การโคลนและการแสดงออกของกลุ่มยีนชีวสังเคราะห์สำหรับไมโคสปอรินจากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*

นางสาววรางคณา โสพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CLONING AND EXPRESSION OF BIOSYNTHETIC GENE CLUSTER FOR MYCOSPORINE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*

Miss Warangkana Sopun

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 2013

Thesis Title	CLONING AND EXPRESSION OF BIOSYNTHETIC GENE CLUSTER FOR MYCOSPORINE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*
By	Miss Warangkana Sopun
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Rungaroon Waditee Sirisattha, Ph.D.
Thesis Co-Advisor	Professor Teruhiro Takabe, 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

(Associate Professor Tanapat Palaga, Ph.D.)

.....Thesis Advisor

(Assistant Professor Rungaroon Waditee Sirisattha, Ph.D.)

.....Thesis Co-Advisor

(Professor Teruhiro Takabe, Ph.D.)

.....Examiner

(Assistant Professor Suchada Chanprateep Napathorn, Ph.D.)

.....External Examiner

(Sophon Sirisattha, Ph.D.)

วรางคณา โสพันธ์ : การโคลนและการแสดงออกของกลุ่มยีนชีวสังเคราะห์สำหรับไมโคสปอรินจากไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*. (CLONING AND EXPRESSION OF BIOSYNTHETIC GENE CLUSTER FOR MYCOSPORINE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รุ่งอรุณ วาดิถี สิริศรัทธา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. Teruhiro Takabe, 82 หน้า.

ไมโคสปอรีน-ไลค์ อะมิโน แอซิด เป็นสารแมทาบอไลท์ทุติยภูมิที่มีความจำเพาะในการดูดซับรังสียูวี-บี ในช่วงความยาวคลื่น 310-362 นาโนเมตร มีหน้าที่หลักในการป้องกันเซลล์จากรังสีที่ได้รับจากแสงอาทิตย์ ในกระบวนการชีวสังเคราะห์สารไมโคสปอรีน พบว่าจะถูกสร้างขึ้นในปริมาณที่สูงภายใต้ภาวะความเครียด จนถึงปัจจุบันได้มีการศึกษาถึงกระบวนการชีวสังเคราะห์สารไมโคสปอรีนเฉพาะเพียงในไซยาโนแบคทีเรียเส้นสาย *Anabaena variabilis* ATCC 29413 และ *Nostoc punctiforme* ATCC 29133 พบว่ามีกลุ่มยีนที่ประมวลรหัสชีวสังเคราะห์สารไมโคสปอรีนอยู่ 4 ยีน ได้แก่ *Ava_3858* หรือ *NpR5600*, *Ava_3857* หรือ *NpR5599*, *Ava_3856* หรือ *NpR5598* และ *Ava_3855* หรือ *NpF5597* ในการศึกษาครั้งนี้ใช้ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* เพื่อศึกษาการชักนำการสร้างสารไมโคสปอรีน วิถีชีวสังเคราะห์และวิศวกรรมแมตาบอลิก การวิเคราะห์ปริมาณสารและการจำแนกชนิดของสารไมโคสปอรีนใน *A. halophytica* ทำโดยวิธีโครมาโตกราฟีของเหลวสมรรถนะสูง และวิธีการวิเคราะห์กรดอะมิโน พบว่าสารไมโคสปอรีนที่พบใน *A. halophytica* คือ ไมโคสปอรีน-2-ไกลซีน จากนั้นได้นำกลุ่มยีนที่ประมวลรหัสชีวสังเคราะห์สารไมโคสปอรีนใน *A. halophytica* ไปแสดงออกในแบคทีเรีย *E. coli* และไซยาโนแบคทีเรียน้ำจืด *Synechococcus* sp PCC 7942 จากการวิเคราะห์ในเซลล์แสดงออกทั้ง 2 ชนิด พบว่าภาวะความเครียดเนื่องจากเกลือ มีผลในการชักนำให้เกิดการสร้างสารไมโคสปอรีนในปริมาณที่สูง และปริมาณของการสะสมสารไมโคสปอรีนสูงสุด ตรวจพบในเซลล์แสดงออก *E. coli* คือ 85.20 ± 2.69 μmol/g DW

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5472258223 : MAJOR BIOTECHNOLOGY
KEYWORDS: SALT STRESS / UV-B STRESS / MAAS / A. HALOPHYTICA

WARANGKANA SOPUN: CLONING AND EXPRESSION OF BIOSYNTHETIC GENE CLUSTER FOR MYCOSPORINE FROM HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*. ADVISOR: ASST. PROF. RUNGAROON WADITEE SIRISATTHA, Ph.D., CO-ADVISOR: PROF. TERUHIRO TAKABE, Ph.D., 82 pp.

Mycosporine-like amino acid (MAA) is secondary metabolite. It has specific ultraviolet B absorption in the range between 310-362 nm. Primary function of MAA is to protect microorganism from harmful effects of UV-B. There have been reported that MAA can be synthesized in high amount under stress condition. Thus far, biosynthetic pathway for MAA has only studied in filamentous cyanobacteria; *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133. In these cyanobacteria, a cluster of genes for MAA biosynthesis comprises of *Ava_3858/NpR5600*, *Ava_3857/NpR5599*, *Ava_3856/NpR5598*, and *Ava_3855/NpF5597*, respectively. In this research, we employed a halotolerant cyanobacterium *Aphanothece halophytica* for the study MAA induction, biosynthesis including metabolic engineering approaches. Analysis and identification of MAA were examined by High Performance Liquid Chromatography and Amino Acid Analysis. We have identified that MAA accumulation in *A. halophytica* is Mycosporine-2-glycine. Furthermore, biosynthetic gene cluster for MAA from *A. halophytica* was expressed into *E. coli* and *Synechococcus* sp. PCC 7942. MAA was highly induced under salt stress condition in both expressing cells. In our tested conditions, *E. coli* expressing cells could produce and accumulate MAA with maximum yield 85.20 ± 2.69 μmol/g DW.

Field of Study: Biotechnology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Assistant Professor Dr. Rungaroon Waditee Sirisattha for her excellent instruction, guidance, encouragement and supported throughout the course of my research. In addition, I would also like to express my thanks to thesis committee, Associate Professor Dr. Tanapat Palaga, Assistant Professor Dr. Suchada Chanprateep Napathorn and Dr. Sophon Sirisattha for their comments and valuable suggestions in this research. My appreciation is also expressed to Professor Dr. Teruhiro Takabe, Professor Dr. Yoshito Tanaka and Assistant Professor Dr. Hakuto Kageyama for their help in laboratory and suggestion during my stay in Japan. Special thanks for all my special friends and members of laboratory room 1904/17 for encouragement and nice friendships. Finally, I would like to express my thanks to my family for their warmest love, care, encouragement, understanding, support and attention throughout my life.

CONTENTS

Page

Page

Page

LIST OF TABLE

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
Kb	Kilo base pair
kDa	Kilodalton
g	Gram
µg	Microgram
hr	Hour
l	Liter
mA	Milliampare
min	Minute
µl	Microliter
ml	Milliliter
mM	Milli Molar
M	Molar
nm	Nanometer
PCR	Polymerase chain reaction
rpm	Revolution per minute

NaCl	Sodium chloride
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
W/m^{-2}	Watts per square meter

CHAPTER I
INTRODUCTION

The increase in ultraviolet (hereafter UV) radiation penetrating the Earth's surface due to industrial released chemicals such as chlorofluorocarbons (CFCs), chlorocarbons (CCs) and organobromides (OBMs) has become a subject of concern these days. These chemicals destroy the ozone layer and UV can be transmitted through the Earth's surface (Crutzen, 1992; Lubin and Jensen, 1995). UV is electromagnetic radiation with a wavelength shorter than that of visible light (<400 nm in wavelength) and can be radiated to the Earth. The UV can be divided into UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm). Among them, UV-C is the most dangerous but the atmosphere can filter out. Therefore, only UV-A and UV-B are harmful for living organisms. The dangers of UV are numerous. For instance, it induces DNA and protein damage that further affected to metabolism of cells, biological molecules of cells or changes in physiology (Singh *et al*., 2008). Accumulating evidence has been shown that marine living organisms such as macroalgae, microalgae, fungi, cyanobacteria and phytoplankton have specific mechanisms to protect detrimental effects from UV (Bebout and Garcia-Pichel, 1995).

Cyanobacteria are photosynthetic microorganisms. It has been reported that some cyanobacteria have the ability to tolerate the exposure to UV in high doses (Singh *et al*., 2010). They have diverse strategies for protection from harmful UV by changing from molecular to behavioral levels. The variety of mechanisms for protecting UV include (1) to produce

antioxidants scavenging reactive for oxygen species (ROS) generated from UV exposure (Singh *et al*., 2010); (2) to synthesize specific secondary metabolite compounds that can absorb UV such as scytonemin, mycosporine-like amino acid (hereafter MAA). Recently, there have been reported that some cyanobacteria can synthesize MAAs in high volume under stress conditions (Gao and Garcia-Pichel, 2011). MAAs are well known as UV-absorbing compounds because of their specific absorbance between 310-362 nm (Rastogi *et al*., 2010; Carreto and Carignan, 2011). They have the primary function to protect cells from radiation received from the sun (Bandaranayake, 1998). The biosynthetic pathway for MAAs has derived from shikimate pathway. This pathway is related to generate aromatic amino acids (i.e. phenylalanine, tyrosine, and tryptophan). It has been shown that 3-dehydroquinate (DHQ) is intermediate compound generating from shikimate pathway. DHQ acts as intermediate to an MAA core, thus this intermediate is crucial for MAA biosynthesis (Shick *et al*., 2002).

Recently, MAA biosynthetic pathway in filamentous cyanobacteria; *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133 has shown to compose of a cluster of genes (Gao and Garcia-Pichel, 2011). They have four genes encoding proteins to synthesize MAA (*Ava_3858-3855* for *A. variabilis* ATCC 29413 and *NpR5600-5598* and *NpF5597* for *N. punctiforme* ATCC 29133). Extensive study reveals that both *A. variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133) synthesize shinorine as a major MAA (Gao and Garcia-Pichel, 2011).

In this research, I aim to study MAA biosynthesis from a halotolerant cyanobacterium *Aphanothece halophytica* (hereafter *A. halophytica*). This extremophile has been shown several novel pathways in response to

salinity stress (Waditee-Sirisattha *et al*., 2014). Thus far, MAA biosynthesis has never been explored in any extremophile. In this study, I have three objectives as follows.

The objective of this research:

1. To study the induction of MAA in *A. halophytica*
2. To express the biosynthetic gene cluster for MAA in expressing cells
3. To analyse MAA production in expressing cells

CHAPTER II
LITERATURE REVIEW

2.1 The dangers of UV

The increase in UV penetrating the Earth's surface due to industrial released chemicals such as chlorofluorocarbons (CFCs), chlorocarbons (CCs) and organobromides (OBMs) has become a subject of concern these days. These chemicals destroy the ozone layer and UV can be transmitted through the Earth's surface (Crutzen, 1992; Lubin and Jensen, 1995). UV is electromagnetic radiation with a wavelength shorter than that of visible light (<400 nm in wavelength) and can be radiated to the Earth. The UV can be divided into UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm) (Figure 2.1). Among them, UV-C is the most dangerous but the atmosphere can filter out. Therefore, only UV-A and UV-B are harmful for living organisms. UV can be absorbed by biomolecules, including nucleic acids, proteins and many low-molecular-weight metabolites. The dangers of UV-A occur through reactive oxygen species (ROS) that is indirect effect. It is dangerous for cell targets. The effect of UV-B has the most potential for cell damage caused by direct effects on DNA and proteins and indirect effects via the production of ROS. It can inhibit the process of translation, transcription and can be mutagenic (Jeffrey *et al*., 1996). Direct effects of UV-B on proteins occur mostly through photon, leading to changes in conformation that carry functional loss (Gao and Garcia-Pichel, 2011). Taken together, the dangers of UV affected to metabolism of cells, biological molecules of cells or changes in physiology including, the survival, growth

pigmentation, photosynthetic oxygen production, motility, N_2 metabolism, phycobiliprotein composition and CO_2 uptake (Singh *et al*., 2008).

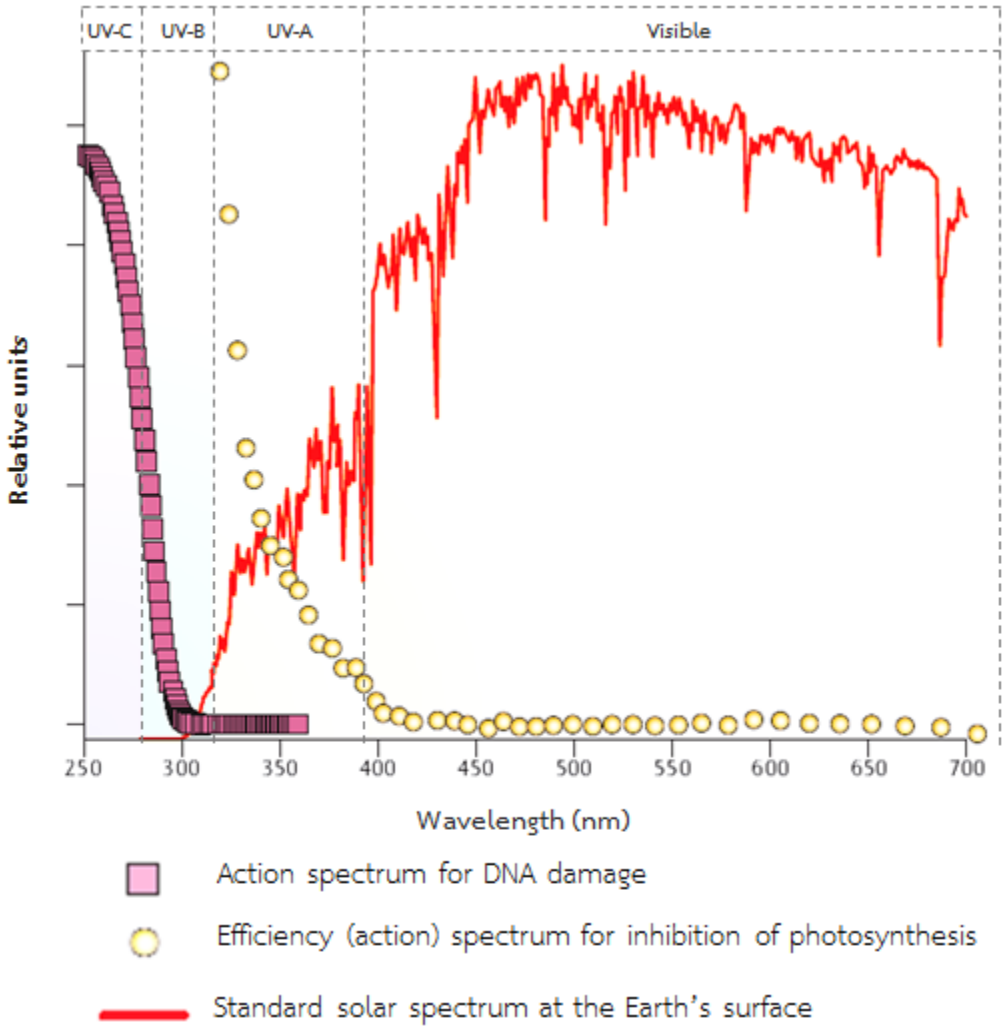


Figure 2.1: Standard solar radiation energy spectrum at the Earth's surface (American Society for Testing Standards) in the visible and ultraviolet ranges (From; Gao and Garcia-Pichel, 2011).

2.2 The response of microorganisms to UV

Many marine microorganisms, such as macroalgae, microalgae, and phytoplankton have specific mechanisms to protect detrimental effects of UV. Similarly, cyanobacteria protect themselves by employing many strategies to avoid dangerous from UV (Bebout and Garcia-Pichel, 1995). They have developed not only physical but also biological responses for UV protection.

The physical response is adaptation of behavior such as:

1. Avoidance exposure to UV (Gao and Garcia-Pichel, 2011)
2. Motiling from UV by finding areas of low exposure (Bebout and Garcia-Pichel, 1995)
3. Creating the biofilm at the surface cell layers

The biological response refers to strategies that have preventive mechanisms including:

1. The system of reducing ROS production during UV stress
2. Use of antioxidants or photoprotective compounds to protect UV (Figure 2.2) such as carotenoids, ascorbate (vitamin C), **α**-tocopherol (vitamin E), superoxide dismutase, glutathione peroxidase, scytonemin and MAA (He and Häder, 2002; Rastogi *et al*., 2010).
3. Use of inorganic compounds (Zinc oxide or Titanium dioxide) which can reduce UV exposure (F'Guyer *et al*., 2003; Rosen, 2003).

Among various strategies mentioned above, the synthesis of UV-absorbing compound is one of the important strategies. UV-absorbing compound known to date are melanin, scytonemin, and MAA (Gao and Garcia-Pichel, 2011). These compounds can prevent damage photons from reaching cellular targets. Especially, MAA is very interesting. There are received much attention in the last few years, because there are strong UV absorption maxima which specific range for UV-B. Thus, MAA is excellent UV-B absorber (Singh *et al*., 2008).

