ผลของรังสีอัลตราไวโอเลตต่อปริมาณรงควัตถุและพอลิเอมีนที่เกี่ยวข้องกับระบบการสังเคราะห์ด้วยแสงในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาวศุภราภรณ์ กันทาสุวรรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

EFFECTS OF ULTRAVIOLET RADIATION ON PIGMENT AND POLYAMINE CONTENTS RELATED TO PHOTOSYNTHETIC SYSTEM IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

Miss Suparaporn Khanthasuwan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2012

Thesis Title	EFFECTS OF ULTRAVIOLET RADIATION ON PIGMENT AND POLYAMINE CONTENTS RELATED TO PHOTOSYNTHETIC SYSTEM IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803
By	Miss Suparaporn Khanthasuwan
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Saowarath Jantaro, Ph.D.
Thesis Co-advisor	Professor Aran Incharoensakdi, Ph.D.

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

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

THESIS COMMITTEE

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

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

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

…….. Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)

…….. External Examiner
(Wuttinun Raksajit, Ph.D.)

ศุภราภรณ์ กันทาสุวรรณ์: ผลของรังสีอัลตราไวโอเลตต่อปริมาณรงควัตถุและพอลิเอ-มีนที่เกี่ยวข้องกับระบบการสังเคราะห์ด้วยแสงในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803. (EFFECTS OF ULTRAVIOLET RADIATION ON PIGMENT AND POLYAMINE CONTENTS RELATED TO PHOTOSYNTHETIC SYSTEM IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.เสาวรัตน์ จันทะโร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.ดร.อรัญ อินเจริญศักดิ์, 84 หน้า.

งานวิจัยนี้ ทำการศึกษาผลของรังสีอัลตราไวโอเลต หรือ รังสียูวีต่อปริมาณรงควัตถุภายในเซลล์และพอลิเอมีนที่สัมพันธ์กับกระบวนการสังเคราะห์ด้วยแสงในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 พบว่า รังสียูวี เอ บี และซี ที่ความเข้มของรังสีโดยเฉลี่ย เท่ากับ 10, 1.3 และ 1.3 วัตต์ต่อตารางเมตร ตามลำดับ มีผลต่อการเจริญเติบโตของเซลล์เพียงเล็กน้อยและมีผลต่อการลดลงของปริมาณรงควัตถุอย่างมีนัยสำคัญภายใน 24 ชั่วโมง โดยปริมาณคลอโรฟิลล์ เอ ลดลงร้อยละ 10 - 40 และปริมาณแคโรทีนอยด์ลดลงร้อยละ 30 - 80 รังสียูวีทุกชนิดทำให้ประสิทธิภาพการสังเคราะห์ด้วยแสงลดลง 90 – 98% ภายในเวลา 3 ชั่วโมง สำหรับผลของรังสียูวีต่อปริมาณพอลิเอมีนพบว่า รังสียูวีทุกชนิดมีผลทำให้ปริมาณพอลิเอมีนรวมภายในเซลล์และพอลิเอมีนที่สัมพันธ์กับเยื่อไทลาคอยด์ลดลงอย่างมีนัยสำคัญภายใน 24 ชั่วโมง โดยปริมาณพอลิเอมีนที่ตอบสนองอย่างชัดเจน ได้แก่ สเปอร์มิดีน นอกจากนี้ยังทำการศึกษาผลของรังสียูวีต่อปริมาณพอลิเอมีนรวมที่สัมพันธ์กับแต่ละระบบแสง โดยการสลายเยื่อไทลาคอยด์ และนำมาแยกโปรตีนที่เกี่ยวข้องกับกระบวนการสังเคราะห์ด้วยแสงด้วยการปั่นเหวี่ยงความเร็วสูงที่ 144,000xg 4 องศาเซลเซียส เป็นเวลา 14 ชั่วโมงในสารละลายเกรเดียนต์ของซูโครส พบว่า พอลิเอมีนที่สัมพันธ์กับระบบแสงที่ 2 ในส่วนที่แยกได้จากแถบความเข้มข้นของซูโครสร้อยละ 30 - 40 (แถบที่ 5) และที่ความเข้มข้นของซูโครสร้อยละ 40 – 50 (แถบที่ 6) มีการเปลี่ยนแปลงอย่างมีนัยสำคัญ ขณะที่ในระบบแสงที่ 1 ในส่วนที่แยกได้จากแถบความเข้มข้นของซูโครสร้อยละ 10 (แถบที่ 2) ไม่พบการเปลี่ยนแปลงของระดับพอลิเอมีน จากผลการทดลองทั้งหมดนี้แสดงให้เห็นว่าพอลิเอมีนมีส่วนสำคัญในการตอบสนองต่อภาวะกดดันเนื่องจากรังสียูวีและเกี่ยวข้องกับระบบการสังเคราะห์ด้วยแสง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5272570823: MAJOR BIOTECHNOLOGY

KEYWORDS : *Synechocystis* sp. PCC 6803/ POLYAMINE/ ULTRAVIOLET RADIATION/ PIGMENT/ PHOTOSYNTHETIC SYSTEM

SUPARAPORN KHANTHASUWAN: EFFECTS OF ULTRAVIOLET RADIATION ON PIGMENT AND POLYAMINE CONTENTS RELATED TO PHOTOSYNTHETIC SYSTEM IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. ADVISOR: ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., 84 pp.

This research studied about the effect of ultraviolet or UV radiations on intracellular pigments and polyamine contents associated to photosynthesis in cyanobacterium *Synechocystis* sp. PCC 6803. All UV radiations (UV-A, UV-B, and UV-C with intensity of 10, 1.3, and 1.3 $w \cdot m^{-2}$, respectively) had slight effects on growth but highly significant changes on intracellular pigments within 24 h-treatment. Chl *a* content was decreased within 24 h about 10 – 40% whereas carotenoid content was decreased about 30 – 80%. All UV radiations reduced the photosynthetic efficiency within 3 h-treatment by 90 -98% decreasing. For total polyamine content, all UV radiations affected decreasingly on polyamine contents in whole cell and polyamines associated with thylakoid membranes within 24 h-treatment. The dominant polyamine responded to UVs was spermidine. Moreover, the polyamines associated with photosystem was determined. The photosynthetic proteins on thylakoid membranes were solubilized from thylakoid membranes and separated those proteins of photosystems using sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h. Polyamines associated with PSII which located at the boundary of 30 – 40% sucrose layer (F_5) and the boundary of 40 – 50% sucrose layer (F_6) were significantly changed under UV treatments. However, the level of polyamines associated with PSI which located at 10% sucrose layer (F_2) was not changed. Altogether, polyamines had the important response to UV stress and were identified their association with photosynthetic systems.

Field of Study : Biotechnology Student's Signature.....

Academic Year : 2012 Advisor's Signature.....

Co-advisor's Signature.....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Saowarath Jantaro, for her beautiful mind, excellent instruction, guidance, attention and support throughout this thesis and my co-advisor, Professor Dr. Aran Incharoensakdi, for his suggestion and support this thesis.

My gratitude is also extended to Professor Dr. Anchalee Tassanakajon, Assistant Professor Dr. Sanit Piyapattanakorn, and Dr. Wuttinun Raksajit for their valuable suggestion and comments and also dedicating valuable time for thesis examination.

I also would like to express my gratitude to the Department of Biochemistry, Faculty of Science Chulalongkorn University that providing chemicals and instruments. My appreciate is also expressed to Dr. Sorawit Powtongsook (Center of excellent for marine biotechnology, NASTDA, Thailand and department of Marine Science, Faculty of Science Chulalongkorn University) for facilitating the modified qubit fluorometer.

Members of Cyanobacterial Biotechnology (CYB) and all lovely friends was really my will supports in many good times of life sharing and discussion.

This study was supported by the Grant from 90th Anniversary Chulalongkorn University Fund, the Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, Chulalongkorn University graduate scholarship, and the CHE (University staff development consortium) to A.I.

Finally, the greatest gratitude is expressed to my parents and my family for their infinite love, encouragement, understanding and everything giving to my life.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
°C	degree Celsius
Chl *a*	chlorophyll *a*
DAB	3,3'-Diaminobenzidine
DM	dodecyl maltoside
DMF	dimethylformamide
g	gram
h	hour
HPLC	high performance liquid chromatography
kDa	kilodalton
M	molar
mA	milliampere
mg	milligram
min	minute
ml	millilitre
mM	millimolar
nm	nanometer
nmole	nanomole
PCA	perchloric acid
PSI	photosystem I
PSII	photosystem II
Put	putrescine
PVDF	polyvinylidene fluoride

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Spd	spermidine
Spm	spermine
TBS	Tris buffer saline
UV	Ultraviolet
V	volt
µl	microlitre
µg	microgram

CHAPTER I

INTRODUCTION

1.1 Ultraviolet radiation

Ultraviolet radiation is an electromagnetic wave at the length between 100 – 400 nm classified mainly as UV-A (320 – 400 nm), UV-B (290 – 320 nm) and UV- C (100 – 290 nm). In natural, all UV radiation ranges come from sunlight, especially UV-A which not affected by ozone while partly UV-B and completely UV-C were absorbed by ozone in stratosphere layer (United States Environmental Protection Agency [USEPA], 2010). In 1985, the first report of ozone depletion phenomenon revealed that ozone layer above Antarctica area had dramatically fallen (Farman, Gardiner, and Shanklin, 1985). Ozone layer was annihilated by ozone-depleting substances (ODSs). At the present, although ODSs are controlled by Montreal protocol, and have declined trend over the last twenty years, the ozone layer is still depleted because of the long lasting ODSs in atmosphere (World Meteorological Organization [WMO], 2011). An increase of UV level on ground level due to the ozone depletion phenomenon is dangerous for all living organisms. By its energy, the UV radiation can penetrate the surface skin of organisms and destroy biomolecules inside the cell such as nucleic acids and proteins (Sinha and Häder, 2002). Especially, photoautotrophs including plants, algae, oxygenic phototrophic bacteria (cyanobacteria) and anoxygenic phototrophic bacteria (i.e. green sulfur bacteria) are the main affected organisms because they use the energy of light for living.

1.2 Photosynthesis

Photosynthesis relates to a high biomass production process which occurs in photoautotroph organisms by converting light energy to organic compounds. The photosynthetic reaction begins with pigments capture the energy of light, then transfer along electron transport chain to final acceptor, and finally produces high energy substance including ATP and NADPH, known as light dependent reaction for sugar production. The reaction involved in photosynthesis and final product are occurred under photosynthetic apparatus and pigments utilization in photoautotrophs.

1.2.1 Photosynthetic apparatus

Photosynthetic apparatus are protein complexes located on thylakoid membrane, including photosystems, cytochrome complex and ATP synthase (Frigerio, Bassi, and Giacometti, 2008). Photosystems contain many protein subunits associated with photosynthetic pigments and electron transport molecules. Moreover, photosystems can be devided into two systems depending on type of chlorophyll reaction center; chlorophyll P_{680} as a photosystem II (PSII) and chlorophyll P_{700} as a photosystem I (PSI). Cytochrome complexes are protein subunits containing metal prosthetic group which efficiently transfer electron from PSII to PSI, like a bridge between two photosystems. ATP synthase is an enzyme which produces high energy substance, ATP.

Photoautotrophs have different patterns of photosynthetic apparatus. Higher plants and algae are the basic model of photosynthetic apparatus study consisted of PSI and PSII, cytochrome b_6f and ATP synthase locating at thylakoid membrane, a

photosynthetic membrane. For cyanobacteria, their photosynthesis model is similar as plant's, but they have phycobilisome as light harvesting complex attaching on PSII. In contrast, anoxygenic phototrophic bacteria, they are distinct from plants and cyanobacteria. They have a different oxygen evolving complex (OEC) protein on photosynthetic membrane and different types of photosystem and cytochrome complex (Figure 1.1). Various environmental stresses highly affect the photosynthetic apparatus such as intense light, heat stress, heavy metal assimilation, and nutrient deprivation (Mishra and Singhai, 1992; Skórzynska-Polit and Baszynski, 1997; Schwarz and Forchhammer, 2005). In particular, UV radiation strongly affected PSII apparatus in oxygenic photoautotrophs. It causes an inactivation of manganese (Mn) cluster in PSII which further result in decreases of PSII activity and photosynthetic rate (Tyystjärvi, 2008). However, photoautotrophs have several mechanisms to protect their cell damage caused by UV radiation and repair photosynthesis processing. For example, UV-B exposed higher plant, *Oenothera stricta* enhanced high flavonoid production in epidermal and mesophyll tissues in order to lower UV-B penetration (Robberecht and Caldwell, 1983). In cyanobacterium *Synechocystis* sp., there are many genes encoding various antioxidant enzymes which responded with high gene expression to environmental stresses such as glutathione peroxidase and superoxide dismutase (Huang et al., 2002). Recently, the research destination studied on the polyamines response against UV radiation in higher plant and cyanobacteria was raised (Lütz et al., 2005; Jantaro et al., 2011).

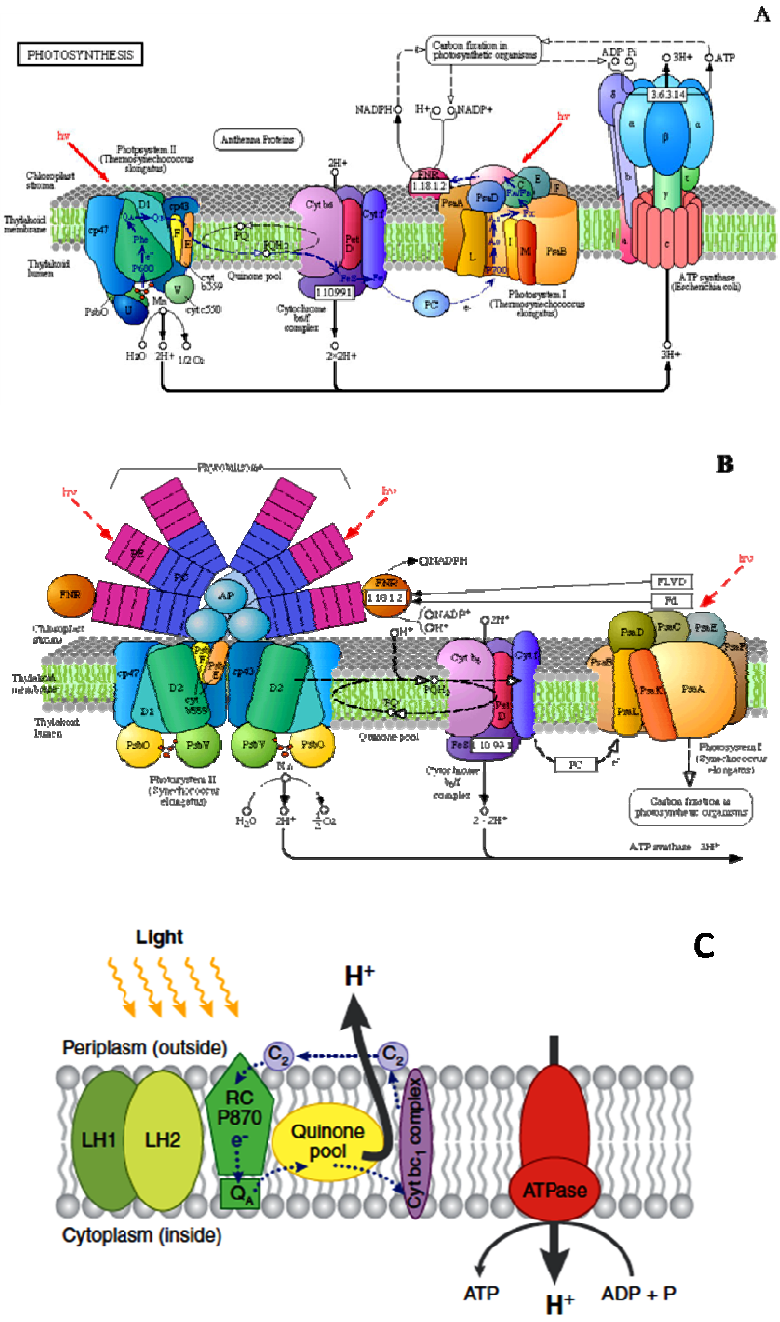


Figure 1.1 The arrangement of photosynthetic apparatus in (A) higher plants and algae, (B) cyanobacteria, and (C) anoxygenic phototrophic bacteria. Source: http://employees.csbsju.edu/hjakubowski/classes/ch331/oxphos/olphotsynthesis.html , http://www.chm.bris.ac.uk/motm/oec/motmc.htm, and Döbler and Biebl, 2006.

1.2.2 Photosynthetic pigments

All photoautotrophs have photosynthetic pigments, molecules that can absorb light energy to produce their foods. There are three types of pigments including chlorophylls, caroteniods, and phycobilins that widely distributed in photoautotrophs (Table 1.1). These pigments associate with protein on photosynthetic membrane and possess the characteristic absorption on spectrum range of visible region with wavelength of 400 – 700 nm (Figure 1.2). On the other hand, there are two categories of photosynthetic pigments. First one is the primary pigments which are reaction centers of light energy absorption existing in photosystems, i.e. chlorophyll *a* in oxygenic photoautotrophs and bacteriochlorophyll in anoxygenic phototrophic bacteria. Secondly, it is the accessory pigments, i.e. other chlorophylls, carotenoids and phycobilins which are pigment molecules capturing light energy and then transferring the energy to the primary pigments. The sufficient photons reaching to chlorophyll reaction center are able to excite an electron and take the electron out of the molecule to further initiate the electron flow within photosynthetic apparatus. In cyanobacteria, long-term of UV radiation stress decreased the level of photosynthetic pigments by several ways, such as directly destroyed pigment-binding proteins (Lao and Glazer, 1996 cited in Huang et al., 2002), increased transcription levels of phycobilisomes degradation, and repressed the expression of chlorophyll biosynthesis genes (Huang et al., 2002).

