METABOLIC ENGINEERING OF *Synechococcus elongatus* PCC 7942 FOR MYCOSPORINE-2-GLYCINE PRODUCTION UNDER SALT STRESS CONDITION

Miss Panwad Pingkhanont

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Microbiology and Microbial Technology

Department of Microbiology

Faculty of Science

Chulalongkorn University

Academic Year 2018

วิศวกรรมเมแทบอลิกของ *Synechococcus elongatus* PCC 7942 สำหรับการผลิตไมโคสปอรีน-2-ไกลซีน ภายใต้ภาวะเครียดจากเกลือ

น.ส.ปานวาด พิงคานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title   METABOLIC ENGINEERING OF *Synechococcus elongatus*
PCC 7942 FOR MYCOSPORINE-2-GLYCINE PRODUCTION
UNDER SALT STRESS CONDITION

By   Miss Panwad Pingkhanont

Field of Study   Microbiology and Microbial Technology

Thesis Advisor   Associate Professor RUNGAROON WADITEE RISATTHA, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.... Dean of the Faculty of Science

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

.... Chairman

(Associate Professor TANAPAT PALAGA, Ph.D.)

.... Advisor

(Associate Professor RUNGAROON WADITEE RISATTHA, Ph.D.)

.... Examiner

(Assistant Professor PANAN RERNGSAMRAN, Ph.D.)

.... External Examiner

(Pongsathon Prapakrangkul, Ph.D.)

ปานวาด พิงคานนท์ : วิศวกรรมเมแทบอลิกของ *Synechococcus elongatus* PCC 7942 สำหรับการผลิต
ไมโคสปอรีน-2-ไกลซีน ภายใต้ภาวะเครียดจากเกลือ. (METABOLIC ENGINEERING OF *Synechococcus
elongatus* PCC 7942 FOR MYCOSPORINE-2-GLYCINE PRODUCTION UNDER SALT STRESS
CONDITION) อ.ที่ปรึกษาหลัก : รศ. ดร.รุ่งอรุณ วาดิถี สิริศรัทธา

ไมโครสปอรีน-ไลก์ อะมิโน แอซิด (MAAs) เป็นกลุ่มของสารเมแทบอไลต์ทุติยภูมิ สังเคราะห์โดยจุลินทรีย์และ
สาหร่ายหลายชนิด สารกลุ่มนี้มีหน้าที่สำคัญในการคัดกรองรังสียูวี และยังมีหน้าที่อื่นๆ ได้แก่ การต้านอนุมูลอิสระ ปกป้อง
เซลล์จากภาวะเครียด และควบคุมการออสโมซิส โดยไมโครสปอรีน-2-ไกลซีน (M2G) เป็นหนึ่งในสารกลุ่ม MAAs ที่มี
ความสามารถในการต้านอนุมูลอิสระมากกว่าสารอื่นๆ ในกลุ่มเดียวกัน ดังนั้นจึงเป็นที่สนใจที่จะเพิ่มการผลิต M2G เพื่อ
นำไปประยุกต์ใช้ในอุตสาหกรรมความงาม และเวชสำอาง ในการศึกษานี้ประสบความสำเร็จในการแสดงออกของยีนในชีว
สังเคราะห์ M2G (*Ap3858-3855*) จาก *Aphanothece halophytica* ในไซยาโนแบคทีเรียน้ำจืด *Synechococcus
elongatus* PCC 7942 จากการวิเคราะห์การถอดรหัสของยีน *Ap3858-3855* พบว่า ยีนทั้ง 4 ยีนนี้เป็น monocistronic
mRNA heterologous expression ของยีน *Ap3858-3855* ใน *S. elongatus* พบว่าทุกยีนมีการแสดงออกมากขึ้น
ภาวะเครียดจากเกลือ NaCl ยีน *Ap3857* แสดงออกเพิ่มมากที่สุด 2.39 ± 0.25 เท่า และภายใต้ภาวะเครียดจากเกลือ
KCl ยีน *Ap3856* แสดงออกเพิ่มมากที่สุด 3.70 ± 0.35 เท่า ระดับการแสดงออกของยีนที่เกี่ยวข้องกับภาวะเครียดออกซิ
เดชั่น (*sodB, cat* และ *tpxA*) ในเซลล์แสดงออก พบว่าภายใต้ภาวะเครียดจากเกลือ NaCl มีเพียงยีน *tpxA* แสดงออก
มากขึ้น 4.58 ± 0.58 เท่า ขณะที่ภาวะเครียดจากเกลือ KCl กระตุ้นการแสดงออกของ *sodB* และ *tpxA* 4.09 ± 0.86
และ 11.81 ± 1.02 เท่า ตามลำดับ ส่วนการแสดงออกของยีนที่เกี่ยวข้องกับสารออสโมไลต์ ได้แก่ *Synpcc7942_0808*
และ *Synpcc7942_2522* พบว่าในเซลล์แสดงออกที่เจริญภายใต้ภาวะเครียดจากเกลือ NaCl มีเพียงยีน
Synpcc7942_0808 มีการแสดงออกมากขึ้น 3.89 ± 0.30 เท่า และภายใต้ภาวะเครียดจากเกลือ KCl พบว่า
Synpcc7942_0808 และ *Synpcc7942_2522* แสดงออกมากขึ้น 9.40 ± 0.88 และ 3.52 ± 0.29 เท่า ตามลำดับ จาก
ตรวจสอบการแสดงออกของโปรตีน โดยวิธีเวสเทิร์น บลอท พบแบนจำเพาะของโปรตีนเป้าหมาย คือ DDG-synthase
(ถอดรหัสจากยีน *Ap3858*) โดยในเซลล์แสดงออกที่เจริญภายใต้ภาวะเครียดจากเกลือ NaCl มีการแปลรหัสได้โปรตีนมาก
ขึ้นถึง 7.20 เท่า ส่วนการวิเคราะห์สารเมแทบอไลต์ พบว่ามีการสะสมของ M2G เพิ่มขึ้นภายใต้ภาวะเครียดจากเกลือทั้ง
สองชนิด โดยมีการสะสมของ M2G เพิ่มมากที่สุดภายใต้ภาวะเครียดจากเกลือ NaCl เมื่อเติมเซอรีน 7.35 ng/mgFW

สาขาวิชา	จุลชีววิทยาและเทคโนโลยีจุลินทรีย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

5972111023 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY

KEYWORD: mycosporine-like amino acids, cyanobacteria, mycosporine-2-glycine, UV-screening compound

Panwad Pingkhanont : METABOLIC ENGINEERING OF *Synechococcus elongatus* PCC 7942 FOR MYCOSPORINE-2-GLYCINE PRODUCTION UNDER SALT STRESS CONDITION. Advisor: Assoc. Prof. RUNGAROON WADITEE RISATTHA, Ph.D.

Mycosporine-like amino acids (MAAs) are a group of secondary metabolite which are synthesized by various microorganisms and algae. These compounds have a major role as UV screening compound. The additional roles of MAAs, such as, antioxidant, protection against stresses, and osmotic regulation are also reported. Mycosporine-2-glycine (M2G) is one of MAAs which has higher antioxidant property than other MAAs. Thus, it is interesting to increase the M2G production for cosmetic and pharmaceutical applications. In this study, M2G biosynthetic gene cluster (*Ap3858-3855*) from *Aphanothece halophytica* was successfully expressed in a fresh water cyanobacterium *Synechococcus elongatus* PCC 7942. Transcriptional analysis of *Ap3858-3855* revealed that these four genes are independently transcribed, which is called monocistronic mRNA. Heterologous expression of *Ap3858-3855* in *S. elongatus* showed that all four genes were highly up-regulated. Under NaCl stress, the highest up-regulation was observed in *Ap3857* for approximately 2.39 ± 0.25 folds. Under KCl stress, the highest up-regulation was observed in *Ap3856* for approximately 3.70 ± 0.35 folds. The expression level of antioxidant-related genes (*sodB, catB* and *tpxA*) in expressing cells revealed that only *tpxA* was up-regulated for 4.58 ± 0.58 folds under NaCl stress. While under KCl stress, *sodB* and *tpxA* were up-regulated for 4.09 ± 0.86 and 11.81 ± 1.02 folds, respectively. The expression of osmolyte-related genes: *Synpcc7942_0808* and *Synpcc7942_2522* revealed only *Synpcc7942_0808* was up-regulated 3.89 ± 0.30 folds in expressing cells culturing under NaCl stress. Under KCl stress, *Synpcc7942_0808* and *Synpcc7942_2522* were up-regulated for 9.40 ± 0.88 and 3.52 ± 0.29 folds, respectively. Moreover, protein analysis by western blotting displayed the specific band of DDG-synthase (encoded by *Ap3858*). In expressing cells culturing under NaCl stress, protein expression was highly induced for 7.20 folds. Metabolite analysis revealed that M2G accumulation was increased in both salinity conditions. The highest M2G level was observed under NaCl stress together with exogenous supplementation of serine in which accounted for 7.35 ng/mgFW.

Field of Study:	Microbiology and Microbial Technology	Student's Signature	
Academic Year:	2018	Advisor's Signature	

ACKNOWLEDGEMENTS

Panwad Pingkhanont

TABLE OF CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The enhancing of ultraviolet radiation (UVR) reaching to the Earth surface becomes an important issue in recent decades. UVR (UV-B, 280–315 nm, UV-C, 100–280 nm) adversely affects mostly to photosynthetic organisms, particularly marine organisms such as algae and cyanobacteria (Platt and Hönninger, 2003). Cyanobacteria present in all habitats from terrestrial, fresh water and marine. As they are photosynthetic microorganisms, cyanobacteria are directly expose to the intense solar radiation. Among the broadly wavelength of UVR, UV-B has the greatest potential for cell damage in various mechanisms (Häder *et al.*, 2015). UVR has many harmful effects on biomolecules (*i.e.* DNA and proteins), physiology and morphology (*i.e.* cellular differentiation and motility) (Rastogi *et al.*, 2014).

Due to the exposure to harmful UVR, cyanobacteria have various strategies to avoid the damage from UVR exposure to survive and grow in high-UV environment. These strategies included UVR avoidance, antioxidative defense system, excretion of extracellular polysaccharide, and synthesis of extracellular polysaccharide (Rastogi *et al.*, 2014). The most important mechanism to prevent the adverse effects of UVR is the synthesis of UV-absorbing/screening compounds. The important biomolecules in this strategy include mycosporine-like amino acids (MAAs) and scytonemin. These compounds acts as the effective photoprotectant in cyanobacteria against UV-A and/or UV-B (Singh *et al.*, 2010).

MAAs are a family of small-molecule secondary metabolite (<400 Da) with colorless and water soluble capacity. MAAs have strong UV protection due to extremely high UV absorption maxima (310-362 nm) and strong molar extinction coefficient (ε = 28,100-50,000 M^{-1} cm^{-1}) (Wada *et al.*, 2015). MAAs structures are composed of a cyclohexanone or cyclohexenimine ring conjugated to the nitrogen substituent by one or two amino acid (s) at the third (and the first) carbon of the core

ring (Singh *et al.*, 2008a; Pope *et al.*, 2015). Variation in attached groups and nitrogen substituents come about the difference among the absorption spectra of MAAs (Sinha *et al.*, 2007).

To date, biosynthetic pathway of MAAs were investigated in some cyanobacteria, such as in *Nostoc punctiforme* ATCC 29133, *Anabaena variabilis* PCC 7937, and *Aphanothece halophytica* (Singh *et al.*, 2008a; Waditee-Sirisattha *et al.*, 2014). MAA is believed to be synthesized by cyanobacteria from two pathways, pentose phosphate pathway and shikimate pathway (Pope *et al.*, 2015). MAA production in cyanobacteria is directly regulated by UVR. UV-B radiation plays the most important role in MAA induction (Bebout and Garcia-Pichel, 1995). Other stresses were found to be inducers for MAA biosynthesis, such as desiccation and hypersalinity.

MAAs play vital role as 'natural sunscreen compound' or primary UV sunscreen with additional biological activities (Shick and Dunlap, 2002). Substantial evidences were shown MAAs contribute as (1) antioxidant compounds, (2) protection against desiccation or thermal stress, (3) osmotic regulation and (4) intracellular nitrogen reservoir.

MAAs are the potential natural sunscreen compound with many beneficial additional biological activities. Therefore, they are promising natural products for use in pharmaceutical and cosmetic industries. One of the distinct MAA is mycosporine-2-glycine (M2G), it has the highest scavenging activity among common MAAs, such as shinorine, porphyra-334, palythine, and mycosporine glycine (Cheewinthamrongrod *et al.*, 2016).

To improve productivity of MAAs, the heterologous expression is one of interesting approaches to MAAs overproduction in potential hosts. Heterologous expression of MAA biosynthesis gene cluster from cyanobacteria has been reported in some organisms, such as *Escherichia coli*, *Streptomyces* and yeast cells. However,

these organisms exhibited the limited succeed in heterologous production of cyanobacterial natural products (Ziemert *et al.,* 2008).

Although the previous description, heterologous expression of MAAs biosynthesis gene cluster have been performed in diverse microorganisms. However, the MAAs production still obtained considerably low contents. Alternatively, cyanobacteria may display as suitable hosts on heterologous expression. This study aimed to examine the heterologous expression of M2G synthetic gene cluster from halotolerant cyanobacterium *A. halophytica* in fresh water cyanobacterium *Synechococcus elongatus* PCC 7942 and demonstrate the expression of M2G biosynthetic gene cluster in heterologous cells and the overproduction of M2G under salt stress.

The objective of this research

1. To express the M2G biosynthetic gene cluster encoding for *Ap3858* (dimethyl 4-deoxygadusol synthase; Ap-DDG), *Ap3857* (*O*-methyltransferase; Ap-OMT), *Ap3856* (C-N ligase; Ap-CN ligase), and *Ap3855* (D-ala-D-ala ligase; Ap-AA ligase) from *A. halophytica* in *S. elongatus* PCC 7942
2. To analyze transcriptional and translational products of M2G biosynthetic gene cluster in heterologous expressing cells under salt stress
3. To analyze M2G in expressing cells under salt stress

The hypothesis in this research

M2G biosynthetic gene cluster from *A. halophytica* can be expressed in *S. elongatus* PCC 7942 and its gene product can be modulated under salt stress conditions.

CHAPTER II

LITERATURE REVIEW

2.1 Effects of UVR on living organisms

The enhancing of UVR reaching to the Earth surface becomes an important issue in recent decades. The damage of the ozone layer causing by anthropogenically released atmospheric pollutants, such as chlorofluorocarbon, chlorocarbons, and reactive nitrogen species (RNS) (Smith *et al.*, 1992). Enhancing of UVR (UV-B, 280–315 nm, UV-C, 100–280 nm) adversely affects mostly to photosynthetic organisms, particularly marine organisms, such as algae and cyanobacteria (Platt and Hönninger, 2003). Substantial evidences on adverse effects of UVR on photosynthetic organisms have been showed. For example, UV-B irradiation induced the format changed in filamentous cyanobacterium (Figure 1) (Ehling-Schulz *et al.*, 1997). In this study, we place special emphasize on the effects of UVR in cyanobacteria.
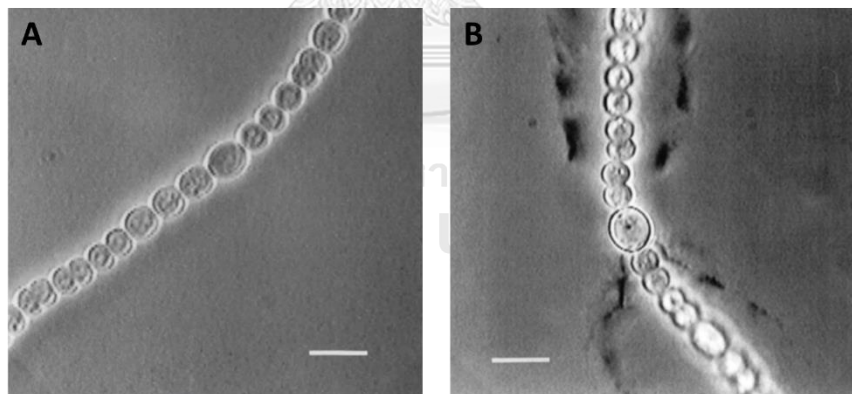
Figure 1. Synthesis of sheath material surrounding the filaments on filamentous cyanobacterium *N. commune* DRH1 grown in liquid culture. Typical appearance of DRH1 grown in liquid culture without UV-B (A) DRH1 cells after exposed to UV-B irradiation (1.0 W m^{-2}) for 72 h. (B) Bars indicate 10 mm (Ehling-Schulz et al., 1997).

2.2 UV-B damage in cyanobacteria

Cyanobacteria present in a variety of habitats including terrestrial, fresh water and marine. Terrestrial cyanobacteria are directly exposed to the intense solar radiation. Marine and fresh water cyanobacteria are also exposed to the high UVR, due to UVR penetrates deep into the water up to several meters. Among the broadly wavelength of UVR, UV-B has the greatest potential for cell damage in various mechanisms (Häder *et al.*, 2015). UVR has many harmful effects on biomolecules (*i.e.* DNA and proteins), physiology and morphology (*i.e.* cellular differentiation and motility) (Rastogi *et al.*, 2014).

Biomolecules

The primary targets of UVR on cellular are proteins and nucleic acids including DNA and RNA (Rastogi *et al.*, 2014). UV-B can cause protein lost and regulate some proteins in many cyanobacteria. For example, in *Synechocystis* sp. PCC 6803, it was reported dramatic protein response to UVR exposure; 66 proteins were up-regulated and 46 proteins were down-regulated (Gao *et al.*, 2009). DNA molecules directly absorb UV-B irradiation. In addition, UVR also induces the formation of thymine dimers or cyclobutane-pyrimidine dimers which disrupt genomic integrity (Häder and Sinha, 2005).

Biochemistry and physiology

Adverse effects of UVR on pigmentation and photosynthesis in cyanobacteria have been reported. For instance, UVR causes the decreasing of chlorophyll a, carotenoids, phycocyanin, and phycobiliproteins. Reductions of photosynthetic pigments result in disassembly of phycobillisomal complex in many cyanobacteria. Thus, UV-B exposure dramatically reduced the efficiency of photosystem II (PSII) (Rastogi *et al.*, 2014). PSII is more sensitive to UVR because the most important target of UVR is in the oxygen-evolving complex of PSII (Jiang and Qiu, 2011). In filamentous

cyanobacteria, UVR effects N$_2$ fixation by inhibit nitrogenase activity (Kumar *et al.*, 2003).

Morphology and cellular differentiation

UVR affects the morphology and cellular differentiation in many cyanobacteria. A significant disruption of the differentiation of heterocysts and akinetes in *Anabaena* sp. PCC 7420 after exposing to UV-B was reported. The changing balance of heterocysts and vegetative cells could have a harmful effect on survival (Gao *et al.*, 2007). Upon the exposure to UVR, the broken of filamentous and spiral in cyanobacteria were shown. The exact mechanism (s) of these breakages still unknown but highly reactive oxygen species (ROS) induced by UV may cause lipid-oxidation in cell membrane (Donkor *et al.*, 1993).

Motility and orientation

Motility and orientation are important mechanisms for avoiding from UVR that protect cyanobacteria from cell damage. UVR seriously reduce in the motile filament of cyanobacteria, resulting in an impaired ability to escape from UVR (Donkor *et al.*, 1993).

2.3 Protective mechanisms in response to UVR in cyanobacteria

Cyanobacteria have various strategies to avoid the damage from UVR exposure to survive and grow in high-UV environment. These strategies included UVR avoidance, antioxidant systems, excretion of extracellular polysaccharides, synthesis of extracellular polysaccharides, and synthesis of UV-absorbing/screening compounds (Rastogi *et al.*, 2014).

UVR avoidance

The first mechanism in many cyanobacteria to avoid from the harmful effect of solar UVR is migration. Motile species of cyanobacteria have ability to move upward or downward related with the incident light intensity to escape high UV levels. A number of reports have shown that UV-A and UV-B radiation act as the guidance controlling for vertical movement. For example, The cyanobacterium *Microcoleus chthonoplastes* and *Oscillatoria cf. laetevirens* gliding vertical downward to escape the UV light (Bebout and Garcia-Pichel, 1995; Bhaya, 2004).

Enzymatic/nonenzymatic antioxidant systems

Cyanobacteria have mechanisms to capture light energy while avoiding oxidative damage from harmful ROS, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxyl radical (HO_2^-) and hydroxyl radical (OH^-) which induce by high irradiance. To respond to these oxidative molecules, cyanobacteria have adapted enzymes as a second line to defense against UVR. These mechanisms involve enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and ascorbate peroxidase (APX) work by breaking down and removing free radicals. Few examples of the nonenzymatic antioxidants are carotenoids, α-tocopherols (vitamin E), ascorbic acid (vitamin C) and glutathione (Ehling-Schulz *et al.*, 2002)

SOD is one of important enzymes in antioxidant scavenging systems. This enzyme scavenges superoxide radical and converts them to H_2O_2 which is then converted H_2O, and O_2 via combined catalase-peroxide system (Miyake *et al.*, 1991). Nonenzymatic antioxidant molecules seem to be an indirect mechanism to prevent cells from oxidative stress. Glutathione protects the thiol groups of many enzymes, such as glutathione-dependent thiol peroxidase and involves in the production of ascorbate (Gaber *et al.*, 2004).

Synthesis of extracellular polysaccharides

Cyanobacterial extracellular polysaccharides compose of high-molecular-mass heteropolysaccharides with various compositions and roles. These productions depend on microorganisms and environments (Pereira *et al.*, 2009). Synthesis of extracellular polysaccharides in cyanobacteria is an important strategy to allow them live and survive in high UV environment. Cyanobacteria having extremely thick polysaccharides sheath can better survive in high solar radiation. Cyanobacteria produce exopolysaccharide to provide a matrix for UV-absorbing/screening compounds, such as MAAs and scytonemin in order to response the high UVR (Ehling-Schulz and Scherer, 1999).

Synthesis of UV-absorbing/screening compounds

Cyanobacteria have ability to produce UV-absorbing or screening compounds as significant strategy to defense the toxicity of solar radiation (Singh *et al.*, 2010). Important biomolecules in this strategy include MAAs and scytonemin. These biocompounds act as the effective photoprotectant in cyanobacteria against UV-A and/or UV-B. (Rastogi *et al.*, 2014).

2.4 Mycosporine-like amino acids (MAAs)

Feature of MAAs

MAAs are a family of small-molecule secondary metabolite (<400 Da) with colorless and water soluble capacity. MAAs have strong UV protection due to extremely high UV absorption maxima (310-362 nm) and strong molar extinction coefficient (ε = 28,100-50,000 M^{-1} cm^{-1}) (Wada *et al.*, 2015). MAAs molecules generally contain a central cyclohexanone or cyclohexenimine ring conjugated to the nitrogen substituent of an amino acids or imino alcohol which is responsible for UV absorption. MAAs generally contain a glycine subunit at the third carbon. Variations in attached groups and nitrogen substituents come about the difference among the absorption

spectra of MAAs (Figure 2) (Sinha *et al.*, 2007). Some MAAs also have sulfate esters or glycosidic linkages at the imine substituent positions (Sinha and Häder, 2008).

Mycosporine-glycine
λmax = 310 nm

Shinorine
λmax = 334 nm

Porphyra
λmax = 334 nm

Mycosporine-2-glycine
λmax = 334 nm

Palythene
λmax = 360 nm

Euhalothece-362
λmax = 362 nm
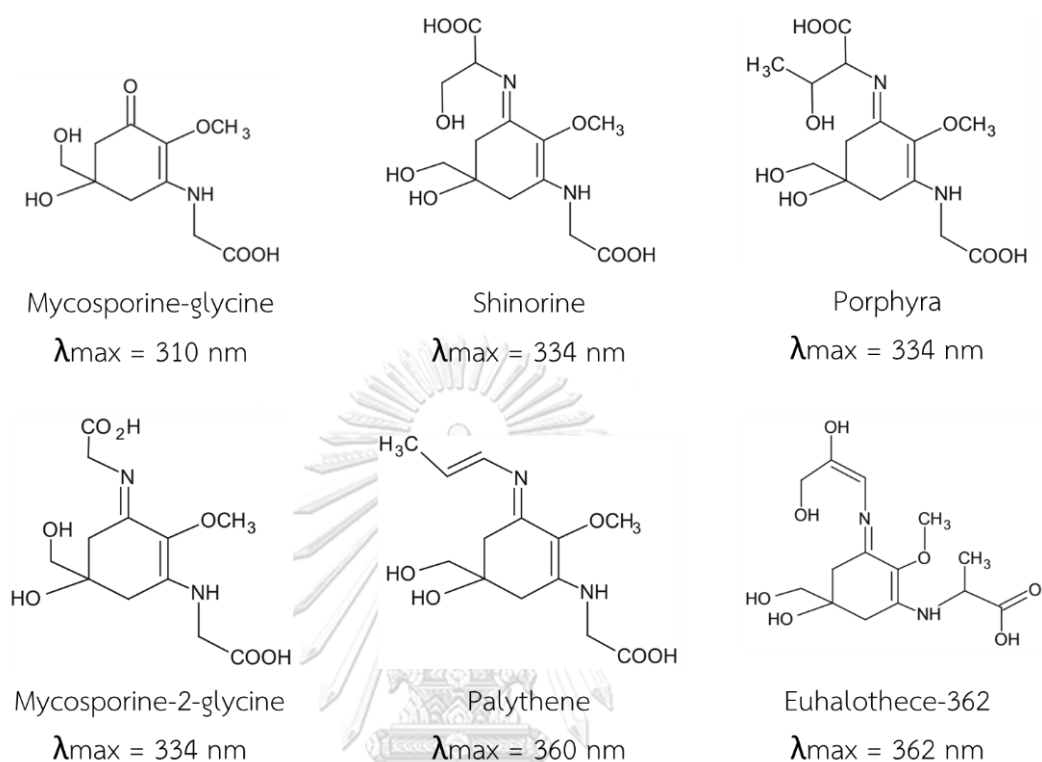
Figure 2. Structures of some common MAAs found in cyanobacteria (Rastogi et al., 2014).

