricine-NaOH, pH7.5, 100 ml

Tricine 8.95 g

Adjusted pH to 7.5 with concentrated NaOH slowly. Added distilled water to the total volume of 100 ml.

0.5 M $CaCl_2$, 100 ml

$CaCl_2$ 5.55 g

Added distilled water to the total volume of 100 ml.

2.5 M sorbitol, 100 ml

Sorbitol 45.54 g

Added distilled water to the total volume of 100 ml.

2.5 M sucrose, 100 ml

Sucrose 85.6 g

Added distilled water to the total volume of 100 ml.

10 mM *ε*-amino-*n*-caproic acid, 10 ml

ε-amino-*n*-caproic acid	13.1 mg

Added distilled water to the total volume of 10 ml.

5 M glycinebetain, 100 ml

Glycinebetain	67.6 g

Added distilled water to the total volume of 10 ml.

Washing buffer, 100 ml

0.5 M HEPES-NaOH, pH7.5	10 ml
0.5 M $CaCl_2$	6 ml
Distilled water	84 ml

Resuspension buffer

0.5 M HEPES-NaOH, pH7.5	10 ml
0.5 M $CaCl_2$	6 ml
2.5 M sorbitol	32 ml
10 mM ε-amino-n-caproic acid	10 ml
Distilled water	42 ml

Storage buffer

0.5 M Tricine-NaOH, pH7.5	10 ml
2.5 M sucrose	24 ml
0.5 M $CaCl_2$	6 ml
5 M glycinebetain	20 ml
Distilled water	40 ml

APPENDIX J

Western blotting reagents

10X TBS (Tris-buffer-saline)

200 mM Tris-HCl buffer pH 7.5

5 M NaCl

TTBS

1X TBS

0.05% Tween-20

Blocking solution

5% Skim milk in TBS

Antibody buffer

1% Skim milk in TTBS

Transfer buffer

Glycine	2.93 g
Tris	5.81 g
SDS	0.38 g
Methanol	200 ml

APPENDIX K

Standard curve of polyamines

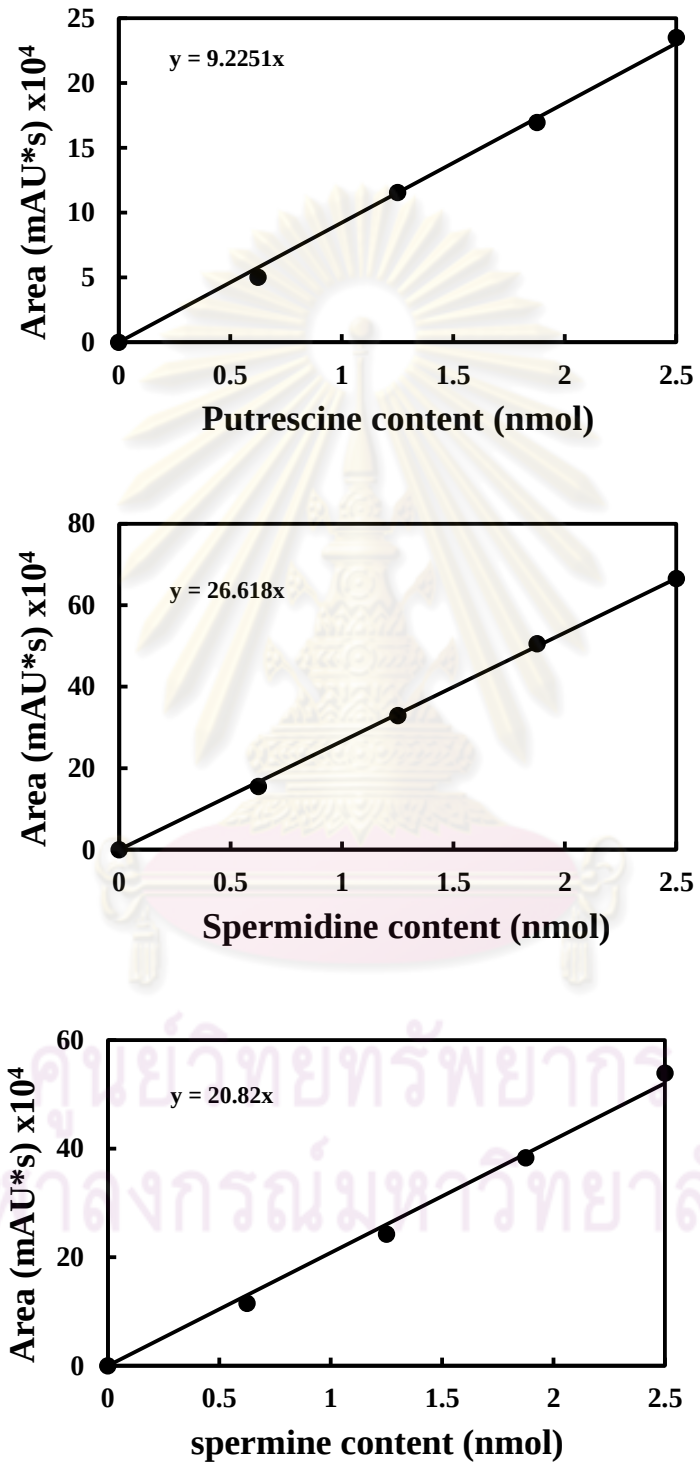


Figure 48 Standard curve of Putrescine (A), Spermidine (B) and Spermine (C).