Table 1.1 The photosynthetic pigment distribution in photoautotrophs

Photosynthetic pigment	Organisms
Chlorophylls	
Chlorophyll *a*	Plants, algae, and cyanobacteria
Chlorophyll *b*	Higher plants and algae
Chlorophyll *c*	Diatoms and brown algae
Chlorophyll *d*	Red algae
Bacterioviridin	Green bacteria
Bacteriochlorophylls	Purple and green bacteria
Carotenoids	
Carotenes	
α-carotene	Higher plants, most algae
β-carotene	Plants, algae, and cyanobacteria
γ-carotene	Green bacteria (some plants)
Xanthophylls	
luteol	Plants, green algae, and red algae
violaxanthol	Plants
fucoxanthol	Diatoms and brown algae
spirilloxanthol	Purple bacteria
Phycobilins	
phycoerythrins	Red algae (some cyanobacteria)
phycocyanins	Cyanobacteria (some red algae)
allophycocyanin	Red algae and cyanobacteria

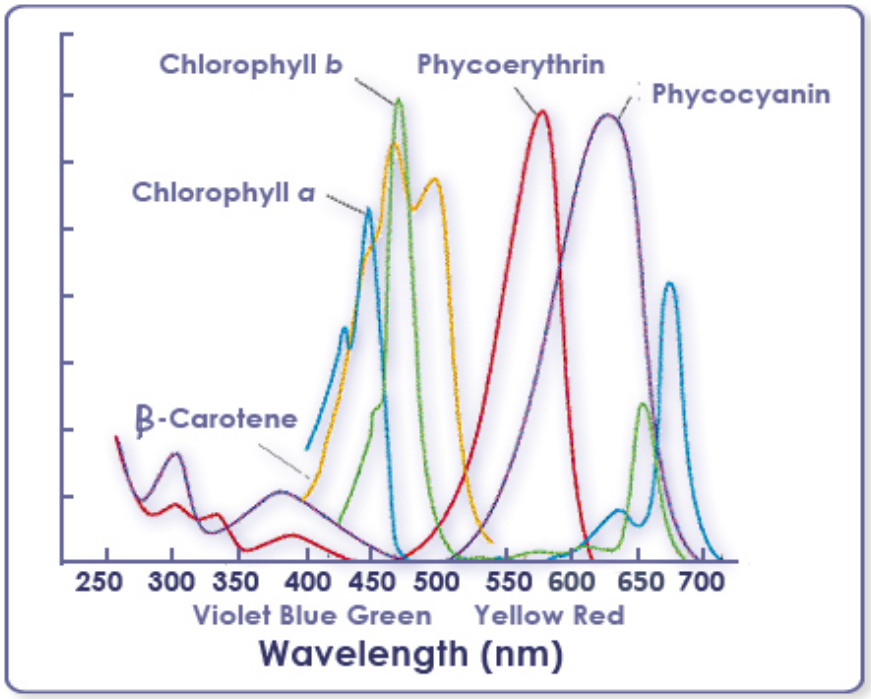


Figure 1.2 The characteristic absorption spectra of some photosynthetic pigments. (http://aventalearning.com/courses/BIOx-CR-A09/a/unit2/section2_03.html)

1.3 Polyamines

Polyamines (PAs) are aliphatic amines found in all organisms. There are many differently natural polyamines which are arisen from the number of carbons in aliphatic chain, the number of amine groups including diamine (two amine groups), triamine (three amine groups), tetramine (four amine groups), etc., and the additionally functional group such as hydroxyl group (Figure 1.3). Each of living organism synthesizes dominant polyamines in different patterns, for example, the major polyamines in higher plant are putrescine (Put), spermidine (Spd), and spermine (Spm) (Sawhney et al., 2003), while the dominant polyamine in cyanobacterium *Synechocystis* sp. is spermidine (Hosoya et al., 2005). The polyamines were suggested

that have many cellular functions in organisms including cell processes such as proliferation and differentiation (Heby, 1981), stabilization of cell membrane (Ballas et al., 1983), action on radical scavengers against oxidative stress inside the cell (Bors et al., 1989), and also signaling response to environmental stresses (Gill and Tuteja, 2010).

1.3.1 Polyamines forms *in vivo*

At the physiological pH, polyamines are mainly free forms and positively charged. Thus, they are able to interact with negatively charged macromolecules, e.g. nucleic acids, proteins or acidic phospholipids (Flink and Pettijohn, 1975; Chapel, Teissie, and Alibert 1984; Mehta et al., 1991 cited in Kotzabasis et al., 1993), consequently generate the bound forms. In addition, polyamines can associate with small anions, such as phenolic acids and then resulted in conjugated forms (Martin-Tanguy, 1997).

1.3.2 Polyamines biosynthesis

The pathway of polyamine biosynthesis in all organisms mainly involves with amino acids catabolism. In higher plants, the polyamine synthesis pathway correlates with ethylene synthesis pathway (Figure 1.4). The diamine (putrescine) was synthesized via two ways, from ornithine and arginine. For the triamine (spermidine) and tetramine (spermidine) were subsequently synthesized by adding aminopropyl groups into putrescine and spermidine molecules, respectively. On the other hand, the polyamine synthesis in mammalians slightly differs from plants and some bacteria. Mammalian polyamine was synthesized via ornithine route (Pegg, 2009).

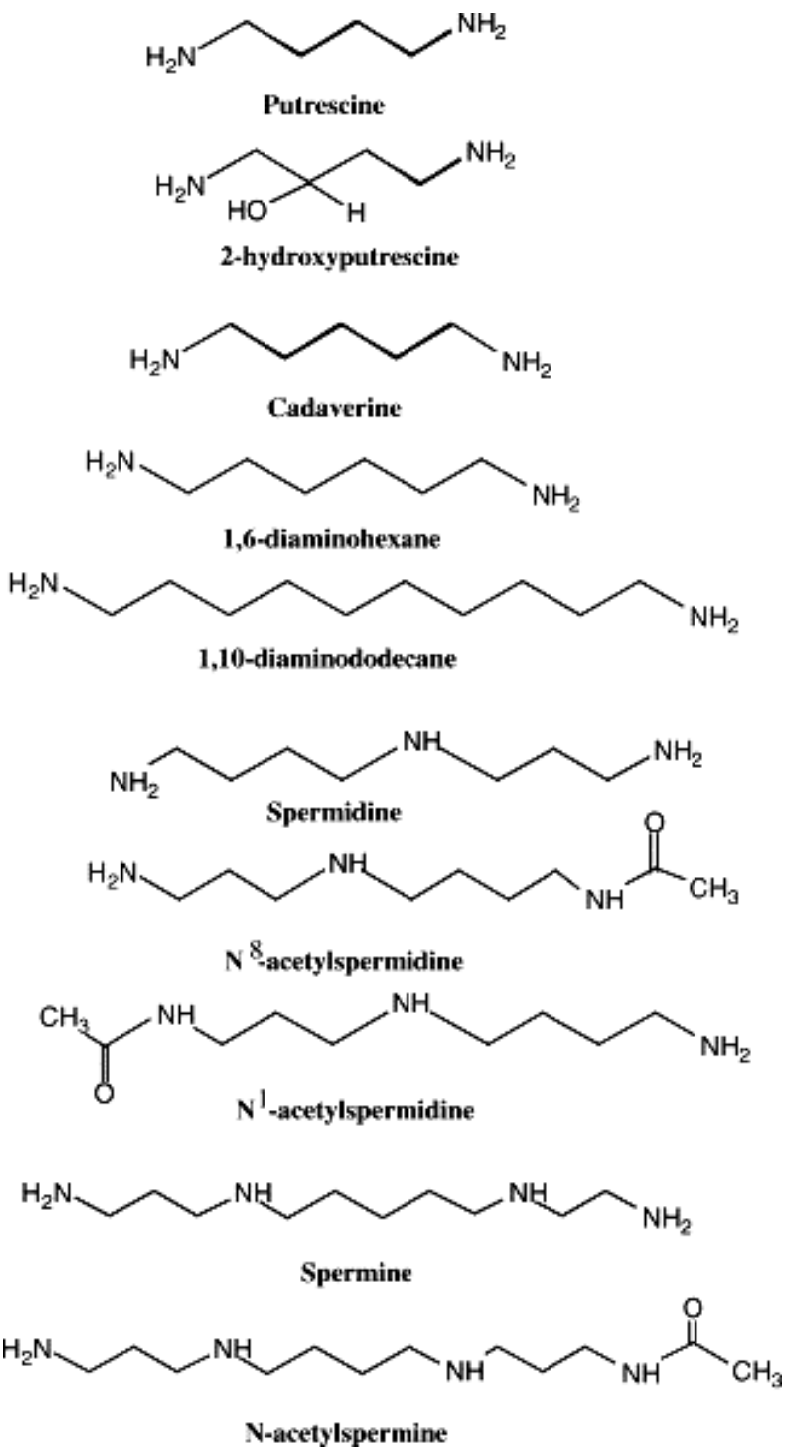


Figure 1.3 The structures of natural polyamines (Gaboriau et al., 2003).

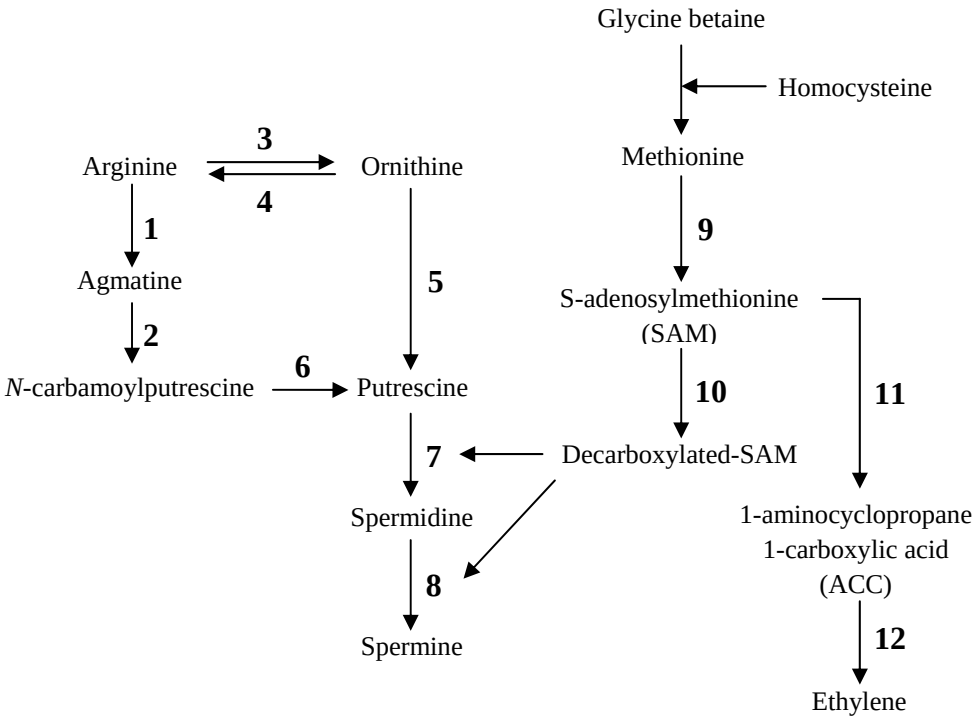


Figure 1.4 The pathway of polyamine biosynthesis in plants (1, arginine decarboxylase (ADC); 2, agmatine iminohydrolase; 3, arginase; 4, arginine synthase; 5, ornithine decarboxylase (ODC); 6, *N*-carbamoyl putrescine amidohydrolase; 7, spermidine synthase; 8, spermine synthase; 9, SAM synthase; 10, SAM decarboxylase; 11, ACC synthase; 12, ACC oxidase) (Bouchereau et al., 1999).

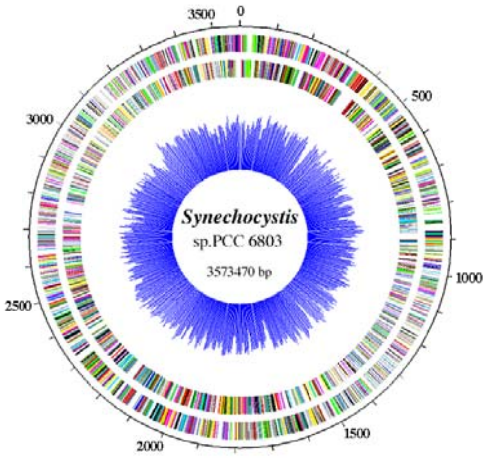
1.3.3 Polyamines and photosynthesis under UV radiations

All UV radiations are very harmful for photosynthetic systems. In higher plants, UV-B radiation severely damaged on 5 key molecules; 1) nucleic acids 2) amino acids and proteins 3) lipids 4) quinines and 5) pigments (Vass, Szilárd, and Sicora, 2005). The considerable damage was core protein of photosystem degradation which resulted in decreases of photosynthesis rate and mass production (Barbato et al.,

1995; Kakani et al., 2003). According to high energy of UV radiation, the UV exposed cell produced more free radicals (Hideg and Vass, 1996) caused of oxidative stress. Higher plant cells could protect themselves from UV damage by several mechanisms including UV absorbance compound accumulation (Lois, 1994), leaf thickness increase (Bolink et al., 2001), and up-regulation of antioxidant enzymes. Recently, many researchers have studied about polyamine response to environmental stresses and their function on photosynthetic membranes. In 2005, Lütz and co worker revealed that polyamines, especially putrescine, on thylakoid membrane were increased after tobacco plants exposed to simulated solar radiation with high UV-B level. They proposed that polyamine function was one of the primary protective mechanisms in photosynthetic apparatus against UV-B radiation (Lütz et al., 2005). Furthermore, Ioannidis and Kotzabasis (2007) found that the diamine, putrescine, stimulated ATP synthesis while spermidine and spermine stimulated on non-photochemical quenching (NPQ). From these results, polyamines were implicated on the functionality of the photosynthetic membrane (Ioannidis and Kotzabasis, 2007). Thus, the polyamines were efficient biomolecules of photoautotroph organism that acted against UV radiation not only served as radical scavengers (Bors et al., 1989), but also stabilized photosynthetic membranes (Besford et al., 1993).

1.4 Cyanobacterium, *Synechocystis* sp. PCC 6803

Cyanobacteria are prokaryotes living in water which were classified into kingdom bacteria (Smith, 2004). They are very well-known as the ancient oxygenic photoautotrophs and popular to use as a model of photosynthesis according to very fast growing and sensibly responding to various stresses. *Synechocystis* sp. PCC 6803 (PCC stood for the Pasteur Culture Collection, USA) is a unicellular cyanobacterium isolated from a freshwater lake in 1968. It is suitable for study with many reasons including its complete genome sequencing (Kaneko and Tabata, 1997), capable response to environmental stresses and similar function of photosynthesis apparatus like higher plants (Glatz et al., 1999).



Kingdom: Bacteria

Phylum: Cyanobacteria

Order: Chroococcales

Genus: *Synechocytis*

Species: *S.* sp. PCC6803

Figure 1.5 Genome map and scientific classification of *Synechocystis* sp. PCC 6803 (http://genome.kazusa.or.jp/cyanobase/Synechocystis)

1.5 The objectives of this study

In higher plants, polyamines were reported to be involved in stabilization mechanism of thylakoid membrane under high light and UV-B stresses (Besford et al., 1993), but there was still no report in cyanobacteria. The hypothesis of this study was proposed that whether the intracellular polyamines are related with photosynthetic systems, which may have an important role responsible to light stress. Thus, the objectives of this study are shown as followings;

1. To study effects of long-term UV radiation on growth of cyanobacterium *Synechocystis* sp. PCC 6803.

2. To study effects of long-term UV radiation on pigment contents in cyanobacterium *Synechocystis* sp. PCC 6803.

3. To study effects of long-term UV radiation on polyamine contents related to photosynthetic system in cyanobacterium *Synechocystis* sp. PCC 6803.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Balances	METTLER PJ360 DeltaRange® GWB, USA
Laminar flow	BVT-124 International Scientific Supply, Thailand
Spectrophotometer	DU® 530 Beckman Coulter, USA
Centrifuge	Mikro 22 R Hettich, Germany
pH meter	ORION model 420A, USA
Ultracentrifuge	Optima™ L-100XP Beckman Coulter, USA
UV-A light bulb	Ecologic® 350 blacklight 15W Sylvania, USA
UV-B light bulb	F15T8 UVP, Japan
UV-C light bulb	G15T8 Sankyo Denki, Japan
UV bench lamp	Blak-Ray® XX-15M UVP, USA
UV meter	UVX Radiometer UVP, USA
HPLC	SCL-10AVP Shimadzu, Japan
C-18 column	4.6 x 150 mm 5 μm i.d. Inertsil® ODS-3 GL Sciences Inc., Japan
Fluorometer	Modified from qubit system fluorometer, Thailand
Autoclave	Model HA-30 Hirayama, Japan
Vortex	Model 232 Touch mixer Fisher Scientific, USA
Semi Dry Electroblotting	Owl HEP-1 Thermo Scientific, USA
Cell disrupter	French® Press Thermo electron corporation, USA

2.2 Chemicals

3,3'-Diaminobenzidine	Sigma, USA
Acetic acid	Lab Scan, Ireland
Acrylamide	Scharlau Chemie S.A., Spain
Ammonium ferric citrate	Ajax Finechem, Australia
Ammonium persulfate	Merck, Germany
Benzoyl chloride	Sigma, USA
Boric acid	Scharlau Chemie S.A., Spain
Bromophenol blue	BDH chemicals Ltd., UK
Calcium chloride	Ajax Finechem, Australia
Citric acid	Ajax Finechem, Australia
Cobalt (II) chloride	Ajax Finechem, Australia
Cobalt (II) nitrate	Carlo ERBA, French
Coomassie blue R-250	Acros, Belgium
Coomassie blue G-250	Fluka, USA
Copper (II) sulfate	Carlo ERBA, France
Diethylether	Labscan, Ireland
Dimethylformamide	RCI Labscan, Thailand
Dipotassium phosphate	Ajax Finechem, Australia
Dithiothreitol (DTT)	Sigma, USA
DM	Sigma, USA
EDTA	Ajax Finechem, Australia
Ethanol	Merck, Germany
Glycerol	Ajax Finechem, Australia
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate	Ajax Finechem, Australia

Manganese chloride	Ajax Finechem, Australia
Mercaptoethanol	Sigma, USA
Methanol	Burdick jackson, USA
Methylene-bis-acrylamide	Amersham Bioscience, Sweden
PCA	Merck, Germany
Pyridoxal-5-phosphate	Sigma, USA
PMSF	Sigma, USA
Putrescine	Sigma, USA
Sodium bicarbonate	Ajax Finechem, Australia
Sodium chloride	Ajax Finechem, Australia
Sodium dithiosulfate	Ajax Finechem, Australia
SDS	Ajax Finechem, Australia
Sodium hydroxide	Ajax Finechem, Australia
Sodium molybdate	Ajax Finechem, Australia
Sodium nitrate	Ajax Finechem, Australia
Spermidine	Sigma, USA
Spermine	Fluka, USA
Sucrose	Ajax Finechem, Australia
TEMED	Bio-Rad, USA
Tris (hydroxymethyl)-aminomethane	USB Corporation, USA
Triton X-100	Packard, USA
Zinc sulfate	Ajax Finechem, Australia

2.3 Antibodies and supplies

0.45 μm PVDF membrane	Immobilon-P Merck, USA
0.45 μm Cellulose acetate membrane	Sartorius stedim biotech, Germany
Anti-PsaA (core PSI protein, rabbit IgG)	Agrisera, Sweden
Anti-PsbA (D1 protein, rabbit IgG)	Agrisera, Sweden
Anti-PsbD (D2 protein, rabbit IgG)	Agrisera, Sweden
Goat anti-rabbit IgG, HRP conjugated	Agrisera, Sweden

2.4 Organism

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

2.5 Culture condition

Synechocystis cells were grown in BG_{11} liquid medium (appendix A) under normal condition; continuous white fluorescent light (40 – 50 μmol photon·m^{-2}·s^{-1}), 27°C and filtered air bubbling for 5 – 7 days. Then, the cells were transferred onto BG_{11} agar medium and cultured on plate for 7 – 8 days under normal condition before treating with UV radiations in various time treatments ranging from 0 – 24 h.

2.6 Growth monitoring

One ml of *Synechocystis* cell culture was taken from either the cultured flask or suspended cells from agar plate. To monitor the growth of cells, we measured their optical density spectrophotometrically at 730 nm.

2.7 Determination of chl *a* and carotenoid contents

Cells suspension (1 ml) was centrifuged at 8,000*xg*, 4ºC, for 15 min. The pellet was further extracted by 1 ml of DMF (dimethylformamide), and incubated for 10 min at room temperature. After centrifugation at 8,000*xg* for 3 min, the supernatant was measured the absorbance at 461, 625, and 664 nm, respectively. Chl *a* and carotenoid contents were calculated using the equation shown below (Jantaro et al., 2006) and reported the total of chl *a* and carotenoid contents in $\mu g \cdot 10^{-8}$ cells.

$$\text{Chl } a \ (\mu g \cdot ml^{-1} DMF) = (12.1 \times A_{664}) - (0.17 \times A_{625})$$

$$\text{Carotenoid } (\mu g \cdot ml^{-1} DMF) = (A_{461} - 0.046 \times A_{664}) \times 4$$

The amount of total cells was calculated from the equation of the standard curve in appendix B.

2.8 Determination of protein content

Protein quantification from the cells was determined by the method of Bradford (1976) using BSA as a protein standard. Sample was incubated with Bradford reagent (appendix C) at room temperature for 15 min, and then measured the absorbance at 595 nm. The total of protein content was represented as $\mu g \cdot 10^{-8}$ cells.