MAAs distribution

The accumulation of MAAs in cyanobacteria was firstly reported in 1969 (Shibata, 1969). Currently, MAA family consists of > 33 compounds. MAAs are widespread in various microorganisms. Form a survey of 152 species of marine microalgae showed that they all contained UV-absorbing compounds (Wada *et al.*, 2015). Various kinds of MAAs have been found in a variety of freshwater and marine organisms, including cyanobacteria, fungi, micro/macroalgae as well as many marine invertebrates and vertebrates such as shrimp, sea urchins including fish through the food chain or symbiotic algae (Shick and Dunlap, 2002). A number of cyanobacteria have been reported on the production of different MAAs in diverse species and

habitats. Representative MAAs and cyanobacterial strains are shown in Table 1 (Conde *et al.*, 2000).

Table 1. Occurrence of MAAs in various cyanobacterial species.
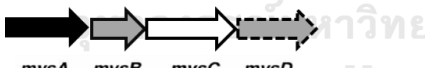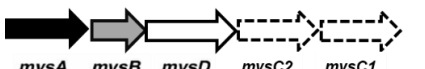
MAAs	λmax (nm)	Cyanobacterial strains
Mycosporine-taurine	309	*Synechocystis* sp. *PCC 6803*
Mycosporine glycine	310	*Scytonema* sp., *Synechococcus* sp.
		N. vommune
		Chlorogloeopsis PCC 6912
Palythene	320	*A. halophytica*
Mycosporine-2-glycine	334	*Euhalothece* sp.
Shinorine	334	*Anabaena* sp., *Gleocapsa* sp.
		Scytonema sp., *Lyngbya aestuarii*
		Microcystis aeruginosa
Porphyra-334	334	*Microcystis aeruginosa*
		N. harveyana
		N. spumigena
Euhalothece-362	362	*Euhalothece* sp.

Biosynthesis of MAAs in cyanobacteria

To date, biosynthetic pathways of MAAs were investigated in *N. punctiforme* ATCC 29133, *A. variabilis* PCC 7937, *A. halophytica*, *Microcystis aeruginosa* PCC 7806, *Scytonema* cf. *crispum* UCFS10 and UCFS15, *Cylindrospermum stagnale* PCC 7417, *N. flagelliforme* CCNUN1 and *Fischerella* sp. PCC9339 (Singh *et al.*, 2008a; Gao and Garcia-Pichel, 2011; Waditee-Sirisattha *et al.*, 2014; Hu *et al.*, 2015; D'Agostino *et al.*, 2016; Katoch *et al.*, 2016; Shang *et al.*, 2018; Yang *et al.*, 2018). As shown in Table 2, in the first six cyanobacteria, four genes were shown to be involved in MAA biosynthesis. Very recently, it was found that genes for MAA biosynthetic consisted of a cluster of five genes in *Cylindrospermum stagnale* PCC 7417 (Katoch *et al.*, 2016) and *N. flagelliforme* CCNUN1 (Shang *et al.*, 2018).

MAA biosynthesis gene cluster in first six cyanobacteria (*i.e. N. punctiforme* ATCC 29133, *A. variabilis* ATCC 29413, *A. halophytica*, *M. aeruginosa* PCC 7806, *Scytonema cf. crispum* UCFS10 and UCFS15, and *Fischerella sp.* PCC 9339) consists of *NpR5600* to *NpF5597*, *ava_3858* to *ava_3855*, *Ap3858* to *Ap3855*, *mysA* to *mysD*, *mysA* to *mysE*, and *FsA* to *FsD*, respectively. The first three genes in these cyanobacteria encode putative proteins for DDG synthase, O-MT, and C-N ligase, respectively. The fourth gene in *A. variabilis* ATCC 29413, *S.* cf. *crispum* and *Fischerella* sp. PCC 9339 encode a nonribosomal peptide synthetase (NPRS ligase) while the fourth genes in other three cyanobacteria encode for AA ligase.

Table 2. MAA biosynthetic gene clusters from six cyanobacterial strains and their MAA products

Strains	MAA biosynthesis gene clusters	MAA products	References
N. punctiforme ATCC 29133	NpR5600 5599 NpR5598 NpF5597	Shinorine	(Singh *et al.*, 2008a)
A. variabilis ATCC 29413	ava_3858 3857 ava_3856 ava_3855	Shinorine	(Gao and Garcia-Pichel, 2011)
A. halophytica	ap3858* ap3857 ap3856 ap3855	Mycosporine-2-glycine	(Waditee-Sirisattha *et al.*, 2014)
M. aeruginosa PCC 7806	mysA mysB mysC mysD	Shinorine, Porphyra-334	(Hu *et al.*, 2015)
S. cf. *crispum* UCFS10 and UCFS15	mysA mysB mysC mysE	Shinorine	(D'Agostino *et al.*, 2016)
Fischerella sp. PCC9339	FsA FsB FsC FsD	Shinorine	(Yang *et al.*, 2018)
C. stagnale PCC 7417	mylA mylB mylC mylD mylE	Shinorine	(Katoch *et al.*, 2016)
N. flagelliforme CCNUN1	mysA mysB mysD mysC2 mysC1	mycosporine-2-(4-deoxygadusolyl ornithine)	(Shang *et al.*, 2018)

Genes are indicated by arrows: black, DDG-synthase; gray, O-MT; black dashed, AA ligase; white, C-N ligase; double-outlined, NRPS-like protein; gray with black dashed, ATP-grasp type ligase. Direction of arrows indicates direction of transcription. *Ap3858** is distal to the other genes in the *A. halophytica* M2G synthetic gene cluster.

MAA is believed to be synthesized in cyanobacteria from two pathways, pentose phosphate pathway and shikimate pathway. Sedoheptulose-7-phosphate (SH7-P), the intermediate derived from pentose phosphate pathway, is supposed to be a common precursor compound utilized in MAA biosynthetic pathway. By the reaction of DDG synthase, SHP is converted to form DDG. Further, DDG is converted to form 4-DG by *O*-MT and can be produced from the shikimate pathway by a dehydroquinate synthase (DHQS). The third step, C-N ligase catalyzes the addition of glycine to 4-DG to produce mycosporine glycine. Further condensation of serine (or glycine, in case of *A. halophytica*) onto mycosporine-glycine yields shinorine (or M2G, in case of *A. halophytica*), which is catalyzed by NRPS or AA ligase (Singh *et al.*, 2008a; Gao and Garcia-Pichel, 2011; Waditee-Sirisattha *et al.*, 2014; Hu *et al.*, 2015; Pope *et al.*, 2015; D'Agostino *et al.*, 2016; Katoch *et al.*, 2016; Shang *et al.*, 2018; Yang *et al.*, 2018).

Regulation of MAA biosynthesis under stress condition

MAA production in cyanobacteria is directly regulated by UVR. Among three ultraviolet wave lengths, UV-B radiation plays an important role in MAA induction (Bebout and Garcia-Pichel, 1995). Moreover, other stresses were found to be inducers for MAA biosynthesis, such as desiccation and hypersalinity.

UV Radiation

UVR is generally the strongest inducer for the biosynthesis of MAAs along with their function as sunscreen compounds. Special photoreceptors appear to be present to sense the need for MAA synthesis induction (Oren and Gunde-Cimerman, 2007). In cyanobacteria *Chlorogloeopsis* PCC 6912, the wavelengths between 280 and 320 nm are the most effective inducers for shinorine production (Portwich and Garcia-Pichel, 2000). The same as observing in *A. doliolum*, the induction of porphyra-334 and shinorine biosynthesis were found under high UV-B radiation (Singh *et al.*, 2008b).

Therefore, a number of cyanobacteria could adapt themselves to overcome UVR via UV-regulation of MAA biosynthesis (Zhang *et al.*, 2007).

Hypersalinity

The higher salt concentration in where microorganisms live, the higher its intracellular solute concentrations have to be. MAAs appear to have an action response to osmotic stress. The highest accumulation of MAAs was reported in a halotolerant cyanobacterium '*Euhalothece*' type, inhabiting in a gypsum crust in a hypersaline saltern pond. MAAs in these cells reached an extremely high concentration ≥98 mM or at least >3% of wet weight (Oren, 1997). Determining the accumulation level of M2G in *A. halophytica* in salt upshock conditions (0.5 to 2.5 M NaCl), the result showed that M2G was significantly increased at the highest level of 28.38 ± 1.2 μmol/g (dry weight) when grown in 2.0 M NaCl (Waditee-Sirisattha *et al.*, 2014).

Desiccation

There is also evident that MAA production can be regulated by desiccation environment. In filamentous sheath forming cyanobacteria including *Lyngbya, Plectonema,* and *Scytonema* habiting in biological soil crusts in India showed that MAA synthesis was stimulated by the combination of desiccation and irradiation (Tirkey and Adhikary, 2005). Cyanobacterium *N. commune,* living in natural habitat, was subjected to simultaneous stress of desiccation. This condition led to a thick extracellular matrix in which glycosylated MAAs were embedded (Wright *et al.*, 2005).

Biological function of MAAs

Substantial reports revealed that MAAs play a role as 'natural sunscreen compound' or primary UV sunscreen with additional biological activities (Shick and Dunlap, 2002). Substantial evidences were also shown MAAs contribute as (1) antioxidant compounds, (2) protection against desiccation or thermal stress, (3)

osmotic regulation and (4) intracellular nitrogen reservoir. The followings are evidence to show that MAA acts as multipurpose secondary metabolites.

Sunscreen compound

The most important function of MAAs is photoprotection in cyanobacteria. They are commonly described as 'microbial sunscreen' (Oren and Gunde-Cimerman, 2007). These compounds have ability to disperse the harmful UVR into heat energy without forming reactive photoproducts (Conde *et al.*, 2000). MAAs strongly absorb UV-A and UV-B radiation with high molar extinction coefficients and resistance to several abiotic stressors, in high solar radiation conditions in particular (Häder and Sinha, 2005; Whitehead and Hedges, 2005). MAAs located in cytoplasm prevent three out of every ten photons from reaching sensitive cellular targets (Garcia-Pichel *et al.*, 1993). Moreover, the biosynthesis of MAAs can be strongly induced by UVR in many cyanobacteria. For example, MAA biosynthesis in *Anabaena* was strongly induced under 280 and 320 nm of UVR (Rozema *et al.*, 2002). These results give the strong evidence that MAAs act as efficient photoprotective compounds in cyanobacteria.

Antioxidant molecules

Although MAA biosyntheses mainly produce in response to UVR but they also play additional roles in UV protection. Recently, they also act as novel antioxidants. MAAs have ability in scavenging ROS, such as singlet oxygen, superoxide anions, hydroperoxyl radicals, and hydroxyl radicals (Oren and Gunde-Cimerman, 2007). The certain MAAs, namely mycosporine glycine and mycosporine taurine, exhibit a strong antioxidant activity by quenching the reactive oxygen species (ROS) (Wada *et al.*, 2015). Mycosporine glycine effectively reduced the amount of singlet oxygen formed under illumination with greater capability to donate electrons to stabilize and inactivate the free radicals (Suh *et al.*, 2003).

Protection against abiotic stress factors

MAAs play another role in protection against abiotic stress factors, such as osmotic pressure, desiccation, and temperature. MAAs were found in cyanobacteria which live in high-salt ecosystems and often called 'osmotic solutes' or 'compatible solutes'. Many microorganisms accumulate MAAs within intracellular space for relieving pressure from salt stress in high salinity environment (Oren and Gunde-Cimerman, 2007). There are many reports that cyanobacteria accumulated high concentrations of MAAs after exposing to drought stress (Whitehead and Hedges, 2005).

2.5 Engineering for UV sunscreen compound synthesis in microorganisms

MAAs are the potential natural sunscreen compound with many beneficial additional biological activities. Therefore, MAAs are promising natural products for use in pharmaceutical and cosmetic industries. One of the distinct MAA is M2G. It has the highest scavenging activity among common MAAs, such as shinorine, porphyra-334, palythine, and mycosporine glycine (Cheewinthamrongrod *et al.*, 2016).

To improve productivity of MAAs, the heterologous expression is one of interesting approaches to overproduction of MAAs in potential host. Heterologous expression of MAA biosynthesis gene cluster from cyanobacteria has been reported in some organisms, such as *E. coli*, *Streptomyces* strains and yeast. The first heterologous production of MAAs was shown in heterologous expression of M2G synthesis gene cluster from *A. halophytica* in *E. coli*. This heterologous expression resulted in the accumulation of M2G under the high-salinity condition (0.30 M NaCl) about 85.2 ± 0.7 μmol/g (dry weight) (Waditee-Sirisattha *et al.*, 2014). This result was consistent with the heterologous expression of M2G biosynthetic gene cluster of *A. halophytica* in *E. coli* in various salt conditions. The result showed the highest transcription level of M2G biosynthesis gene cluster (*Ap3858-3855*) in *E. coli* expressing cells under NaCl stress condition (Cheewinthamrongrod *et al.*, 2016). A shinorine producing *Corynebacterium*

glutamicum strain was constructed for expressing four genes from *Actinosynnema mirum* DSM 43827. The combined deletion of transaldolase (*tal*) gene and overexpression of 6-phosphogluconate dehydrogenase (*gnd*) gene increased the precursor; sedoheptulose-7-phosphate and improved shinorine production (Tsuge *et al.*, 2018). However, in *Corynebacterium glutamicum* expressing cells still showed considerably low amount of desired MAAs (Ziemert *et al.*, 2008). In *E. coli* heterologous expression, cultivation and media costs are rather expensive; therefore, manipulation in cyanobacteria in which a very low cost for cultivation would be one of attractive points.

Cyanobacteria may display as suitable host(s) on heterologous expression for producing photosynthetically cyanobacterial products due to several advantages (1) it has a short doubling time compared with algae (Emlyn-Jones *et al.*, 2006); (2) it is enable to genetic modifications with a variety of available tools (Holtman *et al.*, 2005); (3) it provides products that conveniently to isolate and identify (Weber *et al.*, 2015); and (4) it is cost-effective because cyanobacteria use common medium for their growth (Yang *et al.*, 2018). Therefore, the heterologous expression of foreign gene clusters in cyanobacteria is one of interesting way for MAAs overproduction. The first heterologous production of MAA in photosynthetic cyanobacteria demonstrated by expressing of a shinorine gene cluster from the filamentous cyanobacterium *Fischerella* sp. PCC 9339 in *Synechocystis* sp. PCC 6803 with multiple promoters. Shinorine productivity increased about 10-fold (2.37 ± 0.21 mg/g dry biomass weight), comparable to commercially used shinorine producer (Yang *et al.*, 2018). Since the MAA; M2G has superior biological activities (Cheewinthamrongrod *et al.*, 2016; Tarasuntisuk *et al.*, 2018); therefore, the heterologous expression of M2G in cyanobacteria may be an alternative way to overproduction M2G.

The objectives of this research:

1. To express the M2G biosynthetic gene cluster encoding for *Ap3858* (dimethyl 4-deoxygadusol synthase; Ap-DDG), *Ap3857* (*O*-methyltransferase; Ap-OMT), *Ap3856* (C-N ligase; Ap-CN ligase), and *Ap3855* (D-ala-D-ala ligase; Ap-AA ligase) from *A. halophytica* in *S. elongatus* PCC 7942

2. To analyze transcriptional and translational products of M2G biosynthetic gene cluster in heterologous expressing cells under salt stress

3. To analyze M2G in expressing cells under salt stress

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

Autoclave: Model SS-325 and ES-215, TOMY Digital Biology, Japan

Autopipette: Eppendorf Research plus, Eppendorf, Germany

Biomate 3S UV-visible spectrophotometer: Thermo Scientific™, USA

Biophotometer D30: Eppendorf, Germany

Concentrator 5301: Eppendorf, Germany

Disposable syring: Nipro, Thailand

Gel imaging: Model Gel Doc EZ™, Bio-Rad, USA

Gel electrophoresis: Model MJ-105, Major Science, USA

High Performance Liquid Chromatography (HPLC): Shimadzu, Japan

Incubator shaker: Model innova 4330, New Brunswick Scientific, USA

Laboratory glassware: Pyrex, USA

Laminar flow: Model H1, Microtech, Thailand

Magnetic stirrer: Model MMS-3000, Biosan, Latvia

Microscope: Olympus, Japan

Mini-PROTEAN tetra cell: Bio-Rad, USA

Nanodrop 2000 UV-Vis Spectrophotometer: Thermo Scientific™, USA

Orbital shaker: Model TT-20: Hercuvan Lab Systems, Malaysia

Petri dish 90x15 mm: Biomed, Thailand

pH meter: SevenEasy™, Mettler Toledo, USA

Refrigerated microcentrifuge: Model 5418 R, Eppendorf, Germany

Spectrophotometer cuvette: Spectronic 401, Milton Roy, USA

Trans-Blot® SD Semi-Dry Transfer Cell: Bio-Rad, USA

TT-100H/TT-100C Thermo Shaker Incubator: Hercuvan Lab Systems, Malaysia

Twelve-well cell culture plate: SPL life science, Korea

Ultrasonic bath: Bandelin, Germany

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

3.2 Chemicals and media

Acetic acid: Merck, Germany

Agar powder: Himedia, India

Agarose gel: Bio-Rad Laboratories, USA

Anti-Histidine (Mouse monoclonal IgG): R&D systems, USA

Anti-mouse IgG (AP-linked): Cell signaling technology, USA

Bacto® tryptone: Merck, Germany

BCIP/NBT solution: Amresco, USA

Bio-Rad Protein assay dye reagent concentrate: BioRad, USA

Boric acid: Merck, Germany

Calcium chloride: Merck, Germany

Chloroform: Sigma-Aldrich, USA

Citric acid: Merck, Germany

Cobalt(II) nitrate: Ajax Finechem Pty Limited, Australia

Copper(II) sulfate: Ajax Finechem Pty Limited, Australia

DEPC (Diethylpyrocarbonate), Amresco, USA

Dipotassium phosphate: Ajax Finechem Pty Limited, Australia

DynaMarker DNA low D: BioDynamic laboratories, Japan

DynaMarker Protein MultiColor III: BioDynamic laboratories, Japan

EDTA (Ethylenediaminetetraacetic acid): Amresco, USA

Ethanol: Merck, Germany

Ferric ammonium nitrate: Merck, Germany

GeneAmp® 10 mM dNTP Mix: Applied Biosystems™, USA

Glycerol: Merck, Germany

Glycine: BioRad, USA

Isopropanol: Merck, Germany

L-Serine: Sigma, USA

Magnesium chloride: Merck, Germany

Magnesium sulfate: Merck, Germany

Methanol: Merck, Germany

PCR buffer (10X): Applied Biosystems™, USA

Precision plus protein dual color standard: Biorad, USA

Purified BSA 100x: BioLab, USA

Sodium carbonate: Merck, Germany

Sodium chloride: Ajax Finechem Pty Limited, Australia

Sodium dodecyl sulfate: Ajax Finechem Pty Limited, Australia

Sodium nitrate: Merck, Germany
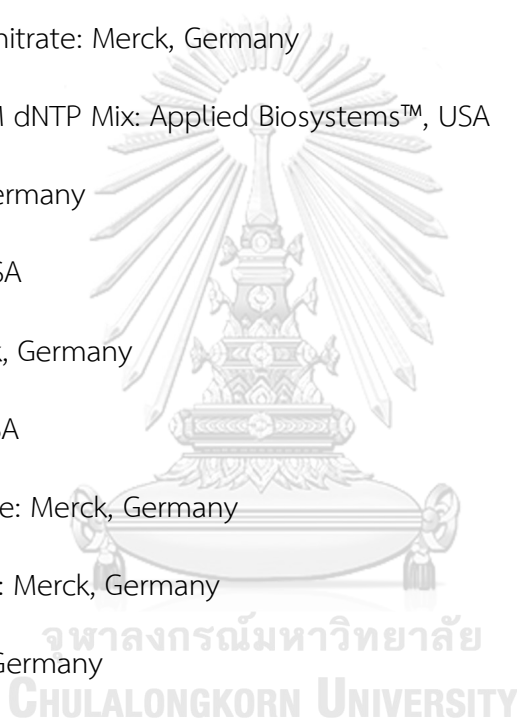
Streptomycin: Sigma, USA

SYBR® safe DNA gel strain: Invitrogen, USA

Thirty percent Acrylamide/Bis Solution: BioRad, USA

Trizma (2-amino-2-(hydroxymethyl)-1,3-propanediol): Sigma, USA

TRIzol® reagent: Invitrogen, USA

Yeast extract powder: Himedia, India

Zinc sulfate: Ajax Finechem Pty Limited, Australia

3.3 Membranes

YM-3 membrane Ultracel®-3K: Millipore, USA

Minisart filters pore size 0.2 μm: Merck, Germany

3.4 Kits

HiYield™ Plasmid Mini Kit, RBC Bioscience, Taiwan

SuperScript™ III First Strand Synthesis system, Invitrogen, USA

3.5 Enzymes

*Bam*HI: New England Biolabs, USA

Taq DNA polymerase: Invitrogen, USA

*Xho*I: New England Biolabs, USA

3.6 Plasmids and microbial strains

Table 3. Plasmids and bacterial strains used in this study

Strains and plasmids	Descriptions	Sources/References
Ap3858-3855/pUC303	2.76 kb *Ap3858* (native promotor and coding region of *Ap3858*) together with 3.63 kb *Ap3857-3855* (native promotor and coding region of *Ap3857-3855)* cloned into pUC303	Waditee-Sirisattha *et al.,* 2014
E. coli DH5α	Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK⁻, mK⁺) phoA supE44 λ– thi-1 gyrA96 relA1	Invitrogen, USA
S. elongatus PCC 7942	Freshwater cyanobacterium	Research Institute of Meijo University, Japan

3.7 Primers

Table 4. Primers used in this study

Primers	Sequences (5′ ⟶ 3′)	Base pairs number
Ap3855_Forward	TTATCCGAGAAACTCTCC	18
Ap3855_Reverse	AGGTCATACTTATCCTGAG	19
Ap3856_Forward	GGATCCAATGCTTCTATTTGTCCGAGG	27
Ap3856_Reverse	ATAGTAACTAGAAACGGGAC	20
Ap3857_Forward	GGATCCAATGACGATCACTAACGATAAAC	29
Ap3857_Reverse	ATGCAGAATAGCCCGTAAAC	20
Ap3858_Forward	GGATCCAATGACGAAAACAACCTCTG	27
Ap3858_Reverse	TGAGGATCGGTTTCCACAAG	20
7942cat_Forward	CTACCGAATTGCCGA	15
7942cat_Reverse	GGGATTGGTGCTTGG	15
7942sodB_Forward	ACCAAGGAAACGCTG	15
7942sodB_Reverse	CGGCTTGTTTGAACTC	16
7942tpxA_Forward	CCGTAAAGAAGGTGGT	16
7942tpxA_Reverse	CTTAACAGGGTCGGG	15
SynPCC 7942_2522_F	CTATCAAGTTGGATTCCG	18
SynPCC 7942_2522_R	CCGGTTATCTAACAACTC	18
SynPCC 7942_0808_F	GAAGTCTTGAAAGAGTGG	18
SynPCC 7942_0808_R	CTGATGGGAATAGATTGAC	19
Syn7942rnpB_Forward	GAGGAAAGTCCGGGCTCCC	19
Syn7942rnpB_Reverse	TAAGCCGGGTTCTGTTCTC	19

3.8 Culture conditions

S. elongatus PCC 7942 cells were grown in blue-green 11 (BG11) liquid medium under photoautotrophically (70 µE m^{-2} s^{-1}) condition (Waditee-Sirisattha *et al.,* 2014) with shaking 150 rpm at 30 °C. *S. elongatus* PCC 7942 cells harboring empty vector (pUC303) or MAA synthetic genes (pUC_Ap3858-3855_303) were grown under the same condition as the wild-type cells. Streptomycin (50 µg/ml) was supplemented in case of growing the expressing cells. The growth of cyanobacterial cells were measured by a UV-visible spectrophotometer (Thermo Scientific™ Biomate 3S, USA) at absorbance 730 nm. *E. coli* DH5α expressing cells were grown in Luria-Bertani (LB) medium with streptomycin (50 µg/ml) at 37 °C.

3.9 Plasmid extraction and analysis

3.9.1 Restriction enzyme analysis

The *E. coli* DH5α cells harboring empty vector (pUC303) and recombinant plasmids (pUC_Ap3858-3855_303; as shown in Figure 3) were used as the materials for plasmid preparation. Plasmids were extracted using Plasmid mini kit (RBC Bioscience). Concentration and purity of plasmids were measured by NanoDrop 2000 (Thermo Scientific™, USA). Restriction enzyme analysis was performed by using *Bam*HI and *Xho*I.

