ระดับของกรดไขมันและลิพิดในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 สายพันธุ์กลายที่มีการแสดงออกของยีน *plsX* เกินปกติ

นางสาวนุชยา สงฆ์รักษ์

จุฬาลงกรณ์มหาวิทยาลัย
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

FATTY ACID AND LIPID LEVELS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 MUTANT OVEREXPRESSING *plsX* GENE

Miss Nutchaya Songruk

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
Department of Biochemistry
Faculty of Science
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Thesis Title	FATTY ACID AND LIPID LEVELS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 MUTANT OVEREXPRESSING *plsX* GENE
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นุชยา สงฆ์รักษ์ : ระดับของกรดไขมันและลิพิดในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 สายพันธุ์กลายที่มีการแสดงออกของยีน *plsX* เกินปกติ (FATTY ACID AND LIPID LEVELS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 MUTANT OVEREXPRESSING *plsX* GENE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อรัญ อินเจริญศักดิ์, หน้า.

การศึกษานี้ได้สร้าง *Synechocystis* sp. PCC 6803 สายพันธุ์กลายที่มีการแสดงออกของยีน *plsX* เกินปกติ (สายพันธุ์ OX_*plsX*) เป็นผลสำเร็จ แล้วศึกษาผลของภาวะเครียดจากเกลือและการปรับสูตรอาหารต่อการเจริญ ปริมาณรงควัตถุคลอโรฟิลล์ เอ และแคโรทีนอยด์ อัตราการเกิดออกซิเจนหรือประสิทธิภาพของการสังเคราะห์ด้วยแสง ปริมาณลิพิดและองค์ประกอบของกรดไขมันและระดับทรานสคริปต์ของยีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมันในไซยาโนแบคทีเรีย *Synechocystis* สายพันธุ์ปกติ (WT) เปรียบเทียบกับสายพันธุ์ OX_*plsX* โดยในช่วงเริ่มต้นเลี้ยงเซลล์ทั้งสองสายพันธุ์ในสูตรอาหาร BG_{11} ปกติ พบว่าสายพันธุ์ OX_*plsX* มีการเจริญเติบโตและปริมาณรงควัตถุภายในเซลล์ไม่แตกต่างจากสายพันธุ์ WT เช่นเดียวกันกับอัตราการเกิดออกซิเจนของทั้งสองสายพันธุ์ อย่างไรก็ตาม ทั้งปริมาณลิพิดรวมและปริมาณลิพิดที่ไม่อิ่มตัวของสายพันธุ์ OX_*plsX* สูงกว่าสายพันธุ์ปกติ ในทำนองเดียวกัน ปริมาณทรานสคริปต์ของยีน *plsX* ของสายพันธุ์ OX_*plsX* ก็สูงกว่าสายพันธุ์ปกติ จากนั้นเมื่อทดสอบผลของภาวะเครียดจากเกลือและการปรับสูตรอาหารต่อการผลิตลิพิดและกรดไขมัน พบว่า โซเดียมคลอไรด์เข้มข้น 100 มิลลิโมลาร์ สามารถเหนี่ยวนำให้สายพันธุ์ OX_*plsX* สะสมลิพิดรวมสูงกว่าสายพันธุ์ปกติ ถึงประมาณ 1.3 เท่า ขณะที่ปริมาณลิพิดที่ไม่อิ่มตัวของสายพันธุ์ OX_*plsX* สูงกว่าสายพันธุ์ปกติในทุกภาวะเครียดจากเกลือที่ศึกษา องค์ประกอบของกรดไขมันจากการวิเคราะห์ด้วย GC-MS ภายในเซลล์สายพันธุ์ปกติและสายพันธุ์ OX_*plsX* ประกอบด้วย กรดไขมันอิ่มตัวและกรดไขมันไม่อิ่มตัว ได้แก่ กรดปาลมิติก (C16:0) กรดสเทียริก (C18:0) กรดปาลมิโทเลอิก (C16:1) กรดโอเลอิก (C18:1) กรดลิโนเลอิก (C18:2) และกรด α-ลิโนเลนิก (C18:3) ภาวะเครียดจากเกลือมีผลต่อการเปลี่ยนแปลงอัตราส่วนขององค์ประกอบของกรดไขมันอิ่มตัวและกรดไขมันที่ไม่อิ่มตัวภายในเซลล์ นอกจากนี้ เมื่อถ่ายโอนเซลล์ที่เจริญถึงเฟสล็อกไปเลี้ยงในสูตรอาหาร BG_{11} ที่ปรับให้ขาดไนโตรเจนและเสริมแหล่งคาร์บอน 0.4% อะซีเทต (เข้มข้น 6.7 มิลลิโมลาร์) พบว่าการเสริมอะซีเทตสามารถเหนี่ยวนำให้สายพันธุ์ OX_*plsX* สะสมปริมาณลิพิดรวม และลิพิดที่ไม่อิ่มตัว สูงกว่าสายพันธุ์ปกติประมาณ 1.3 และ 2.9 เท่า ตามลำดับ เมื่อเปรียบเทียบลำดับกรดอะมิโนของ plsX กับลำดับกรดอะมิโนของ plsX ของไซยาโนแบคทีเรีย *Anabaena* sp. PCC 7120 และ *Synechococcus* sp. PCC 7002 พบว่ามีความเหมือนสูงที่ 65.77% และ 72.70% ตามลำดับ ขณะที่ความเหมือนกับลำดับกรดอะมิโนของกลี-เซอรอล-3-ฟอสเฟต เอซิลทรานสเฟอเรสของพืช *Arabidopsis thaliana* ค่อนข้างต่ำที่ 16.09% สำหรับความเหมือนเมื่อเปรียบเทียบกับลำดับกรดอะมิโนของ plsX ของแบคทีเรีย *Escherichia coli* และ *Bacillus subtilis* มีค่าเท่ากับ 36.72% และ 42.42% ตามลำดับ ผลของ phylogenetic tree อาจแนะนำได้ว่า plsX ของ *Synechocystis* นั้นอยู่ในกลุ่มเอนไซม์ฟอสเฟต เอซิลทรานสเฟอเรส จากผลการทดลองทั้งหมดบ่งชี้ว่ายีน *plsX* มีบทบาทสำคัญในกระบวนการสังเคราะห์กรดไขมัน โดยเฉพาะอย่างยิ่ง การเพิ่มการแสดงออกของยีน *plsX* ทำให้เซลล์ *Synechocystis* สะสมลิพิดเพิ่มขึ้นและสภาวะการปรับสูตรอาหารที่เสริมอะซีเทตสามารถเหนี่ยวนำการผลิตลิพิดได้สูงทั้งในสายพันธุ์ปกติและสายพันธุ์มีการแสดงออกของยีน *plsX* เกินปกติ