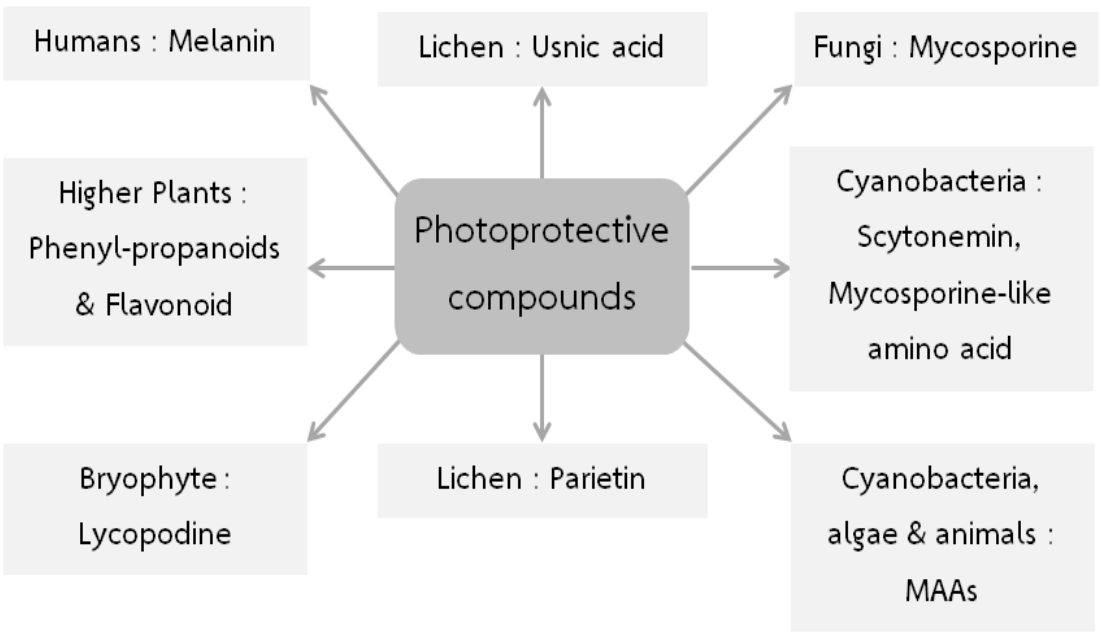


Figure 2.2: The difference of photoprotective compounds in living organisms (Modified from; Rastogi *et al*., 2010)

2.3 MAA

2.3.1 Chemical structure of MAA

MAA composes of 5-hydroxy-5-hydroxymethyl-cyclohex-1,2-ene ring (core structure) linked to an amino acid, amino alcohol or amino group (Figure 2.3.1A). There are linked with methoxy in C2 and linked with an amino compound in C3. In C1 has linked with either an oxo or an imino moiety (Gao and Garcia-Pichel, 2011; Carreto and Carignan, 2011). For example, mycosporine-glycine and mycosporine-taurine are aminocyclohexenones which linked with an amino acid (oxocarbonyl-MAAs). There are C3 of the cyclohexenimine ring linked with glycine (mycosporine-glycine), taurine (mycosporine-taurine), respectively (Figure 2.3.1A) (Volkmann and Gorbushina, 2006). Some MAAs in C2 of the cyclohexenimine ring linked with glycine (porphyra-344, shinorine, mycosporine-2-glycine). Some MAAs in C1 of the cyclohexenimine ring linked with amino alcohol (palythinol, asterina-330) or enaminone system (palythene, usujirene). The difference of the position of carbon linked with an amino acids which make difference of absorption maxima for each MAA (Figure 2.3.1B) (Carreto and Carignan, 2011).

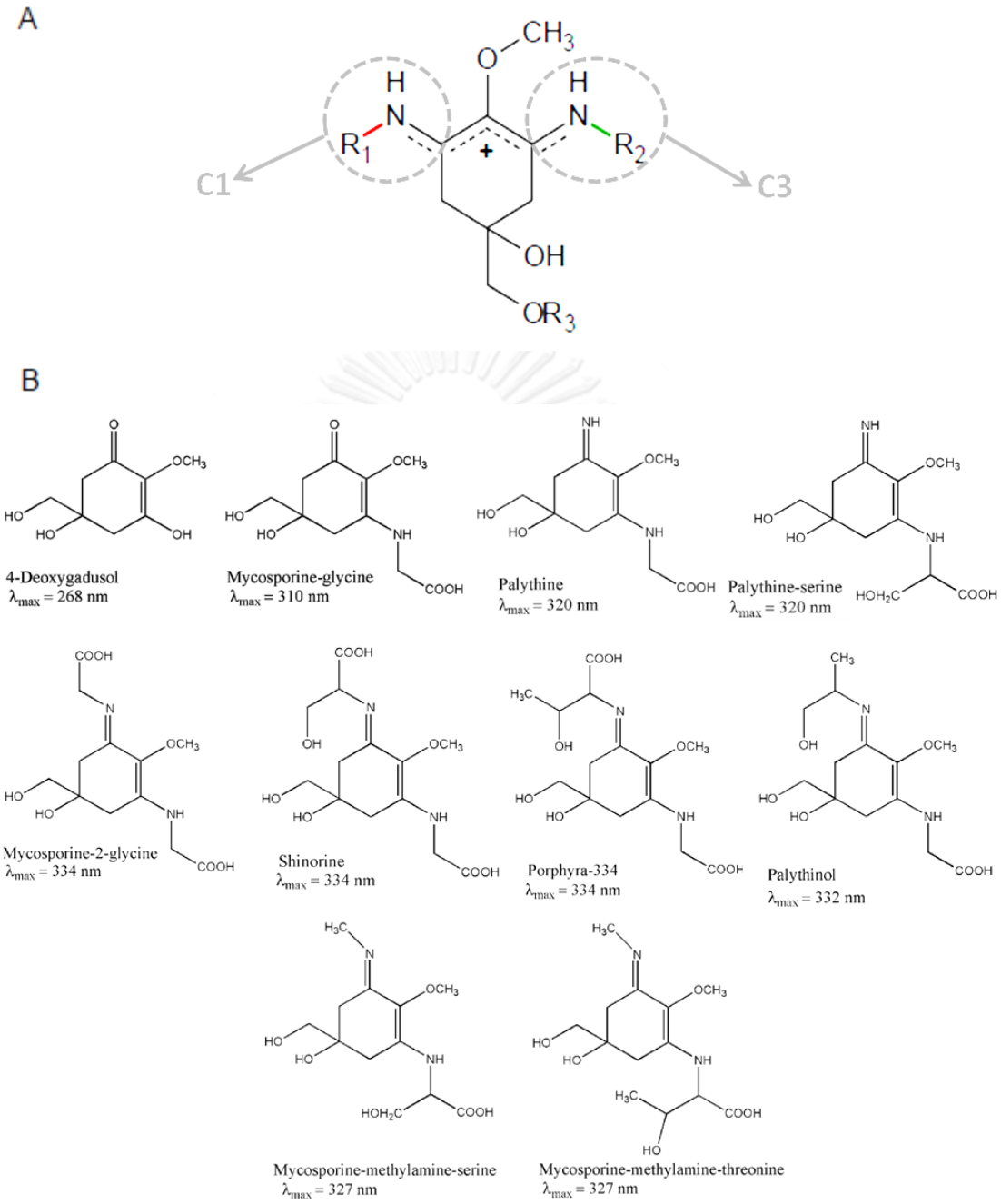


Figure 2.3.1: (A) Structure of MAA; C1 and C3 of core structure linked with amino acid, amino alcohol, and amino group. (Modified from; Carreto and Carignan, 2011); (B) A variety of structures of MAA (From; Rosic, 2012).

2.3.2 Property and biological function of MAA

MAA is valuable molecule that is the basis for important photoprotective constituent. MAA pure form is colorless, water-soluble, highly polar, and small molecular mass (<400 Da) (Rastogi *et al*., 2010). There is average of molecular weight around 300 Da (Bhatia *et al*., 2011). MAAs have different structures but they can be identified the similarity by spectral characteristics. Due to spectral characteristics can identify MAAs at the position of the absorption maximum, the cyclohexenimine ring linked with an amino acid, amino acohol or amino group determine MAA spectral characteristic and the value of their extinction coefficient (**ϵ**). For example, structures of palythene (trans-isomer), usujirene (cis-isomer) and the isomeric pair *E/Z* palythenic acid are different from other MAAs. They have a double bond conjugated with C1 of the cyclohexenimine ring (Figure 2.3.1B) (Uemura *et al*., 1980). The degree of resonance delocalization in structure affect the energy requirements in electronic transition and affect the position of the absorption maximum (Carreto and Carignan, 2011). MAAs can resistant to a wide range of pH. The demonstration in porphyra-334 was stable in solutions with pH 1 to 11 when incubated at room temperature for 24 hour, but unstable at 80 °C (Sinha *et al*., 2000).

2.3.3 Biosynthesis of MAA

The biosynthetic pathway of MAA has studied in cyanobacteria (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011). It has been shown to derive from shikimate pathway. This pathway is related for creating aromatic amino acids (i.e. phenylalanine, tyrosine, and tryptophan). It has DHQ as an intermediate compound. Moreover, DHQ acts as intermediate to an MAA core, thus this intermediate is crucial for MAA biosynthesis (Shick *et al*., 2002) (Figure 2.3.2).

MAA accumulation mostly found in cyanobacteria, phytoplanktons and macroalgae but not in animals because they lack of shikimate pathway (Davidson, 1998; Sinha and Hader, 2008). Recently, MAA biosynthesis has been extensively studied in filamentous cyanobacteria *A. variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133 (Gao and Garcia-Pichel, 2011). There are four steps catalysing MAA biosynthesis. The first step, gene *Ava_3858* or *NpR5600* encodes demethyl 4-deoxygadusol synthase (DDG) which converts sedoheptulose-7-phosphate (SHP) to form 2-demethyl-4-deoxygadusol (DDG). The second step, gene *Ava_3857* or *NpR5599* encodes o-methyltransferase (o-MT) which converts 2-demethyl-4-deoxygadusol (DDG) to form 4-deoxygadusol (4-DG). The intermediate 4-DG is a core structure for mycosporine. The third step, the product of *Ava_3856* or *NpR5598* catalyzes the addition of glycine which converts 4-deoxygadusol (4-DG) to form mycosporine-glycine (Balskus and Walsh, 2010; Singh *et al*., 2010) but the last step of catalysis, in *A. variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133 are difference. In *A. variabilis* ATCC 29413, the product of Ava_3855 catalyzes the addition of serine which convert mycosporine-glycine to form shinorine (Balskus and Walsh, 2010); and *N. punctiforme*

ATCC 29133 the product of NpF5597 catalyzes the addition of serine which convert mycosporine-glycine to form shinorine by another pathway (Gao and Garcia-Pichel, 2011) (Figure 2.3.3).

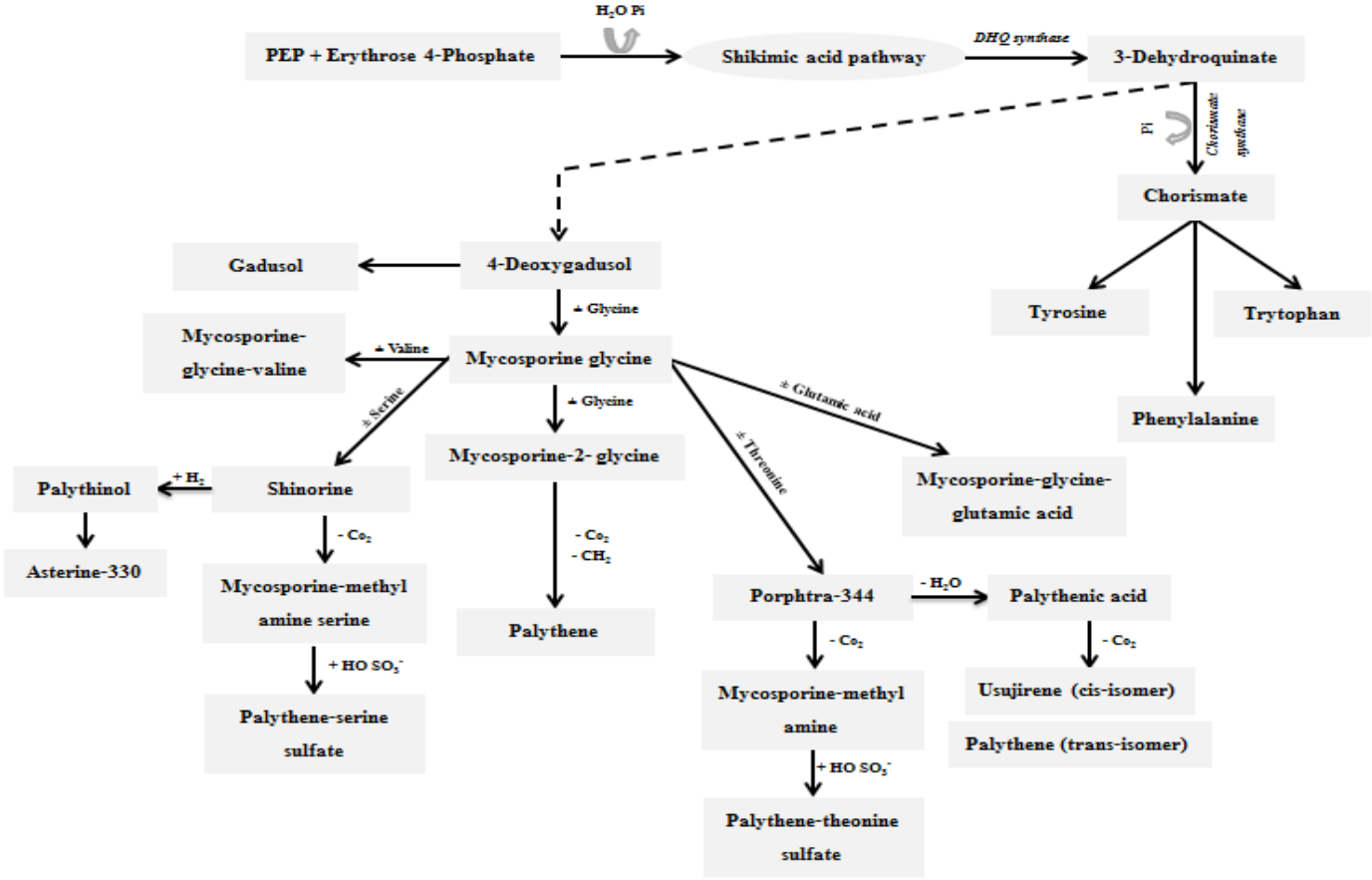


Figure 2.3.2: Conventional biosynthetic pathways for the formation of MAAs via the shikimate pathway (From; Bhatia *et al.*, 2011)

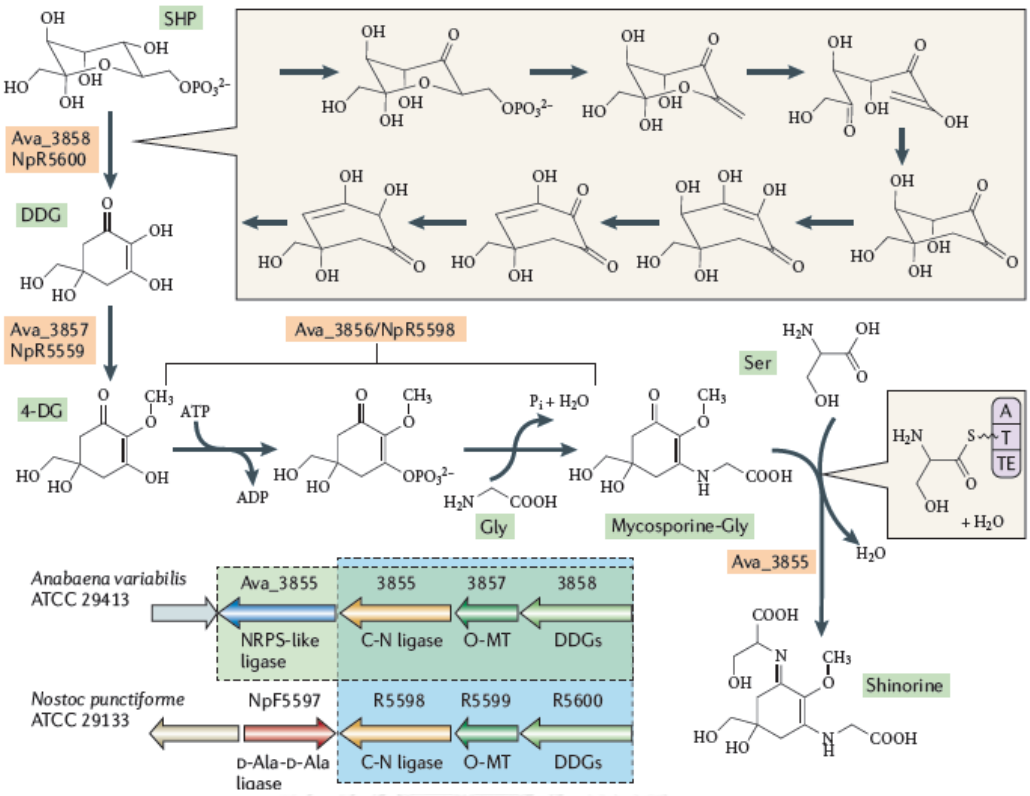


Figure 2.3.3: Biosynthesis of MAA and its genetic basis in cyanobacteria; *A. variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133 (From; Gao and Garcia-Pichel, 2011).