3.9.2 Polycistronic transcription analysis

Polycistronic transcription of *Ap3858-3855* was analyzed by using *A. halophytica* complementary DNA (cDNA) as template. Plasmid pUC303 and pUC_Ap3858-3855_303 were used as negative and positive controls, respectively. *Ap3856* forward primer and *Ap3855* reverse primer were used in polymerase chain reaction (PCR). *A. halophytica* cDNA were used as templates with specific primers: *Ap3856* and *Ap3855*. The PCR products were analyzed onto 1.2% (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml SYBR Safe DNA Gel Stain (Invitrogen, USA). The relative intensity was quantitated by ImageJ (htttp://imagej.nih.gov/ij/).
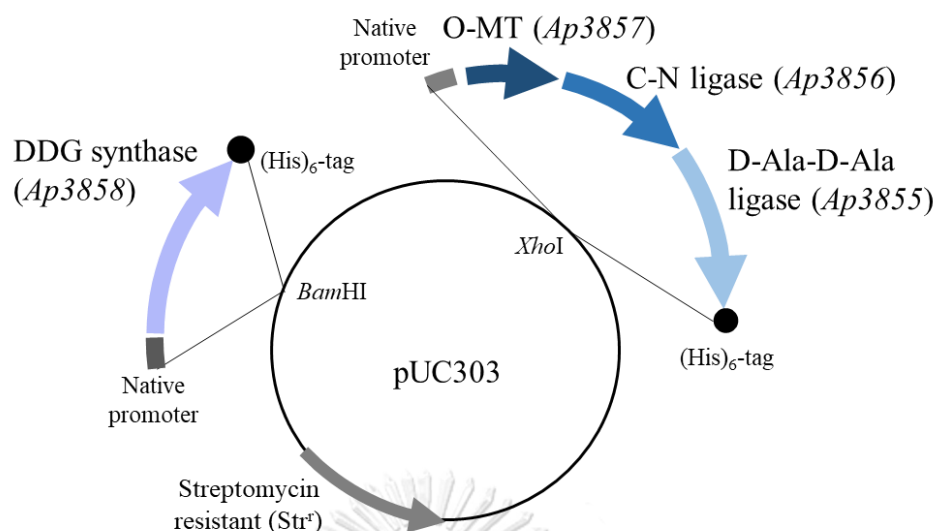
Figure 3 Map of a shuttle vector harboring M2G biosynthetic genes (*Ap3858-3855*) from *A. halophytica.* (*Ap3858*: demethyl-4-deoxygadusol (DDG) synthase; *Ap3857*: *O*-methyltransferase; *Ap3856*: C-N ligase and *Ap3855*: D-ala-D-ala ligase).

3.10 Transformation of *S. elongatus* PCC 7942 and stress treatment

3.10.1 Transformation of M2G biosynthetic gene cluster into *S. elongatus* PCC 7942

S. elongatus PCC 7942 cells were grown in BG11 until reaching to optical density at 730 nm $\cong$ 1.00. The purified pUC303 and pUC_Ap3858-3855_303 plasmids were transformed into *S. elongatus* PCC 7942 by natural transformation protocol (Vioque, 2007). Briefly, One milliliter of cyanobacterial culture was centrifuged at 4,500 rpm for 10 min. Cell pellets were collected and subsequently washed with fresh BG11 for three times. The washed cells were re-suspended in 1 ml BG11. One hundred microliters of cells suspension were mixed with 300 ng purified plasmids. This suspension were incubated at 25 °C under dark condition for overnight. Recovery of the transformed cells were performed in two separated sets. The first set, the transformed cells were laid onto BG11 agar plates. After 10 days, the candidate transformants were selected by supplementing with streptomycin (50 µg/ml). The second set, the transformed cells were cultured in BG11 in 12-well plates. After seven

days, the candidate transformants were selected by transferring onto BG11 agar plates supplemented with streptomycin (50 µg/ml). All of transformed cells were grown at the same condition as wide-type at 30 °C. To analyze the candidate transformants, a single colony was used as a template DNA for colony PCR analysis using specific primer pairs for *Ap3858-3855* genes (Table 2).

3.10.2 Stress treatment

For salt stress treatments, *S. elongatus* PCC 7942 transformant cells were grown in BG11 liquid medium supplemented with 0.35 M NaCl or 0.35 M KCl. Cells were adjusted to optical density at 730 nm $\cong$ 0.8-1.0 prior stress treatment. Transformants growing under these stress treatments were harvested for further analysis.

3.11 Analysis of transformants

3.11.1 Growth profile

S. elongatus PCC 7942 cells harboring empty vector or MAA synthetic genes were grown under the same condition as described in section 3.8. Growth profiles were measured by UV-visible spectrophotometer (Thermo Scientific™ Biomate 3S, USA) at absorbance 730 nm.

3.11.2 Phenotypic analysis

For measurement of chlorophyll and phycobiliprotein contents, control cells and transformants were grown under control and stress conditions. Then, the cultures were used to determine spectrophotometrically for chlorophyll a, phycocyanin, phycoerythrin and allophycocyanin, respectively. Each pigment was calculated its content using the following equations (Colowick & Kaplan, 1988).

$$\text{Chlorophyll a (µg/ml)} \quad = \quad OD_{665} \times 13.9$$

$$\text{Phycocyanin (PC) (mg/ml)} \quad = \quad \frac{A_{620} - (0.7 \times A_{650})}{7.38}$$

$$\text{Allophycocyanin (AP) (mg/ml)} \quad = \quad \frac{A_{650} - (0.19 \times A_{620})}{5.65}$$

$$\text{Phycoerythrin (PE) (mg/ml)} \quad = \quad \frac{A_{565} - 2.8[PC] - 1.34[AP]}{7.38}$$

3.11.3 Gene expression analysis by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

3.11.3.1 RNA extraction and cDNA conversion

Total RNA was extracted from *S. elongatus* PCC 7942 harboring empty vector (control) and pUC_Ap3858-3855_303 (expressing cells) using Trizol® reagent (Invitrogen, USA) according to manufacturer's instructions. Quantity of total RNA was measured by Thermo Scientific™ NanoDrop 2000. RNA integrity was confirmed by agarose gel electrophoresis. Four micrograms of total RNA were converted to cDNA using SuperScript® III First-strand (Invitrogen, USA) according to the manufacturer's instructions. The cDNAs were kept at -20°C for further experiments.

3.11.3.2 Expression of M2G synthetic gene cluster

The cDNA products were used as templates in RT-PCR analysis with specific primer pairs for four M2G biosynthesis genes: *Ap3858, Ap3857, Ap3856,* and *Ap3855* (Table 2). *Synechococcus rnpB* gene (*Syn7942rnpB*) was used as an internal control. The PCR products were analyzed onto 1.2% (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml SYBR Safe DNA Gel Stain (Invitrogen, USA). The relative intensity was quantitated by ImageJ (htttp://imagej.nih.gov/ij/).

3.11.3.3 Expression of antioxidant-related genes

The cDNA products were used as templates in RT-PCR analysis with specific primer pairs of 3 genes: *cat, sodB* and *tpxA* (Table 2) using the same protocol as described in section 3.11.3.2.

3.11.3.4 Expression of glycerol and sucrose synthetic genes

The cDNA products were used as templates in RT-PCR analysis with specific primer of 2 genes: *SynPCC 7942_2522* and *SynPCC 7942_0808* (Table 2) using the same protocol as described in section 3.11.3.2.

3.11.4 Protein expression analysis by western blotting

3.11.4.1 Transformants culture under salt stress for protein analysis

S. elongatus PCC 7942 cells harboring empty vector or MAA synthetic genes were cultured in BG11 liquid medium with streptomycin (50 µg/ml) supplemented with 0.35 M NaCl or 0.35 M KCl. Initial cell concentration was adjusted to optical density at 730 nm $\cong$ 0.8-1.0. Cells were harvested at 0, 24, 48 and 96 hours by centrifugation at 8,000 rpm for 10 minutes. The samples were measured for cell wet weight to get approximately 100 mg.

3.11.4.2 Crude protein extraction

Cell pellets obtained from step 3.11.4.1 were washed with 500 µl of 0.1 M Tris-HCl pH 8.2. Then, cells were centrifuged at 12,000 rpm for 5 minutes. The supernatants were discarded. Cell pellets were resuspended in 200 µl of 0.1 M Tris-HCl pH 8.2 and subjecting to sonitation with 40% amplitude for 10 seconds (repeated for 5 times). Supernatants were collected by centrifugation at 12,000 rpm, 4 °C for 5 minutes. Protein concentration was determined by the Bio-Rad Protein Assay

Kit (Bio-Rad) using BSA as protein standard (appendix 5). Crude protein extracts were kept in -20 °C for further experiments.

3.11.4.3 Sodium dodecyl sulfate polyacrylamind gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was prepared according to standard protocol (Sambrook *et al.*, 2001). Running buffer (appendix 6) was added into mini cell. Protein samples were loaded into gel with final concentration of 200 µg for each sample. SDS-PAGE was performed by using Mini-PROTEIN Tetra cell using 150 voltage for 1.30 hours. The SDS-PAGE gel was stained with 1% Coomassie brilliant blue (CBB). The molecular mass of target protein was determined by comparing to standard protein marker (Biorad, USA).

3.11.4.4 Western blotting analysis

Western blot analysis was performed as described in standard protocol (Sambrook *et al.*, 2001). A polyvinylidene difluoride (PVDF) was treated with 90% methanol for 10 seconds and gently soaking in western blotting buffer. SDS-PAGE-separated protein bands were transferred to PVDF membrane using western blotting buffer. Blotting was performed at 100 mA for 1.30 hours using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA) and then blocking with blocking solution (1X PBS, 0.01% Tween 20 and 5% skim milk) for 1 hour. The blocking membrane was incubated overnight in 30 ml 5% skim milk with primary antibody (antibody raised against Histidine 6X: Mouse monoclonal IgG) (1:500) from R&D systems (USA). The membrane was washed by gently shaking in 5% skim milk in 1X PBS for 3 times (20, 20 and 10 minutes, respectively). Then, the membrane was incubated in 30 ml 5% skim milk in 1X PBS for 2 hours with secondary antibody (anti-mouse IgG) (1:500) from Cell signaling technology (USA). After incubating, the membrane was washed with 1X PBS solution three times as previous step. To detect protein band signal, the membrane was developed with

BCIP/NBT solution (AMRESCO, USA). The molecular mass of target protein were determined by comparing to standard protein marker (BioDynamic laboratories, Japan).

3.12 Metabolite analysis

3.12.1 Analysis of M2G

3.12.1.1 Stress treatment

S. elongatus PCC 7942 cells harboring empty vector or M2G synthetic genes were cultured in BG11 liquid medium with streptomycin (50 μg/ml) supplemented with 0.35 M NaCl or 0.35 M KCl. Initial cell concentration was adjusted to optical density at 730 nm $\cong$ 0.8-1.0. Calls were harvested at 0, 2, 4 and 7 days by centrifugation at 8,000 rpm for 10 min. Cell fresh weights were measured.

3.12.1.2 Exogenous supplementation of amino acid

S. elongatus PCC 7942 cells harboring empty vector or M2G synthetic genes were cultured in BG11 liquid medium with streptomycin (50 μg/ml) supplemented with 0.35 M NaCl or 0.35 M KCl. The culture media were added with glycine and serine (at final concentration of 1 mM). Initial cell concentration was adjusted to optical density at 730 nm $\cong$ 0.8-1.0. Calls were harvested at 0, 7 and 14 days by centrifugation at 8,000 rpm for 10 minutes. Cell fresh weights were measured.

3.12.1.3 M2G extraction

The harvesting cells were extracted by adding 500 μl of 100% high performance liquid chromatography (HPLC)-grade methanol and were disrupted by sonication with 40% amplitude for 30 seconds (repeated for 5 times). The extracted cells were centrifuged at 12,000 rpm for 10 minutes. The supernatants were transferred to new Eppendorf microcentrifuge tubes and were evaporated in a vacuum evaporator at 45 °C. Dried residues were kept in -20 °C for further use in HPLC analysis.

3.12.1.4 High performance liquid chromatography (HPLC)

The extract residues from step 3.12.1.3 were dissolved in 100-200 μl of 1% acetic acid. Dissolved samples were mixed for 15 minutes. To collect the supernatant, samples were centrifuged at 15,000 rpm for 15 min at 25 ℃. After that, the upper water phase was passed through an Ultracel YM-3 membrane (Millipore, USA) by centrifugation at 15,000 rpm at 25 ℃. The flowthroughs were subjected to HPLC analysis.

Ten microliters of each filtrated samples from previous step were injected into Shim-pack FC-ODS reverse phase (3 μm: 150x4.6 ml) with flow rate 0.4 ml/min. The column oven temperature was set at 35 ℃. The mobile phases were 1% acetic acid in H_2O and 100% methanol. The absorption spectra of samples were detected at 330 nm. Shinorine was used as authentic compound. The M2G content of each samples were calculated by comparing with standard calibration curve.

3.12.2 Amino acids analysis

3.12.2.1 Stress treatment

S. elongatus PCC 7942 cells harboring empty vector or M2G synthetic genes were cultured in BG11 liquid medium with streptomycin (50 μg/ml) supplemented with 0.35 M NaCl or 0.35 M KCl. Initial cell concentration was adjusted to optical density at 730 nm ≅ 0.8-1.0. Calls were harvested after 2 days by centrifugation at 8,000 rpm for 10 min. Cell fresh weights were measured.

3.12.2.2 Amino acids analysis

The harvested cells from stress treatment from previous step were extracted with methanol. Cell pellets were broken by sonication. The supernatant was collected by centrifugation at 12,000 rpm for 5 minutes. Then, the pellets were dried by using centrifugal vacuum concentrator 5301 (Eppendorf,

Germany). Dried residue was dissolved in 400 µl of mobile phase (acetonitrile and methanol).

Amino acids content in control and transformant cells were measured by Amino acid analyzer L-8900 (Hitachi, Japan) as manufacture's instruction. Forty microliters of each sample was injected into column with flow rate 0.175 ml/min. The absorption spectra of amino acids in samples were detected at 570 and 440 nm. The content of amino acid in samples were compared with 17 amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine) and ammonia.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Plasmid extraction and analysis

4.1.1 Restriction enzymes analysis

The plasmids used in this study were obtained from the previous work (Waditee-Sirisattha *et al.*, 2014). Empty vector (pUC303) and recombinant plasmids (pUC_Ap3858-3855_303) were extracted and performed restriction enzymes analysis by single digestion using *Bam*HI or *Xho*I. The result showed that the size of inserts that appeared after digestion by *Bam*HI and *Xho*I were 2.3 and 3.6 kb, respectively (Figure 4). These insert sizes confirmed the correct ones. Thus, the purified plasmids were further used in transformation into the fresh water cyanobacteria *S. elongatus* PCC 7942.
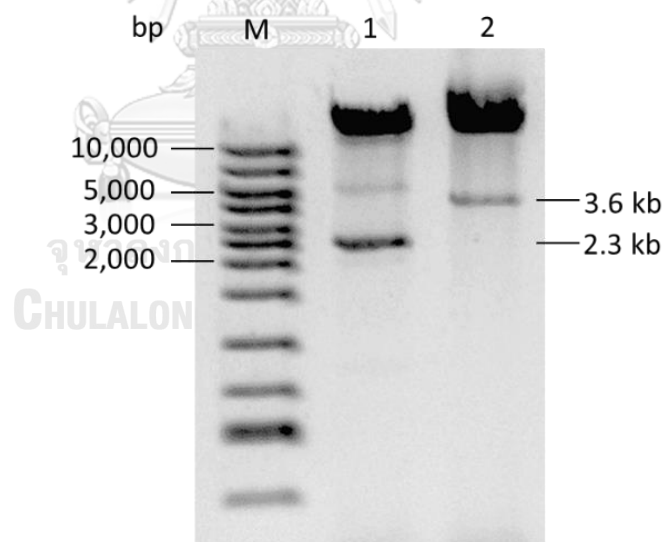
Figure 4. Restriction enzyme analysis of biosynthetic gene cluster for M2G synthesis genes in pUC_Ap3858-3855_303 (map of the expressing vector was described in Materials and Methods section 3.9). Single digestion of pUC_Ap3858-3855_303 with *Bam*HI (lane 1) and *Xho*I (lane 2). Agarose gel electrophoresis 1.2 % (w/v) precasting with 0.1 μl/ml of SYBR® safe DNA gel stain was used to analyze restriction enzyme reaction.

4.1.2 Polycistronic transcription analysis

To analyse mono- or polycistronic transcription of biosynthetic gene cluster for M2G, cDNA derived from *A. halophytica* and the plasmids obtained from section 3.1 were used. The gene organization of *Ap3858-3855* were showed in Figure 5A. The transcription of *Ap3856-3855* genes were expected to be polycistronic transcription as these two genes lining very close in gene organization. The analysis was performed by using specific primer pairs for *Ap3856* and *Ap3855* as showed in black arrow sign in Figure 5A. There was no PCR product when empty vector was used as template. The band size 1,869 bp was detected in expressing vector due to no transcription of gene cluster (Figure 5B). By using cDNA of *A. halophytica,* it showed that there was no PCR product. Therefore, this result confirmed that *Ap3856-Ap3855* genes are not polycistronic transcription. When *A. halophytica* cDNA was used as templates with specific primer pairs for *Ap3856* (forward-reverse) and *Ap3855* (forward-reverse), it clearly showed that PCR product band were detected as 422 and 521 bp, respectively.

Polycistronic transcription analysis revealed that these 4 genes in M2G biosynthetic gene cluster are independently transcribed. Therefore, these genes likely transcript as "monocistronic mRNA".
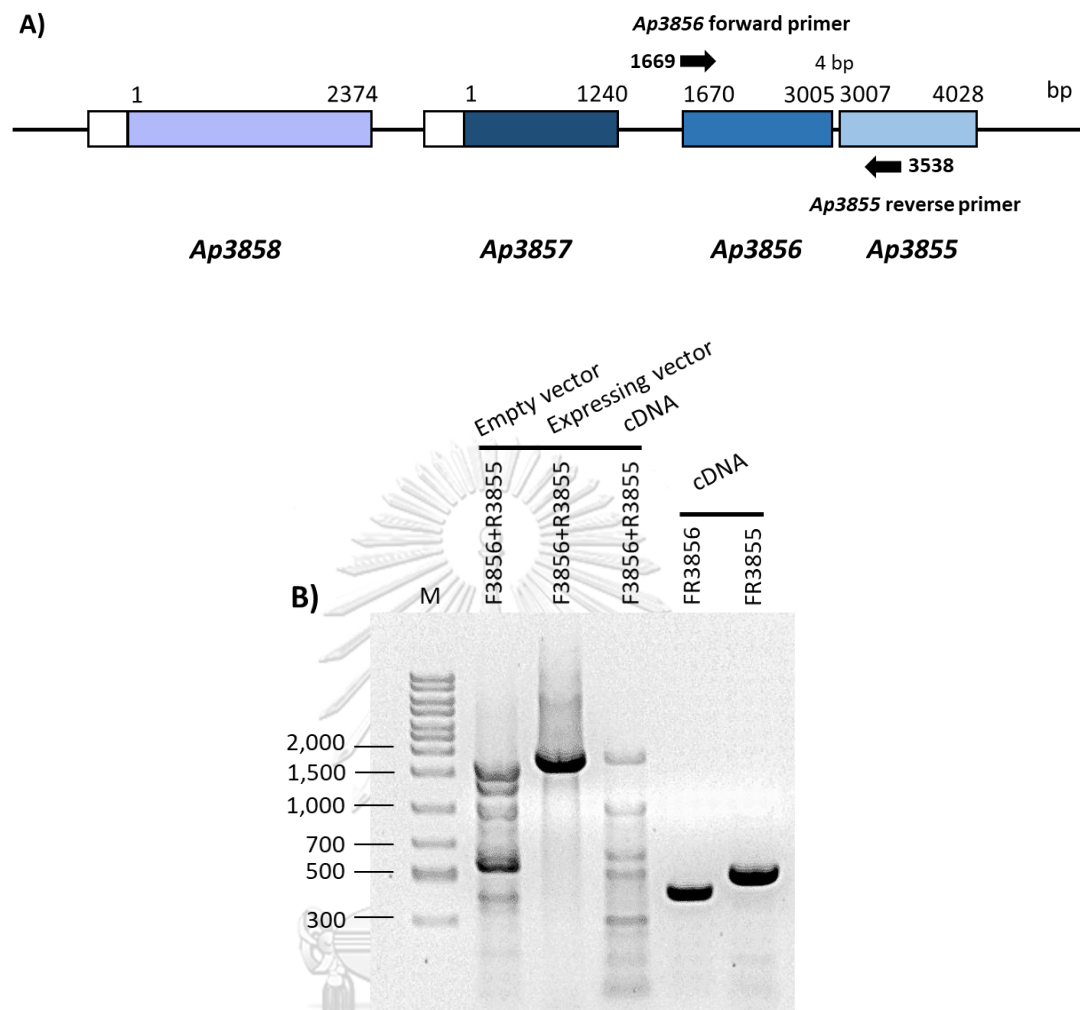
Figure 5. Gene organization of *Ap3858-3855* in *A. halophytica.* The direction of *Ap5856* forward primer and *Ap5855* reverse primer were shown (A). Polycistronic transcription analysis was performed by PCR with specific primer pairs. The first three lanes were analyzed by using *Ap3856* forward primer and *Ap3855* reverse primer. The last two lanes were analyzed by using specific primers: *Ap3856* and *Ap3855*, respectively (B).

4.2 Transformation of *S. elongatus* PCC 7942 and stress treatment

4.2.1 Transformation of M2G biosynthetic gene cluster

The purified pUC303 and pUC_Ap3858-3855_303 plasmids were transformed into *S. elongatus* PCC 7942 by natural transformation for overnight under dark condition at 25 °C. The recovery of transformant cells were performed in 2 separate sets. Streptomycin (50 μg/ml) was used as a selectable marker for transformant cells. The results showed that transformant cells could not grow on BG11 agar plate. However, transformant cells could be recovered in BG11 broth with streptomycin supplementation. As showed in Figure 6A, only transformant cells harboring expressing vector could grow in BG11 supplemented with streptomycin. After 7 days of cultivation, candidate transformants were streak onto BG11 agar plate supplemented with streptomycin to obtain single colony (Figure 6B).
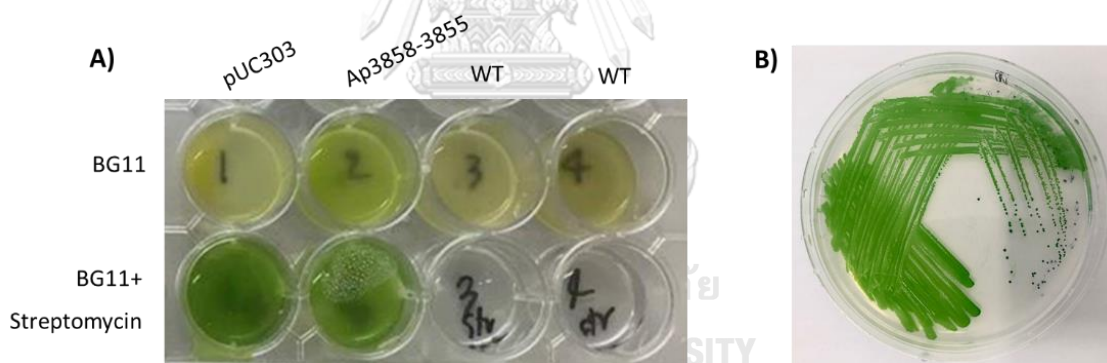
Figure 6. Culture of candidate *S. elongatus* PCC 7942 transformants in 12 wells plates (A); Candidate transformants on BG11 plate supplemented with streptomycin (50 μg/ml) (B).

To confirm the presence of M2G biosynthetic gene cluster in transformant cells, the single colony was used as a template for colony PCR analysis with specific primer pairs for *Ap3858, Ap3857, Ap3856,* and *Ap3855* genes, respectively. Colony PCR analysis revealed the successful of transformation. The specific PCR products of *Ap3858-3855* genes were detected for each gene (Figure 7). While, no band was detected when empty vector was used as template.
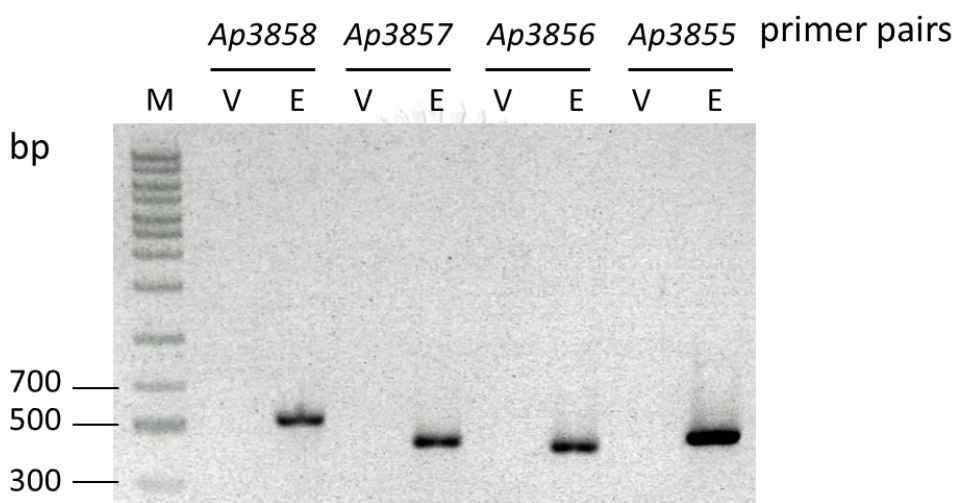
Figure 7. *S. elongatus* PCC 7942 transformant harboring *Ap3858-3855* was analyzed by colony PCR with specific primer sets for *Ap3858* to *Ap3855*. PCR products were analyzed on 1.2 % (w/v) agarose gel precasting with 0.1 µl/ml SYBR Safe DNA Gel Stain. V: *S. elongatus* PCC 7942 harboring pUC303; E: *S. elongatus* PCC 7942 transformant carrying pUC_Ap3858-3855_303.