APPENDIX L

The electron acceptors for oxygen evolution measurement

Stock solutions

1. **250 mM DCBQ (1 ml)**

   DCBQ 45 mg

   Dissolved in absolute ethanol 1 ml

2. **250 mM Ferricyanide (5 ml)**

   Ferricyanide 412 mg

   Dissolved in Milli Q water 5 ml

3. **0.6 M Sodium bicarbornate (10 ml)**

   Sodium bicarbornate 630 mg

   Dissolved in Milli Q water 10 ml

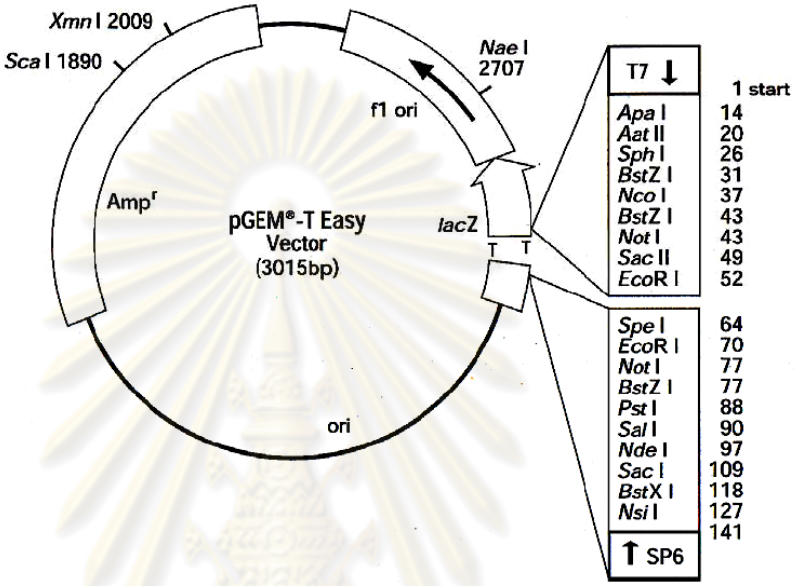
APPENDIX M

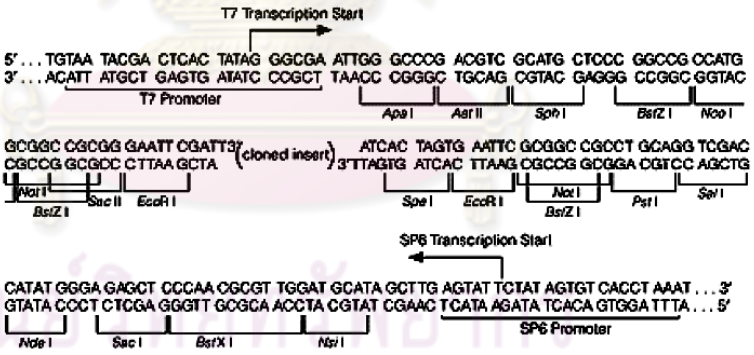
Oxygen concentration of air-saturated water

Temperature (^{o}C)	Oxygen concentration (μmol/ml)
0	0.442
5	0.386
10	0.341
15	0.305
20	0.276
25	0.253
30	0.230
35	0.210