2.3.4 Induction and regulation of MAAs

There are researches that concerned on induction and regulation of accumulation MAA. It has been shown that the accumulation of MAA in cyanobacteria was affected by photon. MAA can synthesize and accumulate by induced or exposed to intense solar radiation which is spectrum for the response varies (Shick and Dunlap, 2002). Some MAAs such as mycosporine-glycine is antioxidant, and can protect creating of ROS

during expose UV, but other MAAs, such as shinorine, does not share this antioxidant property (Dunlap *et al*., 1995). There have been reported on three species of cyanobacterium, *Nodularia spumigena*, *Nodularia baltica* and *Nodularia harveyana* that can produce the MAAs porphyra-334 and shinorine increasingly when they had UV-B exposure (Sinha *et al*., 2003). MAAs in dinoflagellate *Alexandrium excavatum* and *Gyrodinium dorsum* can accumulate increasingly under high intensity of UV-B (Carreto *et al*., 1990; Klisch and Häder, 2002). It has reported on salt treatment and ammonium treatment (Singh *et al*., 2008) promoted MAA level in cyanobacterium *A. variabilis* PCC 7937. This cyanobacteria can produce MAA increasingly when get highly salt and ammonium treatment. Desiccation also has been found to induce accumulation of MAA in cyanobacterium *N. commune*, thus it can tolerate to high desiccation (Matsui *et al*., 2011).

In this research, I aim to study MAA biosynthesis from a halotolerant cyanobacterium *Aphanothece halophytica* (hereafter *A. halophytica*). This extremophile has been shown several novel pathways in response to salinity stress (Waditee-Sirisattha *et al*., 2014). Thus far, MAA biosynthesis has never been explored in any extremophile. In this study, I have three objectives as follows.

The objective of this research:

1. To study the induction of MAA in *A. halophytica*
2. To express the biosynthetic gene cluster for MAA in expressing cells
3. To analyse MAA production in expressing cells

CHAPTER III
MATERIALS AND METHODS

Materials

3.1 Instruments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetteman, Gilson, France

Refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Digital Lux meter FT710: Taiwan

Electrophoresis unit: Model mini protein II cell: Biorad, USA

Erlenmeyer-flask quartz: Witeg, Germany

High performance liquid chromatography: Model Hewlette Packard (1050)

Illuminated/Refrigerated orbital: Sanyo, England

Incubator: Haraeus, Germany

Incubator shaker: Psyco-therm, New Bruncwick Scientific Supply, Thailand

Laminar flow BVT-124: International Scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Power supply: Pharmacia, England

Spectrophotometer UV-240: Shimadzu, Japan

UVB Lamp: Toshiba, Japan

UVP, LLC: Analy Jena AG, USA

Time of flight Mass spectrophotometer: Model Shimadzu, Japan

Vacuum dry: Taitech, Japan

Vortex: Model K-550-GE: Scientific Industries, USA

3.2 Chemicals

Acetonitrile: Katayama Chem, Japan

Acrylamide: Merck, USA

Ammonium persulfate: Katayama Chem, Japan

An antibody raised against 6-histidine: R&D systems, USA

An antibody raised against mouse: Biolab, England

Ampicillin: Katayama, Japan

Bacto tryptone: Merck Ag Darmstadt, Germany

Bacto Yeast extract: Merck Ag Darmstadt, Germany

Calcium chloride: Merck Ag Darmstadt, Germany

Chloroform: Katayama Chem, Japan

Coomasie brilliant blue G-250: Sigma, USA

Coomasie brilliant blue R-250: Sigma, USA

Ethanol: Katayama Chem, Japan

Glycerol: Merck Ag Dramstadt, Germany

Glycine: Sigma, USA

L-serine: Sigma, USA

Magnesium chloride: Merck Ag Dramstadt, Germany

Magnesium sulfate: Merck Ag Dramstadt, Germany

Phenol: Katayama Chem, Japan

Potassium chloride: Merck Ag Dramstadt, Germany

Sodium acetate: Sigma, USA

Sodium chloride: Sigma, USA

Sodium nitrate: Sigma, USA

Streptomycin: Sigma, USA

3.3 Membranes

PVDF/Nitrocellulose membrane: Millipore Cooperation, USA

YM-3 membrane, NMWCO 3 kDa: Sigma, USA

3.4 Kits

Gel extraction kit: Invitrogen, USA

Plasmid extraction kit: Invitrogen, USA

Standard molecular weight: Sigma, USA

3.5 Restriction enzymes

*Bam*HI: Biolabs, USA

*Xho*I: Biolabs, USA

3.6 Bacterial strains and plasmids

Table 1 Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Description	Source/reference
A. halophytica	Halotolerant cyanobacterium	Waditee-Sirisattha *et al*., 2012
E. coli DH5α	(φ80d *lacZ*ΔM15 *recA1endA1 gyrA96 thi*-l *hsd*R17($r_k^- m_k^+$) *sup*E44 *rel*A1 *deoR*Δ (*lacZYA-argF)*U169)	Invitrogen, USA
Ap3858-3855/pUC303	2.76 kb *Ap3858* fragment and 3.63 kb *Ap3857-3855* fragment cloned into pUC303 vector	Waditee-Sirisattha *et al*., 2014

Methods

3.7 Induction of the *Aphanothece* MAA

3.7.1 *A. halophytica* culture condition

A. halophytica cells were grown photoautotrophically in BlueGreen (BG) 11 liquid medium containing 18 mM $NaNO_3$ and Turk Island salt solution (Appendix 1) (Waditee-Sirisattha *et al*., 2012) except that NaCl concentration of the culture medium was adjusted at 0.5 and 2.0 M as desired. Cotton-plugged 500 ml Erlenmeyer-flask containing 150 ml of medium each were used and shaken on reciprocal shaker without supplementation of CO_2. The culture flasks were incubated at 30 $^{\circ}$C under continuous fluorescent white light (70 $\mu Em^{-2}S^{-1}$). The growth of cyanobacterial cells were monitored by measuring absorbance at 730 nm.

3.7.2 Stress condition

A. halophytica cells were grown photoautotrophically under non-stress condition (0.5 M NaCl) for 14 days prior to up-shock by salt (2.0 M NaCl) and UV-B stress experiments. For up-shock experiment, the concentration of NaCl in growth medium was changed from 0.5 to 2.0 M. For UV-B stress experiment, cells were adjusted to an optical density at 730 nm in the culture medium of approximately 0.8 prior to the transfer to Erlenmeyer-flask quartz and further exposed to UV-B radiation at 0.1 W/m^{-2} (Sankyo Denki; GL20SE) (Appendix 2). UV-B exposure was carried out for 6 hour per day.

3.7.3 Effect of exogenous addition of glycine and serine

A. halophytica cells were grown as the same condition as 3.7.2. After 14 days, cells were harvested and transferred to new medium containing 1 mM glycine or serine. Cells were harvested at day 1, 3 and 5 for MAA analysis.

3.8 Extraction, purification and identification of the Aphanothece MAA

3.8.1 Extraction of the *Aphanothece* MAA

Ten milliliters of *A. halophytica* cells were harvested by centrifugation at 4,500 rpm for 10 min at 4 °C. Cell pellets were extracted in 500 µl of methanol overnight at 4 °C. The samples were centrifuged at 15,000 rpm for 10 min at 4 °C and transferred to new Eppendorf

microcentrifuge tubes. Resulting supernatants were evaporated to dryness at room temperature in a vacuum evaporator and re-dissolved in 500 µl Milli-Q water. After that, the extracts were added 2 drops of chloroform and mixed by vortex. Next, the upper water phase was passed through YM-3 membrane (Sigma, USA) by centrifugation at 15,000 rpm for 30 min at 25 °C. Then, the supernatants were measured by spectrophotometer (Spectrophotometer UV-240: Model Shimadzu, Japan) and subjected to high performance liquid chromatography (HPLC).

3.8.2 HPLC analysis

Further analysis was performed using a HPLC system (Model Hewlette Packard series 1050) with Shim-pack FC-ODS reverse phase column (3 µm; 150 × 4.6 mm). MAA was detected at 35 °C by using UV-VIS detector in wavelength at 330 nm. The mobile phase was run at flow rate of 0.4 ml/min: solvent A, 0.1% (v/v) Milli-Q water contained trifluoroacetic acid and solvent B, 0.1% (v/v) acetonitrile containing trifluoroacetic acid.

3.8.3 Amino acid analysis

Before amino acid analysis, MAA was purified by HPLC as follows. The extracts containing MAA (from 3.7.2) were purified by HPLC with Shim-pack FC-ODS reverse phase column (3 µm; 150 × 4.6 mm) connected to a guard column (30 × 4.6 mm) and flow rate at 0.4 ml/min: first 100% solvent A (0.1 M ammonium acetate, pH 6.5), after 15 min 20% solvent B (acetonitrile) in solvent A. After purification, the solvent was

evaporated to dryness and MAA was dissolved in Milli-Q water. Then, MAA was hydrolyzed and further analyzed by amino acid analysis.

Alkaline hydrolysis was carried out according to Stochaj *et al* (1994) with a slight modification. Briefly, 40 µl of the resulting fraction from HPLC analysis was hydrolyzed at room temperature for 12 hour with 4 µl of 10 N NaOH and then neutralized with 4 µl of 10 N HCl for 6 hour. The amino acid composition of the hydrolyzed fraction was identified by amino acid analysis system (Shimadzu, Japan) (Appendix 3).

3.8.4 Quantification of MAA from *A. halophytica*

A. halophytica cells were grown under normal condition photoautotrophically for 14 days (OD_{730} approximately 0.8). After that, 10 ml of cell culture was harvested by centrifugation for quantification of cell gram dry weight. The obtained cell gram dry weight from *A. halophytica* was determined by comparing with cell gram dry weight marker. In parallel, 10 ml of cell culture was extracted by methanol. The methanolic phase containing the *Aphanothece* MAA was analyzed by HPLC analysis. MAA of *A. halophytica* was determined by comparing with standard marker (Appendix 4).

3.9 Expression of the *Aphanothece* MAA biosynthetic genes into *E. coli*

3.9.1 Construction of MAA biosynthetic genes from *A. halophytica*

Coding region of the *Aphanothece* MAA biosynthetic genes and their promoter regions (hereafter *ApMAA*) were amplified and constructed as described (Waditee-Sirisattha *et al*., 2014). Schematic of construction was simply illustrated in Figure 3.9.1.

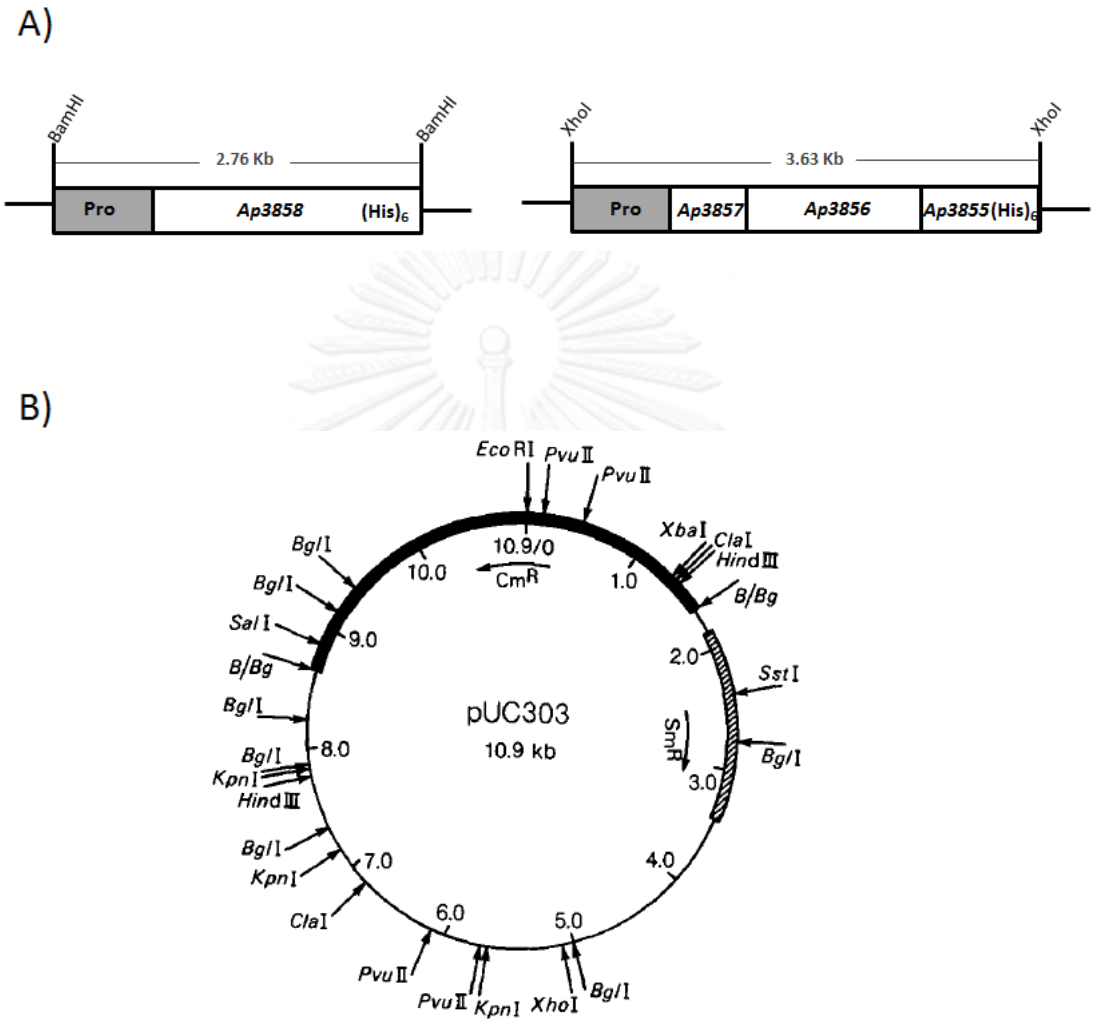


Figure 3.9.1: A) Contruction of the *Aphanothece* biosynthetic genes for MAA expression B) Vector pUC303 (From; Kuhlemeier and Arkel, 1987) used as a shuttle vector for expression of genes *Ap3858-3855* from *A. halophytica*.

3.9.2 Transformation of *ApMAA* genes (*Ap3858-3855*) into *E. coli*

One hundred microliters of competent cells (*E. coli* DH5α) (Appendix 5) were thawed on ice prior to the addition of 3 µl of recombinant plasmid (2.76 kb *Ap3858* fragment and 3.63 kb *Ap3857-3855* fragment cloned into pUC303 vector) mixture. The transformation mixture was flicked 2-3 times and stood on ice for 10 min. After that, the mixture was heated to 42 °C for 90 second and stand on ice for 5 min. Then the mixture was diluted with 900 µl of LB medium and gently shaking at 37 °C for 90 min. Cell suspension was spread and selected on LB agar containing streptomycin (50 µg/ml of concentration). Then the colony was picked and transfer to liquid LB medium for culture large scale (Appendix 6).

3.9.3 Stress condition

Expressing cells from 3.9.2 were grown under non-stress condition in LB medium, incubated at 37 °C and monitored by measuring absorbance at 620 nm until reach to 0.8 for further induction by salt and UV-B stresses. For the induction by salt, cell cultures were added NaCl by varying concentration to a range from 0 to 0.6 M as desired. For UV-B stress experiment, cells were adjusted to an optical density at 620 nm in the culture medium of approximately 0.8 prior to the transfer to Erlenmeyer-flask quartz and exposed to UV-B at 0.1 W/m^{-2}. UV-B exposure was carried out for 2 hour per day for 5 days.

3.9.4 Western Blot analysis

Soluble fraction of protein from expressing cells was prepared from *E. coli* grown under stress condition (from 3.9.3). The *E. coli* pellets were resuspended in 100 mM Tris-Cl (pH 8.0), sonicated, centrifuged at 15,000 rpm for 10 min at 4 °C, and collected clear supernatant transferred to new Eppendorf tubes. Clear supernatant was used to determine expression level of the *Ap3858-3855* products by Western Blotting. Protein content was determined by the method of Bradford (Bradford, 1976) (Appendix 7). SDS-PAGE was carried out as standard protocol (Laemmli, 1970) (Appendix 8). The molecular masses of *Ap3858-3855* products were determined by comparing with protein molecular weight marker (Bio-Rad, USA).

Western Blot analysis was carried out to confirm that the recombinant *Ap3858-3855* was fused in-frame to six histidine tag. For this experiment, 40 µg protein of recombinant *Ap3858-3855* was separated by 15% SDS-PAGE and protein band was transferred to PVDF membrane using blotting transfer buffer. Blotting was done at 150 mA for 1 hour and followed by blocking in blocking solution for 2 hour with 100 ml of PBSt buffer plus 5% skim milk solution. The PVDF membrane was incubated with primary-antibody (an antibody raised against 6-histidine, 6X-His tag) for 2 hour (gently shaking every 30 min) and washed with 100 ml of PBSt buffer for 30 min, 2 times. After washing the membrane, the membrane was incubated with secondary-antibody (an antibody raised against mouse) for 2 hour (gently shaking every 30 min) and washed 2 times with 100 ml of PBSt buffer for 30 min. The PVDF membrane was detected by detection reagent for Western Blotting for 10 min (Appendix 9).