4.3 Transformant analyses

4.3.1 Growth profile

S. elongatus PCC 7942 cells harboring empty vector or M2G synthetic genes were growth under salt stress. Growth rate was measured by UV-visible spectrophotometer at absorbance 730 nm. The results showed that transformant cells harboring MAA synthetic genes had higher growth rate than the cells harboring empty vector in BG11 medium (Figure 8). Along with the cultivation in 0.35 M NaCl, the growth rate was no significantly different in both groups in early period of cultivation. After 6 days, growth rate of transformant cells harboring M2G synthetic genes was significantly faster than empty vector. The growth rate of both sets continuously increased up to 7 days of cultivation. However, the growth rate of transformants cells cultured in both salts (NaCl and KCl) were lower than cultured in BG11 medium. In contrary, cultivation in 0.35 M KCl, the growth rate of both sets could not survive.

Apart from the major role of M2G as UV protective compound, from these results indicated that the cells harboring M2G synthetic genes confer NaCl stress tolerance. It is likely the biosynthesis of M2G in the transformed *S. elongatus* PCC 7942 prevent cells from NaCl stress.
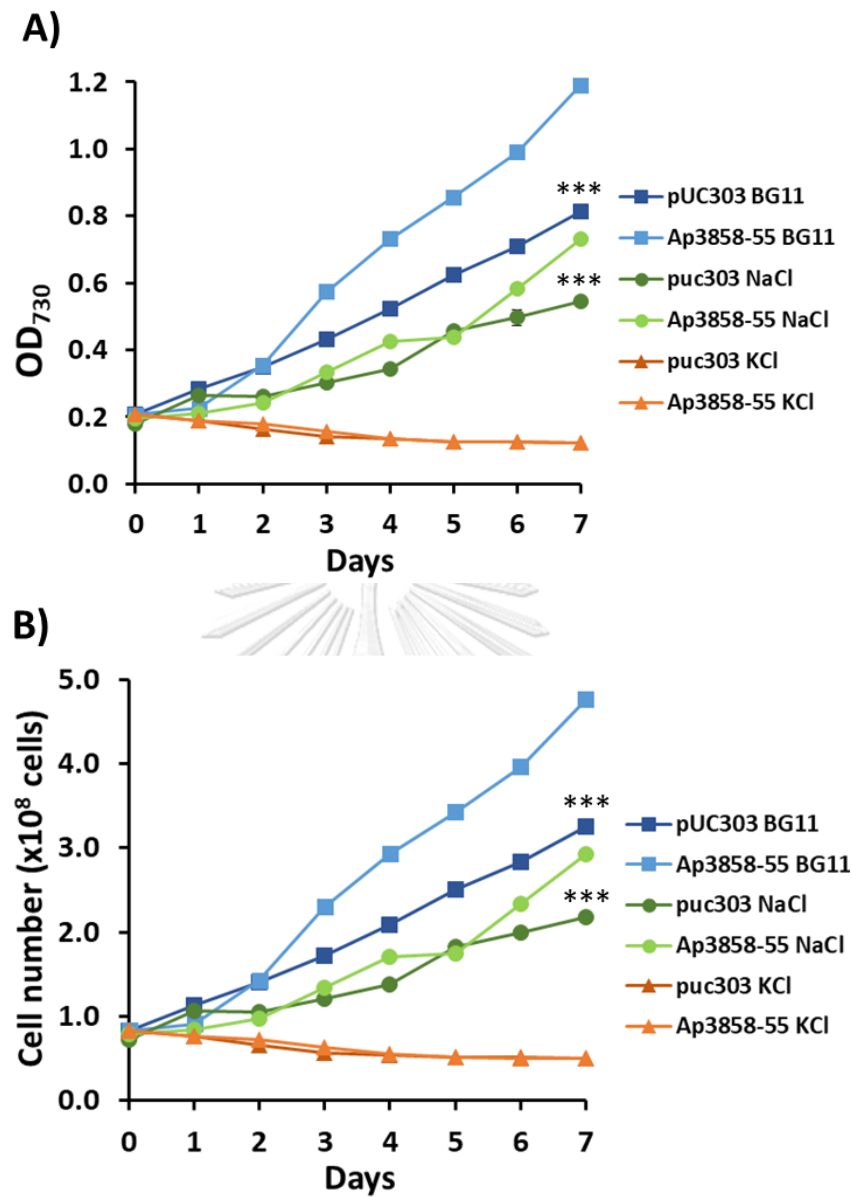
Figure 8. Growth of *S. elongatus* PCC 7942 harboring empty vector (pUC303) or MAA synthetic genes (*Ap3858-3855*) cultured in BG11 under salt stress: 0.35 M NaCl and 0.35 M KCl for 7 days. Growth profile was measured by spectrophotometer at absorbance 730 nm (A). Cell numbers were calculated by standard conversion protocol for unicellular cyanobacteria (Colowick & Kaplan, 1988) (B). Data are mean ± standard error of mean (SEM) from at least three independent experiments. *** denoted significantly differences by unpaired student's t-test (p < 0.0001).

4.3.2 Phenotypic analysis

Since the transformant cells conferred NaCl stress tolerance (Figure 8) so chlorophyll and phycobiliprotein contents were further measured. The result showed that in BG11 and 0.35 M NaCl supplemented media, all of pigments in transformant cells harboring M2G synthetic genes were always higher than those of empty vector ones (Figure 9). The pigments content of both transformants continuously increased up to 7 days of cultivation. However, pigment contents of transformants cells cultured in both salts were lower than culturing in BG11 medium. This is consistent with the growth rate in previous section (Figure 8). The pigments content of both transformants cultured in 0.35 M KCl slightly decreased as the decrease of growth rate. Therefore, the production of pigments in *S. elongatus* PCC 7942 was consistent to the growth rate and was not effect by salt stress.

Under salt stress, chlorophyll and phycobiliprotein contents were decreased due to the degeneration of the cells. In M2G harboring cells, chlorophyll and phycobiliprotein contents were higher than that of empty vector ones (Figure 9A-D). It is probable that the biosynthesis of M2G in the transformed *S. elongatus* PCC 7942 relieves cells from NaCl stress.
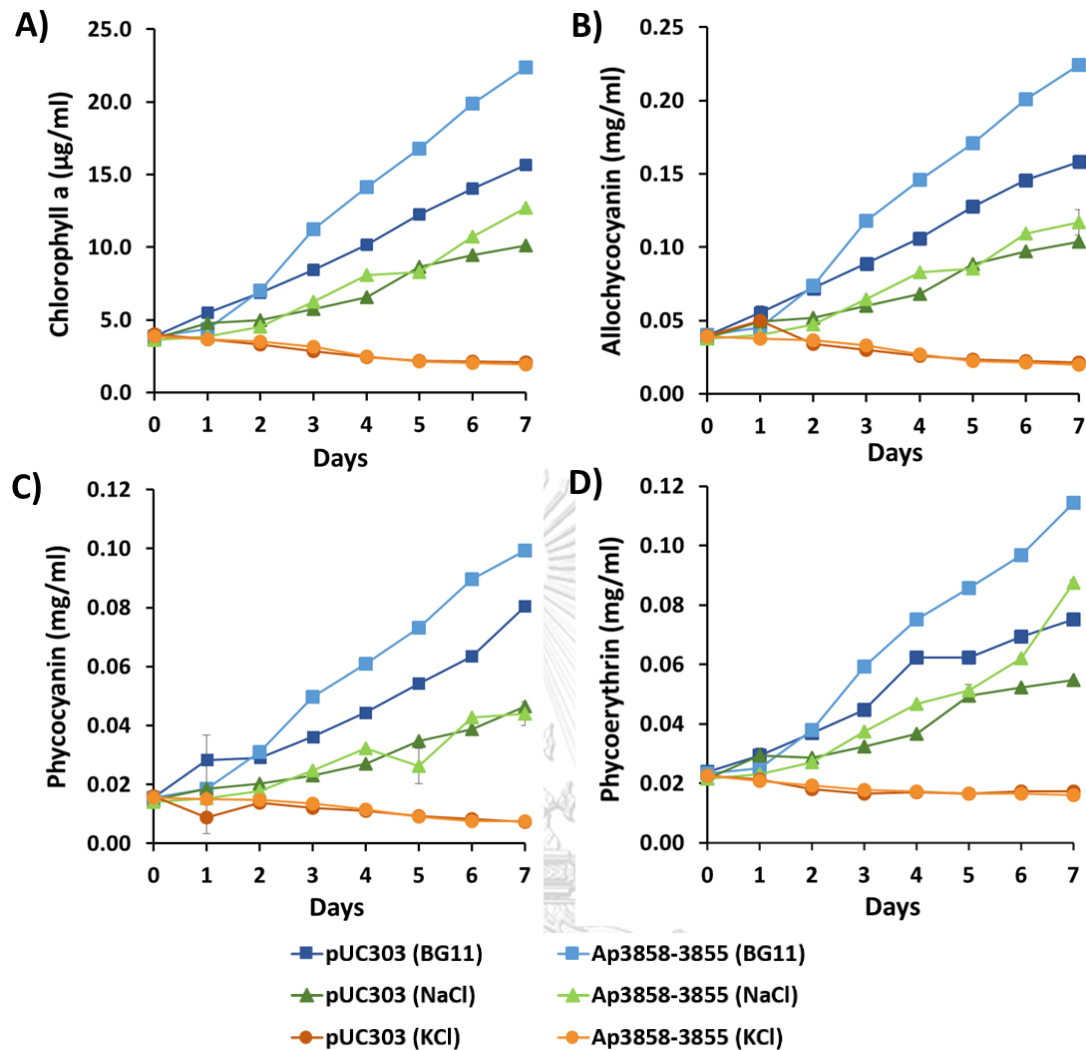
Figure 9. Chlorophyll and phycobiliproteins of *S. elongatus* PCC 7942 harboring empty vector (pUC303) or MAA synthetic genes (*Ap3858-3855*) grown in BG11, 0.35 M NaCl, and 0.35 M KCl, respectively. The content of chlorophyll a (A), allophycocyanin (B), phycocyanin (C) and phycoerythrin (D) were determined follow the standard protocol (Colowick & Kaplan, 1988).

4.4 Gene expression analysis by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

4.4.1 Expression of M2G synthetic gene cluster

M2G biosynthetic gene cluster was analyzed at transcriptional level to monitor the effects of salt stress in heterologous expression. *S. elongatus* PCC 7942 cells harboring empty vector (control) and pUC_Ap3858-3855_303 (expressing cells) were upshocked in concentrations of 0.35 M KCl and 0.35 M NaCl for 0, 4, and 8 hours, respectively. Total RNA was extracted by using Trizol® reagent. RNA integrity was confirmed by agarose gel electrophoresis (Figure 10). *Syn7942rnpB* was served as an internal control.

The transcriptional analysis showed that all of 4 M2G biosynthetic genes (*Ap3858-3855*) were up-regulated under 0.35 M NaCl condition, all of M2G biosynthetic genes (*Ap3858-3855*) were up-regulated. The highest expression levels were found in *Ap3857*, followed by *Ap3856, Ap3858* and *Ap3855*, respectively. Expression of *Ap3857*, *Ap3856*, *Ap3858* and *Ap3855* were increased for 2.39 ± 0.25, 1.68 ± 0.10, 1.25 ± 0.09, and 1.11 ± 0.06 folds, respectively (Figure 11A). The same trend was observed under 0.35 M KCl stress. The highest expression levels was found in *Ap3856*, followed by *Ap3857*, *Ap3858* and *Ap3855*, respectively. Expression of *Ap3856*, *Ap3857*, *Ap3858* and *Ap3855* were increased in transcriptional level for 3.70 ± 0.35, 3.59 ± 0.45, 2.78 ± 0.13, and 2.73 ± 0.47 fold, respectively (Figure 11B).

In accordance with the previous work, transcription of M2G biosynthesis in the halotolerant cyanobacterium *A. halophytica* under salt stress condition (2.50 M NaCl) were up-regulated. An increase up to 8-fold was found in *Ap3858* (Waditee-Sirisattha *et al.,* 2014). Cheewinthamrongrod *et al* (2015) reported the heterologous expression of M2G biosynthetic gene cluster in *E. coli.* The result showed the upregulation of transcription level of all M2G biosynthesis genes and induced by ionic stress but not osmotic stress.

The results obtained in this study suggested that M2G biosynthetic genes from *A. halophytica* can be highly expressed at transcriptional level in photosynthetic microorganisms as well as observing in bacteria.
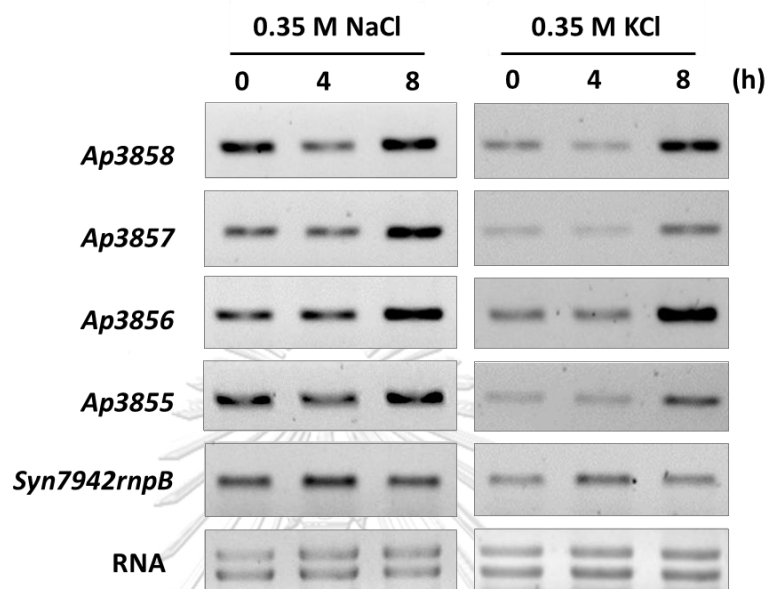
Figure 10. Transcriptional analysis of M2G biosynthesis genes: *Ap3858-3855* in *S. elongatus* PCC 7942 expressing cells after upshocking in salinity stress (NaCl or KCl) for 0, 4, and 8 hours. cDNA was prepared by using SuperScript® III First-strand. RT-PCR analysis was performed as described in Materials and Methods. *Syn7942rnpB* was used as an internal control. PCR products were subjected to 1.2 % (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml of SYBR® safe DNA gel stain.
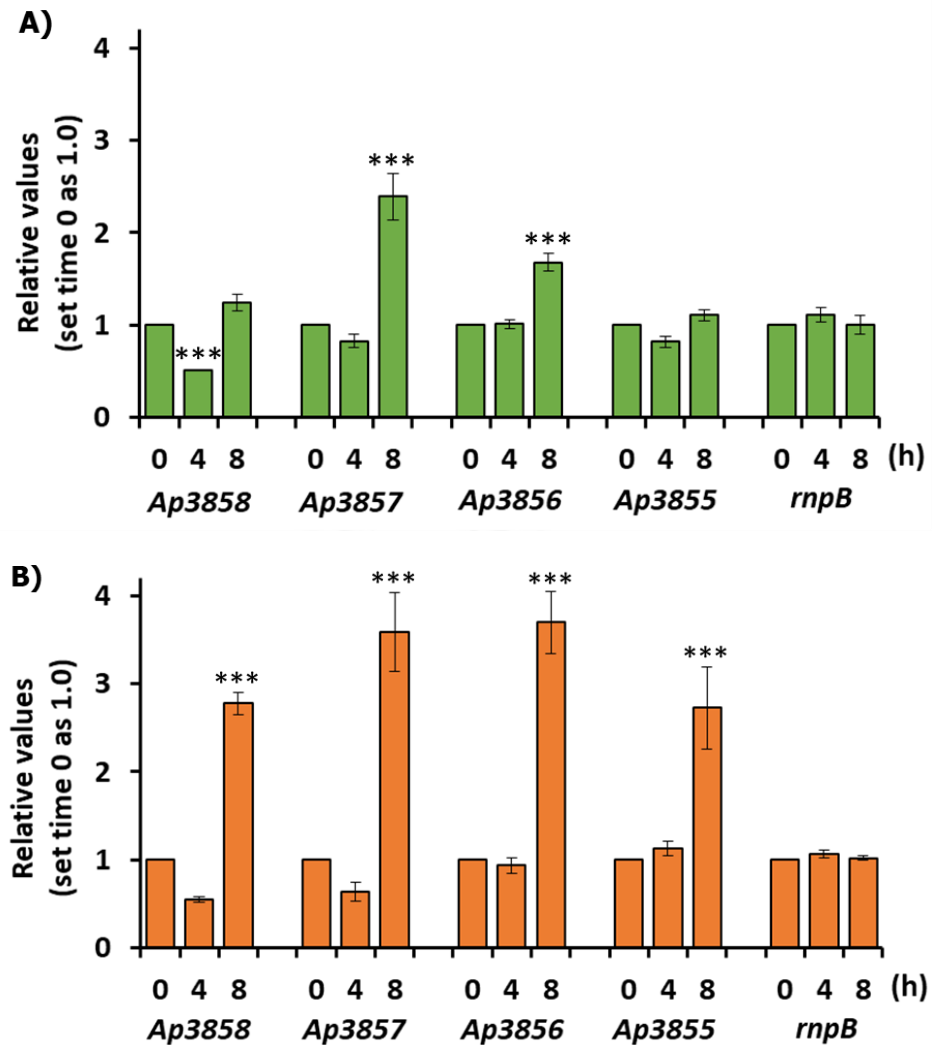
Figure 11. Relative intensity of the specific PCR products of M2G biosynthesis genes: *Ap3858-3855* genes after upshocking for 0, 4, and 8 hours in salinity stress: 0.35 M NaCl (A) and 0.35 M KCl (B). Relative values were analyzed by ImageJ program. Data are mean ± standard error of mean (SEM) from at least three independent experiments (*** $p < 0.001$ by two-way ANOVA).

4.4.2 Expression of antioxidant-related genes

Subjecting to salt stress is eventually induced oxidative stress, we therefore examined transcriptional of antioxidant-related genes. Three antioxidant-related genes were analyzed in this study. NaCl and KCl were used as salt stresses as the same procedures as in section 4.1. An equal internal control was determined by using *Syn7942rnpB* specific primer pairs (Figure 12A, 13A).

Under 0.35 M NaCl condition, the expression of three antioxidant-related genes (*sodB, cat* and *tpxA*) were all up-regulated in empty vector control. The highest up-regulated was observed in *tpxA,* followed *sodB* and *cat.* The expression level of *sodB, cat* and *tpxA* in were increased for 2.09 ± 0.07, 2.05 ± 0.52 and 1.61 ± 0.23 folds, respectively. In expressing cells, there were no statistically significant differences in the expression of *sodB* and *cat* genes. The up-regulated only observed in *tpxA*. The expression level of *tpxA* was increased for 4.58 ± 0.58 folds (Figure 12B, C and D).

Under 0.35 M KCl condition in empty vector control, the expression of *sodB* and *tpxA* were up-regulated. In contrast, the expression of *cat* gene was downregulated. The expression level of *sodB, cat* and *tpxA* at 8 hours of upshocking were 3.37 ± 0.40, 0.45 ± 0.13 and 12.45 ± 0.64 folds, respectively. In expressing cells, the highly up-regulated were observed in *sodB* and *tpxA* genes. The expression level of *sodB* and *tpxA* was increased for 4.09 ± 0.86 and 11.81 ± 1.02 folds, respectively. There was no statistically significant difference in the expression of *cat* gene (Figure 13B, C and D).

This results indicated that the expression of antioxidant related genes tend to be up-regulated in the expressing cells under salt stress. This result was consistent with heterologous expression of M2G biosynthetic genes in *S. elongatus* PCC 7942 under H_2O_2 stress condition (Tarasuntisuk, 2017).
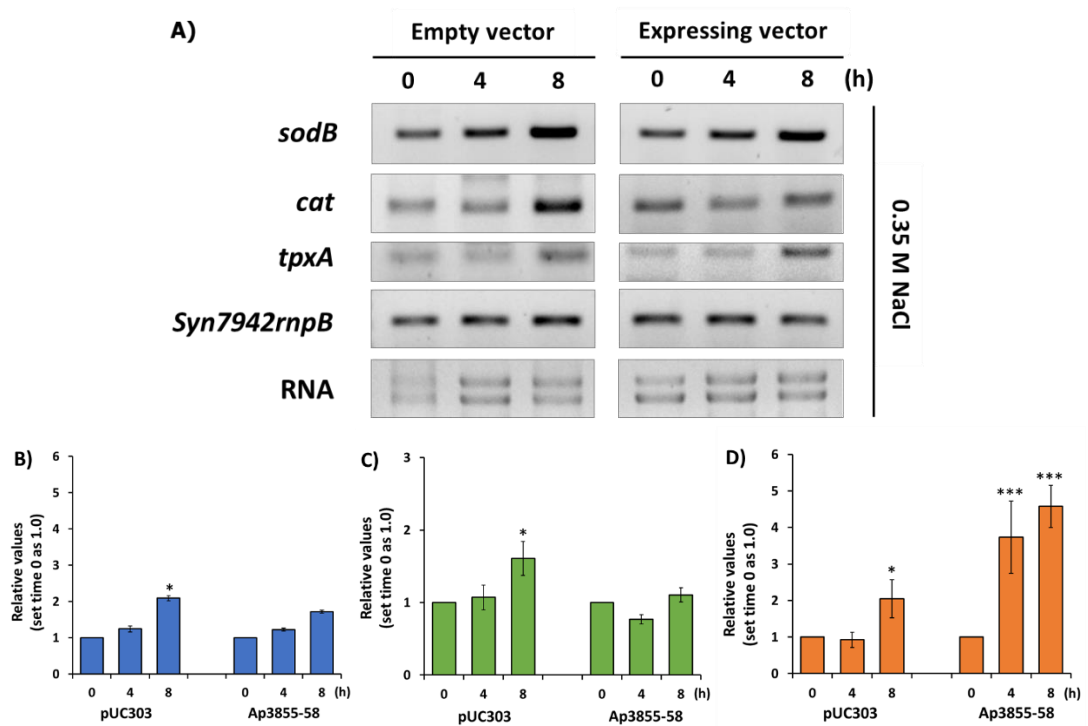
Figure 12. Transcriptional analysis of antioxidant related genes: *sodB, cat* and *tpxA* genes in *S. elongatus* PCC 7942 expressing cells after upshocking in 0.35 M NaCl for 0, 4, and 8 hours (A). cDNA was prepared by using SuperScript® III First-strand. RT-PCR analysis was performed as described in Materials and Methods. *Syn7942rnpB* was used as an internal control. PCR products were subjected to 1.2 % (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml of SYBR® safe DNA gel stain. Relative intensity of the specific PCR products of antioxidant related genes: *sodB* (B), *cat* (C) and *tpxA* (D) were analyzed by ImageJ program. Data are mean ± standard error of mean (SEM) from at least three experiments (* $p < 0.05$, *** $p < 0.001$ by two-way ANOVA).

Figure 13. Transcriptional analysis of antioxidant related genes: *sodB*, *cat* and *tpxA* genes in *S. elongatus* PCC 7942 expressing cells after upshocking in 0.35 M KCl for 0, 4, and 8 hours (A). cDNA was prepared by using SuperScript® III First-strand. RT-PCR analysis was performed as described in Materials and Methods. *Syn7942rnpB* was used as an internal control. PCR products were subjected to 1.2 % (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml of SYBR® safe DNA gel stain. Relative intensity of the specific PCR products of antioxidant related genes: *sodB* (B), *cat* (C) and *tpxA* (D) were analyzed by ImageJ program. Data are mean ± standard error of mean (SEM) from at least three experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA).

4.4.3 Expression of glycerol and sucrose synthetic genes

Glycerol and sucrose are important osmolytes responding to stress condition in cyanobacteria, ionic and osmotic stresses in particular.