2.9 Measurement of photosynthetic efficiency

Cells suspension with total chl *a* concentration of approximately 0.5 mg was filtrated through 0.45 μm cellulose acetate membrane filter. The retentate on membrane was placed under darkness for 30 min. Photosynthetic efficiency (F_v/F_m) was measured by fluorescence induction using the modified fluorometer apparatus. The F_v value equals $F_m - F_0$ which F_m and F_0 are the maximum and minimum values of fluorescence, respectively.

2.10 Thylakoid membrane preparation

Collected cell pellet (100 mg) was resuspended in 2 ml of STNE buffer (appendix D). These suspended cells were disrupted under the pressure of 520 psi using French® Press cell disruptor. Cell debris was discarded after centrifugation at 1000*x*g, 4 ºC, for 4 min. Crude extract of thylakoid membranes was collected in the pellet fraction after centrifugation at 18,000*x*g, 4 ºC, for 20 min.

2.11 Optimization of thylakoid membrane solubilization condition

The thylakoid membrane extract with chl *a* concentration of 0.6 $mg \cdot ml^{-1}$ in STNE buffer was solubilized with various nonionic detergents including triton X-100 and dodecyl maltoside (DM). The various concentration ratios of detergent (mg/l): chl a (mg/l) were tested; consisting of 5:1, 10:1, 20:1 and 40:1, respectively. For the incubation time was varied into 30, 60 and 120 min, respectively. The incubation temperature tested in this experiment was varied at 0 and 4 ºC.

2.12 Sucrose gradient ultracentrifugation

The solubilized thylakoid membranes (from 2.11) were separated into each component by sucrose gradient ultracentrifugation at 144,000*x*g, 4ºC for 16 h. Sucrose gradient was performed in various concentrations of sucrose ranging from 10 – 50% (w/w). The volume addition of each sucrose solution with different %concentration (w/w) of 10%, 20%, 30%, 40% and 50% was 1.0, 2.0, 2.0, 2.0, and 2.0 ml, respectively. Each band separated after centrifugation was collected and kept on ice. Then, protein concentration, western blotting and determination of polyamine contents were detected.

2.13 Protein identification

2.13.1 Protein separation

Protein extract (from 2.12) were separated using SDS-PAGE. Samples (20 μg protein) from each sucrose fraction were resuspended in sample buffer (appendix E) at a ratio of 4:1. The mixture was heated for 5 min in boiling water before loading on a 12% gel. The electrophoresis buffer was used for gel running at constant current of 20 mA per gel. Electrophoresis was run continuously until the dye front reaching the bottom of the gel. The gel was stained with staining solution (appendix E) for 1 h and, then destained three times with destaining solution (appendix E) at room temperature for 30 min each. For western blotting, the gel was not stained but used immediately in next step.

2.13.2 Western blotting

After resolving protein sample by SDS-PAGE, the gel was transferred onto PVDF membrane using semi-dry electrophoretic method. Gel and membrane were attached to each other between transferring buffer-wetted filter papers (appendix F) which directly contacted with flat-plate electrodes. The transferring condition was set as high constant current (5.5 $mA \cdot cm^{-2}$ for 1 h) and low voltage (10 – 15 V). After that the blotting membrane was blocked immediately in blocking buffer for 1 h at room temperature, with agitation. And then, the blotting membrane was rinsed with TBS-T buffer for 4 times with 5 min each at room temperature. Then, the incubation in the appropriate dilution of primary antibody for 1 h at room temperature was performed with agitation. The dilution of primary antibody was as followings; PsaA protein (PSI

core protein) of 1:10000, PsbA (D1 protein of PSII) of 1:20000, and PsbD (D2 protein of PSII) of 1:50000. The blotting membrane was washed in TBS-T buffer once for 10 min and 3 times for 5 min at room temperature. Later, it was incubated in the appropriate dilution of secondary antibody for 1 h at room temperature with agitation. The dilution of secondary antibody was 1:10000. Later, the blotting membrane was rinsed again with TBS-T buffer. Finally, the blotting membrane was detected for the signal by enzymatic reaction of horse radish peroxidase for 3 min using DAB as a substrate (appendix F).

2.14 Polyamine analysis

2.14.1 Polyamine derivatization

Samples (whole cells, crude thylakoid membranes, and solubilized thylakoid membrane fractions from 2.12) were added by 1 ml of 5% cold perchloric acid (PCA), then incubated at 4 ºC for 60 min. After centrifugation at 6,000*xg*, 4 ºC for 20 min, both of supernatant (0.5 ml) and resuspended solution of pellet (0.5 ml) were added by 2 ml of 2M NaOH and mixed gently. Then, the incubation with 20 µl of benzoyl chloride for 30 min was taken place at room temperature. The reaction of derivatization was stopped by adding 2 ml of saturated NaCl, and further separated the obtained derivative by 2 ml of diethyl ether. Diethyl ether from the upper layer containing derivatized polyamines was evaporated. The dry pellet then was dissolved with 1 ml of methanol (HPLC grade) and used further for determination of polyamine contents in next step (2.14.2).

2.14.2 Polyamine quantification

Polyamine contents were measured using high performance liquid chromatography with C-18 reverse phase column (4.6 x 150 mm inertsil®, ODS-3, 5µm i.d., GL Sciences Inc., USA). The chromatogram was detected at an absorbance of 254 nm (diode array detector, SPD-10A*VP*, Shimadzu, Japan). The mobile phase was isocratic using 60% methanol with a flow rate of 1 ml·min^{-1}. Retention time was 15 min. The profile of polyamines standard was shown in appendix G.

2.15 Data analysis

All experiments were repeated independently in three times. Bars on the diagrams represent the average values ± SD. Samples were also analyzed statistically using analysis of variance (ANOVA, P <0.05), after performing Levene's test for equality of error variances. Individual difference was analyzed using Tukey HSD test.

CHAPTER III

RESULTS

3.1 Effect of UV radiation on growth of *Synechocystis* sp. PCC 6803

Synechocystis cells were grown in liquid BG_{11} medium with the initial OD_{730} of 0.2. The growth condition was performed under 40 – 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous fluorescence light (normal growth) at 27 ºC with filtered air bubbling for 7 – 8 days. After that, mid-log phase cells were harvested and used in UV treatment. The cultured cells (OD_{730} of 0.5) were exposed to UV radiations including UV-A (intensity 10 $w \cdot m^{-2}$), UV-B (intensity of 1.3 $w \cdot m^{-2}$), and UV-C (intensity of 1.3 $w \cdot m^{-2}$). The growth of cells was monitored by measuring the OD_{730} with spectrophotometer. The result was shown in Figure 3.1. Within 6 h of UV radiations, the growth of cells was slightly decreased when compared with that under normal growth light. Longer time of UV radiations significantly affected on cell growth. The reduction of cell density under UV-B and UV-C was observed to approximately 40% compared to normal growth while UV-A decreased cell density about 20% compared to normal growth in particular at 24 h.

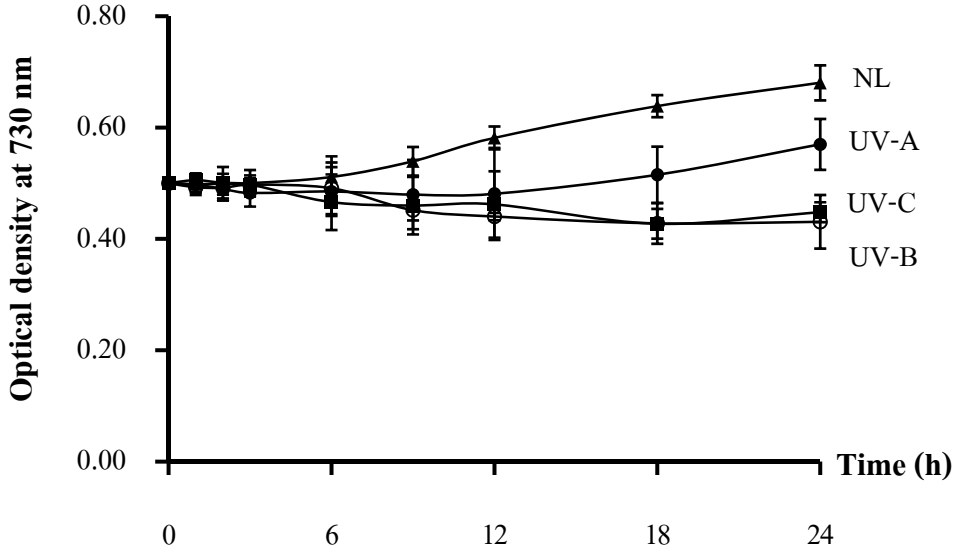


Figure 3.1 The growth of *Synechocystis* cells under normal growth (closed triangle, NL), UV-A (closed circle), UV-B (open circle) and UV-C (closed square) for 24 h. The data represent mean ± SD, n = 3.

3.2 Effect of UV radiation on chl *a* and carotenoid contents

Synechocystis cells were grown on BG_{11} agar medium. Later, cells were exposed to UV-A, UV-B and UV-C at intensity of 10, 1.3 and 1.3 $w \cdot m^{-2}$, respectively for 24 h. The collected cells were extracted with DMF for determination of chl *a* and carotenoid contents. The chl *a* and carotenoid contents inside the cell under UV radiations for 24 h were shown in Figure 3.2 and 3.3, respectively. Within 24 h, chl *a* content was decreased about 40% under UV-B and UV-C and 10% under UV-A. The carotenoid content was obviously decreased by 80% under UV-B and UV-C and 30% under UV-A.

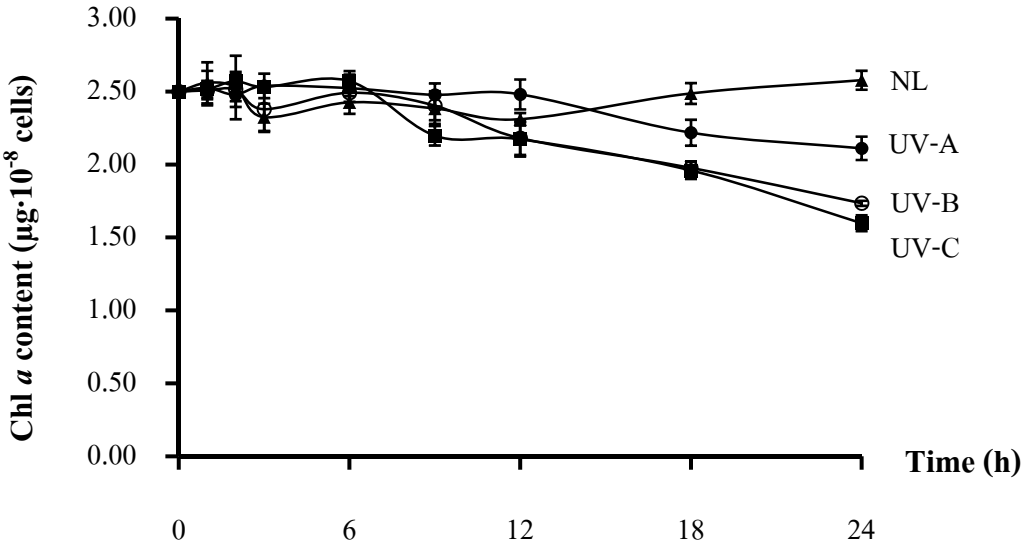


Figure 3.2 The chl *a* content of *Synechocystis* cells under normal growth (closed triangle, NL), UV-A (closed circle), UV-B (open circle) and UV-C (closed square) for 24 h. The data represent mean ± SD, n = 3.

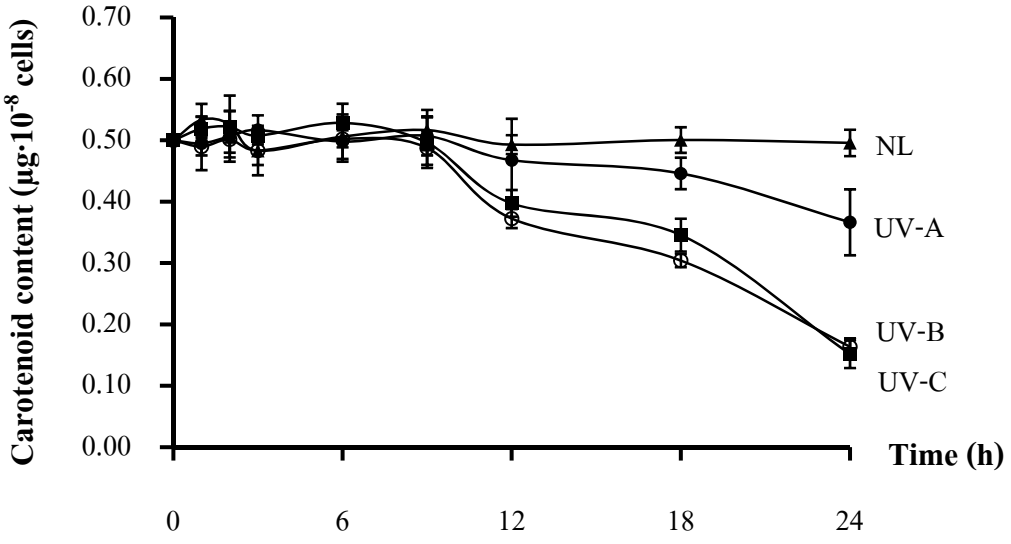


Figure 3.3 Carotenoid contents of *Synechocystis* cells under normal growth (closed triangle, NL), UV-A (closed circle), UV-B (open circle) and UV-C (closed square) for 24 h. The data represent mean ± SD, n = 3.

3.3 Effect of UV radiation on protein content

Cells exposed to UV-A, UV-B and UV-C radiation were collected and measured protein content using Bradford method (Figure 3.4). At first 2 h, UV-A and UV-C treatment enhanced slight contents of protein compared to that under normal growth light, whereas UV-B decreased apparently protein content of *Synechocystis* cells. However, although protein content in all treatments was induced obviously after 12 h-treatment, protein content of normal growth light-treated cells was highest among those three UV treatments.

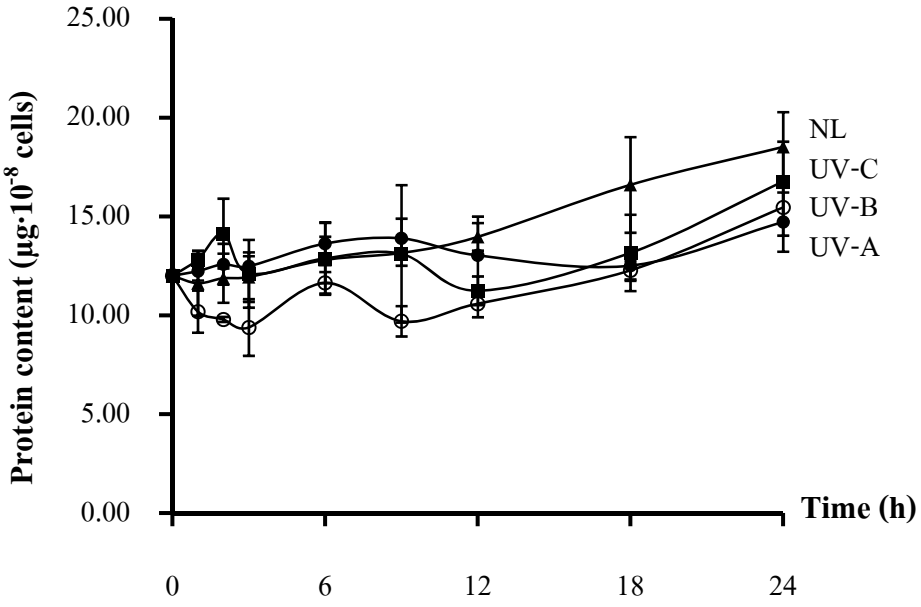


Figure 3.4 Protein content of *Synechocystis* cells under normal growth (closed triangle, NL), UV-A (closed circle), UV-B (open circle) and UV-C (closed square) for 24 h. Data is shown in mean ± SD (n = 3).

3.4 Effect of UV radiation on photosynthetic efficiency

In Figure 3.5, the photosynthetic efficiency (F_v/F_m) was monitored after exposing cells to normal growth light and UV radiations. The cell suspension with total chl *a* content of approximately 0.5 mg was used to measure the photosynthetic efficiency (F_v/F_m) using fluorometer. The F_v/F_m ratio of *Synechocystis* cell under normal condition was about 0.6 (Figure 3.5). The UV-treated cells significantly decreased F_v/F_m ratio, especially under UV-C. After 3 h exposure, photosynthetic efficiency of UV-A and UV-B was around 0.4 whereas that of UV-C was 0.1. Obviously, UV-B and UV-C completely affected on photosynthetic efficiency at 24 h-treatments.

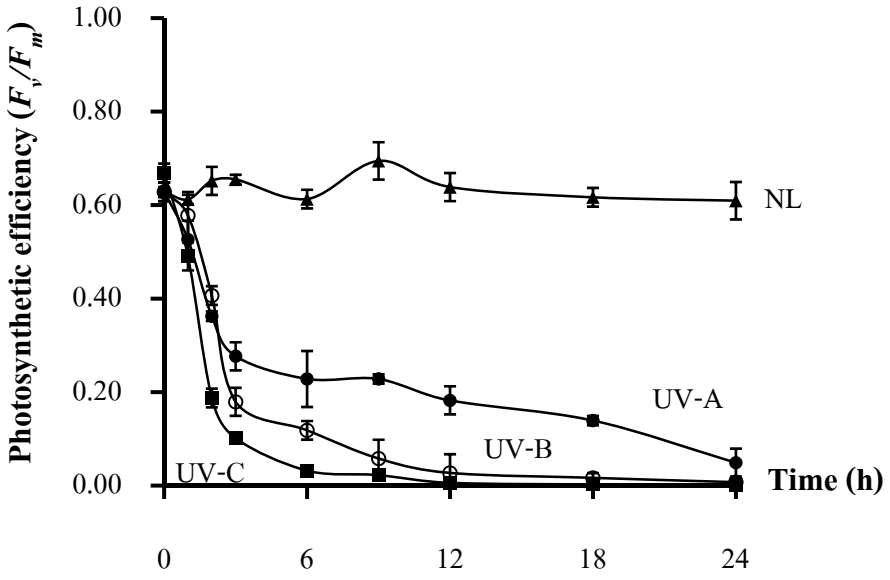


Figure 3.5 Photosynthetic efficiency (F_v/F_m) of *Synechocystis* cells under normal growth (closed triangle, NL), UV-A (closed circle), UV-B (open circle) and UV-C (closed square) for 24 h. The data are mean ± SD, n = 3.

3.5 Effect of UV radiation on polyamine contents in *Synechocystis* cells

The cells on BG_{11} agar medium (age 7 – 8 days under normal condition) were exposed to UV radiation and collected for polyamine determination. The cell pellet was incubated in 5% PCA for 1 h at 4 ºC. Two fractions of supernatant and pellet were separated and continued to polyamine derivative benzoylation. The supernatant fraction consisted of PCA-soluble polyamines, whereas the pellet fraction contained PCA-insoluble polyamines.