3.9.5 Quantification of MAA in *E. coli* expressing cells

E. coli expressing cells were grown in LB medium and incubate at 37 °C prior to induction by salt and UV-B experiment. After that, 10 ml of cell culture was harvested by centrifugation for quantification of cell gram dry weight. The obtained cell gram dry weight from *E. coli* expressing cells was determined by comparing with cell gram dry weight marker. In parallel, 10 ml of cell culture was extracted by methanol. The methanolic phase containing MAA was analyzed by HPLC analysis. MAA of *E. coli* expressing cells was determined by comparing with standard marker.

3.10 Expression of the *Aphanothece* MAA biosynthetic genes into *Synechococcus sp* PCC 7942

3.10.1 Transformation of *ApMAA* genes (*Ap3858-3855*) into *Synechococcus sp* PCC 7942

Log phase of *Synechococcus sp* PCC 7942 growth was used for transformation. One hundred microlitters of *Synechococcus sp* PCC 7942 were mixed with 3 µl of recombinant plasmid (Waditee-Sirisattha *et al*., 2014) and incubated at 30 °C overnight (Natural transformation). After that, cell suspension was spreaded and selected on BG 11 agar containing streptomycin (final concentration; 50 µg/ml). After 14 days, the colony grown on the plate. Then, colonies were picked up and analysed the presence of gene by PCR.

3.10.2 Stress condition

To induce accumulation of MAA in *Synechococcus sp* PCC 7942 system, *Synechococcus sp* PCC 7942 cell were grown photoautotrophically in BG 11 liquid medium and incubated at 30 $^{\circ}$C for 14 days (*Synechococcus sp* PCC 7942 cell were monitored by measuring absorbance at 730 nm until reach to 0.8) prior to up-shock by salt and UV-B stress experiment. For up-shock experiments, the concentration of NaCl in growth medium was changed from 0 to 0.375 M. For UV-B stress experiment, cells were adjusted to an optical density at 730 nm in the culture medium of approximately 0.8 prior to the transfer to Erlenmeyer-flask quartz and exposed to UV-B at 0.1 W/m^{-2}. UV-B exposure was carried out for 3 hour per day.

3.10.3 Quantification of MAA in *Synechococcus sp* PCC 7942

Synechococcus sp PCC 7942 transformants cells were grown photoautotrophically for 14 days prior to up-shock and UV-B stress experiment. After that, 10 ml of cell culture was harvested by centrifugation for quantification of cell gram dry weight. The obtained cell gram dry weight from *Synechococcus sp* PCC 7942 transformant was determined by comparing with cell gram dry weight marker. In parallel, 10 ml of cell culture was extracted by methanol. The methanolic phase containing MAA was analyzed by HPLC analysis. MAA from *Synechococcus sp* PCC 7942 transformants was determined by comparing with standard marker.

CHAPTER IV
RESULTS

4.1 Extraction of the *Aphanothece* MAA

A. halophytica cells were cultured and extracted MAA as described in Materials and Methods. As shown in Figure 4.1A, the absorption spectra of methanolic extracts from *A. halophytica* showed a shoulder of MAA at around 331 nm. In addition to MAA, a considerably amount of photosynthetic pigment was also extracted by this process (Figure 4.1A), for example, chlorophyll a (430 and 664 nm), carotenoid (473 nm).

Absorption spectrum of water phase showed a single peak at 331 nm (Figure 4.1B). A prominent peak at 331 nm suggested the presence of MAA, UV-absorbing compound. There was an increase in the absorbance at 331 nm when the sample was extracted from *A. halophytica* cells grown under 2.0 M NaCl (Figure 4.2).

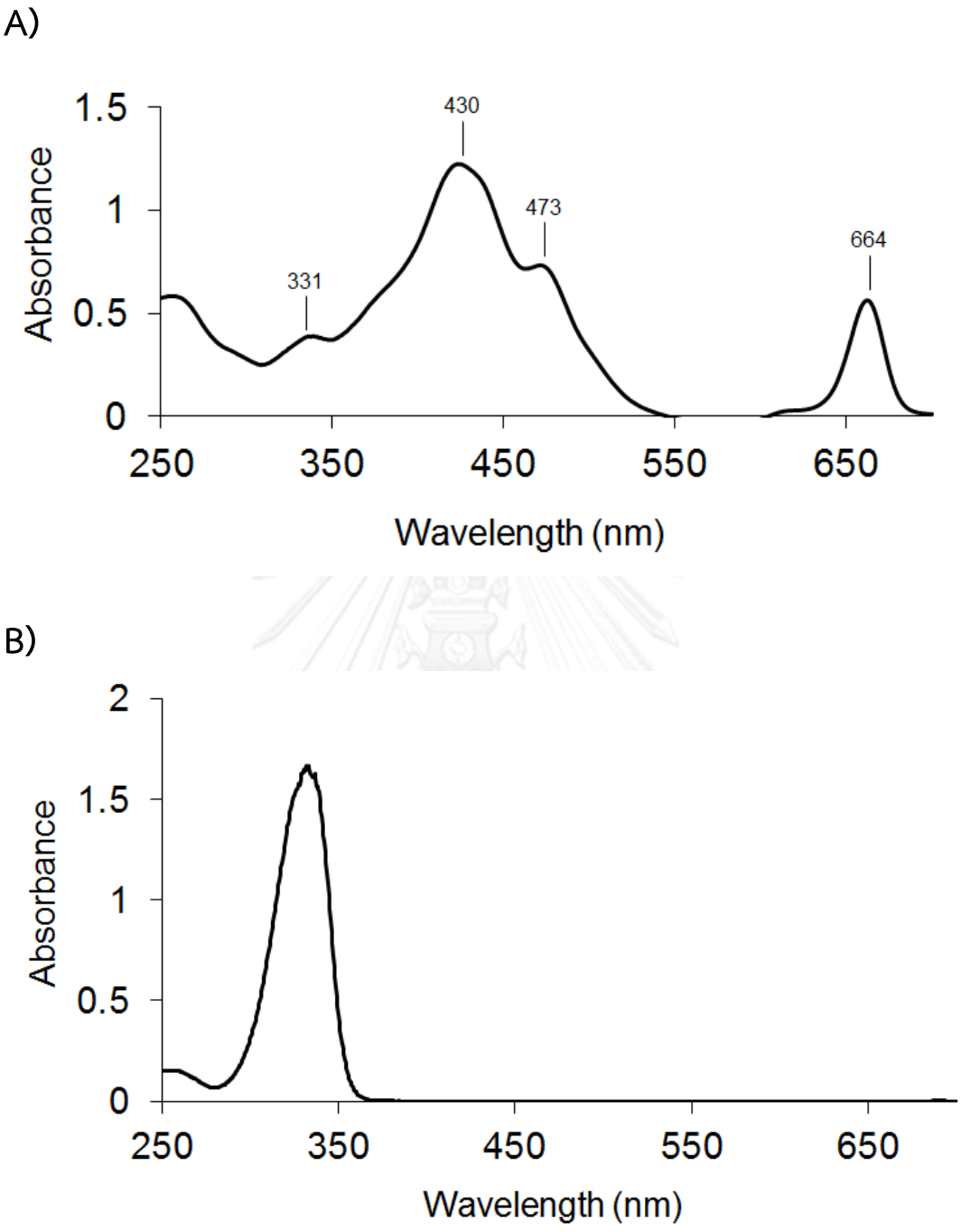


Figure 4.1: The absorption spectra of methanolic and water phases from *A. halophytica*. A) Methanolic phase. B) Water phase. Spectra were scanned from wavelength range 250 nm to 700 nm.

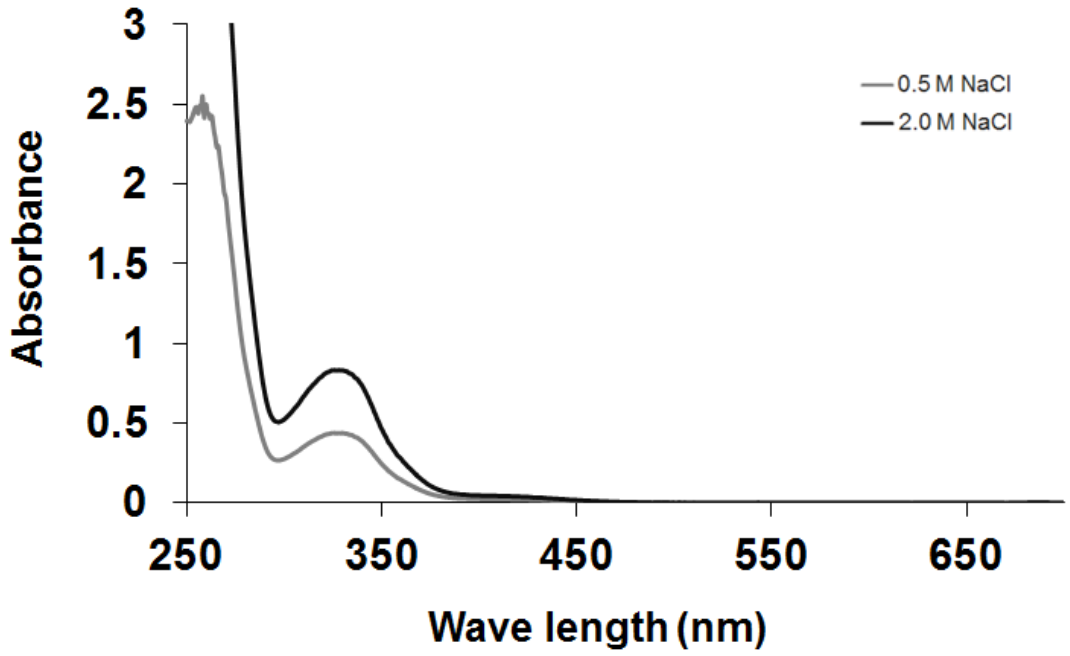


Figure 4.2: The absorption spectra of water phase from *A. halophytica* grown under normal (0.5 M NaCl) and salt stress condition (2.0 M NaCl). Spectra were scanned from wavelength range 250 nm to 700 nm.

4.2 HPLC analysis

Further, analysis and purification of partial purified MAA from *A. halophytica* (from water phase) was done by HPLC. As shown in Figure 4.3, HPLC chromatogram revealed a main single peak at a retention time of about 9.1 minute. A few small unknown peaks were also appeared at about 4.4, 6.6, 8.1, 14.6, 15.1 and 15.8 minute.

Identification of the *Aphanothece* MAA was further performed by amino acid hydrolysis after alkaline hydrolysis. This method was performed to identify the amino acid composition of the *Aphanothece* MAA. As shown in Figure 4.4, amino acid composition analysis revealed the presense of only a single amino acid corresponding to glycine. Therefore, glycine can be considered the sole amino acid in the structure of the *Aphanothece* MAA molecule.

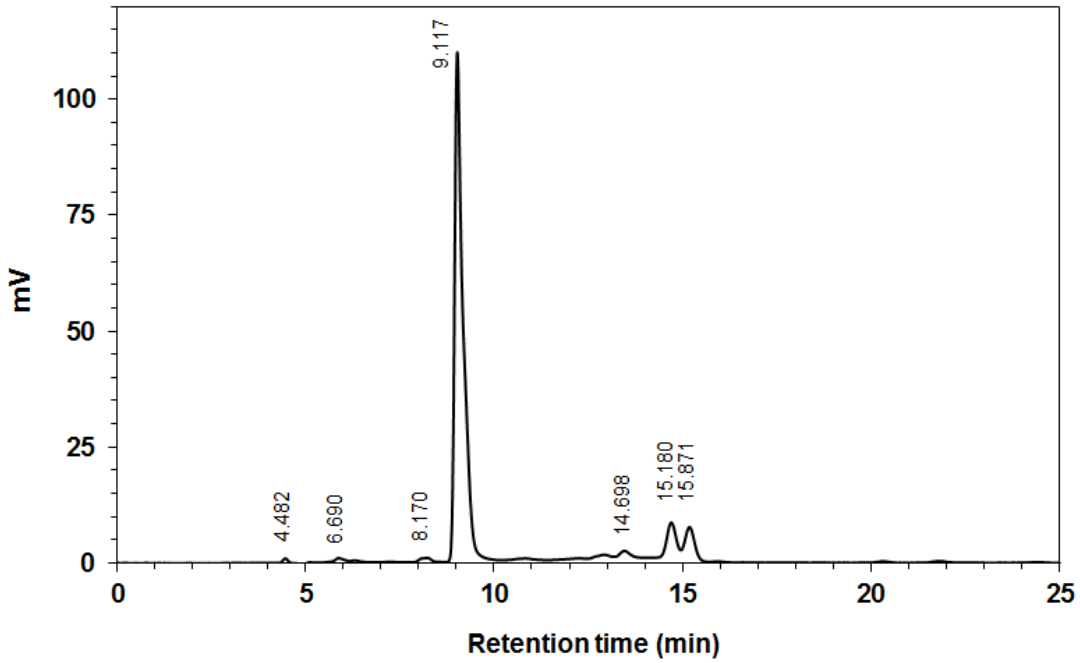


Figure 4.3: HPLC Chromatogram of MAA from *A. halophytica*. Shim-pack FC-ODS reverse phase column; the mobile phase was run at flow rate of 0.4 ml/min: solvent A, 0.1% (v/v) Milli-Q water contained trifluoroacetic acid and solvent B, 0.1% (v/v) acetonitrile containing trifluoroacetic acid and detection by absorbance at 330 nm.

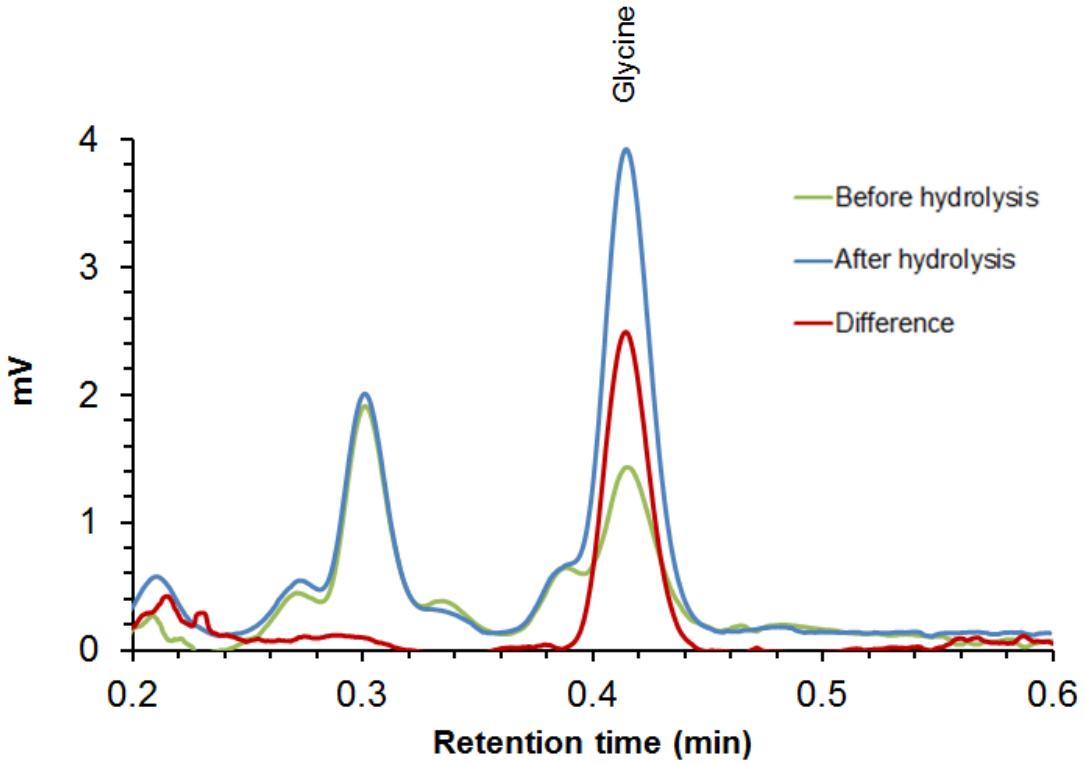


Figure 4.4: Amino acid profile of the hydrolyzed the *Aphanothece* MAA. The amino acid composition of the hydrolyzate was analyzed by HPLC with pre-packaged C18 column and detection by UV.

4.3 Induction of the *Aphanothece* MAA

4.3.1 Salt stress induced accumulation of the *Aphanothece* MAA

Salt up-shock and UV-B stresses were performed to determine the accumulation level of the *Aphanothece* MAA. For salt up-shock, NaCl concentration was increased to 2.0 M. As shown in Figure 4.5, the salt up-shock condition caused a dramatic increase in the *Aphanothece* MAA level. After 48 hour the result showed that the amount of MAA (0.5 M to 2.0 M NaCl) increased from 0.36 ± 0.02 μmol/g DW to 31.97 ± 0.48 μmol/g DW.