In this study, sucrose phosphate synthase (*Synpcc7942_0808*) and glycerol-3-phosphate dehydrogenase (*Synpcc7942_2522*) genes were additionally analyzed at transcriptional level to monitor the effects of salt stress in heterologous expressing cells. NaCl and KCl were used as salt stresses with similar procedures as in section 4.1. An equal internal control was determined by using *Syn7942rnpB* specific primer pairs (Figure 14A, 15A).
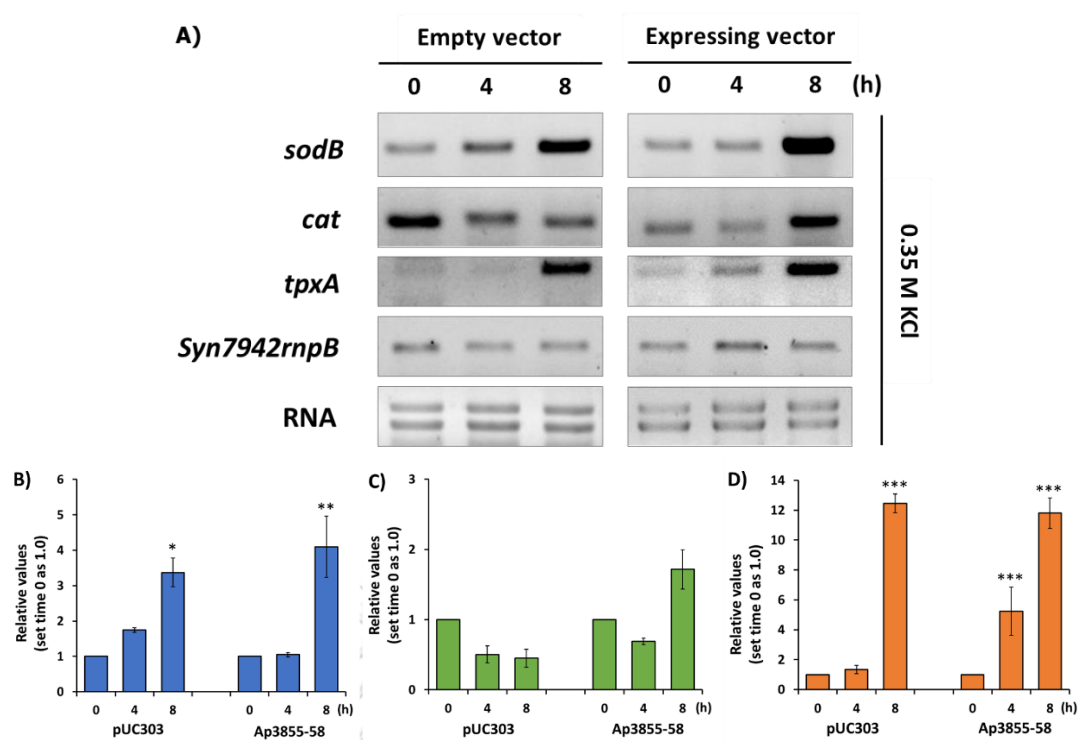
Under 0.35 M NaCl condition, there was no statistically significant difference in the expression of *Synpcc7942_0808* in empty vector harboring cells. While *Synpcc7942_2522* was downregulated. In expressing cells, *Synpcc7942_0808* was highly up-regulated. The expression level was about 3.89 ± 0.30 fold. While *Synpcc7942_2522,* there was no statistically significant difference in the expression level (Figure 14B, C).

Under 0.35 M KCl, the expression of *Synpcc7942_0808* and *Synpcc7942_2522* were modulated in empty vector harboring cells. In contrast, gene expression in expressing cells, *Synpcc7942_0808* and *Synpcc7942_2522* were highly up-regulated. The expression level of *Synpcc7942_0808* and *Synpcc7942_2522* were increased for 9.40 ± 0.88 and 3.52 ± 0.29 fold, respectively (Figure 15B, C). Expression analysis of sucrose phosphate synthase (SPS) in a fresh water cyanobactrium *Synechocystis* sp. PCC 6803 showed that *SPS* was up-regulated after treating with NaCl (Cumino *et al.,* 2010). To date, the upregulation of glycerol-3-phosphate dehydrogenase (G3P-D) under salt stress in cyanobacteria has never been reported.

From our result, both sucrose and glycerol synthetic genes were up-regulated under salt stress. This can be explained that many cyanobacteria naturally synthesize and accumulate glycerol and sucrose in response to abiotic stress. SPS catalyzes the rate-limiting step in sucrose synthesis. Induction of sucrose production in a sucrose-secreting strain of *S. elongatus* PCC 7942 showed that glycogen could serve as a supportive carbon pool for the synthesis of sucrose (Qiao *et al.,* 2018). The

synthesis of low-molecular weight molecules, such as glycerol or sucrose is one of the strategies to control the osmotic balance in cyanobactrial cells (Oren and Gunde-Cimerman, 2007).

M2G biosynthetic gene cluster induced the expression of glycerol and sucrose synthetic genes. This likely promoted expressing cells to be tolerated to salt stress. This result consistent with growth rate and phenotype observing in previous sections (Figure 8 and 9).
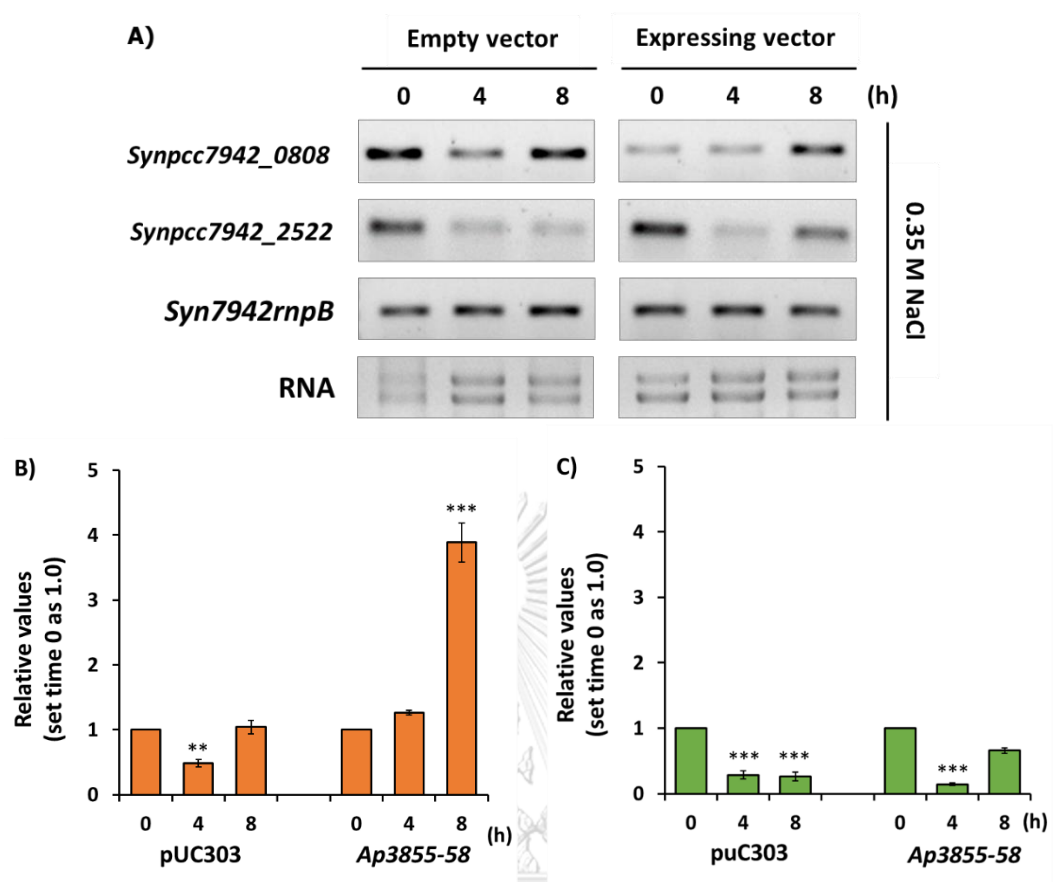
Figure 14. Transcriptional analysis of glycerol and sucrose synthetic genes: *Synpcc7942_0808* and *Synpcc7942_2522* genes in *S. elongatus* PCC 7942 transformant after upshocking in 0.35 M NaCl for 0, 4, and 8 hours (A). The cDNA was prepared by using SuperScript® III First-strand. RT-PCR analysis was performed as described in Materials and Methods. *Syn7942rnpB* was used as an internal control. PCR products were subjected to 1.2 % (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml of SYBR® safe DNA gel stain. Relative intensity of the specific PCR product bands of glycerol and sucrose synthetic genes: *Synpcc7942_0808* (B) and *Synpcc7942_2522* (C) were analyzed by ImageJ program. Data are mean ± standard error of mean (SEM) from at least three experiments (** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA).
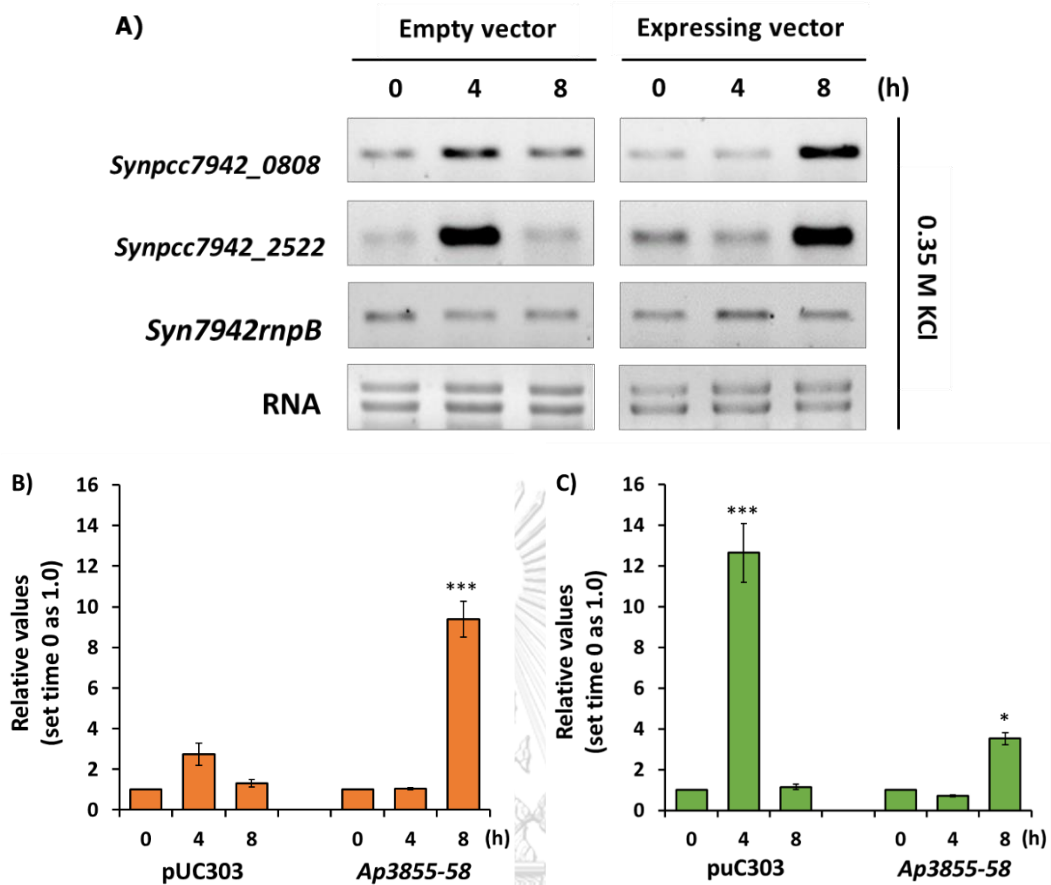
Figure 15. Transcriptional analysis of glycerol and sucrose synthetic genes: *Synpcc7942_0808* and *Synpcc7942_2522* genes in *S. elongatus* PCC 7942 transformant after upshocking in 0.35 M KCl for 0, 4, and 8 hours (A). The cDNA was prepared by using SuperScript® III First-strand. RT-PCR analysis was performed as described in Materials and Methods. *Syn7942rnpB* was used as an internal control. PCR products were subjected to 1.2 % (w/v) agarose gel electrophoresis precasting with 0.1 µl/ml of SYBR® safe DNA gel stain. Relative intensity of the specific PCR product bands of glycerol and sucrose synthetic genes: *Synpcc7942_0808* (B) and *Synpcc7942_2522* (C) were analyzed by ImageJ program. Data are mean ± standard error of mean (SEM) from at least three experiments (* $p < 0.05$, *** $p < 0.001$ by two-way ANOVA).

4.5 Protein expression analysis

In addition to the transcriptional analysis, protein expression analysis was also performed. To analyze the expression of M2G synthetic protein, SDS-PAGE and western blotting were carried out. Control and expressing cells were cultured under salt stress. Cells were harvested at 0, 24, 48 and 96 hours for crude protein preparation. Equal amounts of proteins (200 µg) were subjected to the analysis. SDS-PAGE could not detected the induced or specific bands of target proteins (Figure 16A, B). The expression of target protein was further analyzed by western blotting.

Due to the construction of M2G biosynthetic genes contained 6X Histidine tag at C-terminus of *Ap3858* and *Ap3855* genes (Figure 3). Therefore, the detection of target protein can be probed by using antibody raised against 6X Histidine. Western blotting analysis of expressing cells harboring *Ap3858-3855* genes showed the target protein band of DDG-synthase corresponding to 69.7 kDa (Waditee-Sirisattha *et al.*, 2014) (Figure 16D). However, the target protein band of D-ala-D-ala ligase could not be detected likely due to the low level of expression.

The expression of target protein was highly induced in the M2G expressing cells cultured in 0.35 M NaCl. The target protein band could not be detected at 0 hour (before upshocking). This is likely due to a low translational level. The strongest target protein band was clearly observed at 96 hours of upshocking. In contrast, under 0.35 M KCl condition, this salt was not affected the expression of *Ap3858* (DDG-synthase).

The relative intensity of each protein band was analyzed by ImageJ program. The intensity of target protein band at 24 hour was used to normalize the signals by set as 1. Under 0.35 M KCl condition, the relative intensity of protein bands were equally throughout 96 hours of cultivation. In contrast, using 0.35 M NaCl condition, the relative intensity of protein bands progressively increased until reaching the highest level to about 7.20 folds at 96 hours of upshocking.

These results can be concluded that M2G biosynthetic protein was up-regulated by salt stress (0.35 M NaCl). Our western blotting analysis was consistent with the RT-PCR result, in which showing the upregulation of M2G biosynthesis gene cluster.
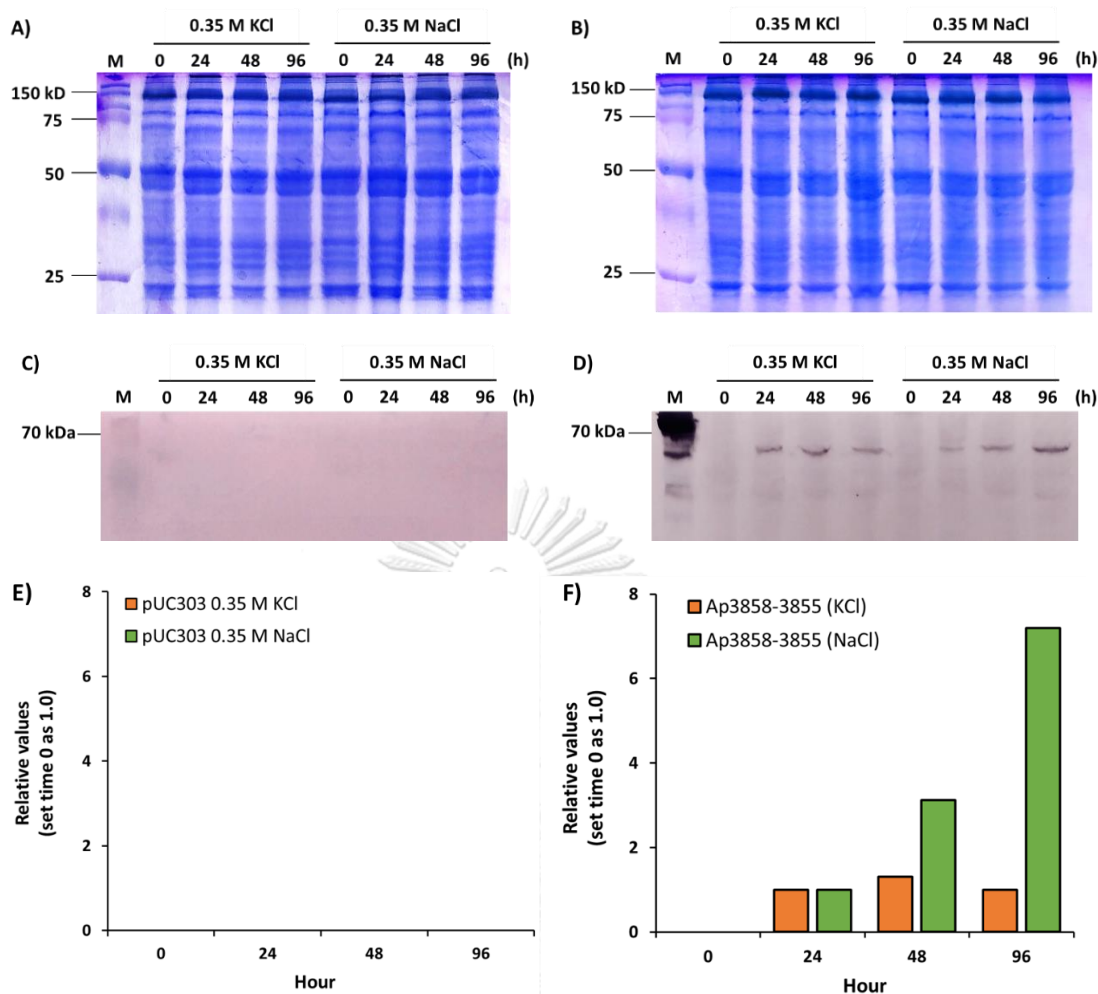
Figure 16. SDS-PAGE and western blotting analyses. Crude protein extracts were prepared and were measured protein concentration. SDS-PAGE and western blotting were carried out as described in section 3.11.4.3-4. Equal amounts of proteins (200 μg) were separated on 12.5% acrylamide gel. Protein bands were stained with CBB. For western blotting, antibody raised against Histidine 6X and anti-mouse IgG were used as primary and secondary antibodies, respectively. The target protein band was visualized by BCIP/NBT solution. Intensity of the target protein band were analyzed by ImageJ. Empty vector (A, C and E) and M2G expressing cells (B, D and F).

4.6 Metabolite analysis

4.6.1 Detection of M2G

4.6.1.1 M2G production under stress treatment

We finally analyzed metabolite production (M2G) by using HPLC. As shown in HPLC chromatogram (Figure 17). Time course measurement of M2G upon salt stress was demonstrated in Figure 18. It should be noted that empty vector could not detect M2G (data not shown). M2G accumulation significantly increased when expressing cells were grown in both salinity conditions. Under 0.35 M NaCl stress condition, M2G accumulation was continuously increased from 0 day to 7 day of upshocking. The highest M2G level was observed about 5.05 ± 2.03 ng/mg fresh weight at 7 day of cultivation. In KCl stress condition, M2G accumulation was slightly increased. M2G level reached to 4.08 ± 1.03 ng/mg fresh weight at 7 day of cultivation, which lower than in 0.35 M NaCl condition. M2G biosynthesis in *S. elongatus* PCC 7942 expressing cells was shown to respond to salinity both 0.35 M KCl and 0.35 M NaCl. Metabolite analysis was consistent with the upregulation of M2G biosynthetic gene cluster under salt stress (Figure 10, 11).

According to Cheewinthamrongrod *et al* (2015), the expression of M2G biosynthesis gene cluster in *E. coli* showed that M2G accumulation in *E. coli* was induced under salt stress (0.5 M KCl and 0.5 M NaCl). M2G highest level was found in 0.5 M NaCl condition which reached 2.88 ± 0.14 mg/g dry weight (2,880 ng/mg DW). This result showed that M2G accumulation in *E. coli* expressing cells was efficient than that of *S. elongatus* PCC 7942 expressing cells in this study. The lower M2G accumulation in this study may cause by several plausible reasons, such as (1) the lower transcriptional level of *Ap3855* (Figure 11), (2) insufficient substrate for M2G production, (2) the condition for inducing M2G accumulation in *S. elongatus* PCC 7942 may be different from *E. coli*. Therefore, exogenous amino acid supplementation (glycine and serine) was performed in the next section.
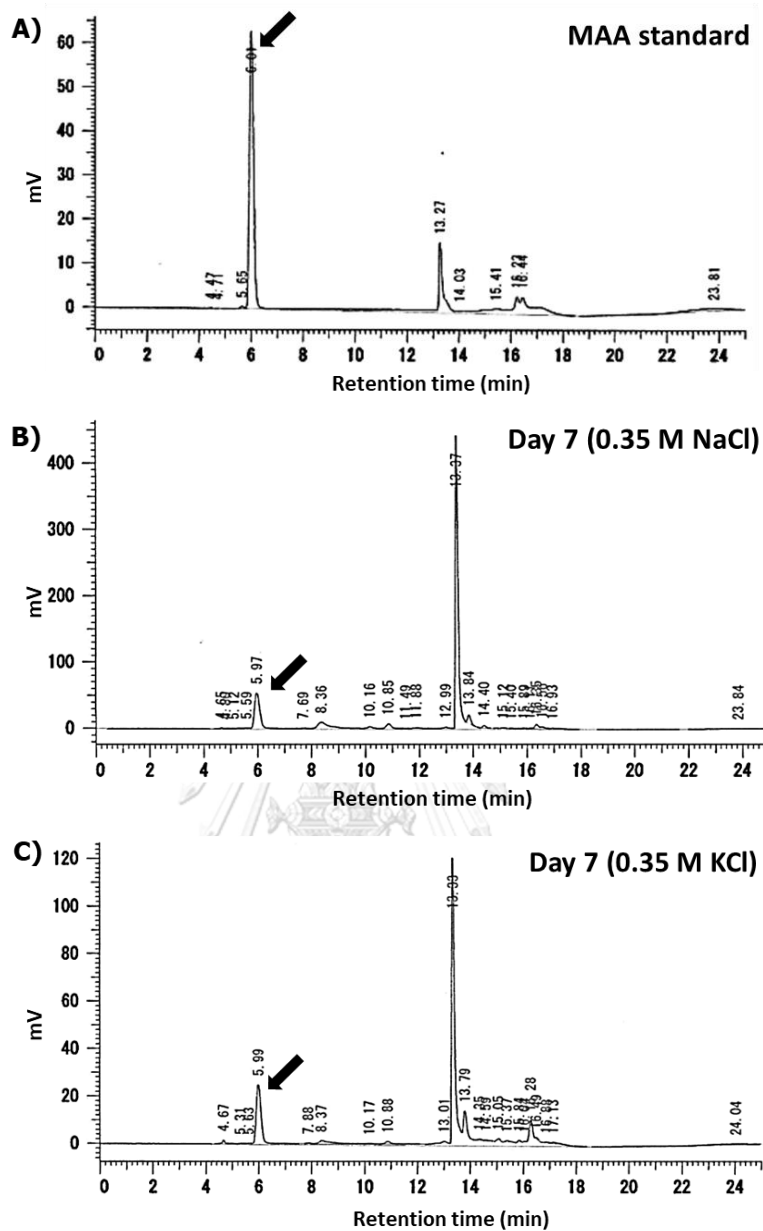
Figure 17. HPLC chromatogram. M2G was extracted and partial purified as described in section 3.12.1. *S. elongatus* PCC 7942 expressing cells cultured in BG11 under salt stress: 0.35 M KCl and 0.35 M NaCl for 7 days. Ten milliliters of each purified was injected into a Shim Pack FC-ODS reverse-phase column. M2G was detected by using a UV-VIS detector at 330 nm. The mobile phase was run at a flow rate of 0.4 ml/min. MAA (shinorine) was use as standard (A). The maximum area peak of M2G were found at day 7 of cultivation under 0.35 M NaCl (B) and 0.35 M KCl (C) conditions. The arrow symbol represents Shinorine and M2G peak.
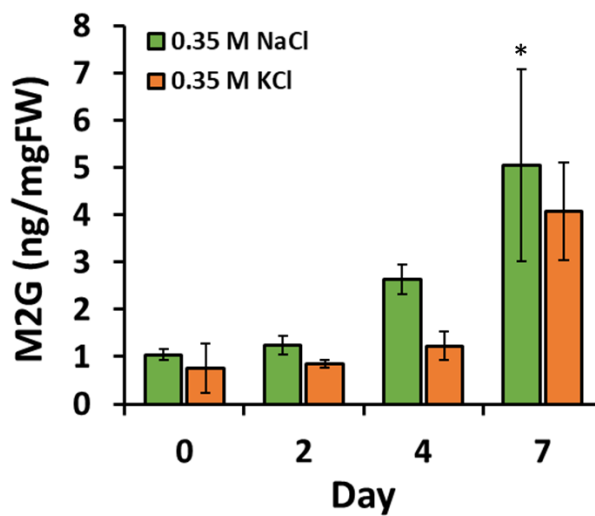
Figure 18. M2G accumulation in *S. elongatus* PCC 7942 expressing cells cultured in 0.35 M KCl and 0.35 M NaCl for 7 days. Data are mean ± standard error of mean (SEM) from at least two experiments (* $p < 0.05$ by two-way ANOVA).
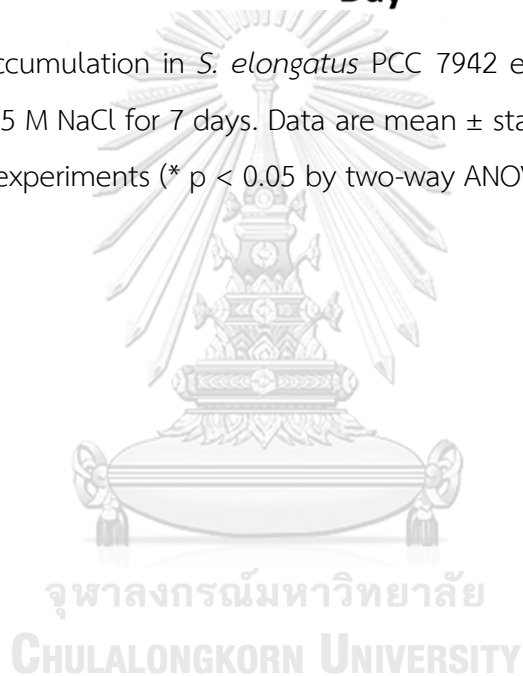
4.6.1.2 M2G production with exogenously supplementation of amino acid

M2G structure composes of two glycine molecules. Thus, glycine can be considered as cruicial substrate in the structure of M2G molecule (Waditee-Sirisattha *et al.*, 2014). Glycine can be synthesized from the interconversion of serine by serine hydroxymethyltransferase (SHMT) (Waditee-Sirisattha *et al.*, 2017). Therefore, glycine and serine were applied in this study. The exogenous supplementation of glycine and serine (final concentration 1 mM) were added into BG11 liquid medium in the presence of 0.35 M NaCl or 0.35 M KCl. The M2G content was analyzed by HPLC.