The highest polyamine content in *Synechocystis* sp. PCC 6803 of three main polyamines was spermidine in both PCA-soluble and PCA-insoluble fractions. *Synechocystis* cells under normal growth light showed a constant level of total polyamines of about 320 nmole·mg^{-1}protein at 24 h (Figure 3.6). After the cell expose to UV-A 3 h, the total polyamine content was significantly increased (413 ± 19.2 nmole·mg^{-1}protein), then decreased at the same level of initial (about 350 nmole·mg^{-1}protein) until 18 h-treatment (Figure 3.7). At 24 h-treatment of UV-A, total polyamine content was decreased significantly. Under UV-B radiation for 24 h (Figure 3.8), the total polyamine content was continuously decreased by 163 ± 13.7 nmole·mg^{-1}protein at 24 h-time point. For the effect of UV-C radiation on the total polyamine content (Figure 3.9), UV-C had a similar effect tendency as UV-B, the total polyamine content was reduced significantly at 24 h-treatment with the content of 129 ± 20.0 nmole·mg^{-1}protein. These results indicate that UV-B and UV-C had a severe damage on polyamine accumulation in long-term exposure, mostly in PCA-soluble form of spermidine.

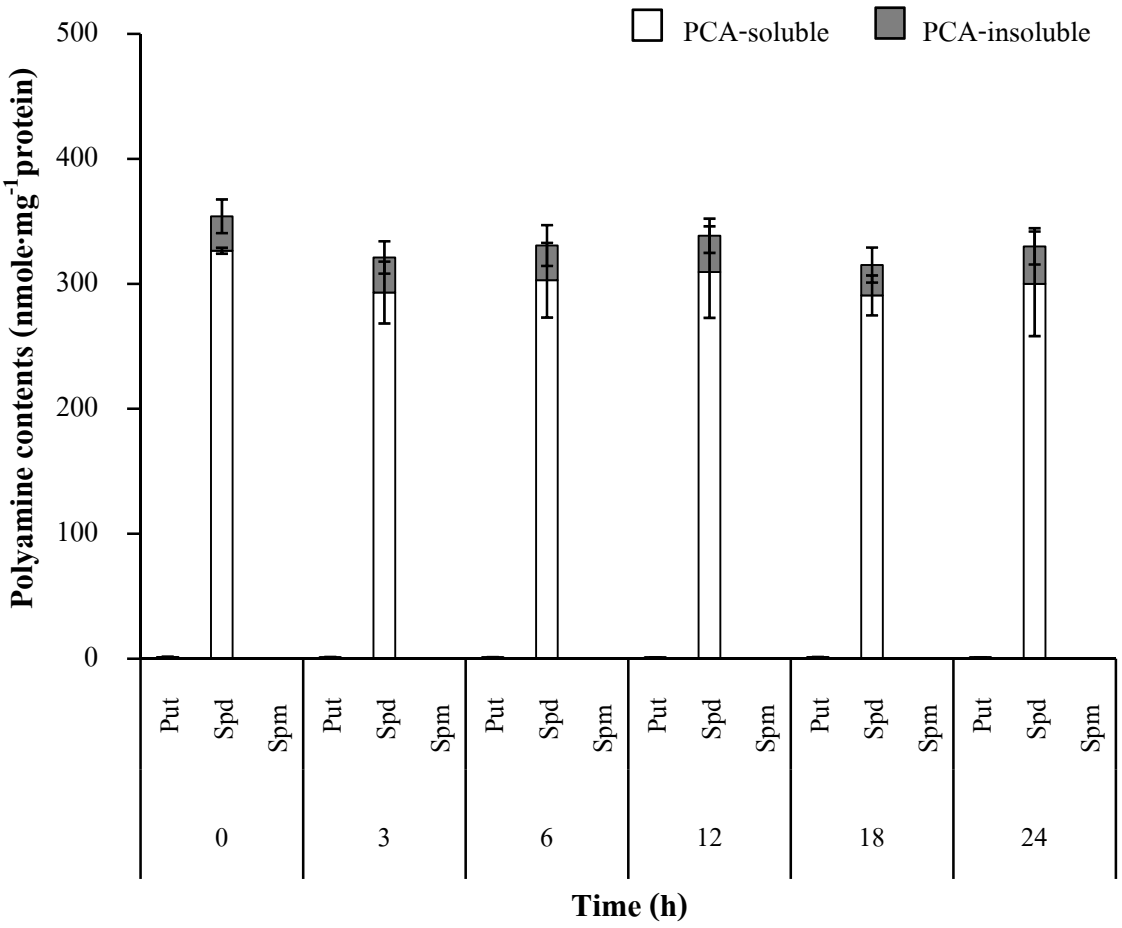


Figure 3.6 Polyamine contents of *Synechocystis* cells under normal growth light (intensity of 50 μmol·m^{-2}·s^{-1}) for 24 h. The data represent mean ± SD, n = 3.

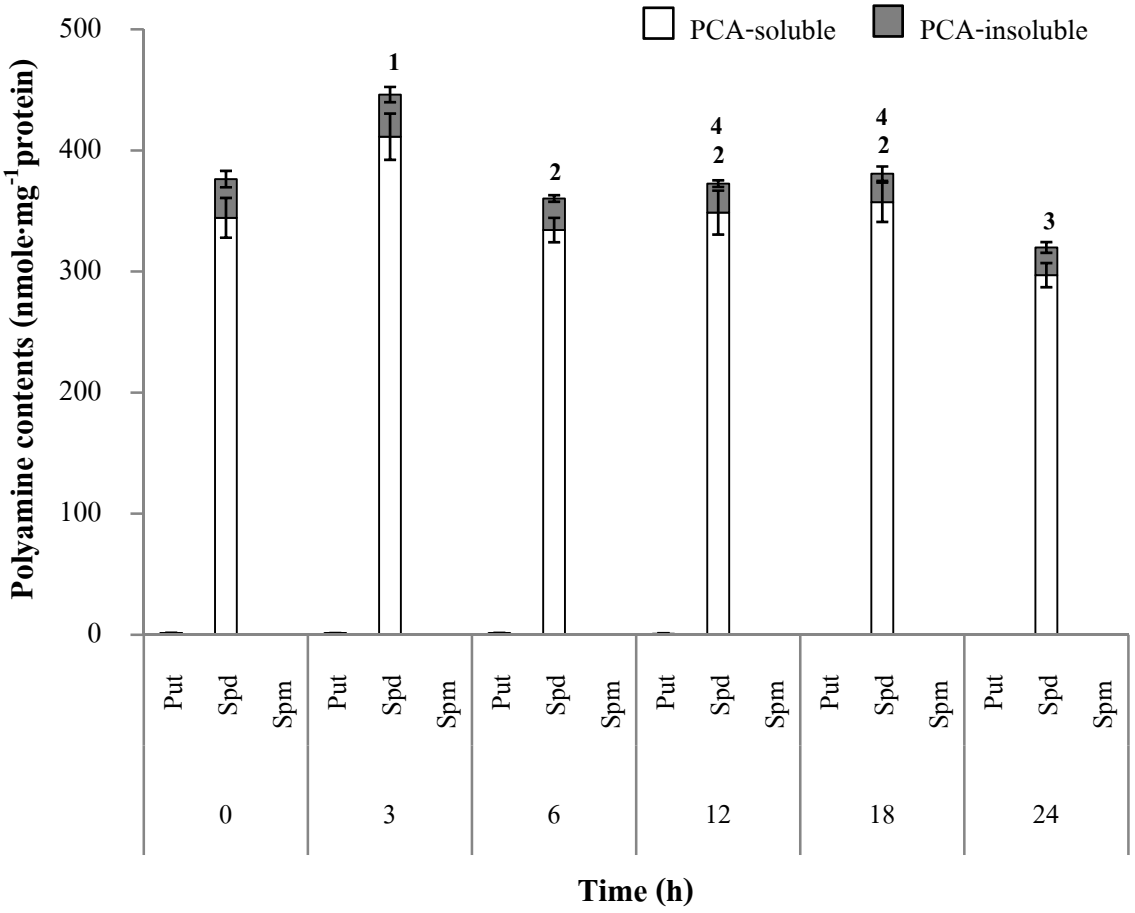


Figure 3.7 Polyamine contents of *Synechocystis* cells under UV-A (intensity of 10 $w \cdot m^{-2}$) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

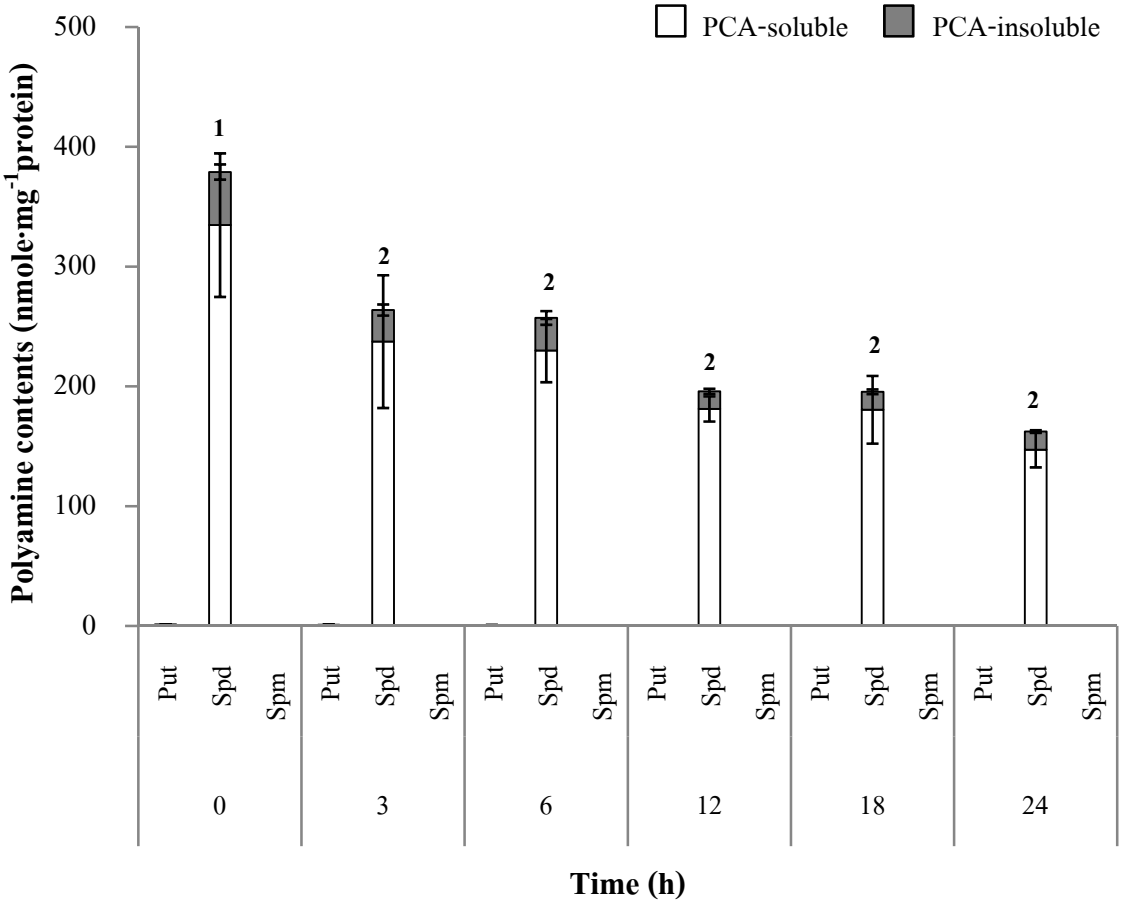


Figure 3.8 Polyamine contents of *Synechocystis* cells under UV-B (intensity of 1.3 $w \cdot m^{-2}$) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

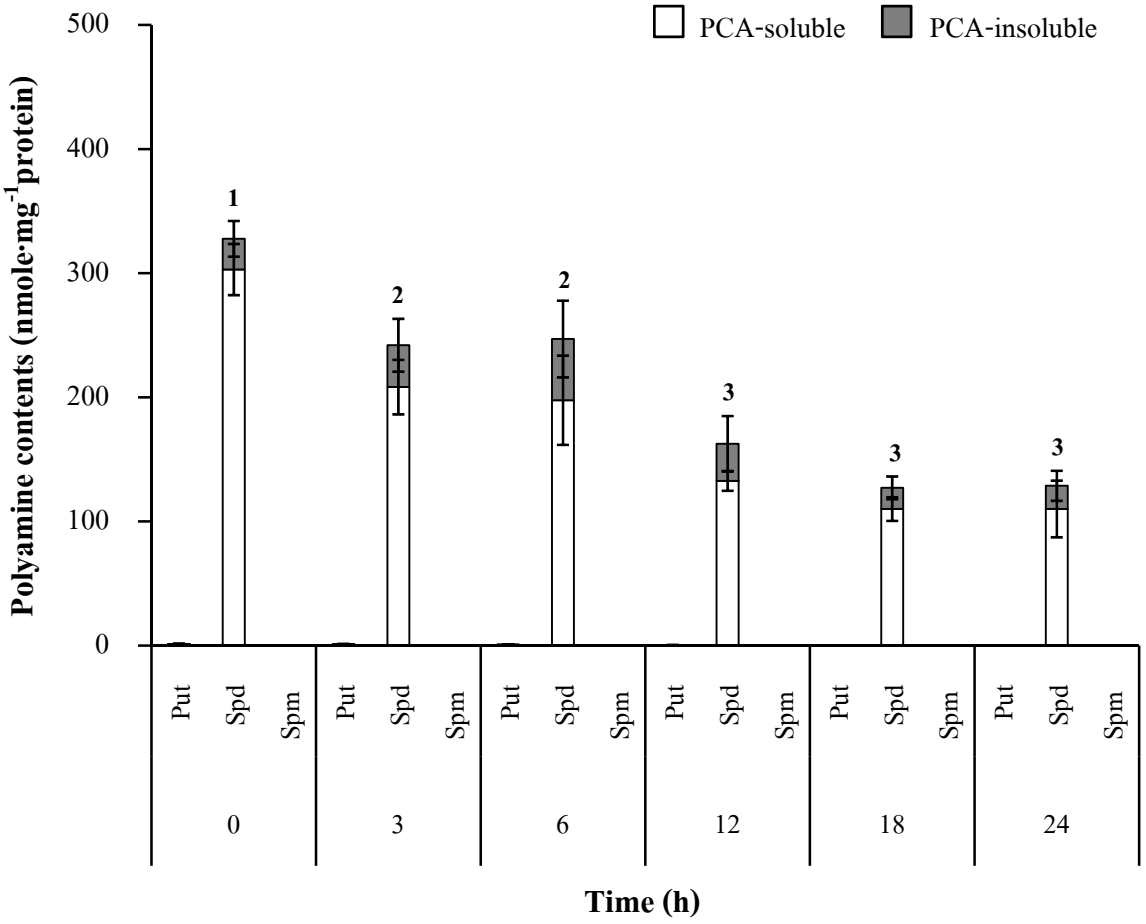


Figure 3.9 Polyamine contents of *Synechocystis* cells under UV-C (intensity of 1.3 $w \cdot m^{-2}$) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

3.6 Effect of UV radiation on polyamine contents associated with thylakoid membranes

After the thylakoid membrane extraction from both cells exposed to normal growth light and UV radiations, polyamine contents, which were associated to thylakoid membranes, were measured. Under normal growth light, polyamine content showed in a steady level (about 280 nmole·mg^{-1}protein) up to 24 h (Figure 3.10). Under UV-A radiation, polyamine content was obviously decreased 1.5 fold for 24 h (Figure 3.11). Under UV-B radiation, polyamine content associated with thylakoid membranes gave an apparent decrease about 40% at 3 h-treatment and a high decrease after 6 h-treatment (Figure 3.12). Similarly, UV-C radiation inhibited the accumulation of polyamines associated with thylakoid membranes after exposing for 3 h-treatment (more than 50%). At 24 h-treatment of UV-C, polyamines associated with thylakoid membranes was highly decreased of about 90% (Figure 3.13).

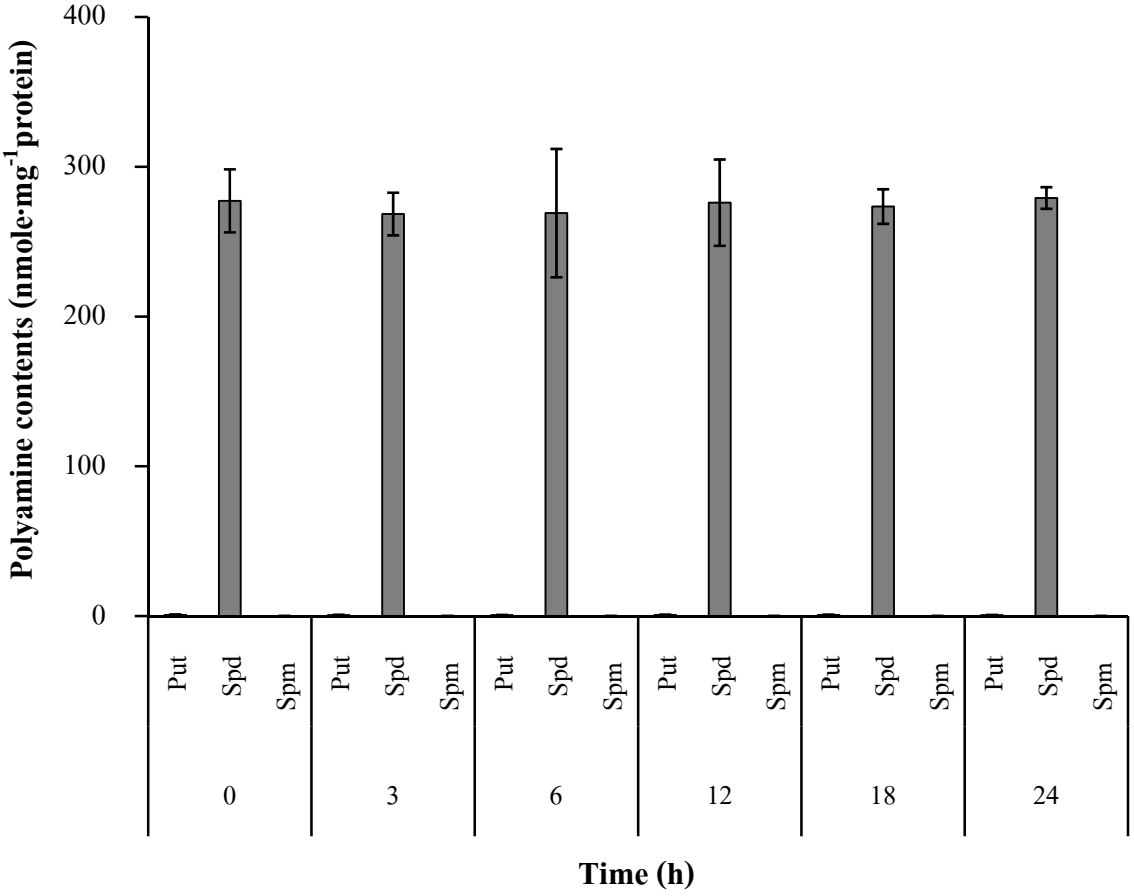


Figure 3.10 Polyamine contents associated with thylakoid membranes of *Synechocystis* cells under normal growth light (intensity of 50 μmol·m^{-2}·s^{-1}) for 24 h. The data represent mean ± SD, n = 3.