ภาควิชา ชีวเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา 2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: LIPID AND FATTY ACID / PLSX GENE/ SYNECHOCYSTIS SP. PCC 6803

NUTCHAYA SONGRUK: FATTY ACID AND LIPID LEVELS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 MUTANT OVEREXPRESSING *plsX* GENE. ADVISOR: ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., pp.

In this study, we successfully constructed the *plsX*- overexpressing strain of *Synechocystis* sp. PCC 6803 (OX_*plsX* strain), and further studied the effects of salt stress and nutrient modifications on growth, pigment contents of chlorophyll *a* and carotenoids, oxygen evolution rate or photosynthetic efficiency, lipid content and fatty acid composition, and transcript level of genes related to fatty acid synthesis compared to *Synechocystis* wild type. Initially, both strains were cultured in normal BG_{11} medium. Cell growth and intracellular pigment contents of OX_*plsX* strain were not different from those of wild type, as well as their oxygen evolution rate. However, both total lipid and unsaturated lipid contents of OX_*plsX* strain were higher than those of wild type. After that, effect of salt stress and nutrient modifications on cell lipid and fatty acid production were studied. It was found that sodium chloride at 100 mM concentration could induce the accumulation of total lipid in OX_*plsX* strain higher than wild type, up to about 1.3 fold whereas unsaturated lipid content of OX_*plsX* strain was higher than wild type in all salt stress studied. The fatty acid composition from GC-MS analysis consisted of saturated and unsaturated fatty acids including palmitic acid (C16:0), stearic acid (C18:0), pailmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3). We found that salt stress effectively influenced the intracellular composition ratio of saturated and unsaturated fatty acids. On the other hand, after transferring log-phase growing cells into adapted BG_{11} medium, with nitrogen deficiency or carbon source supplementation of 0.4% acetate (6.7 mM concentration), acetate supplementation enabled OX_*plsX* strain to accumulate more total lipids and unsaturated lipids of about 1.3 and 2.9 fold, respectively, when compared to wild type. Next, we aligned the amino sequence of *Synechocystis* plsX compared with the amino sequence of cyanobacterium *Anabaena* sp. PCC 7120 and *Synechococcus* sp. PCC 7002 PlsXs. They showed high identity at 65.77% and 72.70%, respectively, whereas the identity comparing with plant *Arabidopsis thaliana* glycerol-3-phosphate acyltransferase was about 16.09%. The identity between *Synechocystis* PlsX and bacterial *Escherichia coli* PlsX and *Bacillus subtilis* PlsX were 36.72% and 42.42%, respectively. Phylogenetic tree analysis suggests that *Synechocystis* plsX may be classified into phosphate acyltransferase enzyme Altogether, *plsX* gene had a crucial role in fatty acid synthesis since the overexpression of *plsX* gene enhanced the lipid accumulation in *Synechocystis* cells. Also, the acetate supplementation could induce the lipid production in both wild type and *plsX*-overexpressing strains.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

aa	Amino acid
bp	Base pair
°C	Degree celsius
EDTA	Ethylenediamine tetraacetic acid
DMF	*N*,*N*-dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides triphosphates
DTT	Dithiotheitol
g	Gram
HAPES	Hydoxyethyl piperazineethanesulfonic acid
kb	Kilobase
L	Liter
mM	Millmolar
min	Minute
µg	Microgram
µl	Microliter
µmol	Micromole
ml	Milliliter
M	Molar
Nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction

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RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse transcriptase- polymerase chain reaction
w/v	Weight by volume
w/w	Weight by weight

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CHAPTER I

INTRODUCTION

1.1 Lipid and fatty acid

Lipids belong to a group of naturally occurring biomolecules that includes fatty acid, sterol, waxes, monoglycerides, diglycerides, triglycerides, phospholipids and others. Lipids are commonly water-insoluble. Fat and oil are the major storage forms of energy in living organisms. Triglycerides are the most common storage lipids and may constitute up to 80 % of the total lipid fraction in cyanobacteria (Klyachko-Gurvich, 1974, Tornabene *et al.*, 1983). The biological functions of lipid are the main structure of membrane containing phospholipid and sterols, enzyme cofactors, electron carriers, hydrophobic anchors for protein as chaperones, hormones and intracellular messengers (Nelson and Cox, 2008).

Fatty acids are classified into three main types consisting of saturated, monounsaturated and polyunsaturated fatty acids. All fatty acid structures are chains of carbon atoms with hydrogen atoms attached to the carbon atoms. The saturated fatty acid has maximally possible number of hydrogen atoms attached to every carbon atoms and the carbons are attached to one another with single bond. Some fatty acids have a pair of hydrogen atoms in the middle of chain that change two carbon atoms connected to a double bond rather than a single bond, so-called unsaturated fatty acid. The fatty acid has one double bond, namely monounsaturated fatty acid whereas more than one bond are namely polyunsaturated fatty acid. The structures of saturated and