The content of M2G in *S. elongatus* PCC 7942 expressing cell culturing in both salt medium supplemented with glycine and serine was shown (Figure 19). Under 0.35 M NaCl condition, there was no different in M2G accumulation between 7 and 14 days of cultivation without amino acid supplementation. In glycine supplementation condition, M2G content was increased and reached the highest level at 7 days with 5.39 ng/mgFW. Its level was decreased at 14 days of cultivation. In serine supplementation condition, M2G content was continuously increased from 7 days and reached the highest level at 14 days of cultivation with 7.53 ng/mgFW of M2G.

Under 0.35 M KCl condition, the results was similar as observing in NaCl stress. In glycine supplementation condition, M2G content was increased to reach the highest level at 7 days with 5.96 ng/mgFW and decreased at 14 days of cultivation. In serine supplementation condition, M2G content was continuously increased from 7 days and reached the highest level at 14 days of cultivation with 6.67 ng/mgFW of M2G.
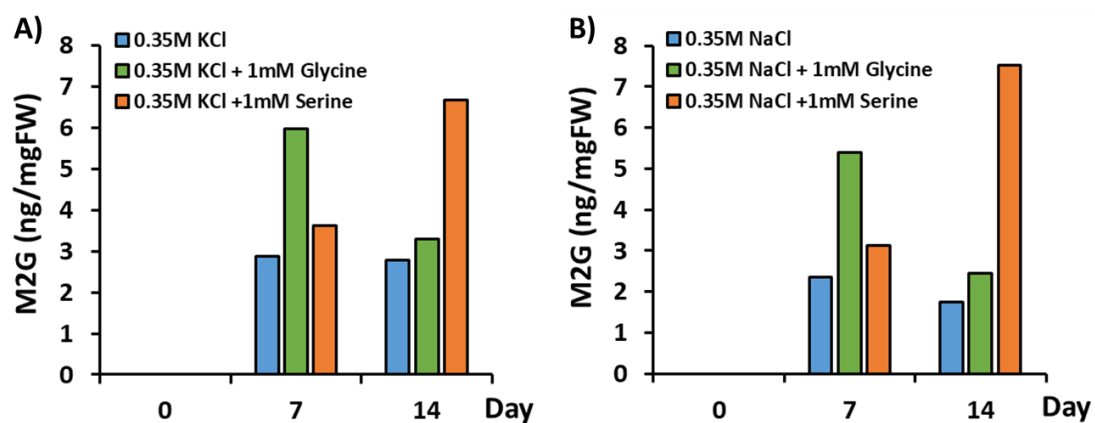
Figure 19. The content of M2G accumulation in *S. elongatus* PCC 7942 expressing cells cultured in 0.35 M KCl (A) and 0.35 M NaCl (B) for 14 days with exogenous supplementation of 1 mM glycine or serine.

4.6.2 Amino acids analysis

Amino acids (glycine and serine) are important precursors in M2G biosynthesis (see section 6.1). Under stress condition, M2G accumulation was clearly increased, suggesting glycine (and serine) must be used *in vivo* (Figure 19). Intracellular amino acid contents in *S. elongatus* PCC 7942 expressing cells were therefore analyzed (Table 5). The result showed that serine content in *S. elongatus* PCC 7942 expressing cells cultured in KCl condition were severely decreased. Lesser decreased was observed in NaCl stress. For glycine content, a slight decrease was observed in KCl condition. Whereas, the glycine content was almost unchanged in NaCl condition. It should be noted that proline content significantly increased under NaCl stress (3 folds), suggesting proline may serve as osmolite in this expressing cell. The result from amino acid analysis revealed that the M2G precursors: glycine and serine in *S. elongatus* PCC 7942 are low level. This may cause the low M2G level in expressing cells (Figure 18). In contrary, exogenious supply of glycine or serine resulted in higher M2G contents.

Table 5. Amino acids composition in *S. elongatus* PCC 7942 harboring M2G synthetic genes (*Ap3858-3855*) grown in BG11 under 0.35 M NaCl and 0.35 M KCl for 2 days. The unit was represented as nmol/mgFW.

Amino Acid	BG11	BG11+KCl	BG11+NaCl	Relative KCl/Control	Relative NaCl/Control
Alanine	25.76	36.45	26.63	1.41	1.03
Arginine	ND	1.14	0.73	ND	ND
Aspartic acid	31.24	18.09	29.29	0.58	0.94
Cystine	28.81	5.85	24.06	0.20	0.83
Glutamic acid	294.17	479.89	770.03	1.63	2.62
Glycine	5.87	3.44	7.70	0.59	1.31
Histidine	0.91	0.87	0.38	0.95	0.42
Isoleucine	0.95	2.72	1.30	2.85	1.37
Lysine	0.72	1.19	1.56	1.65	2.16
Leucine	1.83	2.52	1.82	1.38	0.99
Methionine	3.92	1.34	2.48	0.34	0.63
Phenylalanine	0.99	2.00	0.80	2.03	0.81
Serine	10.53	1.85	8.07	0.18	0.77
Threonine	5.73	4.95	6.08	0.87	1.06
Tyrosine	7.43	10.44	5.26	1.40	0.71
Valine	3.27	2.94	4.04	0.90	1.24
Proline	4.42	5.12	13.54	1.16	3.06
NH_3	5.02	11.80	20.48	2.35	4.08
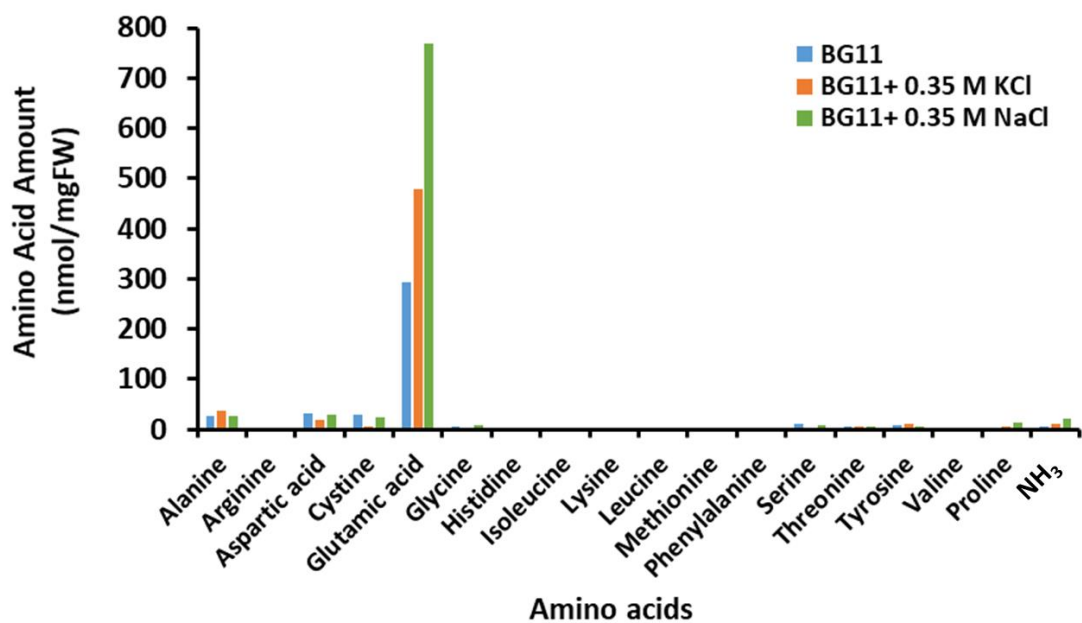
Figure 20. Amino acid analysis of *S. elongatus* PCC 7942 harboring M2G synthetic genes (*Ap3858-3855*) grown in BG11, 0.35 M KCl and 0.35 M NaCl for 2 days. The content of amino acids were determined.

CHAPTER V

CONCLUSIONS

I) M2G synthetic genes (*Ap3858-3855)* in M2G gene cluster are all monocistronic mRNAs in which independently transcribed.

II) M2G synthetic gene cluster from *A. halophytica* was successfully expressed in *S. elongatus* PCC 7942.

III) Transcriptional analysis revealed four M2G biosynthetic genes (*Ap3858, Ap3857, Ap3856,* and *Ap3855*) in expressing cells were highly expressed under salt stress (NaCl and KCl). Heterologous expression of M2G biosynthetic gene cluster in expressing cells enhanced the expression of antioxidant-related genes (*sodB*, *catB* and *tpxA*), glycerol synthetic gene (glycerol-3-phosphate dehydrogenase: *Synpcc7942_2522*), and sucrose synthetic gene (sucrose phosphate synthase: *Synpcc7942_0808*). Upregulation of these genes likely confer stress tolerant in expressing cells.

IV) Protein analysis by western blotting showed that M2G synthetic protein in expressing cells was highly induced under salt stress condition. The highest level was observed about 5.05 ± 2.03 ng/mgFW under 0.35 M NaCl condition at 7 day of cultivation.

V) Metabolite analysis revealed that M2G accumulation increased significantly when expressing cells were grown in both salinity conditions. M2G highest level was observed about 5.05 ± 2.03 ng/mg fresh weight under 0.35 M NaCl condition at 7 day of cultivation.

VI) Exogenous supplementation of amino acids increased M2G content in both salt stress. The highest content was found under the condition of 0.35 M NaCl supplemented with serine at 14 days of cultivation with M2G content was 7.35 ng/mgFW.

REFERENCES

Bebout, B. M., & Garcia-Pichel, F. **(1995).** UV B-induced vertical migrations of cyanobacteria in a microbial Mat. *Applied and Environmental Microbiology,* 61(12), 4215-4222.

Bhaya, D. **(2004).** Light matters: phototaxis and signal transduction in unicellular cyanobacteria. *Molecular Microbiology,* 53(3), 745-754.

Cheewinthamrongrod, V., Kageyama, H., Palaga, T*., et al.* **(2016).** DNA damage protecting and free radical scavenging properties of mycosporine-2-glycine from the Dead Sea cyanobacterium in A375 human melanoma cell lines. *Journal of Photochemistry and Photobiology B: Biology,* 164, 289-295.

Conde, F. R., Churio, M. S., & Previtali, C. M. **(2000).** The photoprotector mechanism of mycosporine-like amino acids. Excited-state properties and photostability of porphyra-334 in aqueous solution. *Journal of Photochemistry and Photobiology B: Biology,* 56(2), 139-144.

Cumino, A. C., Perez-Cenci, M., Giarrocco, L. E*., et al.* **(2010).** The proteins involved in sucrose synthesis in the marine cyanobacterium *Synechococcus* sp. PCC 7002 are encoded by two genes transcribed from a gene cluster. *FEBS Letters,* 584(22), 4655-4660.

D'Agostino, P. M., Javalkote, V. S., Mazmouz, R*., et al.* **(2016).** Comparative profiling and discovery of novel glycosylated mycosporine-like amino acids in two strains of the cyanobacterium *Scytonema crispum. Applied and Environmental Microbiology.*

Donkor, V. A., Amewowor, D. H. A. K., & Häder, D.-P. **(1993).** Effects of tropical solar radiation on the motility of filamentous cyanobacteria. *FEMS Microbiology Ecology,* 12(2), 143-147.

Ehling-Schulz, M., Bilger, W., & Scherer, S. **(1997).** UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune. Journal of Bacteriology,* 179(6), 1940-1945.

Ehling-Schulz, M., & Scherer, S. **(1999).** UV protection in cyanobacteria. *European Journal of Phycology,* 34(4), 329-338.

Ehling-Schulz, M., Schulz, S., Wait, R*., et al.* **(2002).** The UV-B stimulon of the terrestrial cyanobacterium *Nostoc commune* comprises early shock proteins and late acclimation proteins. *Molecular Microbiology,* 46(3), 827-843.

Emlyn-Jones, D., Woodger, F. J., Andrews, T. J*., et al.* **(2006).** A *Synechococcus* PCC 7942 ΔCCMM (cyanophyceae) mutant pseudoreverts to air growth without regaining carboxysomes1. *Journal of Phycology,* 42(4), 769-777.

Gaber, A., Yoshimura, K., Tamoi, M*., et al.* **(2004).** Induction and functional analysis of two reduced nicotinamide adenine dinucleotide phosphate-dependent glutathione peroxidase-like proteins in *Synechocystis* PCC 6803 during the progression of oxidative stress. *Plant Physiology,* 136(1), 2855-2861.

Gao, K., Yu, H., & Brown, M. T. **(2007).** Solar PAR and UV radiation affects the physiology and morphology of the cyanobacterium *Anabaena* sp. PCC 7120. *Journal of Photochemistry and Photobiology B: Biology,* 89(2), 117-124.

Gao, Q., & Garcia-Pichel, F. **(2011).** An ATP-grasp ligase involved in the last biosynthetic step of the iminomycosporine shinorine in Nostoc punctiforme ATCC 29133. *J Bacteriol,* 193(21), 5923-5928.

Gao, Y., Xiong, W., Li, X.-b*., et al.* **(2009).** Identification of the proteomic changes in *Synechocystis* sp. PCC 6803 following prolonged UV-B irradiation. *Journal of Experimental Botany,* 60(4), 1141-1154.

Garcia-Pichel, F., Wingard, C. E., & Castenholz, R. W. **(1993).** Evidence regarding the UV sunscreen role of a mycosporine-like compound in the cyanobacterium *Gloeocapsa* sp. *Applied and Environmental Microbiology,* 59(1), 170-176.

Häder, D.-P., & Sinha, R. P. **(2005).** Solar ultraviolet radiation-induced DNA damage in aquatic organisms: potential environmental impact. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 571(1), 221-233.

Häder, D.-P., Williamson, C. E., Wängberg, S.-Å*., et al.* **(2015).** Effects of UV radiation on aquatic ecosystems and interactions with other environmental factors. *Photochemical & Photobiological Sciences,* 14(1), 108-126.

Holtman, C. K., Chen, Y., Sandoval, P*., et al.* **(2005).** High-throughput functional analysis of the *Synechococcus elongatus* PCC 7942 genome. *DNA Research,* 12(2), 103-115.

Hu, C., Voller, G., Sussmuth, R*., et al.* **(2015).** Functional assessment of mycosporine-like amino acids in Microcystis aeruginosa strain PCC 7806. *Environ Microbiol,* 17(5), 1548-1559.

Jiang, H., & Qiu, B. **(2011).** Inhibition of photosynthesis by UV-B exposure and its repair in the bloom-forming cyanobacterium *Microcystis aeruginosa. Journal of Applied Phycology,* 23(4), 691-696.

Katoch, M., Mazmouz, R., Chau, R*., et al.* **(2016).** Heterologous Production of Cyanobacterial Mycosporine-Like Amino Acids Mycosporine-Ornithine and Mycosporine-Lysine in Escherichia coli. *Appl Environ Microbiol,* 82(20), 6167-6173.

Kumar, A., Tyagi, M. B., Jha, P. N*., et al.* **(2003).** Inactivation of cyanobacterial nitrogenase after exposure to ultraviolet-B radiation. *Current Microbiology,* 46(5), 0380-0384.

Miyake, C., Michihata, F., & Asada, K. **(1991).** Scavenging of hydrogen peroxide in prokaryotic and eukaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria. *Plant and Cell Physiology,* 32(1), 33-43.

Oren, A. **(1997).** Mycosporine‐like amino acids as osmotic solutes in a community of halophilic cyanobacteria. *Geomicrobiology Journal,* 14(3), 231-240.

Oren, A., & Gunde-Cimerman, N. **(2007).** Mycosporines and mycosporine-like amino acids: UV protectants or multipurpose secondary metabolites? *FEMS Microbiology Letters,* 269(1), 1-10.

Pereira, S., Zille, A., Micheletti, E*., et al.* **(2009).** Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiology Reviews,* 33(5), 917-941.

Platt, U., & Hönninger, G. **(2003).** The role of halogen species in the troposphere. *Chemosphere,* 52(2), 325-338.

Pope, M. A., Spence, E., Seralvo, V.*, et al.* **(2015).** O-methyltransferase is shared between the pentose phosphate and shikimate pathways and is essential for mycosporine-like amino acid biosynthesis in *Anabaena variabilis* ATCC 29413. *Chembiochem : a European journal of chemical biology,* 16(2), 320-327.

Portwich, A., & Garcia-Pichel, F. **(2000).** A novel prokaryotic UVB photoreceptor in the cyanobacterium *Chlorogloeopsis* PCC 6912. *Photochemistry and Photobiology,* 71(4), 493-498.

Qiao, C., Duan, Y., Zhang, M.*, et al.* **(2018).** Effects of Reduced and Enhanced Glycogen Pools on Salt-Induced Sucrose Production in a Sucrose-Secreting Strain of *Synechococcus elongatus* PCC 7942. *Applied and Environmental Microbiology,* 84(2).

Rastogi, R. P., Sinha, R. P., Moh, S. H.*, et al.* **(2014).** Ultraviolet radiation and cyanobacteria. *Journal of Photochemistry and Photobiology B: Biology,* 141, 154-169.

Rozema, J., Björn, L. O., Bornman, J. F.*, et al.* **(2002).** The role of UV-B radiation in aquatic and terrestrial ecosystems—an experimental and functional analysis of the evolution of UV-absorbing compounds. *Journal of Photochemistry and Photobiology B: Biology,* 66(1), 2-12.

Sambrook, J., Russell, D. W., & Maniatis, T. **(2001).** Molecular cloning, vol. 1-3. *Cold Spring Habour Laboratory Press, New York.*

Shang, J. L., Zhang, Z. C., Yin, X. Y.*, et al.* **(2018).** UV-B induced biosynthesis of a novel sunscreen compound in solar radiation and desiccation tolerant cyanobacteria. *Environmental microbiology,* 20(1), 200-213.

Shibata, K. **(1969).** Pigments and a UV-absorbing substance in corals and a blue-green alga living in the Great Barrier Reef1. *Plant and Cell Physiology,* 10(2), 325-335.

Shick, J. M., & Dunlap, W. C. **(2002).** Mycosporine-like amino acids and related gadusols: biosynthesis, accumulation, and UV-protective functions in aquatic organisms. *Annual Review of Physiology,* 64(1), 223-262.

Singh, S. P., Klisch, M., Sinha, R. P.*, et al.* **(2008a).** Effects of abiotic stressors on synthesis of the mycosporine-like amino acid shinorine in the cyanobacterium *Anabaena variabilis* PCC 7937. *Photochemistry and Photobiology,* 84(6), 1500-1505.

Singh, S. P., Sinha, R. P., Klisch, M.*, et al.* **(2008b).** Mycosporine-like amino acids (MAAs) profile of a rice-field cyanobacterium *Anabaena doliolum* as influenced by PAR and UVR. *Planta,* 229(1), 225-233.

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

Sinha, R. P., Singh, S. P., & Häder, D.-P. **(2007).** Database on mycosporines and mycosporine-like amino acids (MAAs) in fungi, cyanobacteria, macroalgae, phytoplankton and animals. *Journal of Photochemistry and Photobiology B: Biology,* 89(1), 29-35.

Sinha, R. P., & Häder, D.-P. **(2008).** UV-protectants in cyanobacteria. *Plant Science,* 174(3), 278-289.

Smith, R., Prezelin, B., Baker, K.*, et al.* **(1992).** Ozone depletion: ultraviolet radiation and phytoplankton biology in antarctic waters. *Science,* 255(5047), 952-959.

Suh, H.-J., Lee, H.-W., & Jung, J. **(2003).** Mycosporine glycine protects biological systems against photodynamic damage by quenching singlet oxygen with a high efficiency. *Photochemistry and Photobiology,* 78(2), 109-113.

Tarasuntisuk, S. **(2017).** Functional characterization of natural sunscreen compound mycosporine-2-glycine from extremophilic cyanobacterium *halothece* sp. pcc 7418 in macrophage cell line and fresh water cyanobacterium. Master's Thesis, Department of Microbiology, Faculty of Science, Chulalongkorn University.

Tarasuntisuk, S., Patipong, T., Hibino, T.*, et al.* **(2018).** Inhibitory effects of mycosporine-2-glycine isolated from a halotolerant cyanobacterium on protein glycation and collagenase activity. *Letters in Applied Microbiology,* 67(3), 314-320.

Tirkey, J., & Adhikary, S. P. **(2005).** Cyanobacteria in biological soil crusts of India. *Current Science,* 89(3), 515-521.

Tsuge, Y., Kawaguchi, H., Yamamoto, S.*, et al.* **(2018).** Metabolic engineering of *Corynebacterium glutamicum* for production of sunscreen shinorine. *Bioscience, Biotechnology, and Biochemistry,* 82(7), 1252-1259.

Vioque, A. (2007). Transformation of cyanobacteria *Transgenic microalgae as green cell factories* (pp. 12-22): Springer.

Wada, N., Sakamoto, T., & Matsugo, S. **(2015).** Mycosporine-like amino acids and their derivatives as natural antioxidants. *Antioxidants,* 4(3), 603.

Waditee-Sirisattha, R., Kageyama, H., Sopun, W*., et al.* **(2014).** Identification and Upregulation of Biosynthetic Genes Required for Accumulation of Mycosporine-2-Glycine under Salt Stress Conditions in the Halotolerant Cyanobacterium *Aphanothece halophytica. Applied and Environmental Microbiology,* 80(5), 1763-1769.

Waditee-Sirisattha, R., Kageyama, H., Tanaka, Y*., et al.* **(2017).** Overexpression of halophilic serine hydroxymethyltransferase in fresh water cyanobacterium *Synechococcus elongatus* PCC7942 results in increased enzyme activities of serine biosynthetic pathways and enhanced salinity tolerance. *Archives of Microbiology,* 199(1), 29-35.

Weber, T., Blin, K., Duddela, S*., et al.* **(2015).** antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research,* 43(W1), W237-W243.

Whitehead, K., & Hedges, J. I. **(2005).** Photodegradation and photosensitization of mycosporine-like amino acids. *Journal of Photochemistry and Photobiology B: Biology,* 80(2), 115-121.

Wright, D. J., Smith, S. C., Joardar, V*., et al.* **(2005).** UV irradiation and desiccation modulate the three-dimensional extracellular matrix of *Nostoc commune* (cyanobacteria). *Journal of Biological Chemistry,* 280(48), 40271-40281.

Yang, G., Cozad, M. A., Holland, D. A*., et al.* **(2018).** Photosynthetic production of sunscreen shinorine using an engineered cyanobacterium. *ACS Synthetic Biology,* 7(2), 664-671.

Zhang, L., Li, L., & Wu, Q. **(2007).** Protective effects of mycosporine-like amino acids of *Synechocystis* sp. PCC 6803 and their partial characterization. *Journal of Photochemistry and Photobiology B: Biology,* 86(3), 240-245.

Ziemert, N., Ishida, K., Liaimer, A*., et al.* **(2008).** Ribosomal synthesis of tricyclic depsipeptides in bloom-forming cyanobacteria. *Angewandte Chemie,* 120(40), 7870-7873.

APPENDICES

APPENDIX 1

BG11 medium

BG11 Solution

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

Dissolved all compositions with distilled water up to 1 liter.

Trace element solution

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

Dissolved all compositions with distilled water up to 1 liter.

APPENDIX 2

LB medium

Bacto tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Dissolved all compositions in 800 ml deionized water, adjusted the pH to 7.6 with NaOH. Adjusted volumn of solution to 1 liter with deionized water. Autoclaved at 121 ºC, 15 lb/in^2 for 15 minutes. For agar media, added bactoagar 1.5 g per 100 milliliter.

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

APPENDIX 3

Antibiotic solution

Streptomycin (100 mg/ml)

Streptomycin   1   g

Dissolved streptomycin in 10 ml deionized water, filtrated through the filters

pore size 0.2 μm. Keep solution in -40 °C for further use.