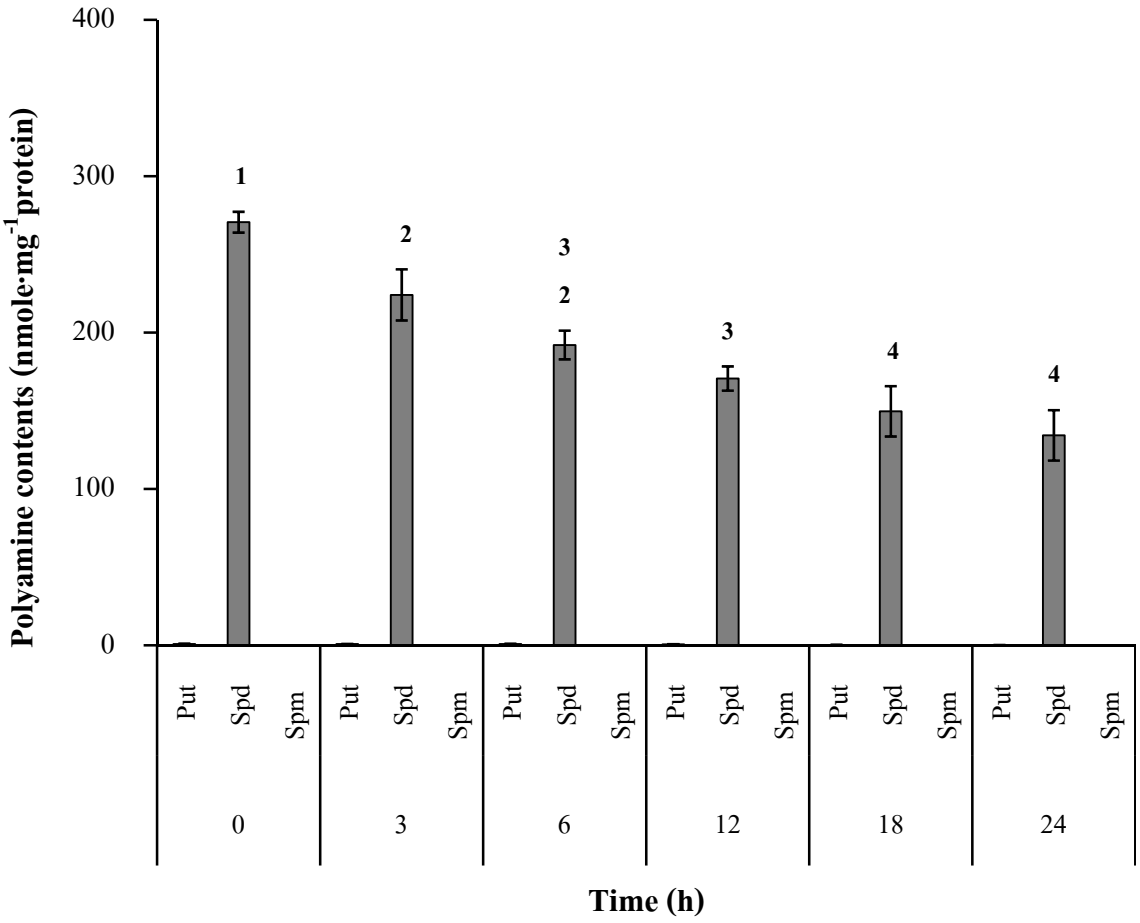


Figure 3.11 Polyamine contents associated with thylakoid membranes of *Synechocystis* cells under UV-A (intensity of 10 $w \cdot m^{-2}$) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

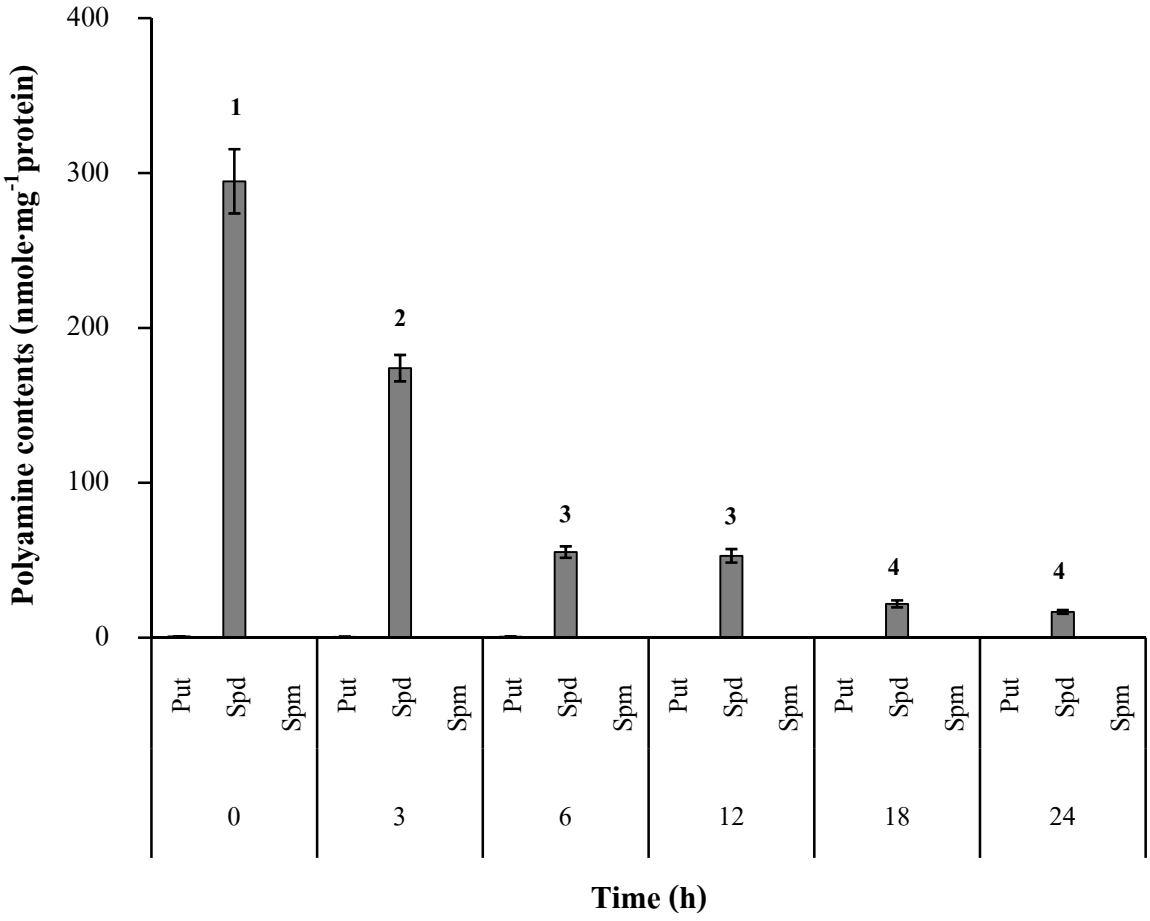


Figure 3.12 Polyamine contents associated with thylakoid membranes of *Synechocystis* cells under UV-B (intensity of 1.3 $w \cdot m^{-2}$) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

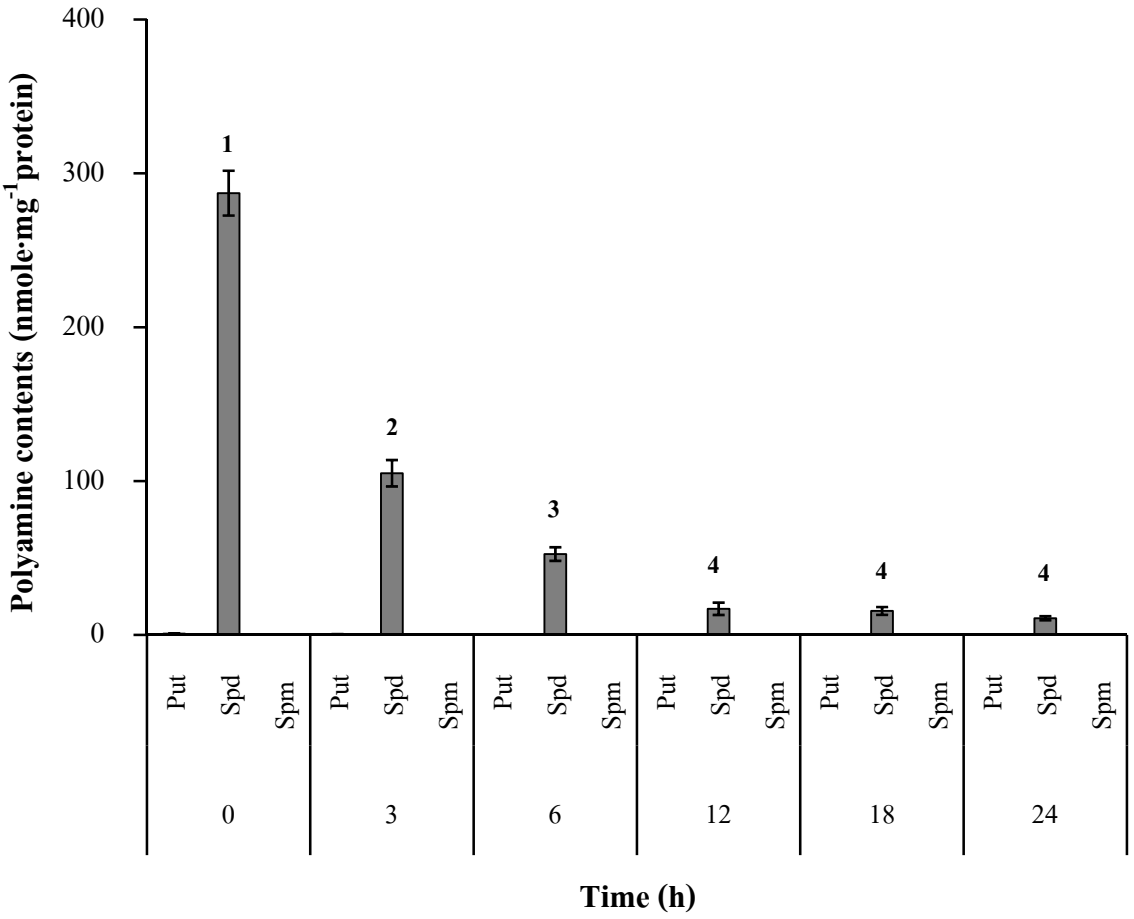


Figure 3.13 Polyamine contents associated with thylakoid membrane of *Synechocystis* cells under UV-C (intensity of 1.3 w·m^{-2}) for 24 h. The different numbers above the bars are used to designate significant differences (ANOVA statistics were performed with the original data $P < 0.05$, $n = 3$).

3.7 Optimization of thylakoid membrane solubilization condition

The optimization factors in this study were consisted of types of nonionic detergents (triton X-100 and DM), incubation time (30, 60, 120 min) and incubation temperature (0 and 4 ºC). The results of optimum condition for thylakoid membrane solubilization were observed from the efficiency of fraction separation after the step of sucrose gradient ultracentrifugation (Figure 3.14).

The triton X-100 solubilized fraction obtained from sucrose gradient step was not well separated (Figure 3.14 A, (1) - (6)) whereas DM detergent effectively solubilized the thylakoid membranes which showed a good separation after sucrose gradient step (Figure 3.14 B, (1) - (6)). Thus, the optimum detergent used to solubilize thylakoid membranes was DM at the ratio of 20 mg DM: 1 mg chl *a*.

Incubation temperature either at 0 (on ice) or 4 ºC did not affect the efficiency of thylakoid membrane solubilization and separation. The incubation temperature at 25 ºC was also tested preliminarily with both triton X-100 and DM but thylakoid membranes were not well solubilized (appendix H). For incubation time of solubilization, it was found that after 120 min-solubilization at either 0 or 4 ºC gave the best separation of each photosynthetic component in each band (Figure 3.14 B, (3)). Then, these obtained optimum conditions were used in next step.

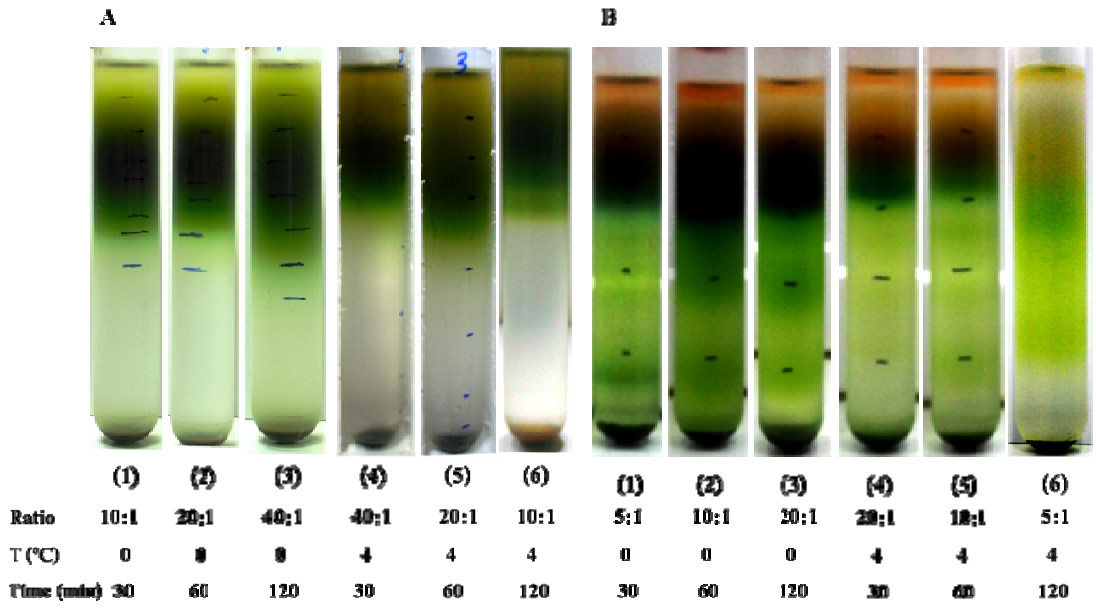


Figure 3.14 Solubilization of thylakoid membrane with **A)** triton X-100 and **B)** DM. Ratio noted as weight (detergent) by weight (chl *a*).

3.8 The separation of solubilized thylakoid membranes

The solubilized thylakoid membranes were loaded onto a set of sucrose gradient in a range of 10 – 50% and centrifuged at 144,000*x*g, 4 ºC for 14 h. After sucrose gradient ultracentrifugation, six bands of solubilized thylakoid membranes were obviously separated (Figure 3.15). Each fraction was collected to determine polyamine contents and identify photosynthetic core proteins in next step. The first fraction, the top of gradient at 10% with orange color band was identified as F_1 (Figure 3.15). The light brown band, F_2, was at the bottom layer of 10% sucrose. For 20% sucrose layer (dark green band) was collected as F_3. The boundary of 20 - 30% sucrose layer, the fresh green band was F_4 fraction. The boundary of 30 - 40% sucrose fraction was the light green band (F_5). And the boundary of 40 - 50% sucrose, the light green ring was collected as F_6.

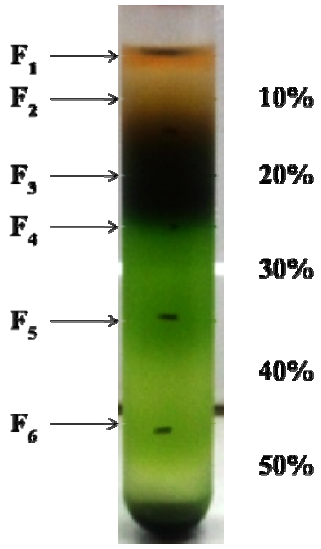


Figure 3.15 The separation of solubilized thylakoid membranes by sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h.

3.9 Protein identification

After the separation of solubilized thylakoid membranes performed using sucrose gradient ultracentrifugation, each fraction (of $F_1 – F_6$) was identified by SDS-PAGE and Western blotting with photosynthetic protein-secondary antibody. We found the bands of photosystem core proteins including PsaA protein (core protein of PSI), PsbA (D1 protein of PSII), and PsbD (D2 protein of PSII) on those separated fractions from solubilized thylakoid membranes.

Firstly, the protein components in each fraction were resolved using SDS-PAGE (Figure 3.17). From the result, protein component was not found in fraction F_1 (lane 2). Thus, F_1 fraction was not continuously transferred to western blotting step.

Secondly, the protein bands were identified using western blotting. Figure 3.18 shows the result of western blotting without F_1 fraction. PSI core protein was found in F_2 fraction that existed at 10% sucrose. D1protein was found in F_3 fraction (20% sucrose), F_4 fraction (between 20 – 30% sucrose) and F_5 fraction (between 30 - 40% sucrose). However, the strong signal was detected in F_3 and F_4 fractions. For D2 protein was found in many fractions including F_3 to F_6 (between 40 - 50% sucrose) fractions. It was previously shown in solubilized thylakoid membranes from *A. thaliana* that the free pigments contained no photosynthetic proteins whereas fractions separated from PSII core protein were monomer and trimeric light harvesting complex (LHC), following by PSII core complex and PSI-LHCI (Dall'Osto et al., 2006). Then, each fraction found PSII core proteins might be represent as the size of core protein cluster. It is consistent with the previous study of Sato and colleagues (2004),

solubilized thylakoid membrane separation in *Synechocystis* sp. PCC 6803 showed the upper band as PSI trimer and monomer, and lower band was PSII monomer.

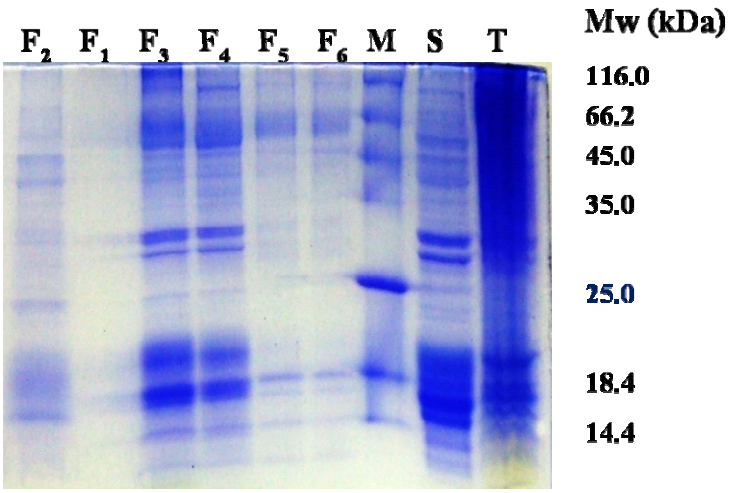


Figure 3.16 Resolved protein components in each fraction after sucrose gradient ultracentrifugation using SDS-PAGE. Protein content was loaded at concentration of F_1; 0.04 µg, F_2; 10 µg; F_3 20 µg; F_4 10 µg; F_5 and F_6; 2 µg, **M;** protein marker, **S;** 20 µg (supernatant of cell disruption after centrifugation at 18,000*x*g, 4ºC for 20 min), **T;** 20 µg (solubilized thylakoid membranes before loading on sucrose gradient ultracentrifugation).

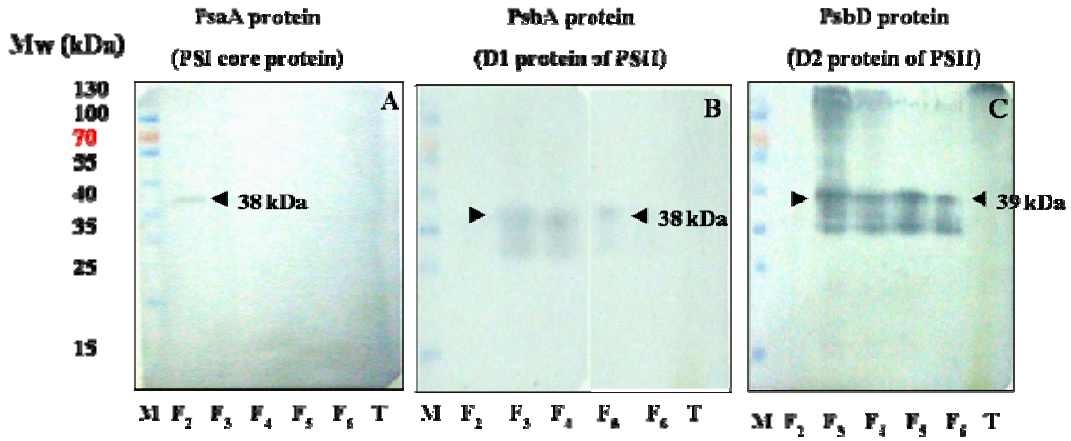


Figure 3.17 Western blotting of **A)** PsaA protein (PSI core protein), **B)** PsbA protein (D1 protein of PSII) and **C)** PsbD protein (D2 protein of PSII). Protein content was loaded at concentration of $\mathbf{F_2}$**;** 10 µg, $\mathbf{F_3}$**;** 20 µg, $\mathbf{F_4}$**;** 10 µg, $\mathbf{F_5}$ and $\mathbf{F_6}$**;** 2 µg, and **T;** 20 µg.