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unsaturated fatty acids are shown in Table 1.1. However, the fatty acid composition of the glycerolipids of cyanobacteria differs from that of most bacteria, which can synthesize only saturated and monounsaturated fatty acids, such as C16:0, C16:1, C18:0 and C18:3. The cyanobacterial cells highly contained families of essential fatty acids including linoleic acid and gamma linolenic acid (Li and Watanabe, 2001). Acyl carrier proteins (ACPs) are essential for the synthesis of fatty acids in prokaryotic cells and chloroplasts from cyanobacteria *Anabaena variabilis* and *Synechocystis* sp. PCC 6803 and higher plants (Froehlich *et al.*, 1990). ACPs from the cyanobacteria are more closely related to ACP from *E. coli* than ACPs from higher plants. The biochemical characteristics of the cyanobacterial enzymes are also similar to those found in most bacterial cells. Elongation of fatty acids from C14:0 to C16:0 and from C16:0 to C18:0 requires ACP and NADPH, but not require to CoA or NADH (Stapleton and Jaworski, 1984b).

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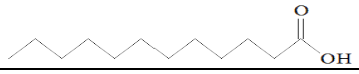
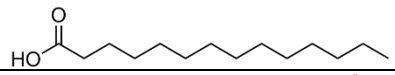
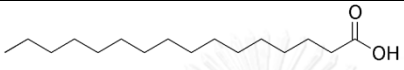
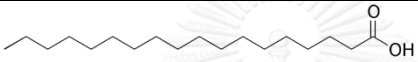
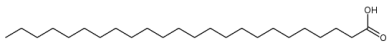
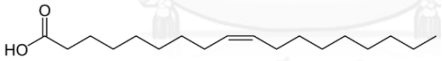
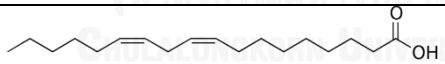
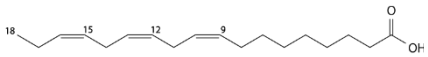
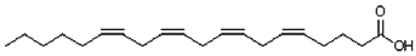
Table 1.1 Naturally occurring saturated and unsaturated fatty acids; structure and nomenclature (Nelson and Cox, 2008).

Carbon skeleton	**Structure**	**Systematic name**	**Common name**
12:0		n-Dodecanoic acid	Lauric acid
14:0		n-Tetradecanoic acid	Myristic acid
16:0		n-Hexadecanoic acid	Palmitic acid
18:0		n-Octadecanoic acid	Stearic acid
20:0		n-Eicosanoic acid	Arachidic acid
24:0		n-Tetracosanoic acid	Lignoceric acid
16:1 (Δ^9)		cis-9-Hexadecenoic acid	Palmitoleic acid
18:1 (Δ^9)		cis-9-Octadecadienoic acid	Oleic acid
18:2 ($\Delta^{9, 12}$)		cis-,cis-9,12-Octadecadienoic acid	Linoleic acid
18:3($\Delta^{9, 12, 15}$)		cis-,cis-,cis-9,12,15-Octadecatrienoic acid	α-Linolenic acid
20:4 ($\Delta^{9, 12, 11, 14}$)		cis-,cis-,cis-,cis-5,8,11,14-Icosatetraenoic acid	Arachidonic acid

1.1.1 Lipid biosynthesis

The lipid biosynthetic pathway exists in different patterns depending on organism species. In plant and bacteria, acetyl-CoA can be converted to malonyl-CoA in a rate-limiting reaction catalysed by acetyl-CoA carboxylase (ACCase EC 6.4.1.2 encoded by *AccC*). In bacteria, ACCase generally contains four proteins of a biotin carboxyl carrier protein, biotin carboxylase and the α and β subunits of carboxyltransferase (Cronan Jr and Waldrop, 2002). In cyanobacterium *Synechocystis* sp. PCC 6803, acetyl-CoA can be converted to malonyl-CoA in a rate-limiting reaction catalysed by a multi-subunit acetyl-CoA carboxylase consisting of AccA (encoded by *slr0728* gene), AccB (encoded by *slr0336* gene), AccC and AccD (Quintana *et al.*, 2011). After that, the malonyl subunit from malonyl-CoA is transferred to ACP by the malonyl-CoA:ACP transacylase (EC 2.3.1.39 encoded by *fabD*). The malonyl-ACP is condensed to acetyl- CoA by 3-ketoacyl-ACP synthase (EC 2.3.1.41 encoded by *fabH*) as shown in Figure 1.1.