APPENDIX 4

Primer design

Ap3858 (*Aphanothece halophytica* Ap-DDG gene for dimethyl 4-deoxygadusol

synthase)

mRNA size: 2,374 bp   Accession number: AB854643.1

AAAAAAGTCTAGCTGTATCTCTACTTTTTTAATAAGCCTGACGGGTTTTTCACCATCTTTGGGCTTGTTCGATT

CTAGCAGACACAGAGGACTAAAGTCCTCCCTTGTCATTCATGAGGGGCTGTCGTCGGAACGAGGGTGACGACG

CTTGTAAGCCCCGTCCATCGGTTTGAAGCCGATGGCTTTCTGACTGAATCGTTGTAATCAGTGCCAGTTTGTTA

TTTCTAGGCGCGATTGCCTTTGGCACTTTACGTCATTTCGAGCGTCCTCACACTTAAGAATTAACCCTGAATTT

TTCAGAAATTAAGTTAAAAATTAATAAAAAATTAGATAAAAATCAGCTAATAATGGCGGTGGAATGATACCACT

GCTAGGAATTTTTTGTCTTAACCCCCTTGCTCGTTTCGTCAATAACACCACTGTTAAGGCTTGTTTTAACTGGT

TTTCAGAAAATCACTCTTTTTGAAAACACCGAGAAAACCGTCTCTATAGAAAGATAGAAATTCCGACTGGAGGG

CTGTTCTAAGATGAGAGGCGTGTTAACTTATATTAAGCAAATTCGCTGCTTTCTGAGTAACACCATCTGTAAAT

TTGTTCTGAGAACTCAATC<mark>ATGACGAAAACAACCTCTGC</mark>TGTAAAAGCGTTTATCGCCACCTATAACAATGAAC

CTTTAACCAAGGAAGACTTAAATGAGGCTGTCGAAGCGATTATTGGCACAACTCCTTTTAGGCATCTACTTTTA

TCACTGATTGATAGTGATGCGTTCTCCCCTGATCTGGGAGAAGAATTTGCTGAAGCAGAAGCGATTGAAGGTT

TAGCAGCGTGTCGCCAACTGCGGACTTGTCTGAATCATTCTCTCAGTCACTTCTTTGGGTTATTAGCGCAATTA

ATCGCAGGTTTTGATGAAAATGCTGCTTCTGAGTGGTATCGCTTTTCCAACCGTATCCATCATTCTGAAATCGC

AAAAACCAAACTGCTGGATTGTCTCTTACAAAGCGAAGATGGGAAATTTTATCAAGAACTTTCCTCGCGC<mark>CTTG</mark>

<mark>TGGAAACCGATCCTCA</mark>TGCGGTTTATCCCACATCCAGTTATCGTCAAAGTCGGGGTTATGTCGTCAGTACAGA

AGATGACCAAACTGTGGAAGCGGTCATGAGTGCGAGCACTTTCACTTCGATTAAAGTGCTGGAAAATTGTCTT

GATCCGCAAGAGACGGTTTTTAAGAGATCTATATATTTCTTTAGGTCGCTGTGTCTGTTTGGTGGATCAGAATGT

AGAACAATACTATGGCGAGCAGATTAACAATTATTTTGAATATCACGAAATTCAGTTAGATAAACTCGTCTATC

GCGCCATGGAAGTGGATAAAGGGATTCATACGGTAGAAAGAATGCTCGGCGACTTTAAACGTCTGGGGGTTTC

TCGGAATGAACCCGTTTTAATTGTCGGTGGAGGCGTTTTAACCGATACAGGTGGCTTAGCTTGTGCGTTATAC

CATCGCAACACGCCTTATGTGATGCTTTCCACGTCTATTGTCGCTGGAATTGATGCGGGTCCGTCTCCTCGCA

CCTGCTGTGATGGCTTTGGTTATAAAAATCTCTTTGGGGCTTACCACGCACCCATTCTTTCTCTCACAGATCGC

TCTTTCTTTAAAACATTACGAGAAGGATGGCTGCGACATGGTATTGCTGAGATTTTGAAGATGGCAGCGGTTA

AAGATGCGGAATTATTCAGTGATCTCGAAGAAGCAGGGGAGGACTTAATAACCACTCGCTTTGGGACACTTAA

CTCCGAACAGAATGACAAAATTAGTGTTCTATCTCAGAAAATTCTCGGTGCAGCGATGCGGAGTTATGTGGAA

GCGGAGTATGATAACCTCTACGAAACCCATCAGTGTCGTCCTCATGCTTACGGACATACTTGGTCTCCTGGAT

TTGAAATTGAAGCAGGATTGTTACACGGACACGCAGTTGCAGTTGGGATGGGCTTTAGCGCGTACTTGAGTTA
TCGCAATAACTGGCTCAGTCACGAAGATTTCCATCGCATTCTTAAGCTGATTAGTTCCTTTGGTTTGAGTCTGT
GGCATGATGTGTTACTGAACGAAGAAACCGTTTGGGCAGCCCAAGAGAAAATGGTGCAGAAACGAGGGGGAAA
TTTAGCAGCACCGATGCCAAAAGGGGAAATTGGCAAATGTGGCTATCTCAATCAGTTGAGTCGTGAGGAGTTA
GGCAGCGCGATCGCGCAATACCAAGCGATTTGTGCAGAATATCCCCGAAAAGGCTTAGGAATTGAAGCCCATT
GTCATGAAGTAGGCTTAGAAAATCCTTCTACGGTTGGTCATCATCTTCCTGTGAACACATCTGAAGAACCAGAA
GAACTGTTATCTACAGTCTAG

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′- GGATCCAATGACGA AAACAACCTCTG -3′	610	27	68.3	5′-TGAGGATCGG TTTCCACAAG-3′	1,050	21	58.4	440

Ap3857-3855 (*Aphanothece halophytica* Ap-AAligase, Ap-CNligase, Ap-OMT genes for

D-ala-D-ala ligase, C-N ligase, O-methyltransferase, respectively)

mRNA size: 4,028 bp   Accession number: AB854644

AAAGGTAAGGGTTGGTCATTTGTCATTAGTCTTTTCGGTTACGGGTACATCACTCTCCCTAAAATTAAAACACG

CTTAAGAGTTCTTATCACTTCTGGCTAAAAATAGGGATTTCTCTGATTTTTAATAAGTTTTCTAAAGGTATTCT

GAGAAAGCGCTGCGTCATCTATCAAGGGAAATACTTTAAGCTCTGAGCACTAAAAACAAGATATAACAGGGAT

TTAGACGTAAACATAGAGAAAAGTTATAAATCAAAAGGAAGATTAATCTGAAAAATGGCAGCGCGATCGTGAC

ATTTTTCTTAACAATATTCTAAGATAAGGACGGCGATGCTTCCCTCGCCAAAGCCAAATTGTAATCATCCTCAT

GTAAAAGCAGTATTAAGAGAGAGTATTGGCA<mark>AATGACGATCACTAACGATAAAC</mark>CCAGTCAAAAAACCGCAAG

ACCTGTAACTCCCTTGGGGATTTTAGTTCAGCAGTTAGAACAGATTGTGGAACAAGGAAAAGAAGAAGGGATT

TCTTCTCAGTTACAAAGCGCGATCGAAAGTGCTTATGAACTGGGTGCTGGACTTGACCCCTATCTGGAACAAA

TCACGACCCAAGAATCAGAAGCCTTAGCTAACTTAGATCGCAAAACCCGTGAAGAAGACTGGAGTCAATCGTT

TTCTGATGGGGAAACGGTACGCCGTTTAGAACAAGAAATGCTGTCAGGTCATATTGAAGGACAAACCCTGAAA

ATGTTTGTTCACATGACAGGCGCAAAACGGATTTTAGAAGTGGGGAT<mark>GTTTACGGGCTATTCTGCAT</mark>TAGCTAT

GGCGGAAGCCCTTCCTGAAAACGGTCGTGTGGTTGCTTTAGAGGTCGATCCTTACACCGCGCAATTGGGTCGC

AACTGCTTTGATGTTTCTCCTCACGGTCAAAAAATCGAAATTATGGTCGATTCCGCGATTAACAGCATGAAGAA

GTTAGCTGCGGATGGTCAGACGTTCCAACTGGCGTTTCTGGATGCAGATAAGACCGAATATAAAGATTACTAT

CGCCTCCTGCTAGATGAAGGGGTTACTAGAAGACGGTGGCTATATTTTTGTCGATAATAGCTTGTTACAAGGAC

AGGCGTATCTCCCGCCAGAAAAACGAACTCTAAATGGTGACGCGATCGCGCAATTTAATCAAATGGTCGCCGA

TGATCCTCGTGTGGAACAAGTTTTGCTTCCCCTCCGTGATGGCTTAACTATGATTAGAAAACTGTAATCTTTGA

CTTGGGTGAGGTACATTTTTTGTTCTTTGTTCTTTGTTCGTTGTTCTTTGACAATCTCAAAAATTTTGGACAGC

AGTTCTAGTAATCAAAAATGAACATGAACATCCACCGACTCCCATTACGTACCTCAACAGACTAAGAAACGCTA

TATAGCAGTATGATGTCATTTGTAAAAAGTAGATTAACTGACGATCCCCCCTATCCCCCCTTACTAAGGGGGGA

ATTAAAGGGGGGATTGCTGGGGGGGGACAACTGATTTAGGATTGCTATAGATCAGATTTTTCTACAGAACACAG

GGAAGGATTGAGCAGAAATGATATCAGCAATTTTTGAGTCTCTTCCCTGTCCCTTTTAACCAATAATGTCAAGA

CGTTCCATAGAACGCCTCAAATAACAAATAACCAAGAACCAAGAACAAATAA<mark style="background:#00ff00">CAATGCTTCTATTTGTCCGAGG</mark>

ACGAATCAATTCCGCAGTCAAAACGATGGGGACATTAACCTTACTGTTAATCGCTTTCCCCATCAATTTAATCA

CCGTCTTGTTCTCTTTTTTTGACTCAGGAGAAACTTAGGCAAGCTGTTACTGATAATCCTAAGCGTATCCTAATT

ACGGGTGGCAAAATGACAAAATCGCTACAGTTAGCGCGATCGTTTTATCAAGCGGGACACACCGTTTTCCTCG

TCGAAACCCATCGTTACTGGCTATCAGGTCATCGCTTCTCCCAAGCTGTAAGCGGATTTTACACCGTTCCCGC

ACCCGAAAAAGACCCAGAAGGCTATCGTCAAGGACTGCTTGATATTGTTAAAAAAGAGCAAATTAATGTTTTTA

TTCCCGTTTGCA<mark style="background:#00ff00">GTCCCGTTTCTAGTTACTAT</mark>GATTCCACCGCAAAACCATTACTCACAGAAGTAGGTTGTGAA

GTCATTCATTTTGATCCAGACATTACCGCAATGCTGGATAATAAATATGAATTTTGTAAACAAGCGCAAGCCCT

AGGACTTGCTGCACCAAAAGTTTTTAAGTTTACTTCTCCCCAAGAAGTTTTAGATTTTGACTTTGAAGCCGATG

GTAGTCGTTACATTATTAAAAGTATTAATTACGATTCCGTTCGCCGTTTAGATCTGACTAAACTTCCCTTTCAA

GGAATGGAAAGTTATATCAAAACTCTTCCTATCTCAAAAGATAATCCTTGGGTGATGCAAGAGTTTATTCGCGG

GAAAGAATTTTGTACTCATAGCACCGTTCGCAATGGCAAAATTCAAGTCCATTGTTGTTCTGAATCTTCTCCCT

TTCAAGTCAATTATCAAAAAGTCGATCATCCAGAAATTTTCGCTTGGGTGAAACACTTTGTCGGGGAATTAAAG

TTAACGGGTCAAATTTCTTTTGACTTTATCGAAACCCCAGATGGCAATATTTATCCCATTGAATGCAACCCTCG

CACTCATTCGGCGATTACGATGTTTCATGATCATCCTAATTTAGCATCTGCTTATTTAGAAGATAATCATTATT

CTGAACCGATTGCGCCTTTACCCAATAGCAAACCCACTTACTGGCTATATCACGAACTGTGGCGACTAACTGA

AATTCGTTCCTTAGAAGACTTAACAAAATGGTGGCAAAAAATCACGCAAGGAACCGATGGAATTTTTCGTAAA

GATGACCCCTTACCCTTTTTATTTGTCCATCACTGGCAAATTCCCCTATTACTTTTAAGTAACTTAAAACGTTT

CCAAGATTGGATTCGCATTGACTTCAACATCGGAAAAATTGTCAAATTAGGAGGAGATTAATTCATGTCTTTAT

TACGAGTTTTACACTTAGCAGGGTCAAGGGTTTCGCAATTTTATTACAATTTGTCCATGCTATATGCAAAAGAA

GTGGTGCAACCTGCTAACATAAAAAGTTACTATGCAGTAGTGCATCCTGATGGGGTTTGGCAACTGGGGTCAT

CTTTAGAGAATTTATCCGAGAAACTCTCCCTGCAAGAAACGATCGCGCAACTTCCCGAGGTTGATGTGGTTGT

TCCTCACTTATTTTGCTTTCCAGGAATGACTAGTTTTCGGGGATTTTTTGAAGACTTACTCGGTTTACCCGTTG

TTGGTTCTCCCGCCCACTGTACCGCATTAGCTAGTAATAAAGCCCACACCAGAAGCGTTGTTGAAACCATGGG

CGTTACCGTCGCAAAAGCACAACAAATCCGTTCTGGGGATACAGTGACAATGAAACCGCCATTTATCGTCAAA

GCCAACTCCGAAGATAACTCTTTAGGGTTAACCTTAGTCAAAACCAAAGATCAAATTGCAGCAGCGTTACAGA

CAGGCTTTGAATATGACGACACTCTACTTGTAGAAGATTATATTCCAGGACGAGAACTGCGTGTGGGAGTCAT

TGAACGGGGGAATGAATTGTATGTTCCCTCCATGATTGAATACGTATTCCCCAAAGATCACACCATTCGCACCA

CTCAGGATAAGTATGACCTACAAGCCGATGGAACGCCTGGAAAACAACCCGATCAACCCGTCGCAAAACCCCA

GTGTCCCGCCGAAGTTACGCCAGAATTATTCGAGAAACTCGCAGATGCAGCAAGAAAAGCCCATATCGCCCTT

GGTTGTCGCGATTATTCCCTGTTTGATTTTCGCGTTCATGAAGAAACAGACGAACCTTATCTTTTAGAAGCGGG

ACTCTTTTGGTCATTCGGAGAAATTAGTATGATCTCTCGGATGCTACTCGCAGATGGAGAAATGTTAGAAGATG

TGGTAGCTGAAGTTTGGCGCAATGCGTCTCAACTAAGAGTTCTGAATGCGGGCTAA

Gene	Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
Ap3857	5′-GGATCCAATGACGATCACTAACGATAAAC-3′	399	29	67.6	5′-ATGCAGAATAGCCCGTAAAC-3′	800	20	56.4	401
Ap3856	5′-GGATCCAATGCTTCTATTTGTCCGAGG-3′	1,668	27	68.3	5′-ATAGTAACTAGAAACGGGAC-3′	2,090	20	54.3	422
Ap3855	5′-TTATCCGAGAAACTCTCC-3′	3,176	18	51.6	5′-AGGTCATACTTATCCTGAG-3′	3,697	19	53	521

cat (Catalase/peroxidase)

mRNA size: 2163 bp   KEGG accession number: Synpcc7942_1656

ATGACAGCAACTCAGGGTAAATGTCCGGTCATGCACGGCGGAGCAACAACCGTTAATATTTCGACTCTGAGTG
GTGGCCAAAGGCACTCAACCTGGATATTTTGAGCCAGCACGATCGCAAGACCAACCCAATGGGGCCAGACTTC
AACTATCAGGAAGAAGTCAAGAAACTGGATGTCGCTGCGCTCAAGCAAGATTTACAGGCGCTGATGACCGATA
GCCAAGACTGGTGGCCGGCAGACTGGGGTCACTACGGCGGTCTGATGATTCGCCTCACTTGGCACGCGGCGG
GCAC CTACCGAATTGCCGA TGGTCGCGGTGGTGCAGGCACGGGGAACCAGCGCTTTGCTCCCCTCAATTCTTG
GCCAGACAACACAAATTTAGACAAAGCGCGTCGCTTGCTTGGCCGATCAAGCAAAAGTACGGCAACAAGTTGA
GTTGGGCAGATTTAATTGCCTATGCCGGCACGATCGCCTACGAATCGATGGGGCTTAAAACCTTTGGTTTTGC
CTTTGGACGAGAAGATATTTGGCATCCTGAGAAAGATATCTACTGGGGGCCTGAGAAGGAATGGGTTCCC CCA
AGCACCAATCCC AACAGTCGCTATACGGGCGATCGCGAACTTGAAAATCCGCTAGCAGCCGTGACAATGGGGC
TGATTTACGTCAACCCCGAAGGCGTGGATGGCAATCCTGATCCGCTCAAAACCGCCCATGACGTGCGCGTCAC
CTTTGCGCGGATGGCGATGAACGATGAGGAAACGGTGGCGCTAACTGCTGGTGGACACACCGTTGGCAAATGT
CATGGCAATGGCAATGCTGCTTTGCTAGGACCCGAACCGGAAGGGGCGGATGTGGAAGATCAAGGCTTGGGC
TGGATCAATAAAACCCAGAGCGGTATTGGTCGCAACGCTGTCACCAGTGGGCTGGAAGGGGCTTGGACACCCC
ACCCGACTCAATGGGACAACGGCTATTTCCGTATGCTCCTGAACTATGACTGGGAACTGAAGAAAAGCCCTGC
AGGCGCATGGCAGTGGGAACCGATTAATCCCCGAGAAGAAGATCTACCGGTCGATGTCGAAGATCCATCGATT
CGCCGCAACTTGGTGATGACCGACGCCGACATGGCCATGAAGATGGACCCAGAGTATCGGAAAATCTCGGAGC
GCTTCTACCAAGATCCGGCCTACTTTGCGGATGTGTTTGCACGGGCTTGGTTCAAGTTAACCCACCGCGATAT
GGGGCCGAAAGCCCGTTACATTGGCCCGGATGTGCCACAGGAAGACCTGATTTGGCAGGATCCAATTCCGGCG
GGCAACCGCAACTATGACGTGCAAGCGGTGAAAGATCGGATTGCTGCCAGTGGACTAAGTATCAGTGAGCTAG
TCAGCACAGCTTGGGATAGCGCCCGTACTTATCGAAATTCGGATAAGCGGGGCGGGGCGAATGGGGCACGGA
TTCGGTTAGCACCCCAGAAAGATGGGAAGGGAATGAACCCGATCGCTTGGCGAAAGTGCTGGCTGTGCTGGAA
GGAATTGCTGCCGCTACTGGGGCGAGCGTTGCCGACGTCATTGTTCTGGCTGGCAATGTTGGGGTGGAGCAAG
CAGCCAGGGCTGCCGGTGTTGAAATTGTGCTTCCCTTTGCGCCGGGTCGTGGCGATGCAACGGCTGAGCAAAC
GGATACGGAATCCTTTGCAGTGCTGGAGCCGATTCACGATGGCTATCGCAACTGGCTCAAGCAGGACTATGCG
GCAACGCCTGAAGAATTGCTGCTTGATCGCACGCAACTGTTGGGTCTGACGGCTCCAGAGATGACGGTGTTGA
TTGGTGGCCTGCGTGTCTTGGGAACCAACCATGGCGGTACGAAGCACGGTGTCTTCACCGATCGCGAAGGGGT
GTTAACGAATGACTTTTTCGTGAATCTGACCGACATGAATTATCTGTGGAAACCGGCTGGAAAAAACCTGTATG
AAATCTGCGATCGCAAGACGAATCAGGTGAAGTGGACGGCAACGCGAGTCGATTTGGTCTTTGGATCAAATTC
GATTCTGCGAGCCTACTCAGAGCTCTATGCACAAGACGACAACAAAGAGAAGTTTGTGCGAGACTTTGTCGCT
GCCTGGACGAAGGTGATGAATGCCGATCGCTTTGAT CTGGACTAA

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-CTACCGAATTGCCGA-3′	295	15	52.46	5′-CCAAGCACCAATCCC-3′	595	15	53.86	300

sodB (Fe/Mn-superoxide dismutase)

mRNA size: 690 bp   KEGG accession number: Synpcc7942_0801

GTGCTGCAGGAGACGCTCAGACGAGGGAAGCGTCCGGTTTTTTATTAATCTAGGTAAAGACAACCTGCTCAAGA
GAACACACTGCATGTCCTACGAATTGCCAGCATTGCCCTTTGACTACACGGCACTGGCGCCTTACATC<mark>ACCAA
GGAAACGCTG</mark>GAGTTCCACCACGATAAGCACCACGCGGCCTACGTCAATAACTACAACAACGCCGTCAAAGAC
ACCGACCTCGATGGCCAGCCGATCGAAGCCGTGATCAAAGCGATCGCGGGTGACGCTAGCAAAGCCGGTCTG
TTCAACAATGCGGCTCAAGCTTGGAACCACAGCTTTTACTGGAACTCGATCAAGCCCAATGGCGGTGGCGCTC
CCACCGGCGCGTTGGCCGACAAAATCGCCGCTGACTTCGGCAGTTTCGAGAACTTCGTGACC<mark>GAGTTCAAACA
AGCCG</mark>CAGCAACCCAGTTCGGCAGCGGCTGGGCTTGGTTGGTGCTGGACAATGGCACCCTCAAAATCACCAAA
ACCGGCAACGCCGACACCCCGATTGCCCATGGTCAAACCCCGCTACTGACCATCGATGTCTGGGAACACGCTT
ACTACCTCGACTACCAAAACCGTCGTCCCGACTACATCAGCACCTTCGTTGAGAAGCTGGCGAACTGGGACTT
CGCCTCT GCCAACTACGCAGCTGCGATCGCTTAG

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-ACCAAGGAAACGCTG-3′	141	15	53.34	5′-GAGTTCAAACAAGCCG-3′	441	16	52.94	300

tpxA (Thioredoxin peroxidase)

mRNA size: 597 bps   KEGG Accession number: Synpcc7942_2309

ATGACCGAAGGAGCCCTGCGCGTCGGCCAATTGGCCCCCGATTTTGAAGCGACTGCAGTCGTTGATCAGGAAT
TCCAGACGATCAAGCTATCCAATTACCGGGGCAAATACGTCGTTCTGTTCTTCTATCCCCTCGACTTCACCTTT
GTTTGCCCGACCGAAATTACTGCTTTTAGCGATCGCTATGCAGACTTTTCAGCCCTGAACACCGAAATCTTGG
GTGTCTCGGTCGATAGCCAATTCAGCCACTTGGCTTGGATTCAAACCAGCCGTAAAGAAGGTGGTTTGGGTGA
CTTGGCTTACCCGCTGGTTGCTGACCTCAAGAAAGAAATCAGCACTGCCTACAACGTGCTTGATCCGGCTGAA
GGCATTGCCCTGCGCGGTCTGTTCATCATCGACAAAGAAGGTGTGATCCAGCACGCCACCATCAACAACCTGG
CGTTTGGCCGCAGCGTTGATGAAACCCTGCGGGTGCTGCAAGCCATTCAGTACGTCCAAAGTCACCCCGATGA
AGTTTGCCCCGCCAATTGGCAACCGGGTGCAGCGACGATGAACCCCGACCCTGTTAAGTCGAAAGAGTTCTTC
GCTGCAGTCTAG

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-CCGTAAAGAAGGTGGT-3′	269	16	52.56	5′-CCCGACCCTGTTAAG-3′	569	15	52.28	300

Synpcc7942_2522 (Glycerol-3-phosphate dehydrogenase (NAD(P)$^+$))

mRNA size: 927 bp   KEGG Accession number: Synpcc7942_2522

ATGCAAGAGGCAAAAGTTGCGGCAATTTTGGGAGCCGGTGCTTGGGGAACGACCCTGGCTCAGCTCTTGCGCA
GCAATGGCCTTGAGGTGCGGCAATGGTCTCGCCGAAGTGAGACCTCTTTGGCAGCAACATTGGCAGAGGCCGA
TCTCTGGATTATGGCGGTCTCCATGGCCGGCTTGGCTTCCGTGGCGGATCAAGTGGCAGCCCTCCAACTGGGC
GATCGCGCGATTTGGGTGAGTGCAACTAAAGGACTGGCGGATCTGGGCTGGCGGACTCCCTCTCAGGTTCTCA
GCGATCGCTTCCCGCTGCAGCCGATTACGGTGCTCTCAGGGCCCAATCTTTCCAAGGAAATTAGCCAGGGATT
ACCCGCAGCGACCGTGATTGCCAGTCGCGATCGCCATGCAGCGGCCGTGGTTCAGCAGGCGTTTGCCAGCGA
TCGCTTTCGGGTCTATACCAATCGCGATCCCCTCGGAACCGAGCTGGGCGGGGCTCTCAAAAATGTGATTGCG
ATCGCTGTCGGGGTCTGTGATGGTCTCTGTCTGGGTGCCAATGCGCGATCGGCCTTGGTAACGCGAGCCTTGG
CAGAAATTCTGCGCGTGGGGGCCTATTTCGGTGCCCGCACGGAAACGTTCTTTGGACTGTCAGGGCTAGGTGA
TTTACTGGCGACCTGCACCAGTCCGCTCAGTCGCAA<mark>CTATCAAGTTGGATTCCG</mark>GCTCGCGCAAGGTGAGAGC
TTGGCTGCGGCACTAACGGCGATCGCAGCCACGGCTGAAGGCGTATCTACCGCTCGAGTGCTGGCCCAGCTG
GCCAGCCGTGAGGGACTAGAGTTGCCGATCGCAGCCTGTGTGGCG<mark>GAGTTGTTAGATAACCGG</mark>ATTTCGCCAA
CAACCGCCATTGAGCGGCTGATGGCTCGCGATCTGAAAGCGGAATTGGTCTGA