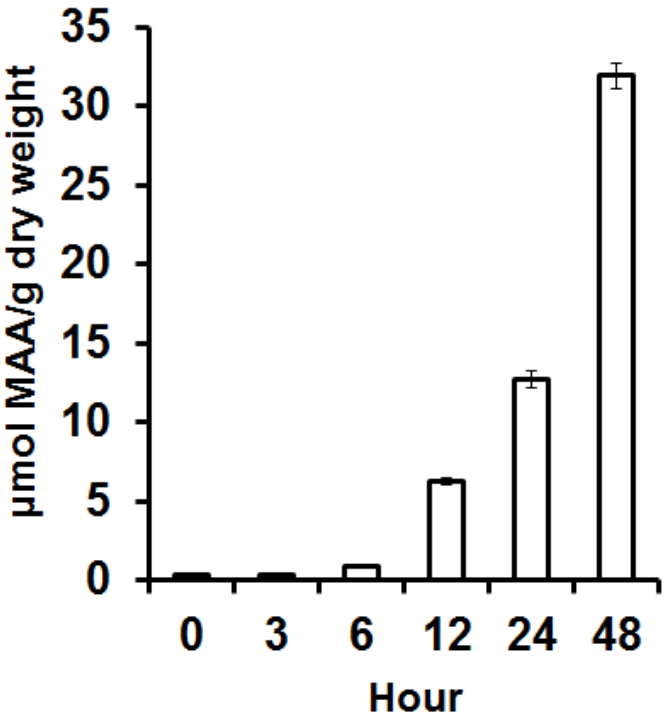


Figure 4.5: Time course of the *Aphanothece* MAA accumulation under salt stress. MAA was extracted and quantified by HPLC as described in Materials and Methods. Data are means ± standard deviation of three independent experiments.

4.3.2 Effect of UV-B radiation for accumulation the *Aphanothece* MAA

UV-B radiation was one important stress to induce the accumulation of MAA. In this study, effect of UV-B radiation for MAA accumulation also was observed. After exposing to UV-B radiation for 5 days, amount of MAA increase from 0.49 ± 0.04 μmol/g DW to 16.64 ± 0.6 μmol/g DW when *A. halophytica* grown under 0.5 M NaCl. Amount of MAA increased from 9.18 ± 1.37 μmol/g DW to 28.39 ± 0.45 μmol/g DW when *A. halophytica* grown under 2.0 M NaCl (Figure 4.6). Comparing the amount of MAA under induced by salt and UV-B stress experiments (Figure 4.5 vs Figure 4.6). It was found that MAA was much more induced by salt rather than UV-B stress experiment, approximately 2 folds after culturing for 2 days.

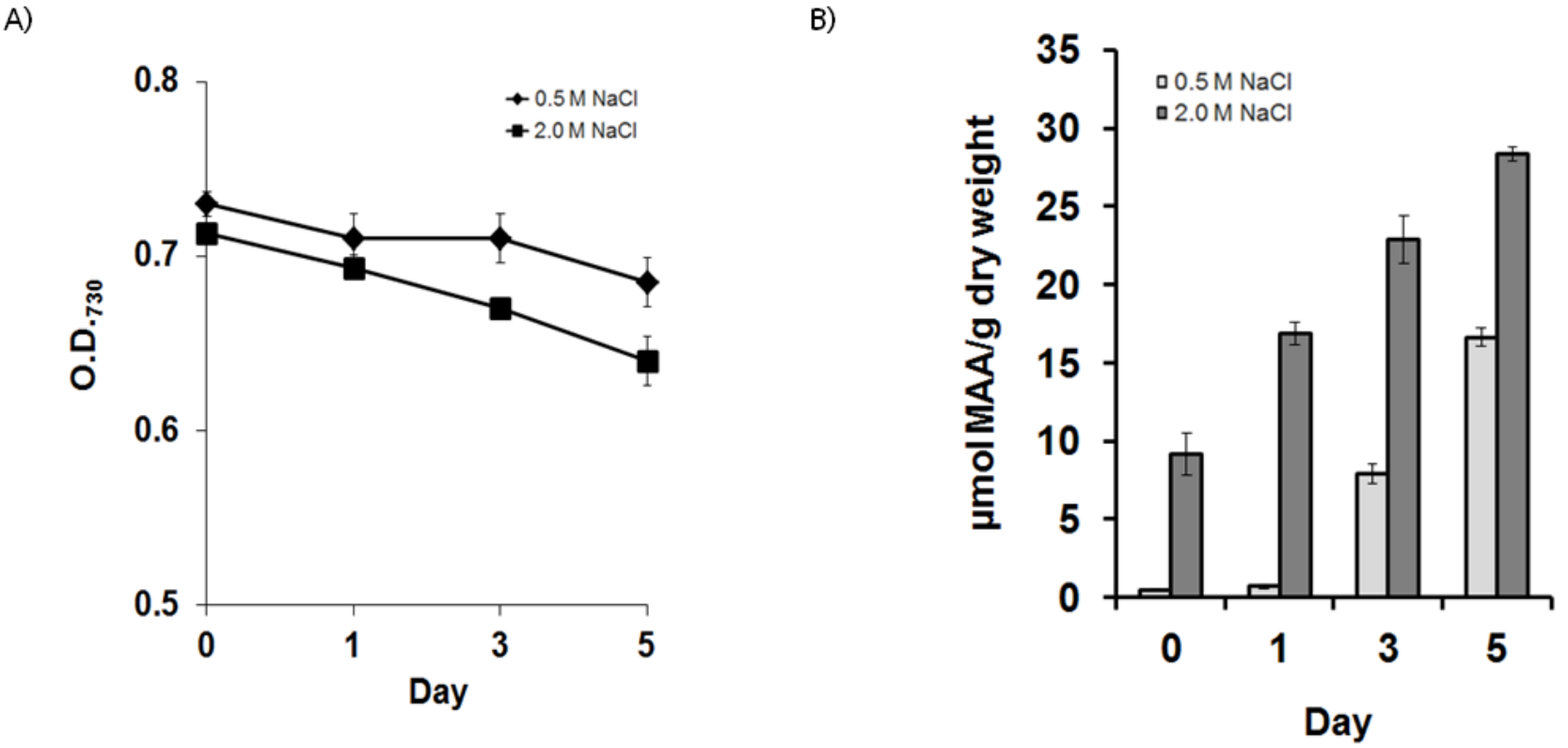


Figure 4.6: A) Growth profile of *A. halophytica* after UV-B stress experiment (exposed UV-B at 0.1 W/m^{-2} for 6 hours per day) for 5 days. B) Time course of MAA accumulation under UV-B (expose UV-B at 0.1 W/m^{-2} for 6 hours per day) for 5 days. Data are means ± standard deviation of three independent experiments.

4.3.3 Effect of exogenous addition of glycine and serine

Because the structure of the *Aphanothece* MAA has glycine linked to a core structure; therefore, the *Aphanothece* MAA was examined whether addition of glycine induced its accumulation. Furthermore, serine can interconvert to glycine. So, the effect of the addition of serine was also examined. After 5 days of the addition of glycine and serine, relative amount of MAA increased approximately 170 ± 11.46% and 150 ± 8.97% respectively (Figure 4.7).

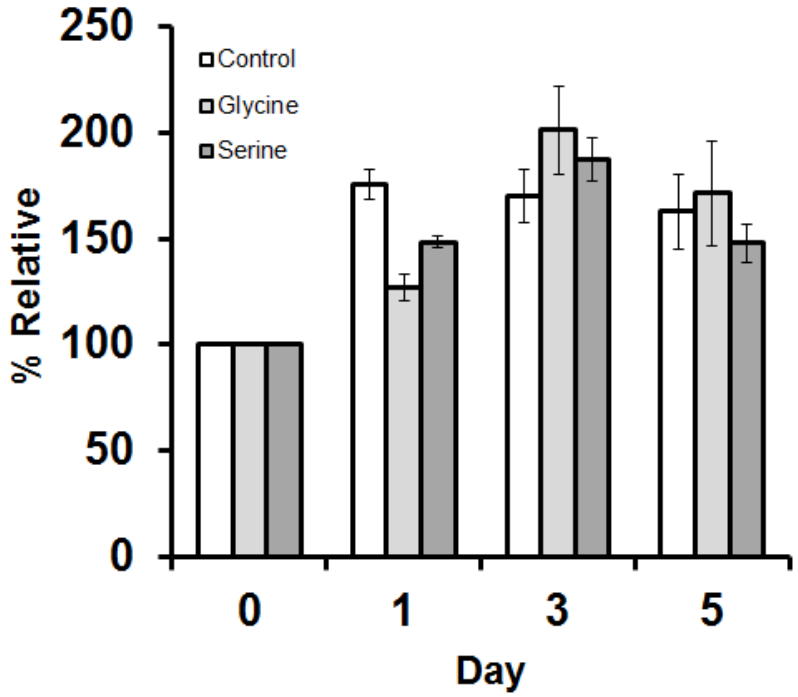


Figure 4.7: Relative amount of MAA from *A. halophytica* under the supplementation of glycine and serine (final concentration at 1 mM). Data are means ± standard deviation of three independent experiments.

4.4 Expression of biosynthetic gene cluster for MAA from *A. halophytica* into expressing cells

4.4.1 Expression of *Ap3858-3855* in *E. coli*

A cluster of the putative *Aphanothece* MAA biosynthetic genes was constructed and expressed in *E. coli* and *Synechococcus sp* PCC 7942, respectively. Both host cells were unable to synthesize any MAAs. Expression levels of Ap3858-3855 proteins in soluble fraction were analyzed by SDS-PAGE (Figure 4.8A). Western Blotting was carried out to analyze fusion protein at C-terminus (Ap3858xHis6, Ap3857-3855xHis6). Western Blotting of the *E. coli* transformed with *Ap3858-3855* genes revealed the cross-reacting band. The molecular masses of Ap3858 and Ap3857-3855 in linear polypeptide showed approximately 64.5 KDa and 37.3 KDa (Figure 4.8B). In this study, amount of protein for analysis was used only 40 µg. In Western Blotting can detect only fusion protein of Ap3858xHis6. The fusion protein of Ap3857-3855xHis6 can be detected if adding protein over 100 µg (data not shown).

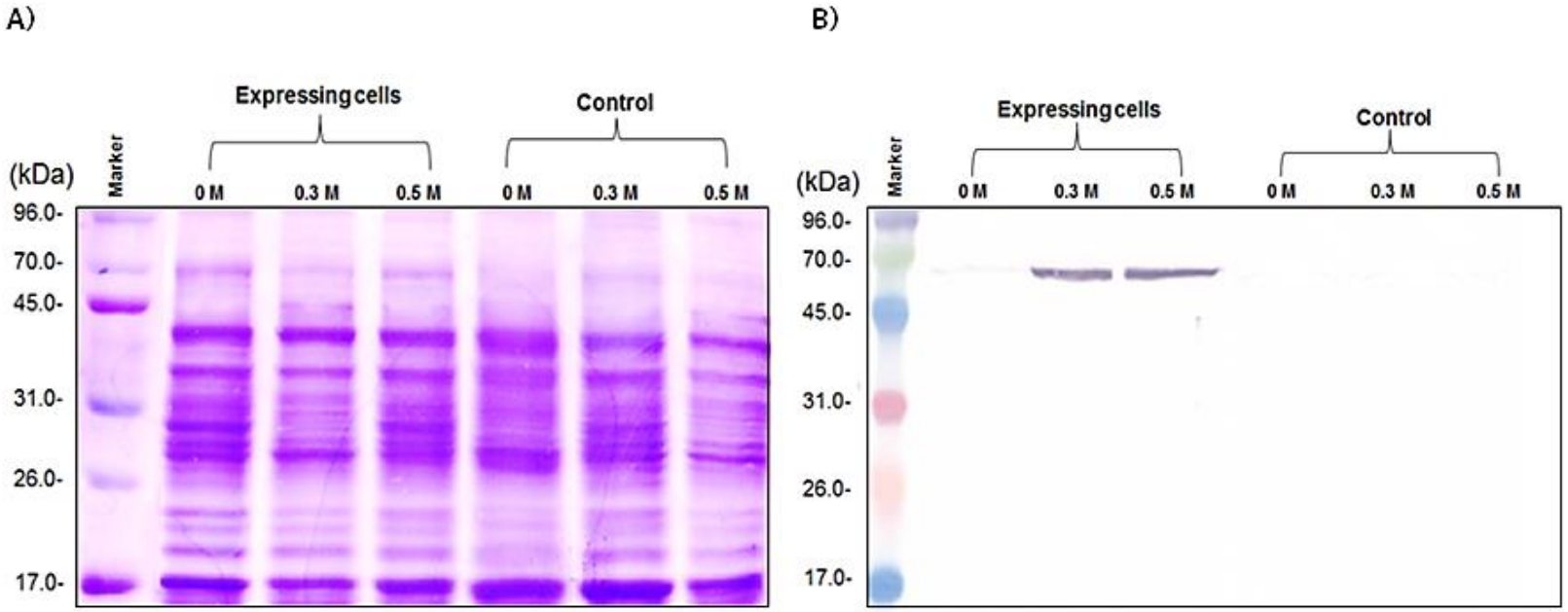


Figure 4.8: SDS-PAGE and Western Blotting analysis of Ap3858-3855 protein. A) SDS-PAGE of soluble fraction from control and expressing cells under the supplementation of 0 M, 0.3 M, 0.5 M. of NaCl respectively. B) Western Blotting analysis of soluble fraction from control and expressing cells under the supplementation of 0 M, 0.3 M, 0.5 M. of NaCl respectively. Equal amount of protein (40 µg) were applied on SDS-PAGE. Western Blotting was carried out as described in Materials and Methods. Antibody raised against 6 His was used. Numerals on the left indicated molecular mass.

4.4.2 *E. coli* cells expressing *Ap3858-3855* biosynthetic genes are capable of producing MAA

4.4.2.1 Salt stress induced accumulation of MAA in E. coli expressing cells

The inductions by salt and UV-B radiation stresses were performed to determine the accumulation level of MAA. For the induction by salt, NaCl concentration was increased to 0.3, 0.5, and 0.6 M, respectively. As shown in Figure 4.10, the induction by salt condition caused a dramatic increase in MAA level. After expressing cells were cultured for 16 hours the result showed that amount of MAA increased from 0 ± 0 μmol/g DW to 17.01 ± 1.96 μmol/g DW, 59.82 ± 2.41 μmol/g DW and 85.20 ± 2.69 μmol/g DW, respectively (Figure 4.10). Growth profiles of control and expressing cells were also performed and shown here (Figure 4.9).

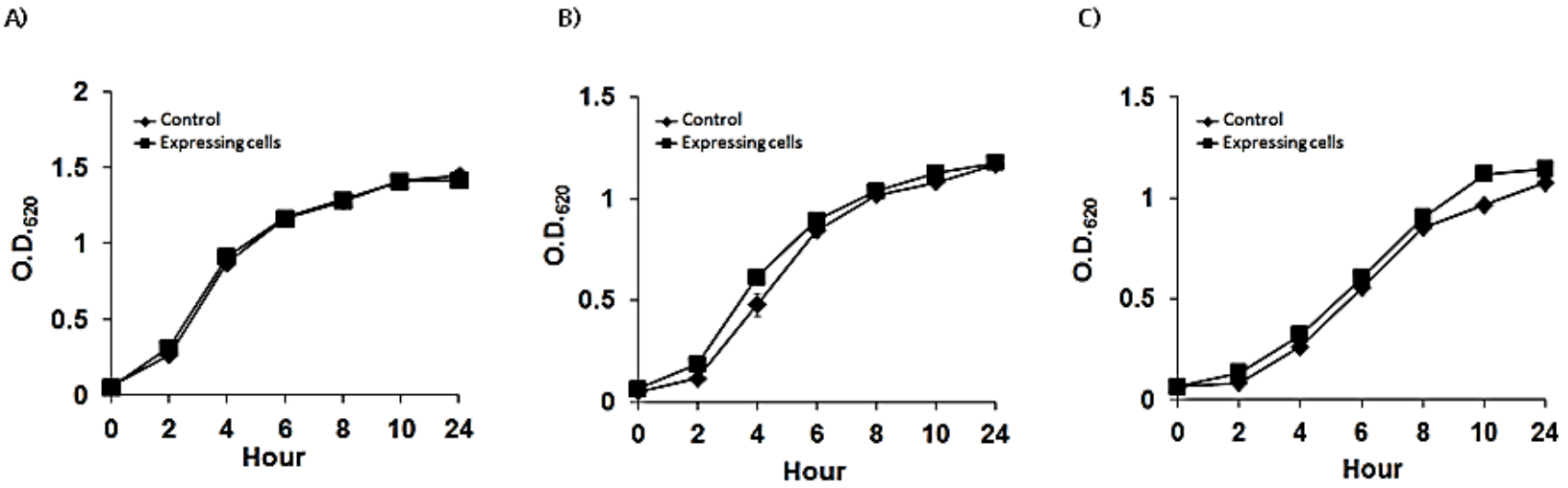


Figure 4.9: Growth profile of control and *E. coli* expressing cells (*Ap3858-3855*) in LB medium supplemented with 0.3 M and 0.5 M NaCl. A) Control and *E. coli* expressing cells in LB medium for 24 hours. B) Control and *E. coli* expressing cells in LB medium supplemented with 0.3 M of NaCl for 24 hours C) Control and *E. coli* expressing cells in LB medium supplemented with 0.5 M of NaCl for 24 hours. Data are means ± standard deviation of three independent experiments.