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In prokaryote organisms, Acyl-ACP synthesis from malonyl-CoA involves in five different reactions catalyzed by the type II complex of fatty acid synthase (FAS II) whereas eukaryote organisms possess a single multifunctional enzymatic entity (FAS I). The fatty acyl-ACPs, the products of FAS II, are later direct to the synthesis of membrane glycerolipids including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Mizusawa and Wada, 2012). In microorganisms, the fatty acyl-ACP is directly added into a PG molecule (backbone for the glycerolipid synthesis) by a sn-glycerol-3-phosphate acyl- transferase (EC

2.3.1.15 encoded by *GPAT* or *PlsB*;) or by a newly discovered two-reaction system catalyzed by the enzymes, PlsX and PlsY (Zhang and Rock, 2008). Phosphate is added into the fatty acyl group derived from a fatty acyl-ACP chain by *PlsX* catalyzing and then transferred into G-3-P molecule catalyzed by PlsY (Lu and Rock, 2006)

In *Bacillus subtilis*, the roles of three genes consist of *plsX*, *plsY* and *plsC* were investigated (Figure 1.2). In order to figure out the functions of those three genes, the constructions of knockout strains of those three genes in *B. subtilis* were performed. The *plsX*-depleted cells were inactivated on fatty acid and phospholipid systems that *B. subtilis* mutant could not produce long chain acyl-ACP end product of fatty acid synthesis. However, *plsY*-depleted cells also blocked phospholipid synthesis whereas *plsC*-depleted cells accumulated monoacylglycerol content and high amount of fatty acid (Paoletti *et al.*, 2007).The plsC enzyme catalyzed the transfer of fatty acid to the 2-position of Acyl-G3P via acyl-ACP into phosphatidic acid (PtdOH), the key intermediate of phospholipid synthesis.

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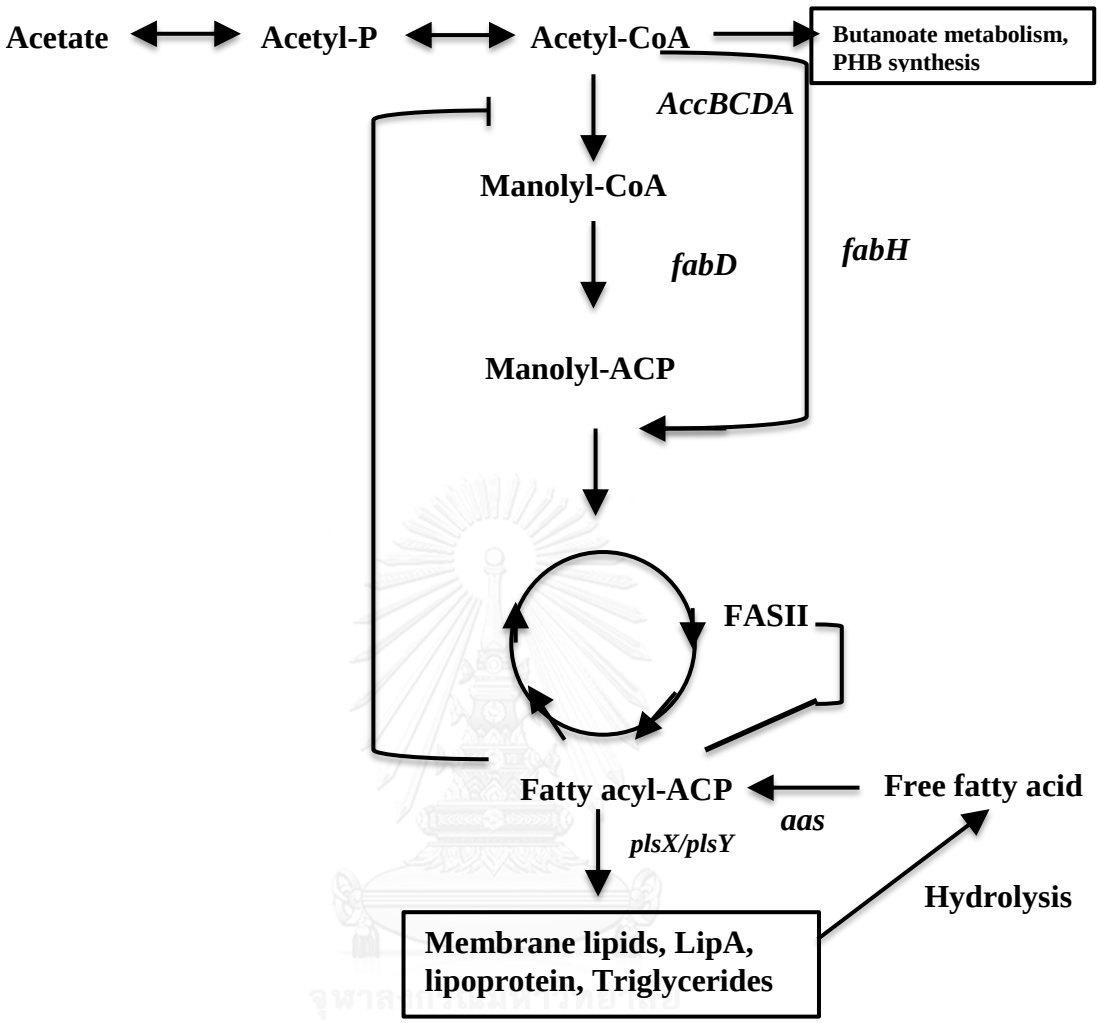