3.10 Effect of UV radiation on photosystems

The solubilized thylakoid membranes of cell under normal growth within 6 h after sucrose gradient ultracentrifugation were not changed by visual observation (Figure 3.16). Under UV-A radiation, increasing time obviously affected on protein components in F_6 layer (the lowest layer of PSII protein component). Under UV-B, the green color of F_4 (PSII protein component) was slightly destroyed at 6 h-treatment. Under UV-C treatment, after 3 h-treatment showed no different pattern of fractions whereas after 6 h-treatment evidently affected the amounts of photosynthetic proteins in all fractions. Then, within 6 h of UV-C exposure strongly damaged the

photosynthetic protein components of thylakoid membranes when compared with normal growth light (NL), UV-A and UV-B.

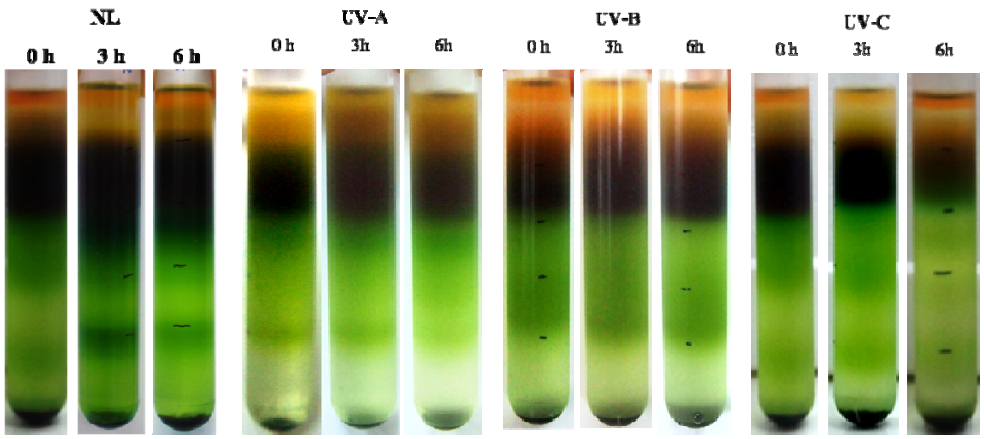


Figure 3.18 Sucrose gradient ultracentrifugation of solubilized thylakoid membranes from *Synechocystis* cells under normal growth (intensity of 50 μmol·m^{-2}·s^{-1}), UV-A (intensity of 10 W·m^{-2}), UV-B (intensity of 1.3 W·m^{-2}) and UV-C (intensity of 1.3 W·m^{-2}).

3.11 Effect of UV radiation on polyamine associated with photosystems

All fractions (F_1 – F_6) of sucrose gradient ultracentrifugation under normal growth condition and UV radiations were determined on polyamine contents. Fraction F_1 with the orange colored pigment contained also polyamine contents (appendix G) but the fraction's protein content was, very few, undetectable. Thus, the polyamine contents of F_1 fraction were not calculated. Furthermore, the sharp peak of one kind of polyamines was detected in all fractions (appendix G) which its retention time was

about 6.4 – 6.5 min, located in front of the Spd peak. Then, we defined this peak as the spermidine-like polyamine.

In addition, Figure 3.19 showed the polyamine contents in fraction F_2 – F_5 obtained from solubilized thylakoid membranes under normal growth light, the highest of total polyamine was found in fraction F_6 (about 1100 nmol·mg^{-1}protein) which D2 protein of PSII was identified. Under UV-A (Figure 3.20), polyamine content was increased 2-fold in fraction F_5 at 3 h-treatments and stable at the same level as initial. In fraction F_6, total polyamine contents in 3 h-treatments was significantly decreased by 50% and continued decreasing in 6 h-treatments. Within 3 h-treatments, Put was rapidly decreased whereas Spd was stable. However, Spd was significantly decreased after exposing to UV-A for 6 h-treatment. The changes of total polyamine contents in sucrose fraction of solubilized thylakoid membranes from cells under UV-B are shown in Figure 3.21. The significant change was observed in fraction F_5 and F_6. In fraction F_5, total polyamine content was increased according to the increase of Put, whereas Spd was stable within 6 h of UV-B exposure. In contrary, polyamine content in fraction F_6 was suddenly decreased after 3 h-treatments by significantly decreasing Put and Spd levels, and then recovering about 80% at the 6 h-time-point of treatment. Under UV-C (Figure 3.22), the total polyamine content was significantly decreased in F_6 fraction after 3 h-treatments (mostly on Put but slightly decreasing on Spd). All UV radiations did not apparently affect on total polyamine contents in F_2, F_3, and F_4 fractions.

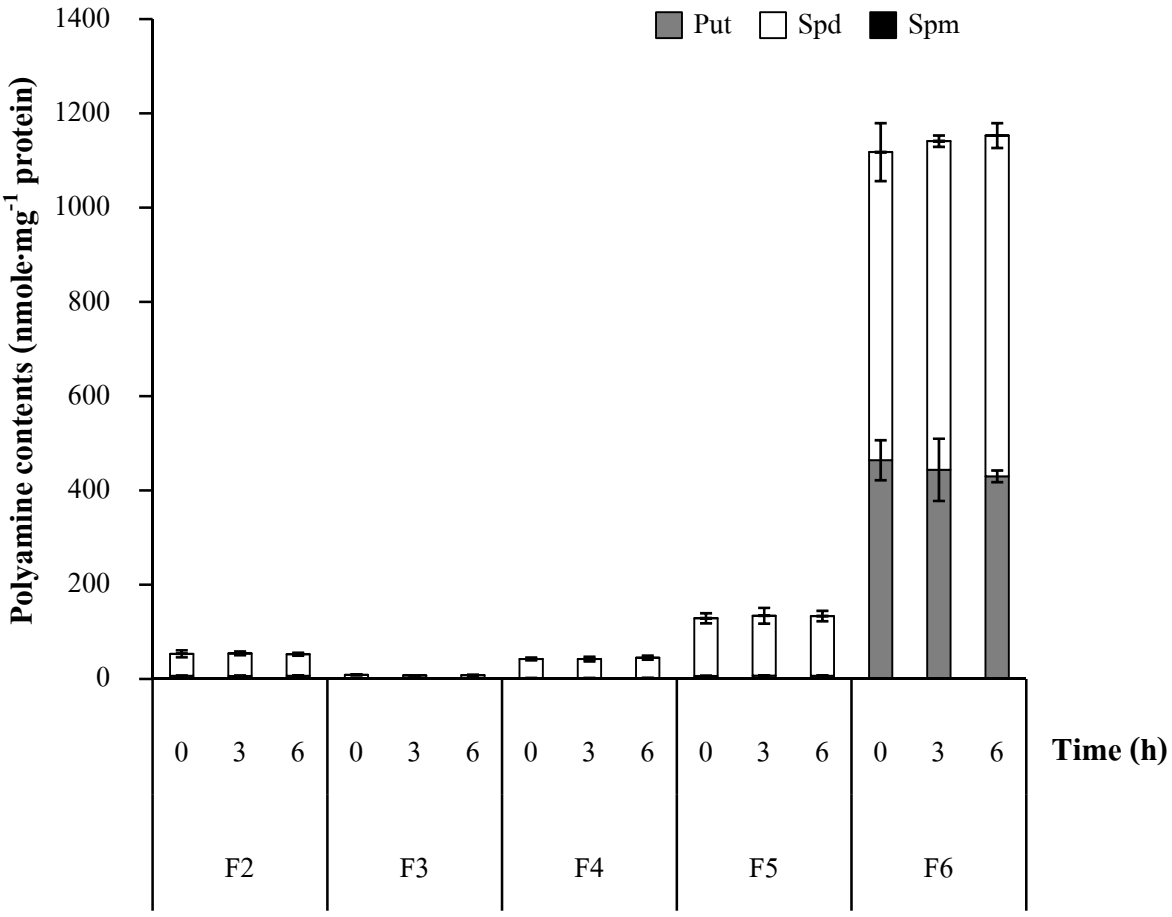


Figure 3.19 Polyamine contents in each fraction of solubilized thylakoid membranes from *Synechocystis* cells under normal growth (intensity of 50 μmol·m^{-2}·s^{-1}) after sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h. The data represent mean ± SD, n = 3.

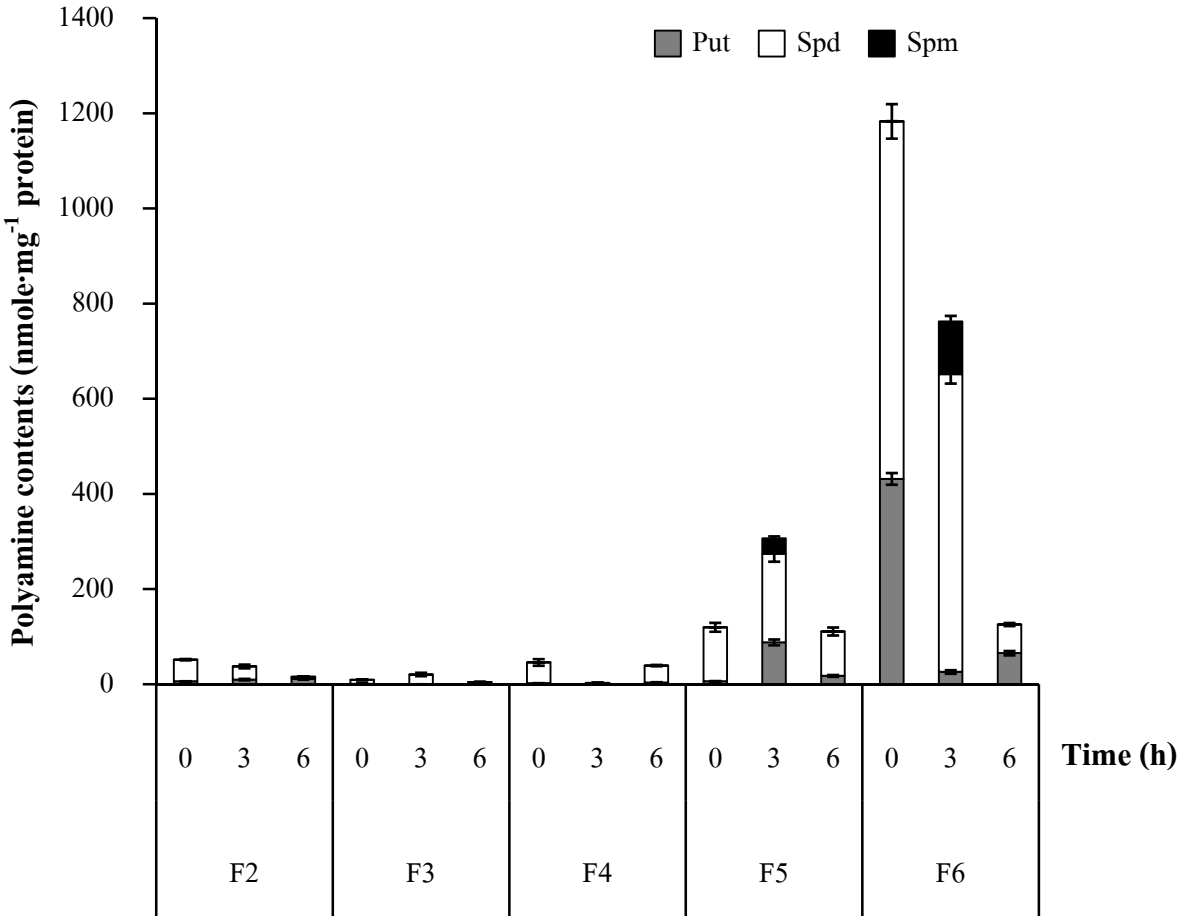


Figure 3.20 Polyamine contents in each fraction of solubilized thylakoid membranes from *Synechocystis* cells under UV-A (intensity of 10 $w \cdot m^{-2}$) after sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h. The data represent mean ± SD, n = 3.

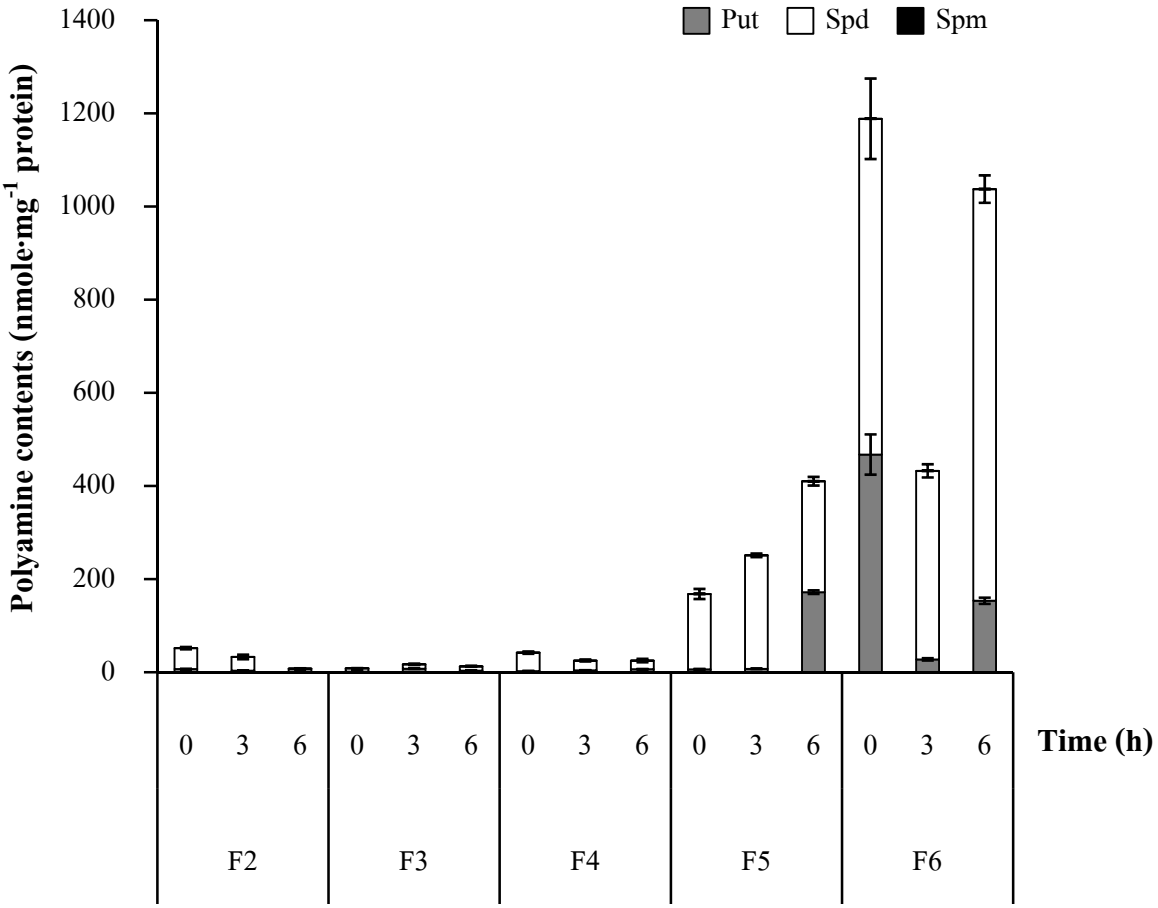


Figure 3.21 Polyamine contents in each fraction of solubilized thylakoid membranes from *Synechocystis* cells under UV-B (intensity of 1.3 w·m^{-2}) after sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h. The data represent mean ± SD, n = 3.

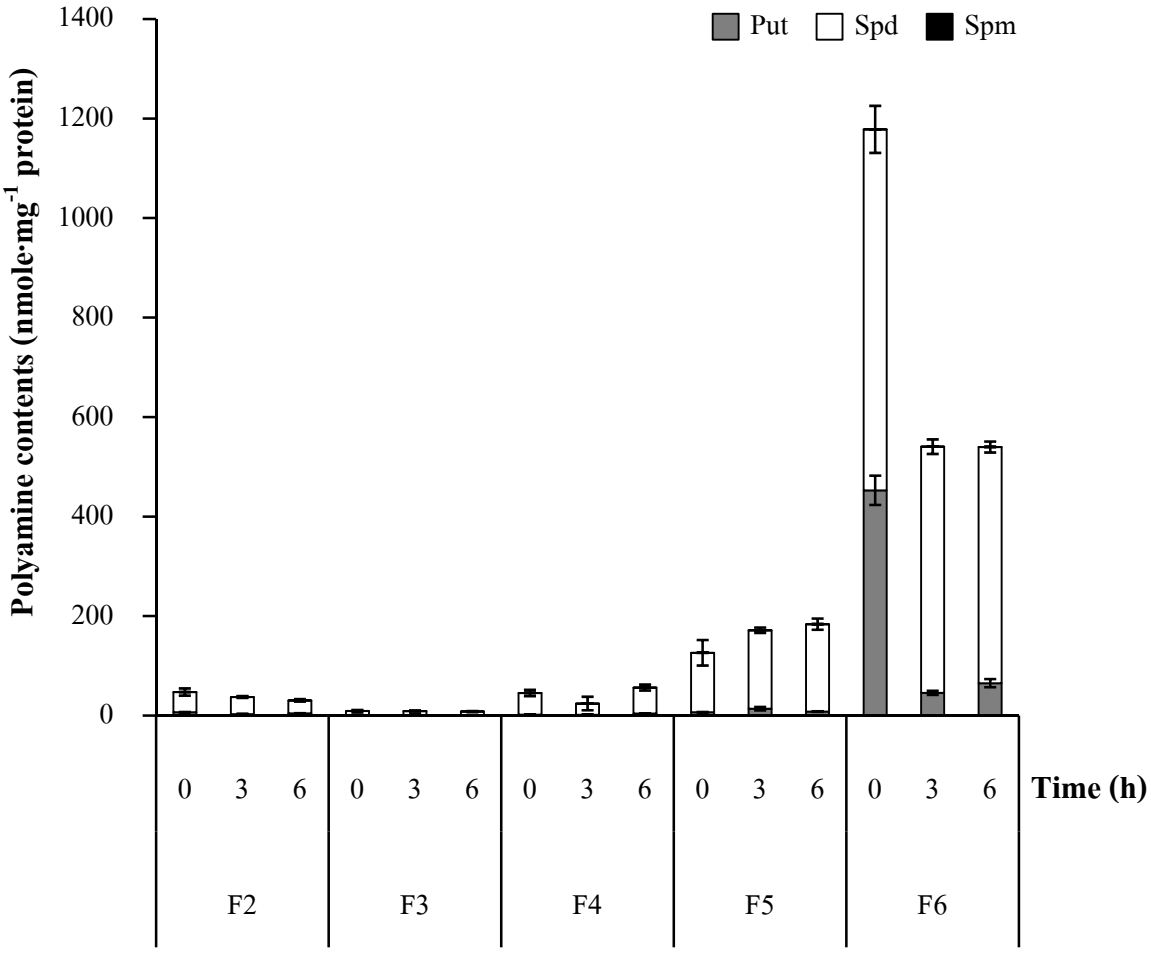


Figure 3.22 Polyamine contents in each fraction of solubilized thylakoid membranes from *Synechocystis* cells under UV-C (intensity of 1.3 $w \cdot m^{-2}$) after sucrose gradient ultracentrifugation at 144,000*x*g, 4 ºC for 14 h. The data represent mean ± SD, n = 3.