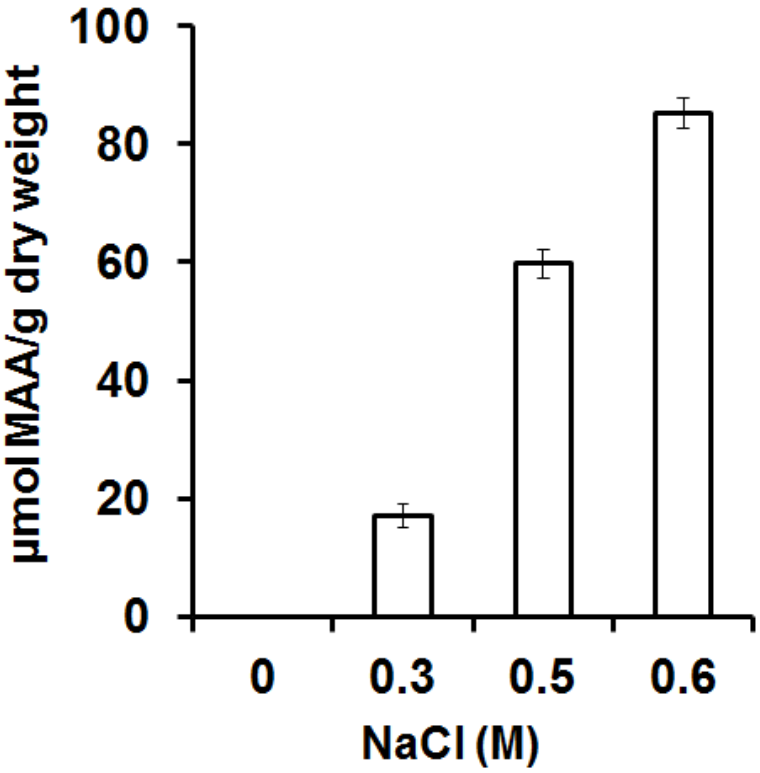


Figure 4.10: Amount of MAA in *E. coli* expressing cells after induction by NaCl at 0.3, 0.5, and 0.6 M, respectively for 24 hours. Data are means ± standard deviation of three independent experiments.

4.4.2.2 Effect of UV-B radiation for accumulation MAA in *E. coli* expressing cells

UV-B radiation experiment was performed to determine the accumulation level of MAA into *E. coli* expressing cells. In this study, after exposing to UV-B radiation for 3 days, amount of MAA increased from 0 ± 0 μmol/g DW to 0.57 ± 0.02 μmol/g DW (Figure 4.11).

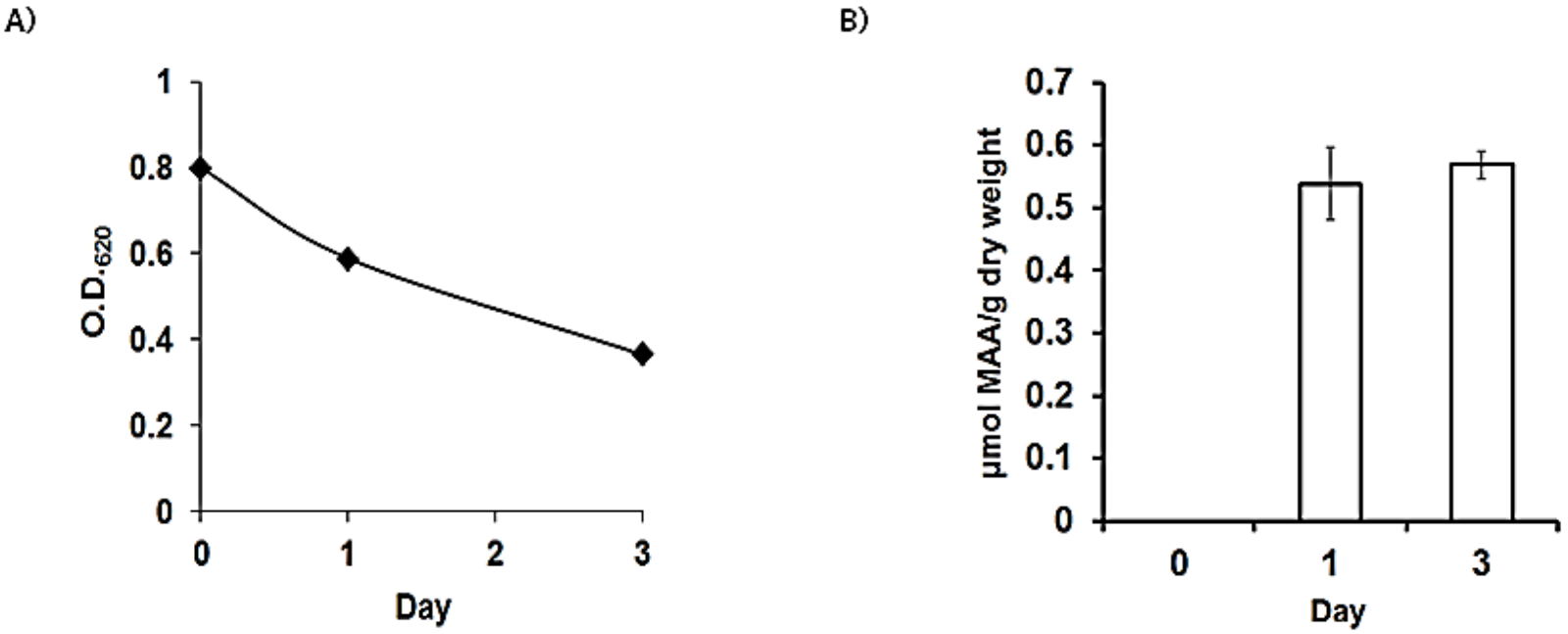


Figure 4.11: A) Growth profile of *E. coli* expressing cells after UV-B stress experiment (expose UV-B at 0.1 W/m^{-2} for 2 hours per day) for 3 days. B) Amount of MAA from *E. coli* expressing cells after UV-B stress experiment (expose UV-B at 0.1 W/m^{-2} for 2 hours per day) for 3 days. Data are means ± standard deviation of three independent experiments.

4.4.3 *Synechococcus sp* PCC 7942 cells expressing MAA biosynthetic genes are capable of producing MAA

4.4.3.1 Salt stress induced accumulation of MAA in *Synechococcus* sp PCC 7942 expressing cells

The induction by salt in *Synechococcus sp* PCC 7942 expressing cells, NaCl concentration was increased from 0 M to 0.375 M. As shown in Figure 4.12B. After expressing cells were cultured for 5 days, the result showed amount of MAA increased from 0 ± 0 μmol/g DW to 9.22 ± 0.81 μmol/g DW. Growth profiles of expressing cells were also performed (Figure 4.12A).

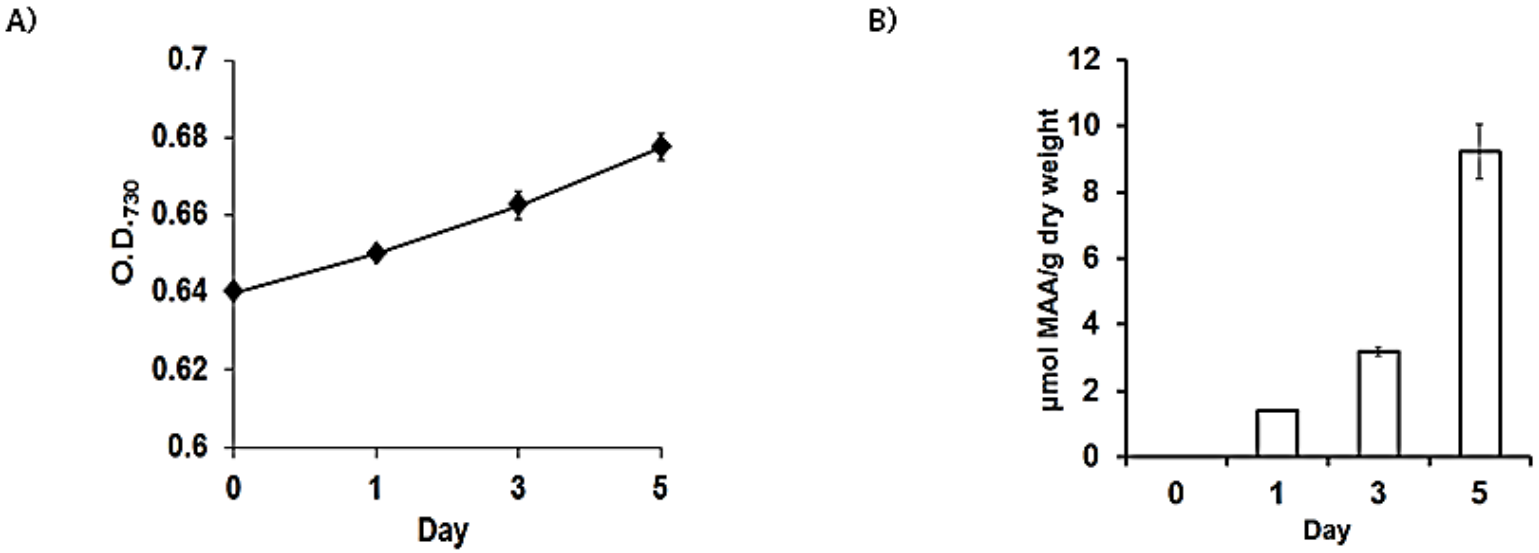


Figure 4.12: A) Growth profile of *Synechococcus sp* PCC 7942 expressing cells after induction by salt (0.375 M NaCl) for 5 days. B) Amount of MAA from *Synechococcus sp* PCC 7942 expressing cells after induction by salt experiment (0.375 M NaCl) for 5 days. Data are means ± standard deviation of three independent experiments.

4.4.3.2 Effect of UV-B radiation for accumulation MAA in *Synechococcus* sp PCC 7942 expressing cells

UV-B radiation experiment was performed to determine the accumulation level of MAA into *Synechococcus sp* PCC 7942 expressing cells. In this study, after exposing to UV-B radiation for 5 days, the amount of MAA increased from 0 ± 0 μmol/g DW to 2.95 ± 0.23 μmol/g DW (Figure 4.13).

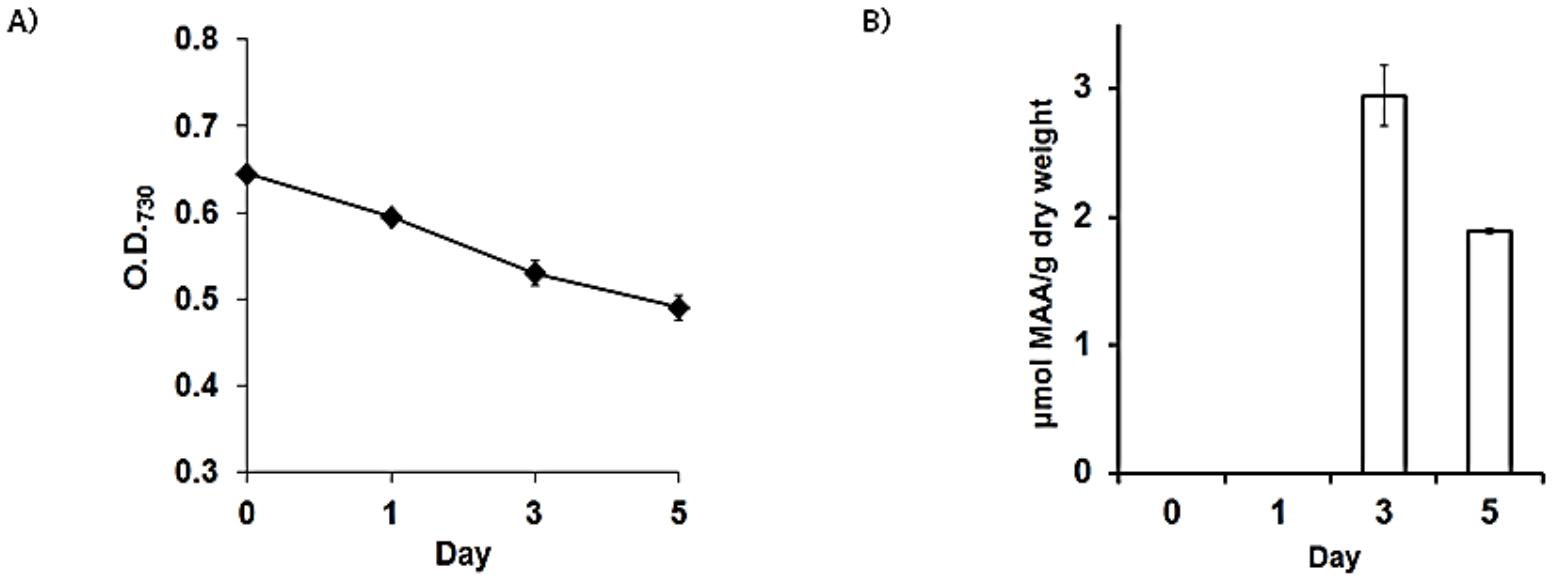


Figure 4.13: A) Growth profile of *Synechococcus sp* PCC 7942 expressing cells after UV-B stress experiment (expose UV-B at 0.1 W/m^{-2} for 3 hours per day) for 5 days. B) Amount of ApMAA from *Synechococcus sp* PCC 7942 expressing cells after UV-B stress experiment (expose UV-B at 0.1 W/m^{-2} for 3 hours per day) for 5 days. Data are means ± standard deviation of three independent experiments.

In this study, *A. halophytica* cells, *E. coli* expressing cells and *Synechococcus sp* PCC 7942 expressing cells were cultured under salt stress conditions as desired. When comparing the amount of MAA under induction by salt, the amount of MAA from *E. coli* expressing cell was higher than *A. halopthytica* and *Synechococcus sp* PCC 7942 expressing cells approximately 2.7 and 8 folds, respectively (Figure 4.13).

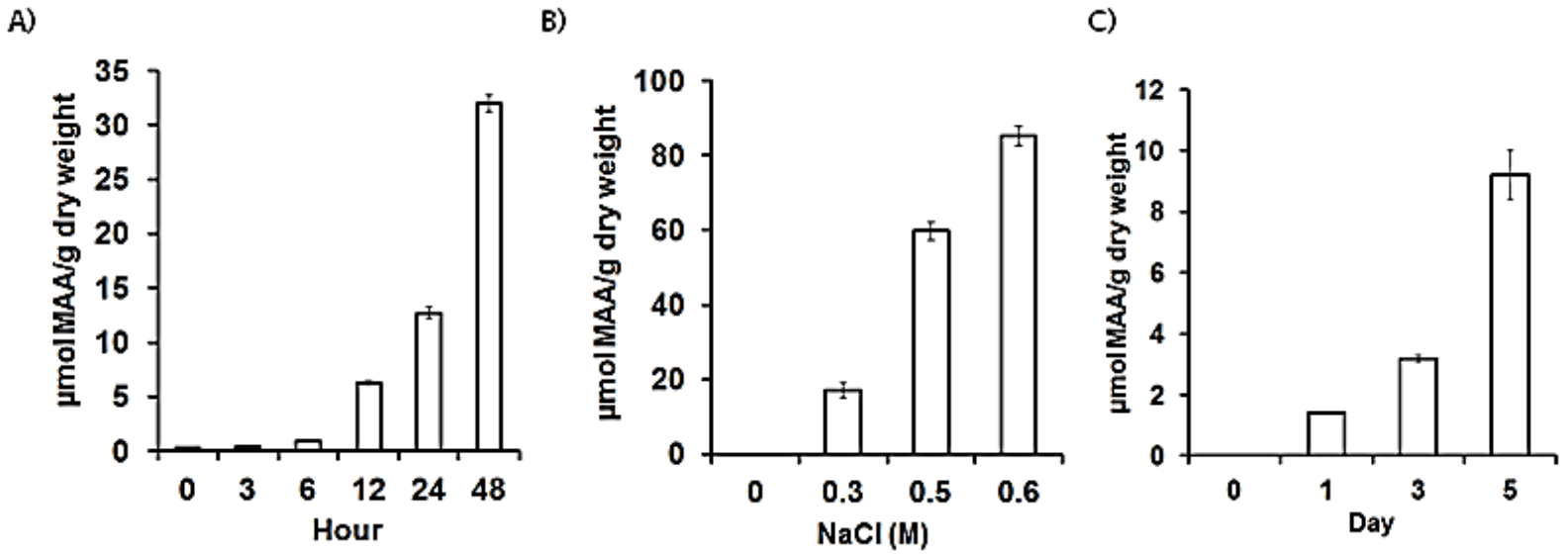


Figure 4.14: Amount of MAA after induction by salt condition as described in Materials and Methods in three species. A) Amount of MAA from *A. halopthytica* cells. B) Amount of MAA from *E. coli* expressing cells. C) Amount of MAA from *Synechococcus sp* PCC 7942 expressing cells. Data are means ± standard deviation of three independent experiments.