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Figure 1.1 Simplified overview of the lipid biosynthesis and neighbouring pathways in cyanobacteria (modified from (Liu *et al.*, 2011). Genes encoding metabolic enzymes: *accBCDA* (acetyl-CoA carboxylase subunit BCDA), *fabD* (ACP transacylase), *fabH* (3-ketoacyl-ACP synthase), FASII (fatty acid synthase II), *plsX/plsY* (fatty acid/phospholipid synthesis protein PlsX) and *aas* (acyl-acyl carrier protein synthetase).

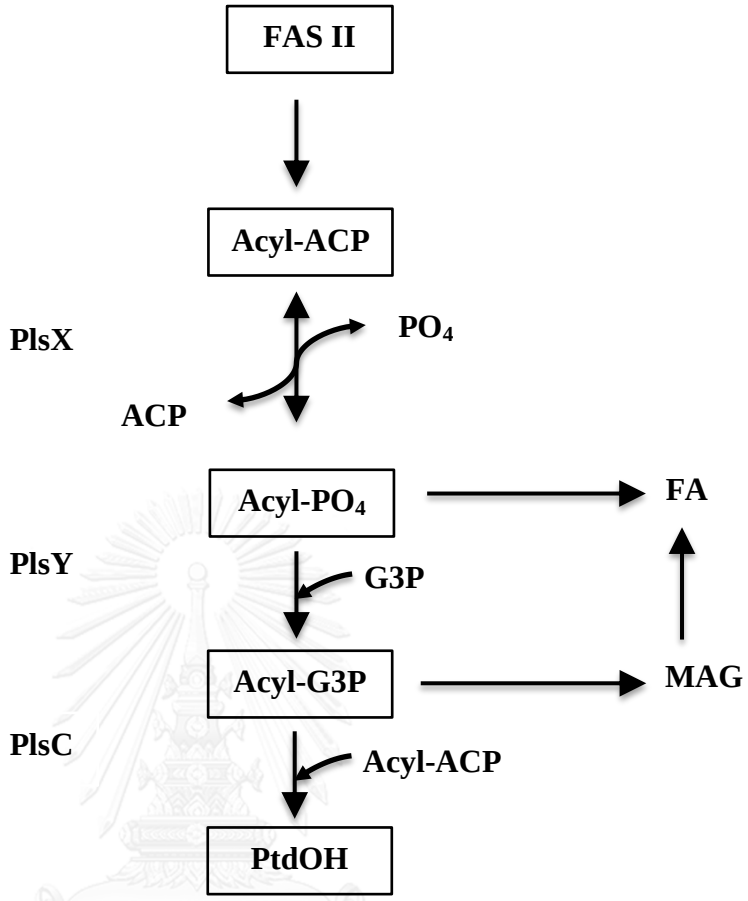

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Figure 1.2 Coupling of fatty acid and phospholipid syntheses in *B. subtilis*. Long-chain acyl-ACPs are the end products of the bacterial dissociated type II fatty acid synthase system (FAS II). Genes encoding metabolic enzymes: PlsX (phosphotransferase), PlsY (acylglycerol-phosphate-acyltransferase) encoded by *yneS* gene and PlsC (1-acylglycerol-Phosphate-acyltransferase) encoded by *yhdO* gene. The intermediates of phospholipid synthesis: Acyl-PO_4 (acyl phosphate), Acyl-G3P (acyl glycerol-3-phosphate), PtdOH (phosphatidic acid), FA (fatty acid), Acyl-ACP (acyl-acyl carrier proteins) and MAG (monoacylglycerol) (Paoletti *et al.*, 2007).

1.1.2 Structural membrane lipids

Membrane lipids are groups of compounds, which form the double layered surface of cells, which act as barrier to the passage of molecules and ion. Membrane lipids are amphipathic, which means one end of the molecular is hydrophobic and their other hydrophilic. There are hydrophobic interactions with each other and hydrophilic interactions with water direct packing into sheets called membrane bilayers. Membrane lipids can be classified into five types consisted of glycerophospholipids, sphingolipids, galactolipids, sulfolipids and archaeal ether lipids (Figure 1.3).