CHAPTER IV

DISCUSSION

The objective of this study was to investigate effects of UV radiations on intracellular pigments and polyamine contents related to photosynthetic systems in cyanobacterium *Synechocystis* sp. PCC 6803. Firstly, we started to figure out the effect of various UV radiations, including UV-A, -B and –C, on growth of *Synechocystis* cells. The result obviously showed an unchanged level of cell growth under all UV radiations within first 3 h of exposure. For longer exposure time up to 24 h, UV radiation affected apparently on cell growth by slightly decreasing under UV-B and UV-C treatments compared to cell growth under normal growth light. Although it was found the slight increase of cell growth under UV-A radiation, *Synechocystis* cell growth was inhibited by UV-A compared to cells exposed to normal growth light. This finding result was as similar as that of our previous report which studied in short-term (1 h) response of *Synechocystis* cells to UV radiations (UV-A, -B and –C) (Jantaro et al., 2011). Although 1h-UV treatments upon *Synechocystis* cells did not affect cell growth, the extended exposure up to 3 h of UV-B treatment (intensity of about 1.1-1.3 $W \cdot m^{-2}$) could inhibit growth of *Synechocystis* cells (Pothipongsa et al., in press). Thus, *Synechocystis* cells were able to adapt themselves and survive under moderate severity of all UV radiations studied to last 24 h.

Secondly, UV stress on pigment contents was demonstrated. The contents of intracellular pigments, chl *a* and carotenoid are normally collaborated with cell growth. The corresponding data within 12 h of UV-exposure, *Synechocystis* cells had potentially maintained their pigment contents but started rapidly to decrease at 24 h-treatment, especially carotenoid content. Similarly, the amounts of carotenoids in plant cells exposed to high light stress were faster decreased than chlorophyll content (Pallet and Young, 1993 cited in Xunzhong et al., 2005). Previously, the function of carotenoids was reported in the photoprotective role by quenching the excited triplet state chlorophyll via triplet-triplet energy transfer from Chl *a* to carotenoid (Durchan et al., 2012) and scavenging the singlet oxygen generated by UV stress from excited triplet chlorophyll. In this study, it was clearly shown that *Synechocystis* cells could maintain their accumulation of chl *a* and carotenoid pigments within first 6 - 12 h-treatment of all UV radiations, and started to decrease significantly in long-term of UV exposure.

Thirdly, we found that the protein content inside the cell was increased significantly after 24 h of UV-exposure. Recently, proteomics approach revealed the changed of protein expression in *Synechocystis* sp. PCC 6803 under UV-B radiation (Yang et al., 2009). They reported that protein levels in *Synechocystis* sp. during long-term response, within 8 h-UV-B treatment, were up-regulated whereas those during longer-term response, within 84 h-UV treatment, were down-regulated. The up-regulated proteins were encompassed among groups of DNA repairing, heat shock protein family, NADPH generation and cellular antioxidative reactions. For the down-

regulated proteins were consisted of groups of amino acid biosynthesis, photosynthesis and protein biosynthesis (Yang et al., 2009).

We also studied on the photosynthetic efficiency (F_v/F_m value) of the cell under UV stress. It was obviously demonstrated a significant decrease of F_v/F_m value after 3 h of each UV exposure. Not only F_v/F_m value but also oxygen evolution rate could monitor the photosynthetic efficiency. This result was then corresponding to that from oxygen evolution detected in *Synechocystis* cells exposed to 1h-treatment of each UV type (Jantaro et al., 2011), as well as, F_v/F_m value of UV-B treated cells for 3 h-treatment (Pothipongsa et al., in press). It is indicated that UV radiation affected severely on photosynthesis efficiency and function but moderately on photosynthesis components, such as intracellular pigments.

For the effect of UV radiations on polyamine contents, we focused on polyamine contents comparing in three parts; including total polyamines in whole cells, polyamine contents associated with thylakoid membranes, and polyamine contents associated with photosynthetic protein complex of photosystems.

Total polyamine contents in whole cells was decreased greatly by UV stress. At 3 h of UV-A exposure, total polyamine contents were significantly increased and after that up to 18 h-treatment, cells efficiently maintained the accumulation of total polyamine contents as existed in the initial state, following by decreasing until last 24 h-treatment. On the other hand, at treatments under UV-B and –C exposures, total polyamine contents were decreased after 3 h UV exposure and highly decreased until 24 h-treatment. Our results were coincident with published report of Mapelli and

colleagues (2008) which showed the decreased accumulation of polyamine contents from four plant species exposed to UV-B radiation for 24 h in various intensities (Mapelli et al., 2008).

For polyamine associated with thylakoid membranes, we found that the dominant polyamine type which associated with thylakoid membranes was Spd whereas a previous report studied in spinach leaves, higher plant, found the diamine Put as a major polyamine associated to thylakoid membranes (Kotzabasis et al., 1993). It was surprised that the amounts of polyamines associated with thylakoid membranes in all treatments gave in high levels as similar as those of whole cells. This provides us an assumption that whether the binding interaction of polyamines associated to the abundant thylakoid membranes in *Synechocystis* cells could be destroyed by perchloric acid (PCA; a chemical used in first step of polyamine extraction) and released those polyamines bound with thylakoid membranes into the PCA-soluble fraction of whole cells, according to no chloroplast protection as in higher plant. Moreover, the accumulation of total polyamines in whole cell was decreased during UV-exposures, especially UV-B and UV-C.

Furthermore, we firstly demonstrate that polyamines were associated with photosynthetic protein complex of photosystems (pigments, D1, D2, PSI core protein) in *Synechocystis* cells using Western blot approach. The dominant polyamine associated with either PSI or PSII was Spd whereas Put and Spm were in trace amounts. Interestingly, we showed that the highest level of polyamines was found in D2 core protein of PSII. It was revealed previously in higher plants that polyamines associated with D1 and D2 proteins were able to stabilize the protein and retard PSII

protein degradation under drought and osmotic stresses (Duan et al., 2006; Besford et al., 1993). Moreover, Ioannidis and Kotzabasis (2007) demonstrated that the indicated Put was an efficient stimulator of ATP synthesis whereas Spd was an efficient stimulator of non-photochemical quenching. Both common types of polyamines played an important role in photosynthesis. Thus, our study results suggest that polyamine associated with each photosynthetic components existing in *Synechocystis* may involve with response mechanisms against UV radiations.

On the other hand, the polyamine contents of three parts studied were decreased during 24 h-UV exposure, especially UV-B and UV-C. Surprisingly, PSII core protein, D2 protein fraction showed a high reduction of polyamines affected by UV exposure. This might be due to many reasons whether the upstream inhibition of polyamine biosynthesis, the increase of polyamine degradation and excretion, the increase of polyamine translocation between free and bound forms, or the change of polyamine binding. Recently, one possible assumption of the changed level of polyamine contents was involved with tranglutaminase enzyme, which regulates the physiological actions via modification of protein-polyamine complexes (Ohtake et al., 2007). This enzyme efficiently catalyzes both dissociation and association reactions of *N*-(γ-glutamyl) polyamine (Gln-polyamine) bond in protein. To prove this assumption, transglutaminase transcripts and/or enzyme activity in *Synechocystis* sp. PCC 6803 should be monitored.

CHAPTER IV

CONCLUSION

1. All UV radiations had down-regulation effect on growth, intracellular pigments, photosynthetic efficiency and polyamine contents.

2. The dominently detected polyamine in *Synechocystis* sp. PCC 6803 was spermidine.

3. UV-A at intensity of 10 $w \cdot m^{-2}$ had a slight effect on total polyamine content.

4. UV-B and UV-C at intensity of 1.3 $w \cdot m^{-2}$ had remarkable effect on total polyamine contents which associated with thylakoid membrane.

5. The PsaA protein (core protein of PSI) which separated in sucrose gradient ultracentrifugation was found in fraction F_2 at the density of 10% sucrose, which confirmed by using western blotting technique.

6. The PsbA (D1 protein of PSII) which separated in sucrose gradient ultracentrifugation were found in three fractions F_3, F_4, and F_5 at the density of 20%, between 20 - 30%, and between 30 – 40% sucrose, respectively which confirmed by using western blotting technique.

7. The PsbD (D2 protein of PSII) which separated in sucrose gradient ultracentrifugation were found in four fractions F_3, F_4, F_5, and F_6 at the density of 20%, between 20 - 30%, between 30 – 40% , and between 40 – 50% sucrose, respectively which confirmed by using western blotting technique.

8. The polyamine was found in both photosysthetic component and free pigment layers.

9. All UV radiations affected on total polyamine contents in F_5 and F_6 fractions that contained D1 and D2 protein (PSII core protein).

10. The high peak on polyamine chromatogram with shifted retention time from photosynthetic component fractions was observed, and was later defined as Spd-like polyamine.

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APPENDICES

APPENDIX A

Cyanobacterial culture medium

BG_{11} medium (1,000 ml)	**Liquid medium (ml)**	**Agar medium* (ml)**
15%w/v $NaNO_3$	10	10
3.6%w/v $CaCl_2 \cdot 2H_2O$	1	1
0.6%w/v Citric acid	1	1
7.5%w/v $MgSO_4 \cdot 7H_2O$	1	1
0.1%w/v Na-EDTA	1	1
2%w/v Na_2CO_3	1	1
3%w/v K_2HPO_4	1	1
0.6%w/v Ammonium iron(III) citrate	1	1
Trace element**	1	1
30%w/v $Na_2S_2O_3 \cdot 5H_2O$	-	10
H_2O	982	972

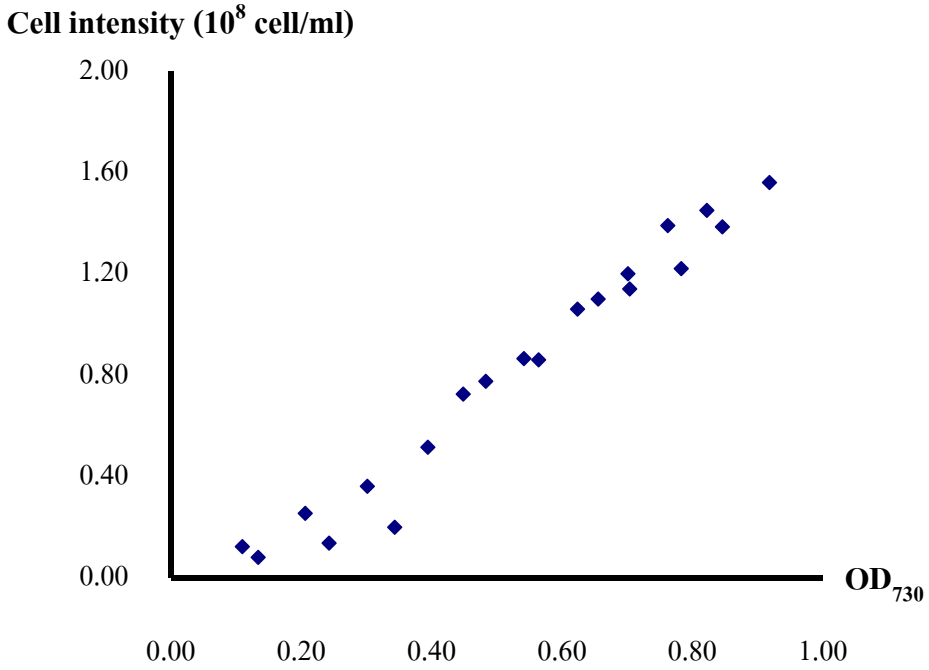
*Agar medium contains 1.5% agar

**Trace element (1,000 ml) consist of

H_3BO_3	2.86 g	$Na_2MoO_4 \cdot 2H_2O$	0.39 g
$MnCl_2 \cdot 4H_2O$	1.81 g	$CuSO_4 \cdot 5H_2O$	0.08 g
$ZnSO_4 \cdot 7H_2O$	0.22 g	$Co(NO_3)_2 \cdot 6H_2O$	0.05 g

APPENDIX B

The standard curve of *Synechocystis* cell intensity



Cell intensity (10^8 cell/ml) = 2 × OD_{730} (R^2 = 0.93)

APPENDIX C

Preparation for protein assay

Bradford reagent (1,000 ml)

Coomassie Brilliant Blue G-250	0.1 g
95% ethanol	50 ml
85% (w/v) phosphoric acid	100 ml

Add distilled water up to 1,000 ml, completely dissolved and filter through Whatman#1 filter paper before use.

APPENDIX D

Preparation of STNE buffer

STNE buffer (100 ml)

Sucrose	68.4 g
0.1 M Tris-HCl pH 8.0	10 ml
0.1 M NaCl	10 ml
0.2 M Na-EDTA	10 ml

Add distilled water up to 100 ml

APPENDIX E

Preparation for Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE)

1. Stock reagents

30% acrylamide solution (30%T, 2.67%C)

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

Add distilled water up to 100 ml and stir until completely dissolved.

1.5M Tris-HCl pH 8.8

Tris (hydroxymethyl) aminomethane	18.17 g

Adjust pH to 8.8 with 12 M HCl and add distilled water up to 100 ml.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl) aminomethane	6.06 g

Adjust pH to 6.8 with 12 M HCl and add distilled water up to 100 ml.

10% ammonium persulfate

Ammonium persulfate	0.1 g

Add distilled water up to 1 ml (fresh daily).

10% SDS

SDS	10 g

Add distilled water up to 100 ml (store at room temperature).

0.5% bromophenol blue

Bromophenol blue	0.05 g

Add distilled water up to 10 ml.

2. Sodium dodecyl sulfate polyacrylamide gel (SDS gel)

12% seperating gel (2 slap gel)

30% acrylamide solution	4.0 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
10% SDS	100 µl
10% ammonium persulfate	50 µl
TEMED	5 µl
Distilled water	3.35 ml

5% stacking gel (2 slap gel)

30% acrylamide solution	0.84 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	50 µl
10% ammonium persulfate	25 µl
TEMED	5 µl
Distilled water	2.83 ml

3. Loading buffer (5X)

0.5 M Tris-HCl pH 6.8	1.2 ml
Glycerol	0.8 ml
10% SDS	2 ml
β-mercaptoethanol	0.5 ml
0.5% bromophenol blue	0.5 ml
Distilled water	5.2 ml

Make aliquots and store at -20 ºC.

4. Electrophoresis running buffer

Tris (hydroxymethyl) aminomethane	3 g
Glycine	14.4 g
SDS	1 g

Add distilled water up to 1,000 ml (pH should be approximately 8.3).

5. Stianing solution

Coomassie Blue R-250	10 g
Methanol	450 ml
Glacial acetic acid	100 ml

Add distilled water up to 1,000 ml (store at room temperature and dark).

6. Destaining solution

Methanol	100 ml
Glacial acetic acid	100 ml

Add distilled water up to 1,000 ml (store at room temperature).

APPENDIX F

Preparation for protein blotting

1. Transfer buffer (Fresh preparation)

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

2. TBS buffer

Tris (hydroxymethyl) aminomethane	2.42 g
NaCl	8.8 g

Adjust pH to 7.5 with 12 M HCl and add distilled water up to 1,000 ml (store at 4 ºC)

3. TBS-T buffer

Tween-20	1 ml
TBS buffer	999 ml

Store at 4 ºC

4. Blocking buffer

Skim milk	5 g

Add TBS-T buffer up to 100 ml and stir until completely dissolved at room temperature.

5. Antibody dilution

Primary and secondary antibody were diluted with blocking buffer

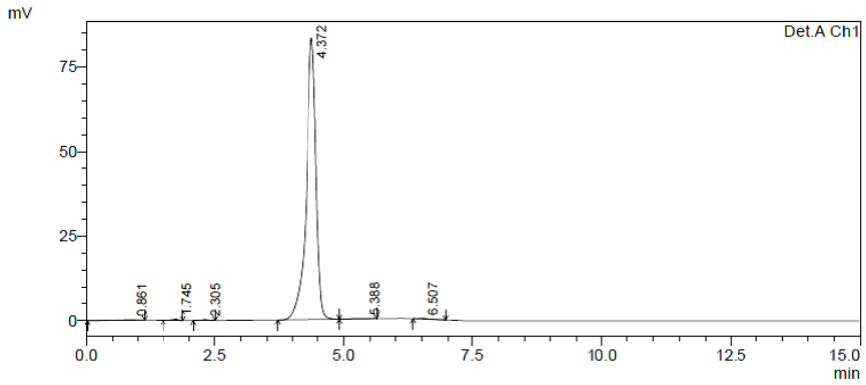
6. Signal detection solution

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

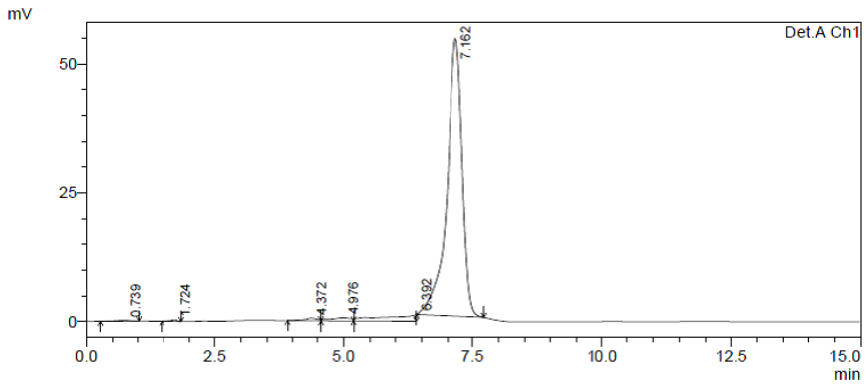
APPENDIX G

Chromatogram of polyamines analysis by HPLC

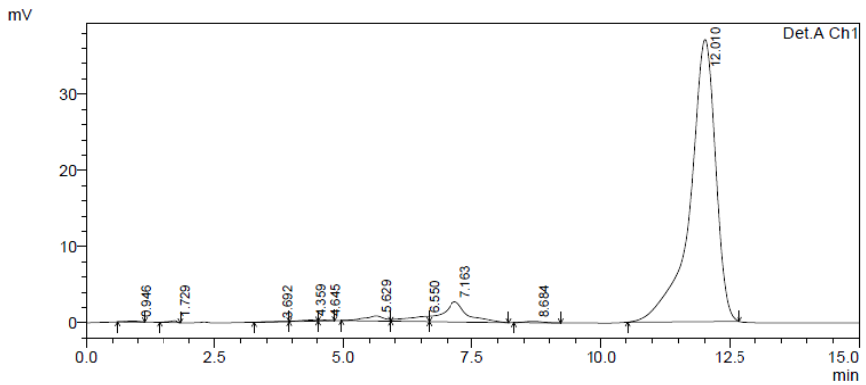
1. Chromatogram of standard putrescine (Put)



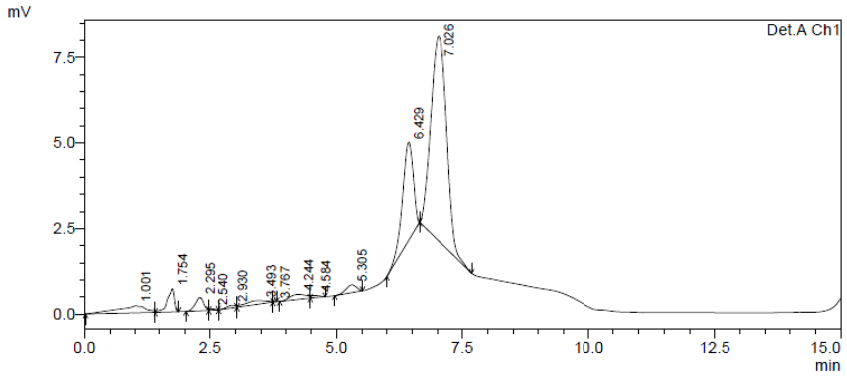
2. Chromatogram of standard spermidine (Spd)