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-CTATCAAGTTGGATTCCG-3′	693	18	51.6	5′-CCGGTTATCTAACAACTC-3′	864	18	51.6	171
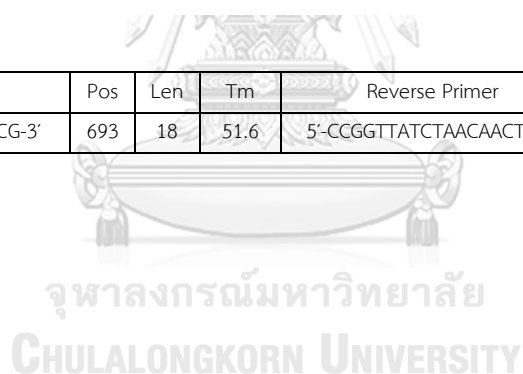
Synpcc7942_0808 (Sucrose-phosphate synthase)

mRNA size: 2,130 bp   KEGG Accession number: Synpcc7942_0808

GTGGCAGCTCAAAATCTCTACATTCTGCACATTCAGACCCATGGTCTGCTGCGAGGGCAGAACTTGGAACTGG
GGCGAGATGCCGACACCGGCGGGCAGACCAAGTACGTCTTAGAACTGGCTCAAGCCCAAGCTAAATCCCCACA
AGTCCAACAAGTCGACATCATCACCCGCCAAATCACCGACCCCCGCGTCAGTGTTGGTTACAGTCAGGCGATC
GAACCCTTTGCGCCCAAAGGTCGGATTGTCCGTTTGCCTTTTGGCCCCAAACGCTACCTCCGTAAAGAGCTGC
TTTGGCCCCATCTCTACACCTTTGCGGATGCAATTCTCCAATATCTGGCTCAGCAAAAGCGCACCCCGACTTG
GATTCAGGCCCACTATGCTGATGCTGGCCAAGTGGGATCACTGCTGAGTCGCTGGTTGAATGTACCGCTAATT
TTCACAGGGCATTCTCTGGGGCGGATCAAGCTAAAAAAGCTGTTGGAGCAAGACTGGCCGCTTGAGGAAATTG
AAGCGCAATTCAATATTCAACAGCGAATTGATGCGGAGGAGATGACGCTCACTCATGCTGACTGGATTGTCGC
CAGCACTCAGCAGGAAGTGGAGGAGCAATACCGCGTTTACGATCGCTACAACCCAGAGCGCAAGCTTGTCATT
CCACCGGGTGTCGATACCGATCGCTTCAGGTTTCAGCCCTTGGGCGATCGCGGTGTTGTTCTCCAACAGGAAC
TGAGCCGCTTTCTGCGCGACCCAGAAAAACCTCAAATTCTCTGCCTCTGTCGCCCCGCACCTCGCAAAAATGT
ACCGGCGCTGGTGCGAGCCTTTGGCGAACATCCTTGGCTGCGCAAAAAAGCCAACCTTGTCTTAGTACTGGGC
AGCCGCCAAGACATCAACCAGATGGATCGCGGCAGTCGGCAGGTGTTCCAAGAGATTTTCCATCTGGTCGATC
GCTACGACCTCTACGGCAGCGTCGCCTATCCCAAACAGCATCAGGCTGATGATGTGCCGGAGTTCTATCGCCT
AGCGGCTCATTCCGGCGGGGTATTCGTCAATCCGGCGCTGACCGAACCTTTTGGTTTGACAATTTTGGAGGCA
GGAAGCTGCGGCGTGCCGGTGGTGGCAACCCATGATGGCGGCCCCCAGGAAATTCTCAAACACTGTGATTTCG
GCACTTTAGTTGATGTCAGCCGACCCGCTAATATCGCGACTGCACTCGCCACCCTGCTGAGCGATCGCGATCT
TTGGCAGTGCTATCACCGCAATGGCATTGAAAAAGTTCCCGCCCATTACAGCTGGGATCAACATGTCAATACC
CTGTTTGAGCGCATGGAAACGGTGGCTTTGCCTCGTCGTCGTGCTGTCAGTTTCGTACGGAGTCGCAAACGCT
TGATTGATGCCAAACGCCTTGTCGTTAGTGACATCGACAACACACTGTTGGGCGATCGTCAAGGACTCGAGAA
TTTAATGACCTATCTCGATCAGTATCGCGATCATTTTGCCTTTGGAATTGCCACGGGGCGTCGCCTAGACTCTG
CCCAA**GAAGTCTTGAAAGAGTGG**GGCGTTCCTTCGCCAAACTTCTGGGTGACTTCCGTCGGCAGCGAGATTCA
CTATGGCACCGATGCTGAACCGGATATCAGCTGGGAAAAGCATATCAATCGCAACTGGAATCCTCAGCGAATT
CGGGCAGTAATGGCACAACTACCCTTTCTTGAACTGCAGCCGGAAGAGGATCAAACACCCTTCAAAGTCAGCT
TCTTTGTCCGCGATCGCCACGAGACTGTGCTGCGAGAAGTACGGCAACATCTTCGCCGCCATCGCCTGCGGCT
GAA**GTCAATCTATTCCCATCAG**GAGTTTCTTGACATTCTGCCGCTAGCTGCCTCGAAAGGGGATGCGATTCGC
CACCTCTCACTCCGCTGGCGGATTCCTCTTGAGAACATTTTGGTGGCAGGCGATTCTGGTAACGATGAGGAAA
TGCTCAAGGGCCATAATCTCGGCGTTGTAGTTGGCAATTACTCACCGGAATTGGAGCCACTGCGCAGCTACGA
GCGCGTCTATTTTGCTGAGGGCCACTATGCTAATGGCATTCTGGAAGCCTTAAAACACTATCGCTTTTTTGAGG
CGATCGCTTAA

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-GAAGTCTTGAAAGAGTGG-3′	1,540	18	51.6	5′- CTGATGGGAATAGATT GAC -3′	1,848	19	53.0	308

rnpB (RNA component of RNaseP)

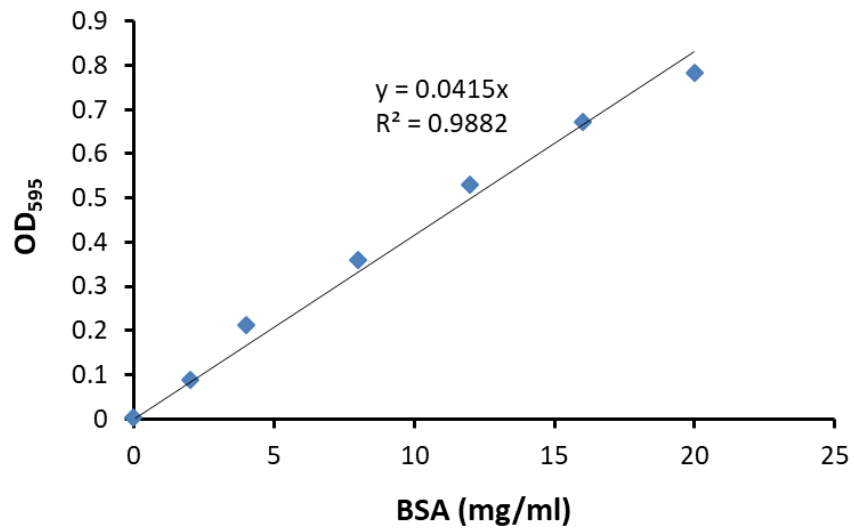mRNA size: 312 bp   KEGG Accession number: Synpcc7942_R0036

GAGGAAAGTCCGGGCTCCCAAAAGACCAGACTTGCTGGGTAACGCCCAGTGCGGGTGACCGTGAGGAGAGTGCCACAGAAACATA
CCGCCGATGGCCTGCTTGCAGGCACAGGTAAGGGTGCAAGGGTGCGGTAAGAGCGCACCAGCAACATCGAGAGGTGTTGGCTCG
GTAAACCCCGGTTGGGAGCAAGGTGGAGGGACAACGGTTGGTCTTTTACCTGTTCCGTTTATGGACCGCTAGAGGTGGCTAGTAA
TAGCCATCCCAGAGAGATAACTGCCCTCTGTCTTCGACAGAGAACAGAACCCGGCTTA

Forward Primer	Pos	Len	Tm	Reverse Primer	Pos	Len	Tm	Amp
5′-GAGGAAAGTCCGGGCTCCC-3′	1	19	63.80	5′-TAAGCCGGGTTCTGTTCTC-3′	312	19	57.3	312

APPENDIX 5

BSA standard curve

$$y = 0.0415x$$
$$R^2 = 0.9882$$

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

APPENDIX 6

Preparation for polyacrylamide gel electrophoresis

1. Tris-HCl Buffer 1.5 Molar pH 8.8

Trisma base ($C_4H_{11}NO_3$)   18.17   g

Dissolve Trisma base in distilled water 80 ml and adjust pH to 8.8 with concentration HCl. Adjust to final volume at 100 ml.

2. Tris-HCl Buffer 0.5 Molar pH 6.8

Trisma base ($C_4H_{11}NO_3$)   6.06   g

Dissolve Trisma base in distilled water 80 ml and adjust pH to 6.8 with concentration HCl. Adjust to final volume at 100 ml.

3. 10% Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate (SDS)   10   g

DI water   100   ml

4. 10% Ammonium persulphate (APS)

Ammonium persulphate (APS)   10   g

DI water   100   ml

5. Separating gel 10% (recipe for 1 gel)

Distilled water	3.56	ml
30% Acrylamide	3	ml
Tris-HCl Buffer 1.5 Molar pH 8.8	2.25	ml
10% Sodium dodecyl sulfate	90	µl
10% Ammonium persulphate	75	µl
TEMED	7.5	µl

6. Stacking gel 5% (recipe for 1 gel)

Distilled water	1.4	ml
30% Acrylamide	830	µl
Tris-HCl Buffer 1.5 Molar pH 8.8	625	µl
10% Sodium dodecyl sulfate	25	µl
10% Ammonium persulphate	25	µl
TEMED	2.5	µl

7. 10X running buffer

Glycine	144	g
Trisma base ($C_{14}H_{11}NO_3$)	30.2	g
Sodium dodecyl sulfate	10	g

Dissolved all compositions into 1,000 ml of deionized water. Keep in room temperature.

8. 1X running buffer

10X running buffer	100	ml

Dissolved in 900 ml of deionized water. Keep in room temperature.

9. Coomassie blue staining solution

Coomassie blue staining	1	g
50% Methanol	500	ml
10% Acetic acid	100	ml
DI water	400	ml

10. Destaining solution

40% Methanol	400	ml
10% Acetic acid	100	ml
DI water	100	ml

APPENDIX 7

Preparation for western blotting analysis

3. Western blotting buffer

Tris(hydroxymethyl)aminomethane	3	g
Glycine	14.4	g
Methanol	200	ml
DI water	800	ml

2. Phosphate-buffer-saline: PBS 1X

Sodium Phosphate 10 mM pH 7.4

Sodium Chloride 150 mM

Adjust to final volume at 1,000 ml

3. Blocking buffer

5% skim milk and 0.01% Tween 20 dissolved in 1X PBS

APPENDIX 8

HPLC chromatogram of M2G produced by *S. elongatus* PCC 7942 expressing cells

Shinorine (3.93 µg/ml) standard curve (2 replicates) retention time at 6.01 min
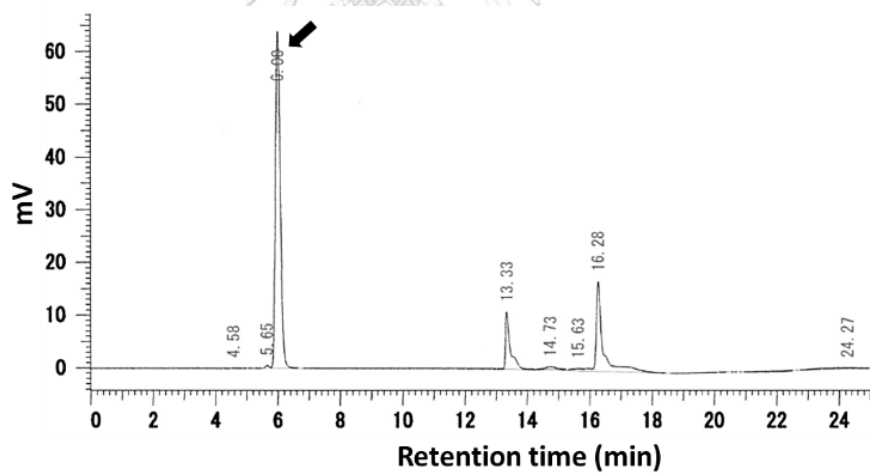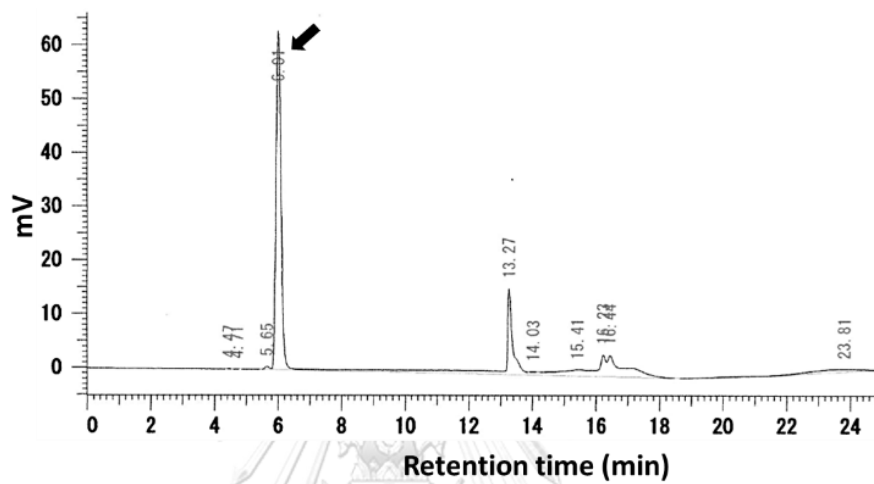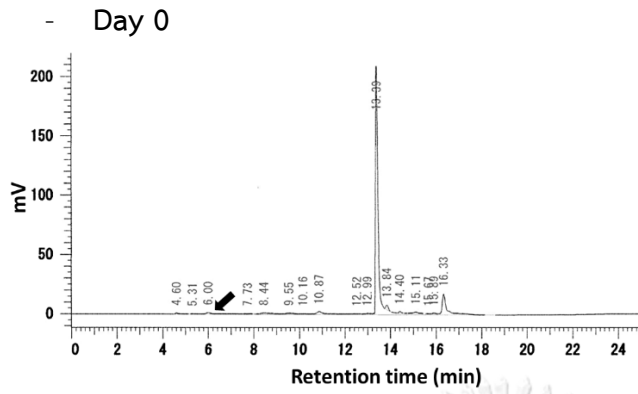
Cultured in 0.35 M KCl (2 replicates) retention time at 5.99 min
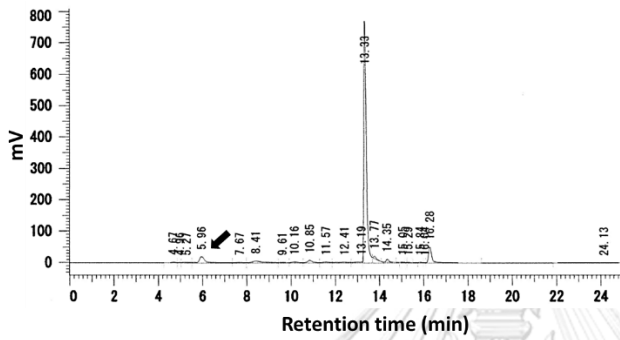
– Day 0

– Day 2
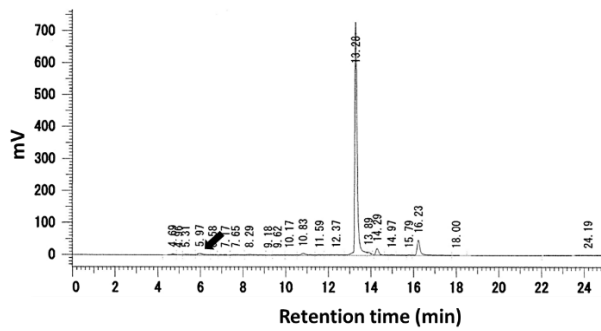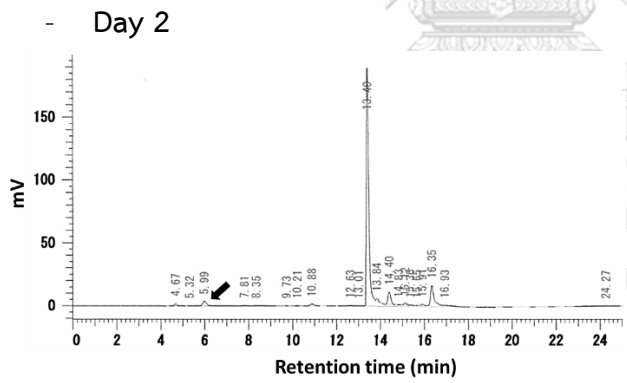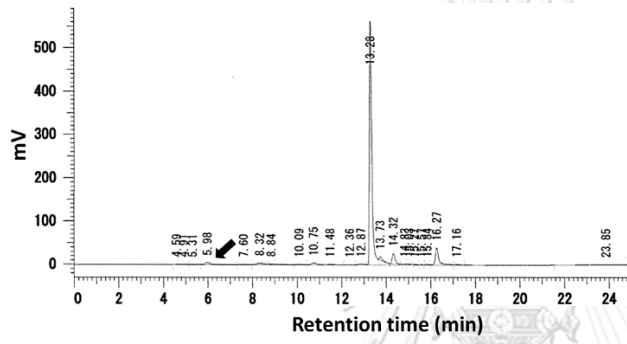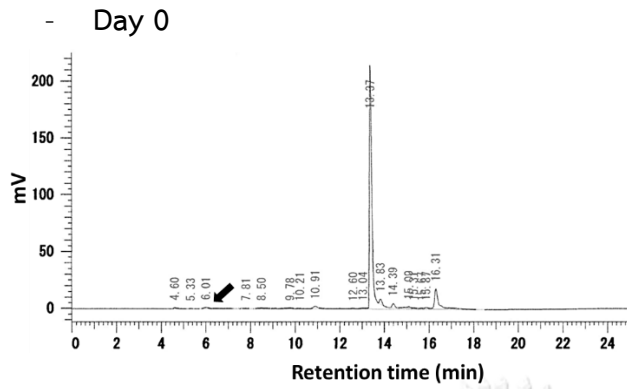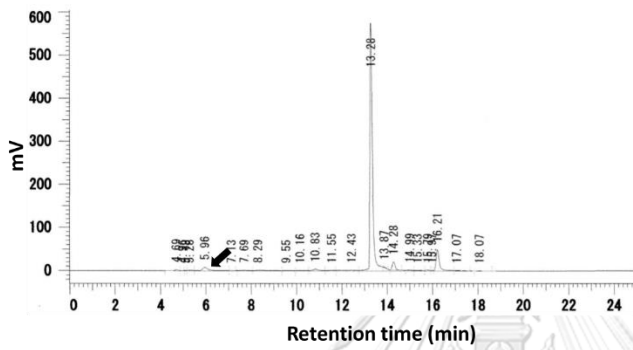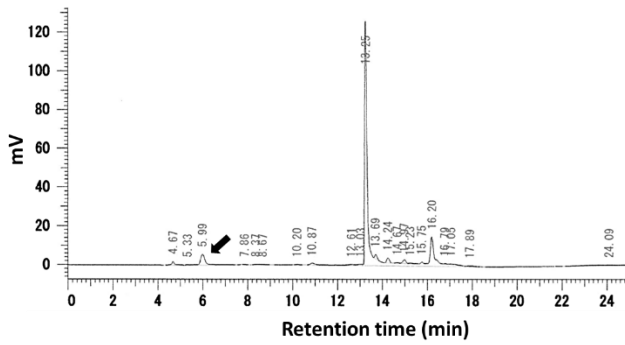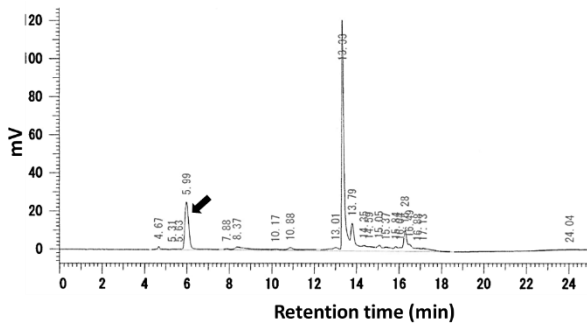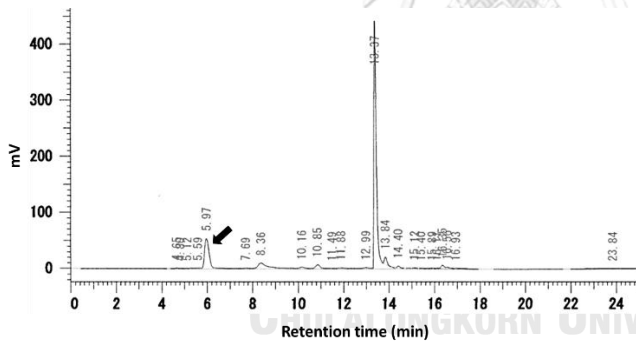
- Day 4

- Day 7

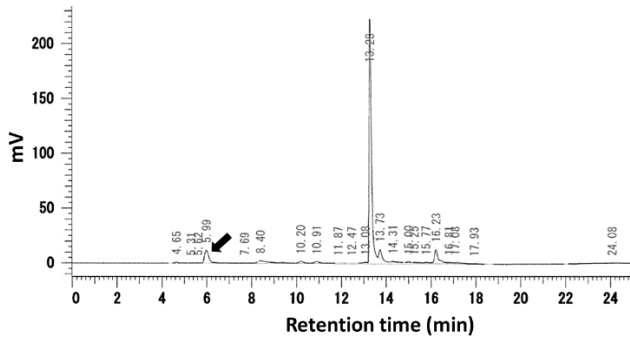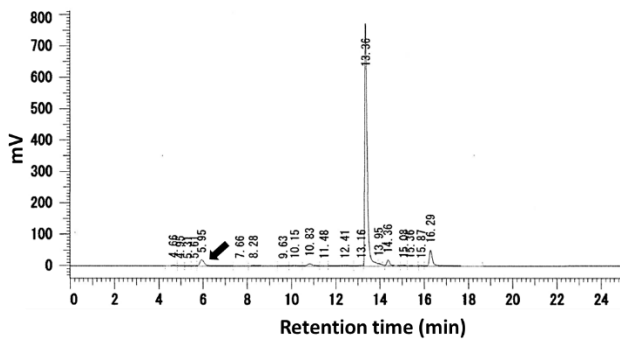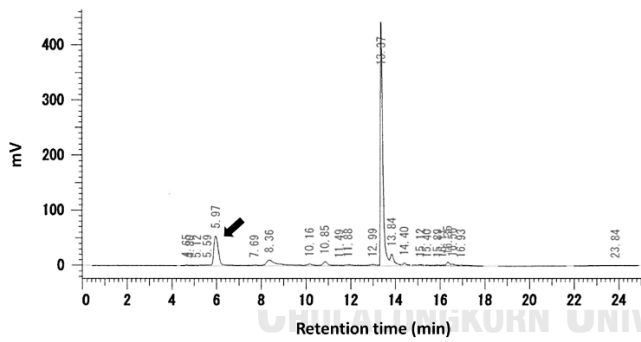**Cultured in 0.35 M NaCl (2 replicates)** retention time at 5.99 min

- Day 0

- Day 2
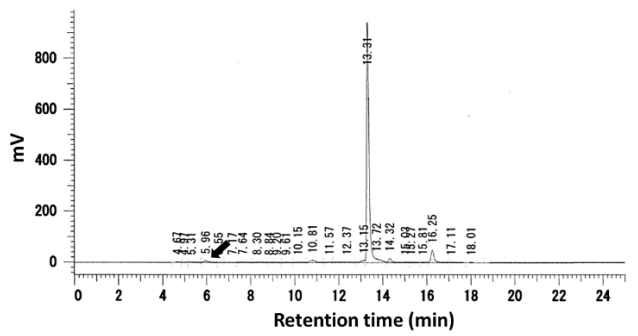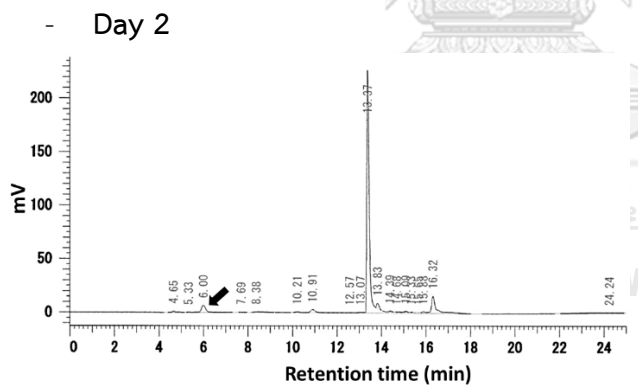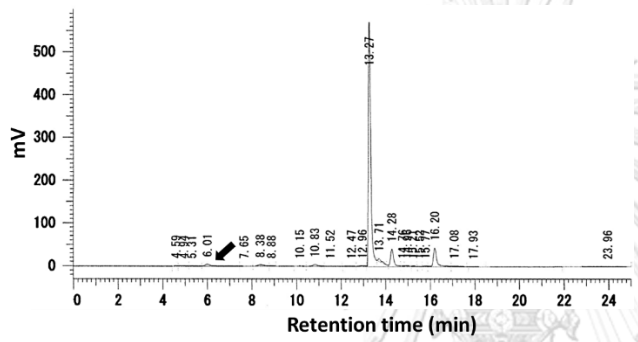
- Day 4

- Day 7

VITA

NAME   Panwad Pingkhanont

DATE OF BIRTH   11 September 1993

PLACE OF BIRTH   Surin, Thailand

INSTITUTIONS ATTENDED   Department of Microbiology, Microbiology and Microbial
                        Technology Program, Faculty of Science, Chulalongkorn
                        University

HOME ADDRESS   608/102 Dindaeng Road, Khwaeng Dindaeng, Khet
                        Dindaeng, Bangkok 10400