The glycerophospholipids (GPLs) (Figure 1.4A) also called phosphoglycerides are the main constituents of cellular membranes, consisting of a polar head group with a phosphate moiety and two fatty acids that are attached to the glycerol backbone, which two fatty acid are attached in ester linkage to first and second carbons of glycerol. Then, the high polar or charged group is attached through a phosphodiester linkage with three carbons of glycerol. The common of GPLs are phosphatidylethanolamine and phosphatidylcholines (PC).

Phosphatidylcholines (PC) are derivatives of GPLs in mammalian cells, accounting for more than 30% of the total lipid content and are mainly located on the outer leaflet of the plasma membrane (Renooij *et al.*, 1976). In cyanobacteria, there was classified as one phospholipid of phosphatidylglycerol (PG) in thylakoid membranes as well as of the chloroplast of higher plants (Block *et al.*, 1983).

The membrane lipids in cyanobacteria consist of inner- and outer-envelope membranes and thylakoid membranes (Pushparaj *et al.*, 2008). The thylakoid

membranes of cyanobacteria contain three glycolipids, namely, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG), and one phospholipid, phosphatidylglycerol (PG) (Figure 1.4B) as major components of the thylakoid membranes. MGDG accounts for about 50% of the total lipid and the other three lipids such as DGDG, SQDG and PG ranging accounted for about 5–20% of the total in *Synechocystis* sp. PCC 6803 (Wada and Murata, 1998). The compositions in most cyanobacteria are similar to those of the inner envelope membranes and thylakoid membranes of chloroplasts in higher plants (Block *et al.*, 1983, Joyard and Douce, 1997). Then, the major components of phospholipids are PC, PE and PG in higher plant and cyanobacterial cells.