APPENDIX N

pGEM-T easy vector

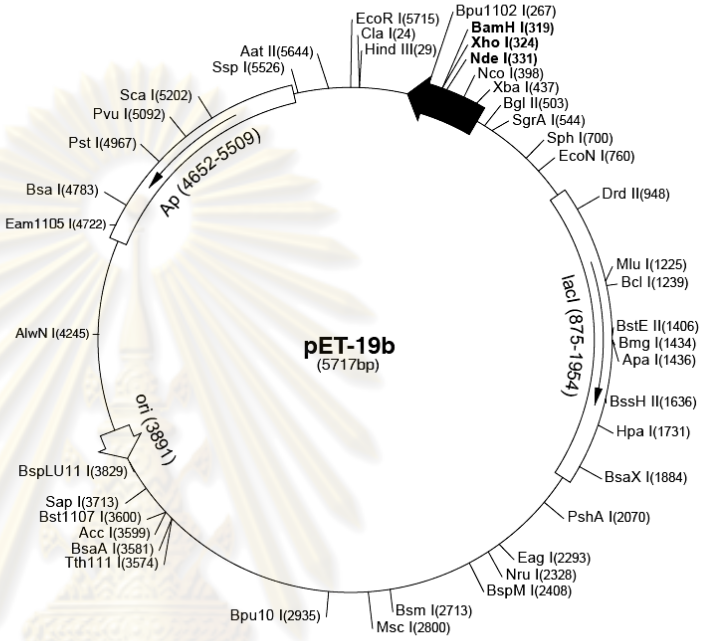


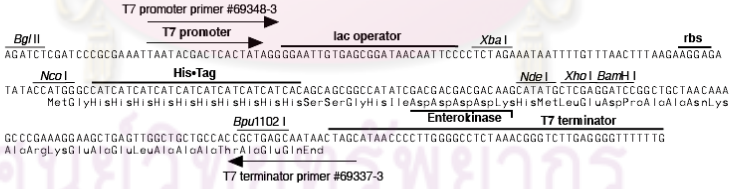


APPENDIX O

pET-19b vector

pET-19b sequence landmarks	
T7 promoter	472-488
T7 transcription start	471
His• Tag coding sequence	366-395
Multiple cloning sites	
(*Nde* I -*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	875-1954
pBR322 origin	3891
bla coding sequence	4652-5509

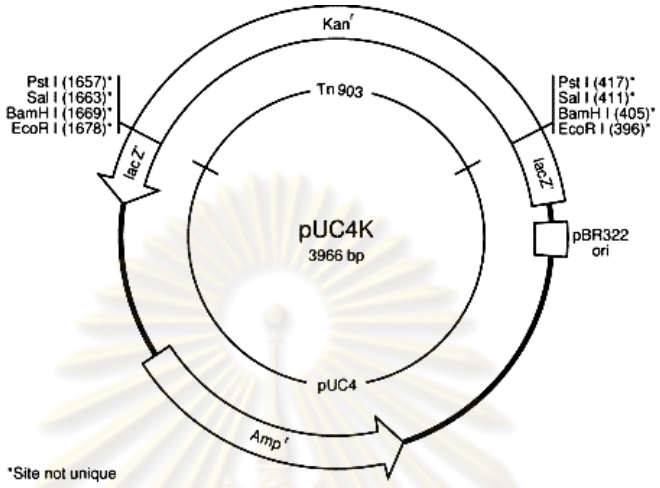




pET-19b cloning/expression region

APPENDIX P

pUC4K vector



BIOGRAPHY

Miss Panutda Yodsang was born on May 31, 1981 in Bangkok, Thailand. She has graduated with a Bachelor of Science degree in Biochemistry and Master of Science degree in Biotechnology from Faculty of Science, Chulalongkorn University in 2003 and 2006, respectively. She has further studied for the Doctor of Philosophy (Ph.D.) degree in Biochemistry, Faculty of Science, Chulalongkorn University since 2007.

PUBLICATIONS

1. **Yodsang, P**., Raksajit, W., Brandt, A.M., Salminen, T., Mäenpää, P., and Incharoensakdi, A. (2011) Recombinant Polyamine-binding Protein of *Synechocystis* sp. PCC 6803 Specifically Binds to and Is Induced by Polyamines. Biochemistry (Moscow). 76 *(In Press*).
2. Raksajit, W., **Yodsang, P**., Mäenpää, P., and Incharoensakdi, A. (2009) Characterization of spermidine transport system in a cyanobacterium *Synechocystis* sp. PCC 6803. J. Microbiol. Biotechnol. 19(5): 447-454.
3. Brandt, A.M., Raksajit, W., **Yodsang, P**., Mulo, P., Incharoensakdi, A., Salminen, T., and Mäenpää, P. (2010) Characterization of the substrate-binding PotD subunit in *Synechocystis* sp. strain PCC 6803. Arch. Microbiol. 192:791-801.

Academic Experiences

1. Commission on Higher Education Congress I: University Staff Development Consortium CHE-USDC Congress, Ambassador City Jomtien hotel, Chonburi, Thailand, 5 September - 7 September 2008. (proceeding), “Polyamine-Binding Protein D (PotD) Mutagenesis in *Synechocystis* sp. PCC 6803”.
2. 34th Congresses on Science and Technology of Thailand, Queen Sirikit National Convention Center, Bangkok, Thailand, 31 October - 2 November 2008. (proceeding) “Mutagenesis of Polyamine-Binding Protein D (PotD) in Cyanobacterium *Synechocystis* sp. PCC 6803”.
3. 13th Biological Sciences Graduate Congress, National University of Singapore, Singapore, 15 - 17 December 2008, (oral presentation) “Characterization and Mutagenesis of Periplasmic Polyamine-Binding Protein (PotD) in Cyanobacterium *Synechocystis* sp. PCC 6803”.