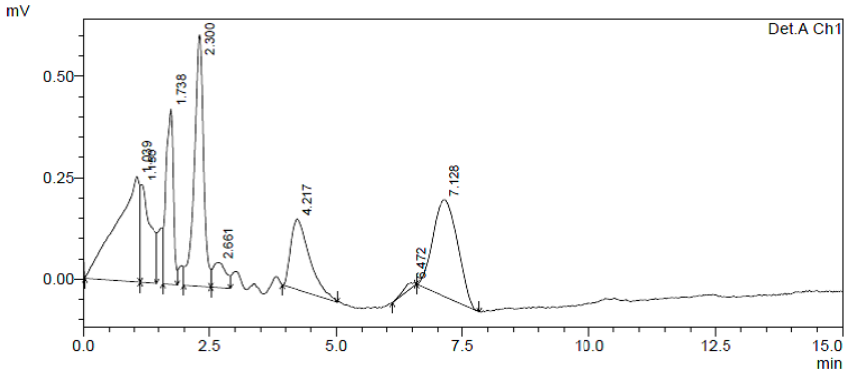
3. Chromatogram of standard spermine (Spm)



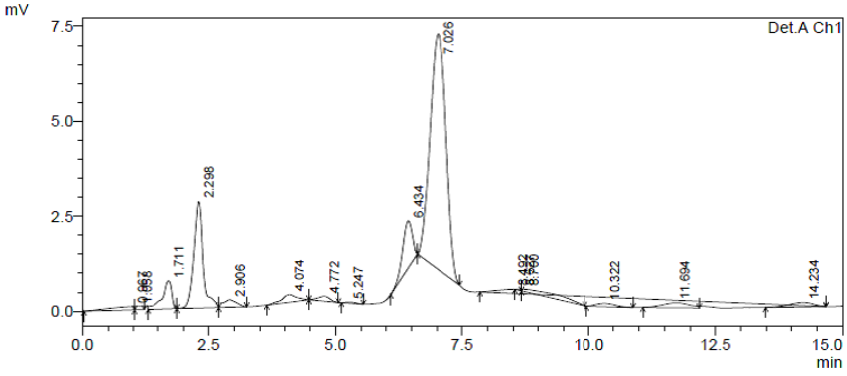
4. Chromatogram of extracted polyamines PCA-soluble fraction from normal *Synechocystis* cell



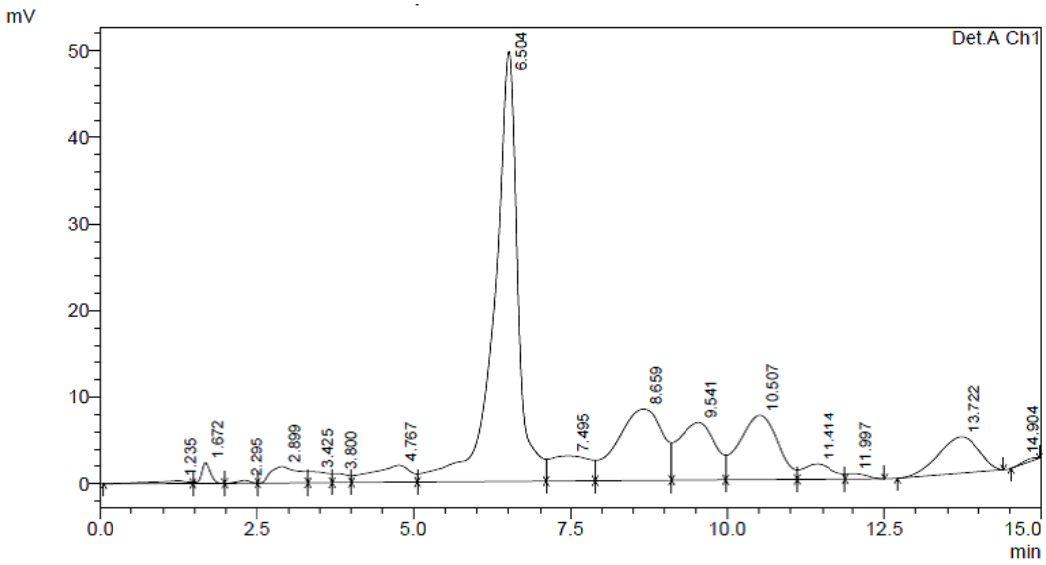
5. Chromatogram of extracted polyamines PCA-insoluble fraction from normal *Synechocystis* cell



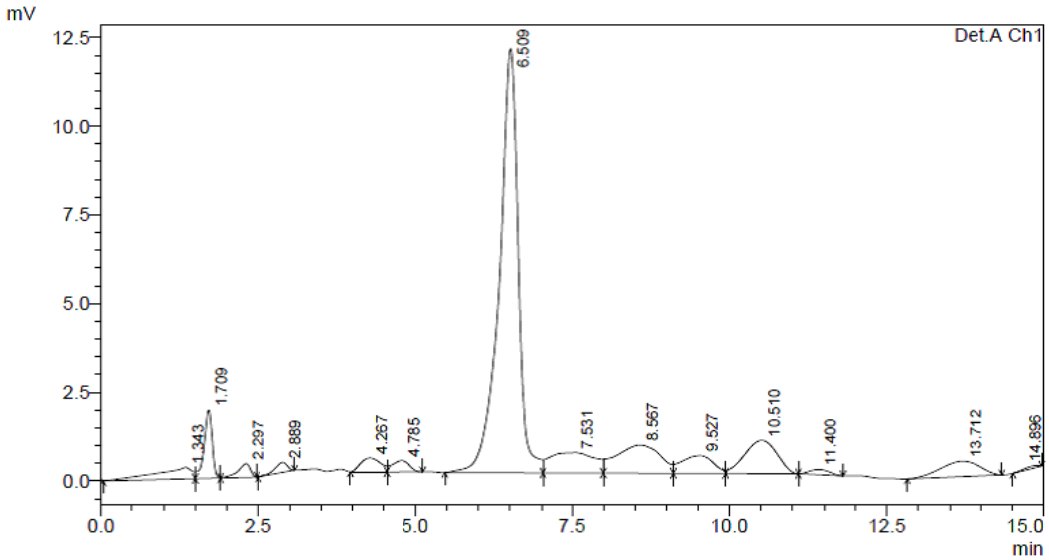
6. Chromatogram of extracted polyamines associated with thylakoid membrane from normal *Synechocystis* cell



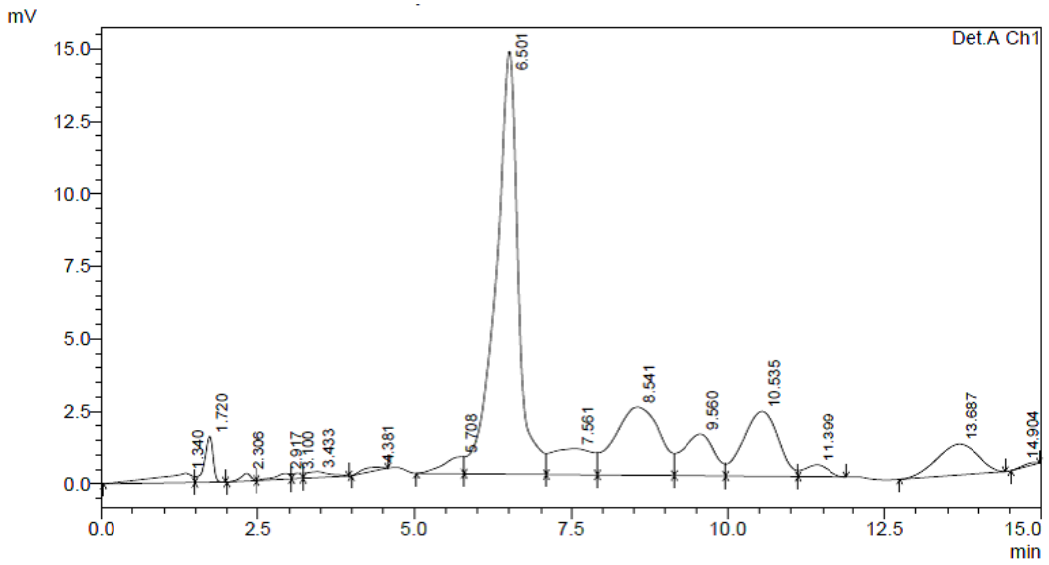
7. Chromatogram of extracted polyamines in solubilized thylakoid membrane at 10% sucrose upper layer from normal *Synechocystis* cell (F_1 fraction)



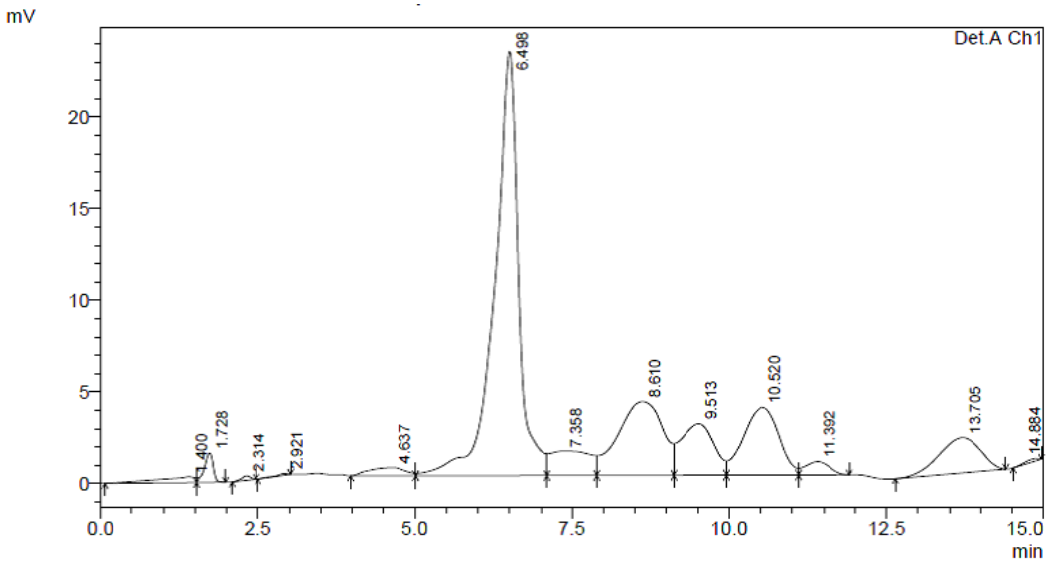
8. Chromatogram of extracted polyamines in solubilized thylakoid membrane at 10% sucrose layer from normal *Synechocystis* cell (F_2 fraction)



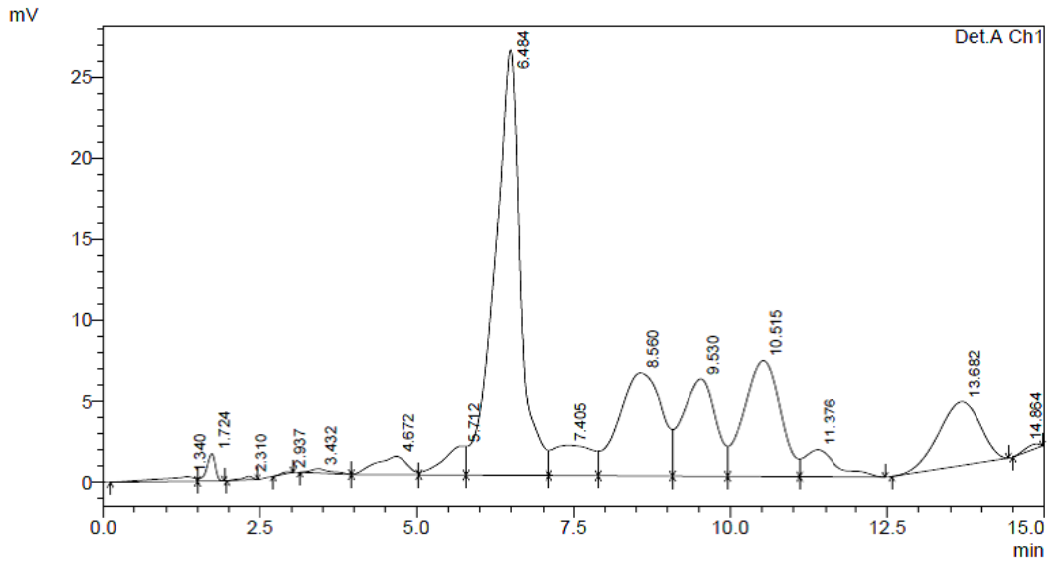
9. Chromatogram of extracted polyamines in solubilized thylakoid membrane at 20% sucrose layer from normal *Synechocystis* cell (F_3 fraction)



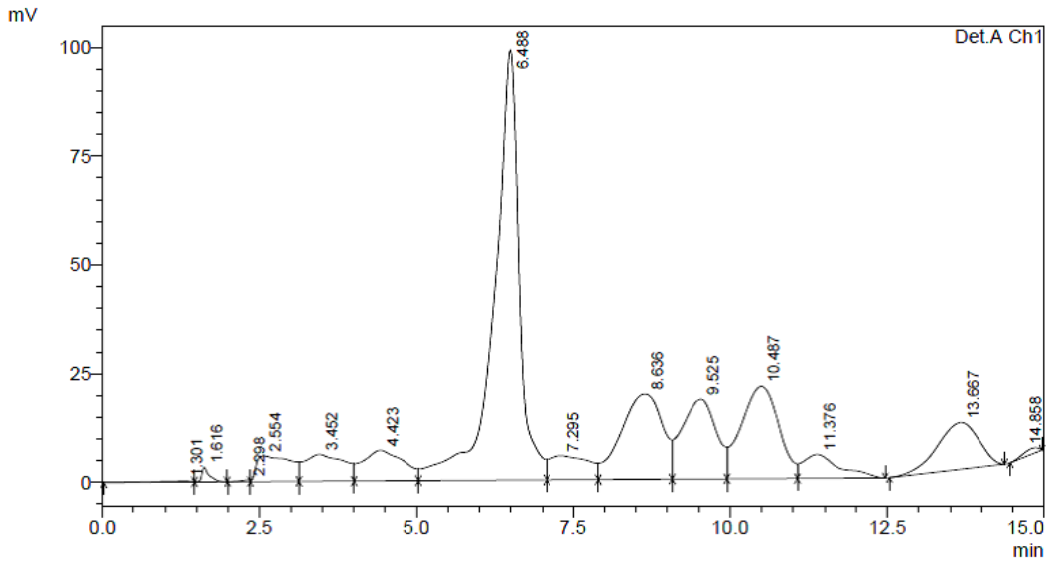
10. Chromatogram of extracted polyamines in solubilized thylakoid membrane between 20 - 30% sucrose layer from normal *Synechocystis* cell (F_4 fraction)



11. Chromatogram of extracted polyamines in solubilized thylakoid membrane between 30 - 40% sucrose layer from normal *Synechocystis* cell (F_5 fraction)

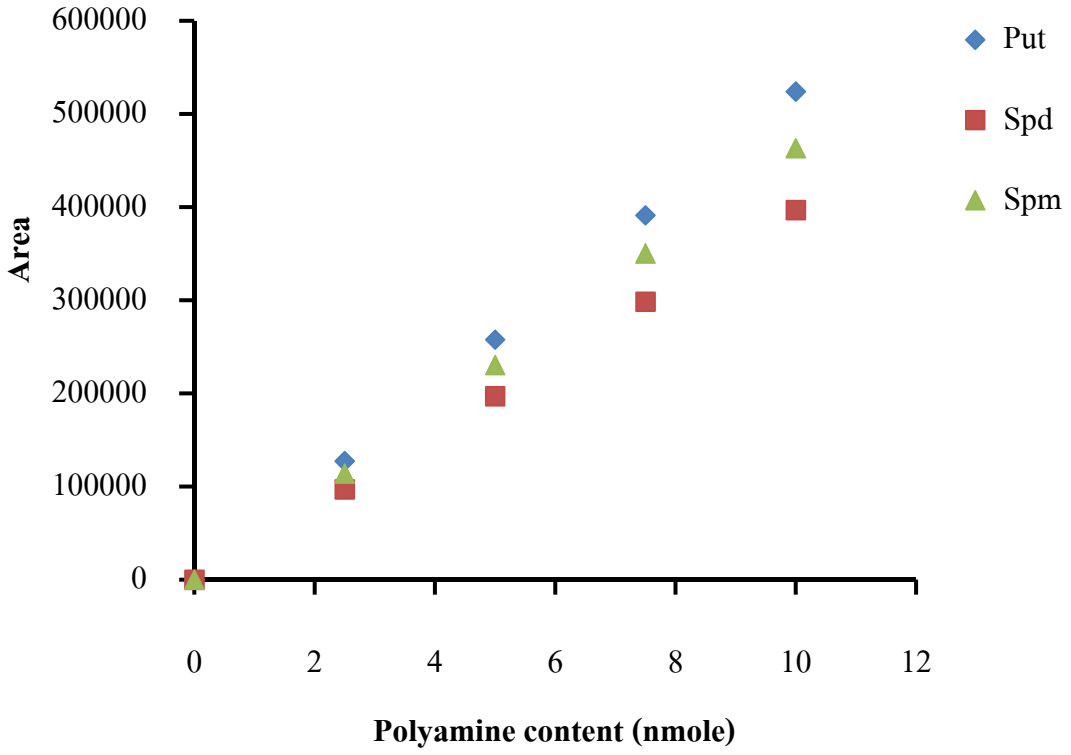


12. Chromatogram of extracted polyamines in solubilized thylakoid membrane between 40 - 50% sucrose layer from normal *Synechocystis* cell (F_6 fraction)



APPENDIX H

Stand curve of polyamine contents



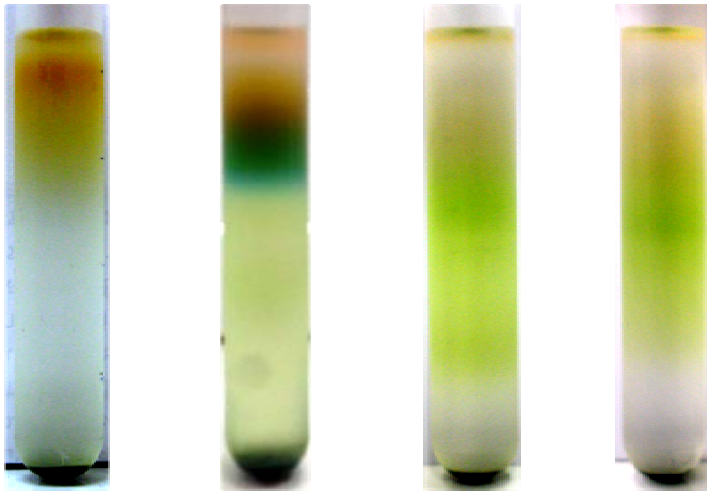
Put content (nmole) = Area ÷ 52,157 (R^2 = 0.999)

Spd content (nmole) = Area ÷ 39,645 (R^2 = 0.999)

Spm content (nmole) = Area ÷ 46,363 (R^2 = 0.999)

APPENDIX I

Optimization of thylakoid membranes solubilization condition (preliminary results)



Detergent	Triton X-100	Triton X-100	DM	DM
Ratio	20:1	20:1	20:1	20:1
T (°C)	25	25	25	25
Time (min)	30	120	30	120

BIOGRAPHY

Miss Suparaporn Khanthasuwan born on January 15, 1985. She graduated with Bachelor Degree of Science in Biochemistry from Chulalongkorn University in 2007. Then she further studied for the Master Degree of Science in Biotechnology, Chulalongkorn University until 2012.