CHAPTER V
DISCUSSION

Detrimental effects of UV-B in living organisms have been well documented. Direct effects are harmful to membrane proteins, photosystem II, DNA, enzymes, growth regulation, survival, motility and other metabolic processes (Häder *et al*., 2007). Moreover, UV-B causes indirect effect through the generating of ROS. They have been reported on growth and survival of cyanobacteria when exposing to UV-B. Gao *et al* (2007) found that UV-B can inhibit growth of *Anabaena* sp. PCC7120 up to 40% under solar UVR. To avoid detrimental effects from UV exposure, cyanobacteria have developed several strategies for UV protection. Several strategies for UV protection are described previously in Chapter II, 2.2.

One of the most important UV-B protections in cyanobacteria is the production of secondary metabolite acting as UV-absorbing compounds. These molecules must have the properties to protect UV-B; for example, it can absorb in the UV range with a high absorption coefficient, and it should optimal for screening UV-B exposure. Among various UV-absorbing compounds, MAA is thought to be excellent UV-B absorber (Sinha and Häder, 2008). Moreover, MAAs have several alternative roles aparting from UV-absorbing compound. MAA can function as osmoprotectant under salinity stress (Oren, 1997; Kogej *et al*., 2006). Keda *et al* (2002) reported that the halophilic cyanobacterium accumulate mycosporine-2-glycine

under salinity stress. In halophilic fungus *Hortaea werneckii* also can accumulate mycosporine-glutaminol-glucoside under high salinities (Kogej *et al*., 2007). Antioxidant property has been reported in some MAAs. Mycosporine-glycine and phorphyra-334 have found to be an efficient antioxidant. They can inhibit the lipid peroxidation that induced by singlet oxygen and can scavenge ROS produced during UV exposure (Tao *et al*., 2009).

The halotolerant cyanobacterium *A. halophytica* inhabit in highly salty and alkaline environments and it was used in this study. The biosynthesis of MAA in *A. halophytica* was found to have different gene organization, when comparing with MAA biosynthetic genes of filamentous cyanobacteria (Waditee-Sirisattha *et al*., 2014) (Figure 5.1). The *DDG* gene (*Ap3858*) was separated from the other three genes of MAA biosynthetic genes of *A. halophytica* including, *OMT* gene (*Ap3857*), *CNligase* gene (*Ap3856*), and *AAligase* gene (*Ap3855*). To date, MAA biosynthetic pathway has been reported for *A. variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133 as described previously in chapter II, 2.3.3 (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011). MAA biosynthetic genes in the two cases are arranged consecutively. Therefore, the study in *A. halophytica* is the first case of MAA biosynthetic genes showing a far-separated first gene. Moreover, MAA biosynthetic pathway of *A. variabilis* ATCC 29413 or *N. punctiforme* ATCC 29133 produced shinorine as a main MAA but *A. halophytica* is different. It biosynthesized mycosporine-2-glycine as a main MAA. So, the difference of MAA accumulation in *A. variabilis* ATCC 29413, *N.*

punctiforme ATCC 29133 and *A. halophytica* are interesting and needs to be discussed in relation of MAA synthesis genes.

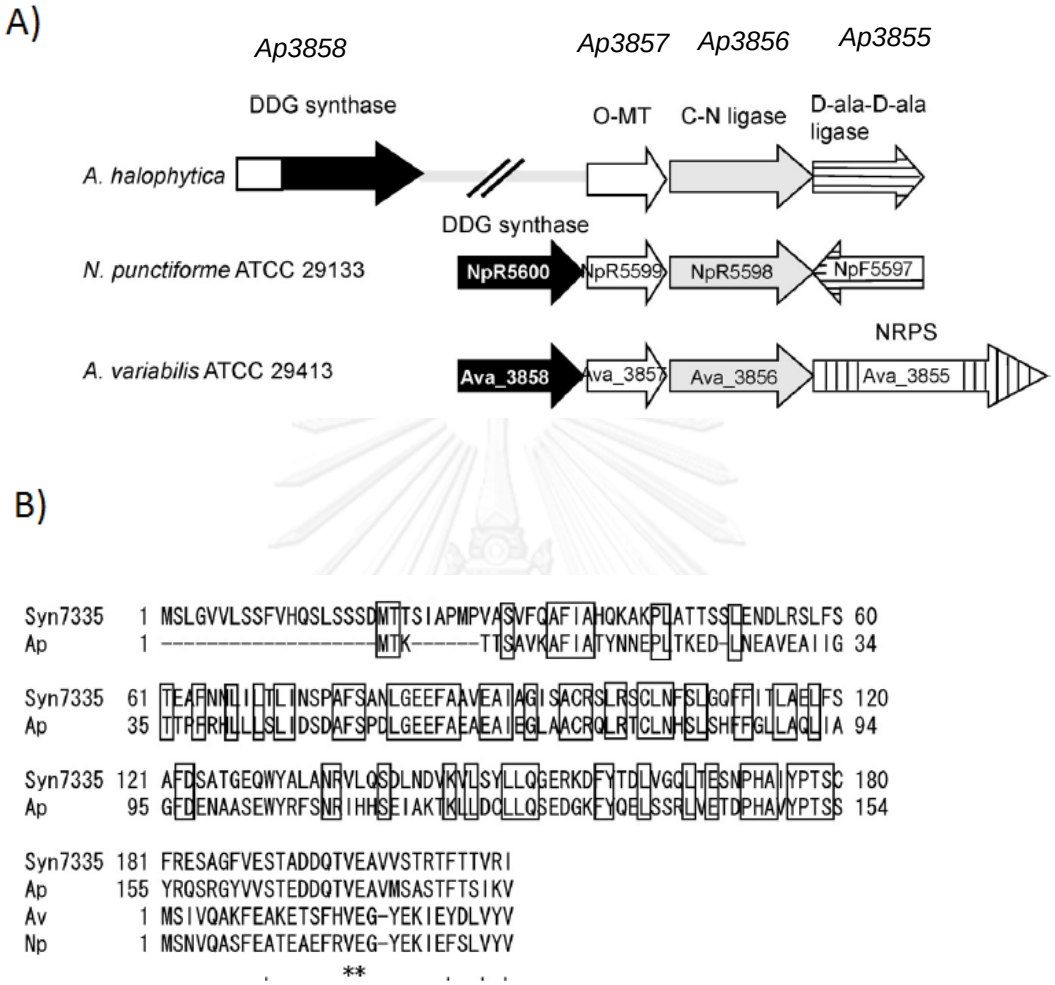


Figure 5.1: (A) Genomic region of MAAs from *A. halophytica*, *N. punctiforme* ATCC 29133, and *A. variabilis* ATCC 29413. (B) Alignment of N-terminal region of DDG synthase. The same amino acid residues in the upper region in *A. halophytica* (1 to 154) and *Synechococcus* sp. PCC 7335 (1 to 180) are boxed. The same amino acid residues among four DDGs after the first Met of *A. variabilis* ATCC 29413 DDG are shown by asterisks (From; Waditee-Sirisattha *et al*., 2014).

We have previously showed that *A. halophytica* cells have several adaptive mechanisms under stressful conditions. For example, *A. halophytica* can synthesize and accumulate a large amount of glycine betaine through three-strep glycine methylation (Waditee *et al*., 2012). *A. halophytica* contains Na^+/H^+ antiporter that has novel ion specificity (Waditee *et al*., 2001). In this study, we examined MAA accumulation in *A. halophytica* under stress condition. These are salt and UV-B stresses. We have identified that MAA accumulation in *A. halophytica* is mycosporine-2-glycine (Figure 4.4). Previously research, mycosporine-2-glycine was identified only in the halophilic cyanobacterium *Euhalothece*, the sea slug *Aplysia dactylomela*, and the green sea urchin *Strongylocentrotus droebachiensis* which are marine organisms (Kedar *et al*., 2002; Corredor *et al*., 2000; Adams and Shick, 2001).

Accumulation level of mycosporine-2-glycine in *A. halophytica* was induced more under high salinity than UV-B stress conditions (Figure 4.5 vs Figure 4.6). In this study, we have successfully expressed the *Aphanothece* MAA biosynthetic genes in two different host cells, *E. coli* and *Synechococcus sp* PCC 7942.

To summarize, we report the accumulation of mycosporine-2-glycine in *A. halophytica* and expressed recombinant MAA in *E. coli* as well as *Synechococcus* cells to produced mycosporine-2-glycine. The large amount of produced mycosporine-2-glycine found in recombinant MAA from *E. coli* (85.20 ± 2.69 μmol/g DW) was found to be induced under salt stress condition.

CHAPTER VI
CONCLUSION

I) UV-absorbing compound can detect in *A. halophytica*. It was identified by HPLC, amino acid analysis and TOF-MS as Mycosporine-2-glycine.

II) Mycosporine-2-glycine is induced by salt stress rather than UV-B exposure.

III) Expression of heterologous genes for MAA in *E. coli* leads to the production of MAA with maximum yield 85.20 ± 2.69 μmol/g DW under salt stress condition.

REFERENCES

Adams, N.L., Shick, J.M. 2001. Mycosporine-like amino acids prevent UVB induced abnormalities during early development of the green sea urchin *Strongylocentrotus droebachiensis*. Biol. 138: 267–280.

Balskus, E.P., and Walsh C.T. 2010. The genetic and molecular basis for sunscreen biosynthesis in cyanobacteria. Science. 329(5999): 1653-1656.

Bandaranayake, W.M. 1998. Mycosporines: are they nature's sunscreens? Nat Prod Rep. 15(2): 159-172.

Bebout, B.M., and Garcia-Pichel, F. 1995. UV B-Induced Vertical Migrations of Cyanobacteria in a Microbial Mat. Appl Environ Microbiol. 61(12): 4215-22.

Bhatia, S., Garg, A., Sharma, K., Kumar, S., Sharma, A., Purohit, A.P. 2011. Mycosporine andmycosporine-like amino acids: A paramount tool against ultra violet irradiation. Pharmacogn Rev. 5(10): 138-46.

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

Carreto, J.I., Carignan, M.O. 2011. Mycosporine-like amino acids: relevant secondarymetabolites. Chemical and ecological aspects. 9(3): 387-446.

Carreto, J.I., Lutz, V.A., Dc Marco, S.G., Carignan, M.O. 1990. Fluenceand wavelength dependence of mycosporine-like amino acid synthesis in the dinoflagellate *Alexandrium excavatum*. In: Graneli E, Edler L, Sundstro¨m B, Anderson DM (eds) Toxic marine phytoplankton. Elsevier. 275–279.

Crutzen, P.J. 1992. Ultraviolet on the increase. Nature. 356: 104-105.

Corredor, J.E., Bruckner, A.W., Muszynski, F.Z., Armstrong, R.A., García, R., Morell, J.M. 2000. UV-absorbing compounds in three species of Caribbean *zooxanthellate corals*: depth distribution and spectral response. Sci. 67:821–830.

Davidson, A.T. 1998. The impact of UVB radiation on marine plankton. Mutat Res. 422: 119-29.

Dunlap, W.C., Yamamoto, Y. 1995. Small-moleculeantioxidants in marine organisms- antioxidant activityof mycosporine-glycine. Comp. Biochem. Phys. BBiochem. Mol. Biol. 112: 105–114.

F'guyer, S., Afaq, F., Mukhtar, H. 2003. Photochemoprevention of skin cancer by botanical agents. Photodermatol Photoimmunol Photomed. 19(2): 56-72.

Gao, Q., Garcia-Pichel, F. 2011. An ATP-grasp ligase involved in the last biosynthetic step of the iminomycosporineshinorine in *Nostocpunctiforme* ATCC 29133. J Bacteriol. 193: 5923-5928.

Gao, Q., Garcia-Pichel, F. 2011. Microbial ultraviolet sunscreens. Nat Rev Microbiol. 9(11): 791-802.

Gao, K., Yu, H., Brown M.T. 2007. Solar PAR and UV radiation affect the physiology and morphology of the cyanobacterium *Anabaena* sp. PCC 7120. J. Photochem. Photobiol. B Biol. 89: 117-124.

Häder, D.P., Kumar, H.D., Smith, R.C., Worrest, R.C. 2007. Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. Photochem. Photobiol. Sci. 6: 267-285.

He, Y.Y., Häder, D. 2002. Reactive oxygen species and UV-B: effect on cyanobacteria. PhotochemPhotobiol Sci. 1(10): 729-36.

Hirata, Y., Uemura, D., Ueda, K., Takano, S. 1979. Several compounds from Palythoatuberculosa (Coelenterata). Pure Appl. Chem. 51: 1875–1883.

Jeffrey, W.H., Peter, A., Maille Lyons, M., Richard, B., Ralph, J., David, L. 1996. Ambient solar radiation induced photodamage in marine bacterioplankton. Photochem. Photobiol. 64: 419–427.

Kedar, L., Kashman, Y., Oren, A. 2002. Mycosporine-2-glycine is the major mycosporine-like amino acid in a unicellular cyanobacterium (*Euhalothece* sp.) isolated from a gypsum crust in a hypersaline saltern pond. FEMS Microbiol Lett. 208(2): 233-237.

Klisch, M., Häder, D.P. 2002. Wavelength dependence of mycosporine-like amino acidsynthesis in *Gyrodinium dorsum*. J PhotochemPhotobiol B. 66(1): 60-66.

Kogej, T., Gostincar, C., Volkmann, M., Gorbushina, A.A., Gunde-Cimerman, N. 2006. Mycosporines in extremophilic fungi: Novelcomplementary osmolytes? Environ Chem. 3: 105-10.

Kogej, T., Stein, M., Volkmann, M., Gorbushina, A.A., Galinski, E.A., Gunde-Cimerman, N. 2007. Osmotic adaptation of the halophilic fungus *Hortaea werneckii*: role of osmolytes and melanization. Microbiology. 153(12): 4261-4273.

Kuhlemeier, C.J., Van Arkel, G.A. 1987. Host-vector systems for gene cloning in cyanobacteria. Methods in Enzymology. 153: 199-215.

Laemmli, U.K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 227: 680–685.

Lubin, D., Jensen, E.H. 1995. Effects of clouds and stratospheric ozone depletion on ultraviolet radiation trends. Nature. 377: 710-713.

Matsui, K., Nazifi, E., Kunita, S., Wada, N., Matsugo, S., Sakamoto, T. 2011. Novelglycosylated mycosporine-like amino acids with radical scavenging activity fromthe cyanobacterium *Nostoc commune*. J PhotochemPhotobiol B. 105(1): 81-89.

Oren, A. 1997. Mycosporine-like amino acids as osmotic solutes in a community of halophilic cyanobacteria. Geomicrobiol. J. 14: 231-240.

Rastogi, R.P., Richa-Sinha, R.P., Singh S.P., Häder, D.P. 2010. Photoprotective compoundsfrom marine organisms. J Ind Microbiol Biotechnol. 37(6): 537-58.

Rosen, C.F. 2003. Topical and systemic photoprotection. Dermatol Ther. 16(1): 8-15.

Rosic, N.N. 2012. Phylogenetic analysis of genes involved in mycosporine-like amino acid biosynthesis in symbiotic dinoflagellates. Appl Microbiol Biotechnol. 94(1): 29-37.

Shick, J.M., Dunlap, W.C. 2002. Mycosporine-like amino acids and related Gadusols: biosynthesis, acumulation, and UV-protective functions in aquatic organisms. AnnuRev Physiol. 64: 223-262.

Shick, J.M., Dunlap, W.C., Pearse, J.S., Pearse, V.B. 2002. Mycosporine-like amino acid content in four species of sea anemones in the genus

Anthopleura reflects phylogenetic but not environmental or symbiotic relationships. Biol Bull. 203(3): 315-330.

Singh, S.P., Häder, D.P., Sinha, R.P. 2010. Cyanobacteria and ultraviolet radiation (UVR) stress: mitigation strategies. Ageing Res Rev. 9(2): 79-90.

Singh, S.P., Klisch, M., Sinha, R.P., Häder, D.P. 2008. Effects of abiotic stressors onsynthesis of the mycosporine-like amino acid shinorine in the Cyanobacterium *Anabaena variabilis* PCC 7937. PhotochemPhotobiol. 84(6): 1500-1505.

Singh, S.P., Klisch, M., Sinha, R.P., Häder, D.P. 2010. Genome mining of mycosporine-like amino acid (MAA) synthesizing and non-synthesizing cyanobacteria: A bioinformatics study. Genomics. 95(2): 120-128.

Singh, S.P., Kumari, S., Rastogi, R.P., Singh, K.L., Sinha, R.P. 2008. Mycosporine-like aminoacids (MAAs): chemical structure, biosynthesis and significance as UV-absorbing/screening compounds. Indian J Exp Biol. 46(1): 7-17.

Sinha, R.P., Hader, D.P. 2008. UV-protectants in cyanobacteria. Plant Sci. 174: 2782-89.

Sinha, R.P., Klisch, M., Gröniger, A., Häder, D.P. 2000. Mycosporine-like amino acids in the marine red alga *Gracilaria cornea*-effects of UV and heat. Environ. Exp. Bot. 43: 33–43.

Sinha, R.P., Ambasht, N.K., Sinha, J.P., Klisch, M., Ha¨der, D.P. 2003. UVB-induced synthesis of mycosporine-like amino acids in three strains of *Nodularia* (cyanobacteria). J PhotochemPhotobiol B Biol. 71: 51–58.

Stochaj, W.R., Dunlap, W.C., Shick, J.M. 1994. Two new UV-absorbing mycosporine-like aminoacids from the sea anemone *Anthopleura elegantissima* and the effects of *zooxanthellae* and spectral irradiance on chemical composition and content. Biol. 118: 149-156.

Tao, C., Sugawara, T., Maeda, S., Wang, X., Hirata, T. 2009. Antioxidative activities of a mycosporine-like amino acid, porphyra-334. Fish. Sc. 74: 1166-1172.

Uemura, D., Katayama, C., Wada, A., Hirata, Y. 1980. Crystal and molecular structure of palythene possessing a novel 360 nm chromophore. Chem. 9: 755–756.

Volkmann, M., Gorbushina, A.A. 2006. A broadly applicable method for extraction and characterization of mycosporines and mycosporine-like amino acids of terrestrial, marine and freshwater origin. FEMS Microbiol Lett. 255(2): 286-295.

Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., Takabe, T. 2001. Halotolerant cyanobacterium *Aphanothece halophytica* contains an $Na^{(+)}/H^{(+)}$ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. J Biol Chem. 6(40): 36931-36938.

Waditee-Sirisattha, R., Kageyama, H., Sopun, W., Tanaka, Y., Takabe, T. 2014. Identification and up-regulation of biosynthetic genes required for accumulation of mycosporine-2-glycine under salt stress in halotolerant cyanobacterium *Aphanothece halophytica*. Appl Environ Microbiol. 80(5): 1763–1769.

Waditee-Sirisattha, R., Sittipol, D., Tanaka, Y., Takabe, T. 2012. Overexpression of serine hydroxymethyltransferase from halotolerant cyanobacterium in *Escherichia coli* results in increased accumulation of choline precursors and enhanced salinity tolerance. FEMS Microbiol Lett. 333(1): 46-53.

APPENDIX

APPENDIX 1

Modified BG11 medium

Trace element solution

H_3BO_3	2.8	g
$MnCl_2.4H_2O$	1.81	g
$ZnSO_4.7\ H_2O$	0.22	g
$CuSO_4.5\ H_2O$	0.079	g
$Co(NO_3)_2.6H_2O$	0.049	g

Dissolved all compositions with distilled water to 1 liter.

BG11 solution

$NaNO_3$	1.5	g
K_2PO_4	40	mg
$MgSO_4.7H_2O$	75	mg
$CaCl_2.H_2O$	36	mg
Na_2CO_3	20	mg
EDTA*2Na	1	mg
Citric acid	6	mg
Ferric ammonium nitrate	6	mg
Trace element	1	ml

Dissolved all compositions with distilled water to 1 liter.

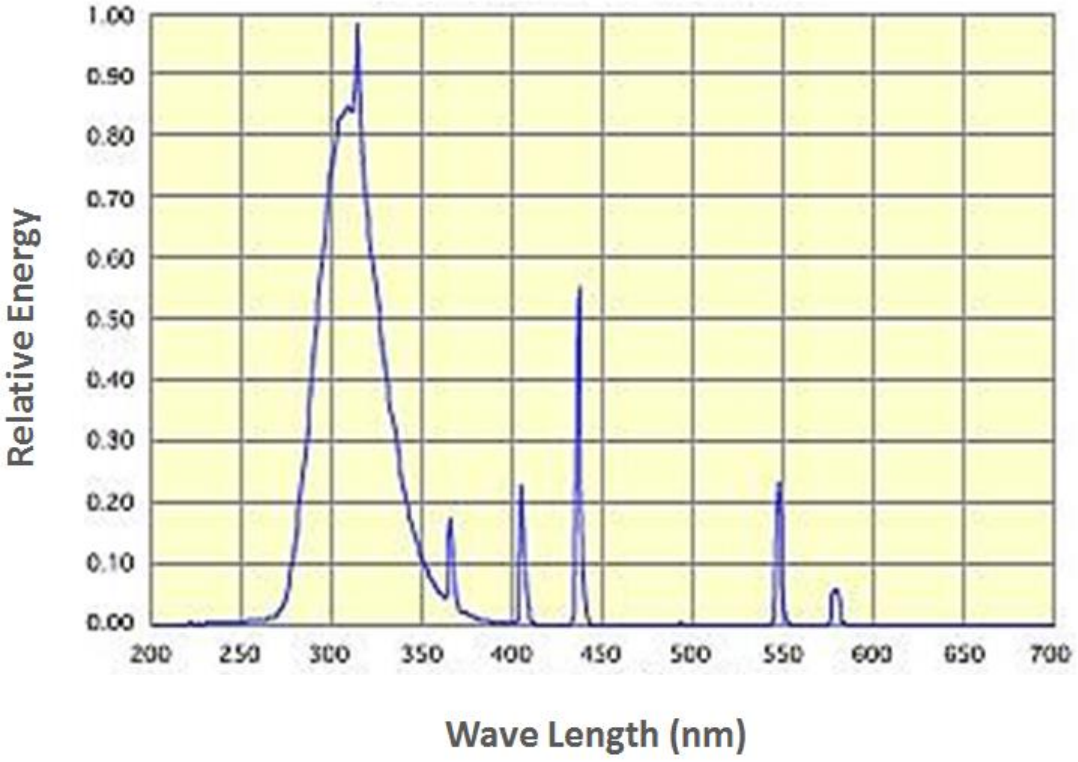
BG11 Tark solution

NaCl	28.17	g
KCl	0.67	g
$MgSO_4.7H_2O$	6.92	g
$MgCl_2.6H_2O$	5.50	g
$CaCl_2.2H_2O$	1.47	g

Modified BG11 medium was prepared by dissolved all compositions with distilled water to 1 liter adjust at pH 8.2.

APPENDIX 2

Spectral energy distribution of UV-B lamp



UV-B lamps (Sankyo Denki; GL20SE): UV-B lamps emit ultraviolet rays between 280nm and 360nm (at peak 306nm). The spectral energy distribution suits to test the ultraviolet resistance of paints, plastics and rubbers. They are also used for the special light sources for inspection and analysis.

APPENDIX 3

Amino acid analysis

Solution for analysis (Lithium column)

4 N LiOH

$LiOH.H_2O$	42.0	g

Dissolved with distilled water to 250 ml and store in plastic bottom.

MA solution

Trilithium citrate tetrahydrate	14.1	g
Ethyleneglycol monoethylether	70	ml
60% $HClO_4$	13	ml
H_2O	800	ml

Adjust pH 2.6 using 60% $HClO_4$ then fill up water to 1000 ml and then filtration MA solution using whatman No.1.

MB solution

Trilitium citrate tetrahydrate	28.2	g
Borate	12.4	g
4 N LiOH	30	ml
H_2O	800	ml

Adjust pH 10.0 using 4 N LiOH then fill up water to 1000 ml and filtration MB solution using whatman No.1.

MC solution

$LiOH.H_2O$	4.2	g
H_2O	450	ml

Fill up water to 500 ml and filtration MC solution using whatman No.1.

Remark: MA, MB and MC solution store at 4 °C at least 3 weeks.

Buffer for preprartion RA and RB solution

Na_2CO_3	40.7	g
K_2SO_4	18.8	g
H_3BO_3	13.6	g
H_2O	830	ml

Dissolve complete step by step and fill up water to 1000 ml.

Brij -35 10%

Brij-35	10	g
H_2O	50	ml

Fill up water to 100 ml.

RA solution

Mixed buffer with 400 µl NaClO and then filtration RA solution using whatman paper No.1.

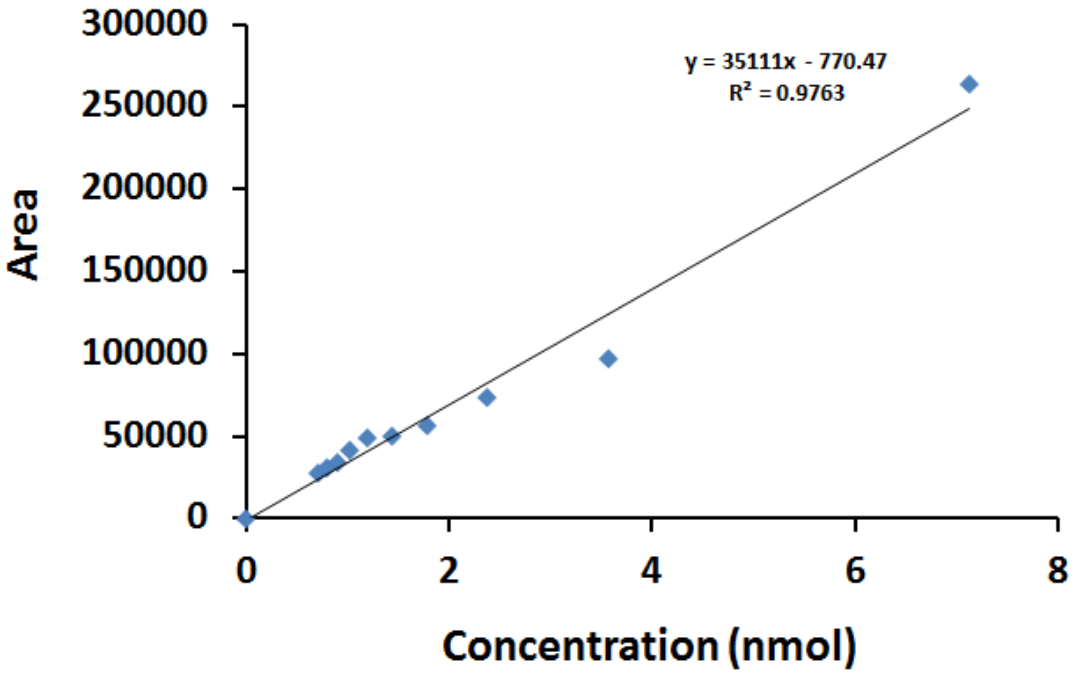
RB solution

O-pathaldehyde	400	mg
Ethanol	7	ml
2-mercaptoethanol	1	ml
Brij-35 10%	2	ml
Buffer	490	ml

Rinse 10-25 ml ethanol in flask before use. Put O-pathaldehyde 400 mg resolved with ethanol then Pore above solution into 450 of buffer. Add 2-mercaptoethanol 1 ml and Brij-35 10% 2 ml and fill up buffer to 500 ml. Filtration RB solution using whatman No.1.

APPENDIX 4

Standard ApMAA



APPENDIX 5

Transformation, Chang-Miller Method

1. Preparation of competent cells ($CaCl_2$ method)

A single colony of *E. coli* DH5α was incubated to 2 ml of LB medium and incubation at 37 °C overnight with vigorous shaking. This culture was re-inoculated to fresh LB medium and incubated at 37 °C with vigorous shaking for 3-4 hours until the OD_{620} reach 0.4-0.6. The culture was stand on ice for 10 min and centrifuged at 4,000 rpm for 10 min at 4 °C. Cell pellet was resuspend in 0.05 of 15% of glycerol in $CaCl_2$ and stand on ice for 10 min. This cell suspension was dispensed in 100 µl aliquots into 1.5 ml microcentrifuge tubes and stored at -80 °C.

2. Transformation

One hundred microlitres of competent cells was thawed on ice prior to the addition of 1-3 µl of plasmid DNA or ligation mixture. The transformation mixture was flicked 2-3 times and stand on ice for 10 min. Subsequently, the mixture was heated to 42 °C for 90 second futher on ice for 5 min. The mixture was diluted with 900 of LB medium and gently shaking at 37 °C for hour. Cell suspension was spread on selection medium as desired.

APPENDIX 6

LB medium

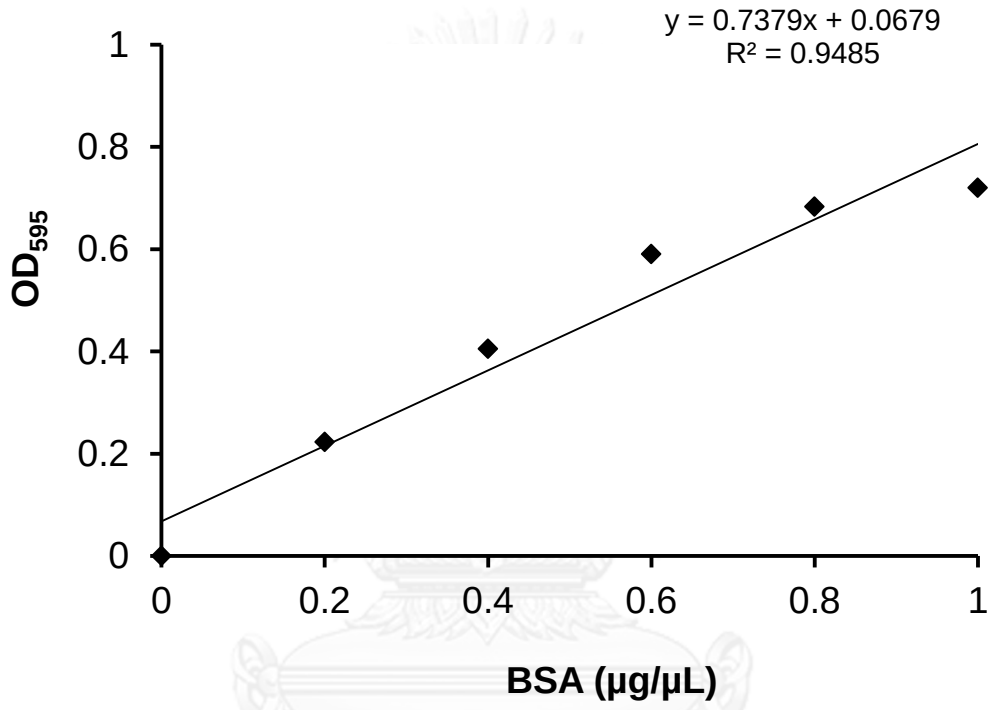
Composition per 1 liter

10 g	Bacto tryptone
5 g	Yeast extract
10 g	NaCl

Dissolve all compositions with 800 ml deionizaed water, adjust the pH to 7.0 with 6 M NaOH. Adjust volume of solution to 1 liter with deionizaed water. Autoclave at 121 °C, 15 lb/in^2 for 15 min. For media containing agar add bactoagar 15 g per liter.

APPENDIX 7

Standard protein



APPENDIX 8

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

30% Acrylamide, 0.8% bis acrylamide, 100 ml

Acrylamide	29.2	g
N, N´ methylene bis acrylamide	0.8	g

Adjust volume to 100 ml with distill water.

1.5 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	18.17	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water.

2 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	24.2	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water.

0.5 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	6.06	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water.

1 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	12.1	g

Adjust pH to 8.8 and adjust volume to 100 ml with distill water.

Solution B (SDS-PAGE)

2 M Tris Cl pH 8.8	75	ml

10% SDS	4	ml
Distill water	21	ml

Solution C (SDS-PAGE)

2 M Tris Cl pH 6.8	50	ml
10% SDS	4	ml
Distill water	46	ml

2. SDS-PAGE

10% separating gel

30% acrylamide solution	3.33	ml
Solution B	2.5	ml
Distill water	5.0	ml
10% Ammonium sulfate	50	µl
TEMED	10	µl

5% stacking gel

30% acrylamide solution	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium sulfate	30	µl
TEMED	5.0	µl

Sample buffer

1M Tris Cl pH 6.8	0.6	ml
50% glycerol	5.0	ml
10% SDS	2.0	ml
2 mercaptoethanol	0.5	ml
1% bromphenol blue	1.0	ml
Distilled water	0.9	ml

4x of sample buffer is mixture sample to 1x. The mixture heated 5 min in boiling water before loading to the gel.

Electrophoresis buffer for 1 litre

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

Adjust volume to 1 litre with distilled water (pH 8.3).

APPENDIX 9

Buffer for western blotting

PBS buffer (Phosphate-buffer-saline)

Final concentration per 1 lite

10 mM sodium phosphate pH 7.4

150 mM NaCl

Blocking buffer

5% (w/v) skim milk and 0.01% Tween20 in 1x PBS buffer

Blotting transfer buffer

Final concentration per 1 liter

39 mM glycine

48 mM Tris base

0.037 % SDS

20 % methanol

Detection reagent for western blotting

Detection reagent for western blotting

150 mM Barbital pH 9.6	18	ml
0.1% NTB (Nitro Blue Tetrazolium)	2	ml
1M MgCl2	80	µl
0.5% BCIP (5-bromo-4-chloro-3-indolyl phosphate)	200	µl

Detection reagent for western bloting should be freshly prepared and used within 30 min. When the bands are desired intensity, wash the nitrocellulose membrane with deionized water 2-3 times.

VITA

Miss Warangkana Sopun was born on April 27, 1988 in Udontani, Thailand. She graduated from Department of Biology, Faculty of Science, Mahidol University in 2011 with a Bachelor degree of Science (Biology). Recently, she has pursued her Master degree of Program in Biotechnology, Faculty of Science, Chulalongkorn University and then this work has been published in Applied and Environmental Microbiology.