The major of membrane lipid in *Gloeobacter violaceus* sp. PCC 7421 consisted of MGDG, DGDG, PG and phosphatidic acid were 51, 24, 18 and 4% respectively, but a lack of SQDG affected on growth rate and impaired photosystem II function (Selstam and Campbell, 1996). In *Spirulina platensis*, it was found that the total lipid content contained MGDG of about 43.6% (C18:3, C16:0), 13.9% (C16:0, C16:0), 9.9% (C18:2, C16:0), and 8.9% (C18:3, C16:1). DGDG contained 37.6% (C18:3, C16:0), 8.7% (C16:0, C16:0), and 8.7% (C18:2, C16:0). Molecular species composition of SQDG contained 52.5% (C18:2, C16:0) (Xue *et al.*, 2002). However, in *Synechocystis* sp. PCC6803 showed the glycerolipids in thylakoid membrane including MGDG, DGDG, SQDG and PG of about 62, 14, 18 and 6 %, respectively (Wada and Murata, 1998).

The membrane lipids in *Gloeobacter violaceus* sp. PCC 7421 found esterified to C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 (Selstam and Campbell, 1996). Fatty acids compositions in *Spirulina platensis* UTEX 1928

included C16:0, C17:0, C18:0, C18:1, C18:2 and C18:3 which the major of fatty acids composition was C16:0 accounted for about 55.3% (Kaneko *et al.*, 1996). Also, in *Synechocystis* sp. PCC6803, it had been accounted up to 60% of C16:0 (Sheng *et al.*, 2011). Moreover, the fatty acid composition in *Arthrospira* were two polyunsaturated C18 acids including linoleic acid formed 13.1–31.5% and γ -linolenic acid formed 12.9–29.4% of total fatty acids. The palmitic acid (C16:0) was about 42.3-47.6% (Mühling *et al.*, 2005).

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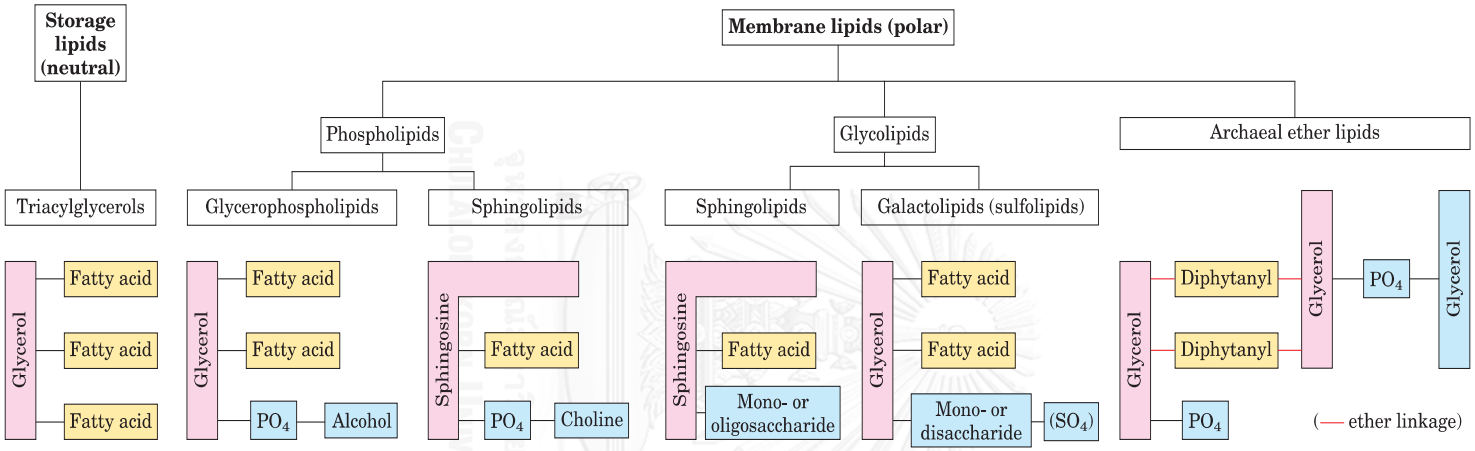

Figure 1.3 Types of storage and membrane lipids (Nelson and Cox, 2008). The lipid types shown glycerol and sphingosine are the backbone represent in pink color, which attach one or more long chain alkyl groups are fatty acid represent in yellow color and a polar head group represent in blue color

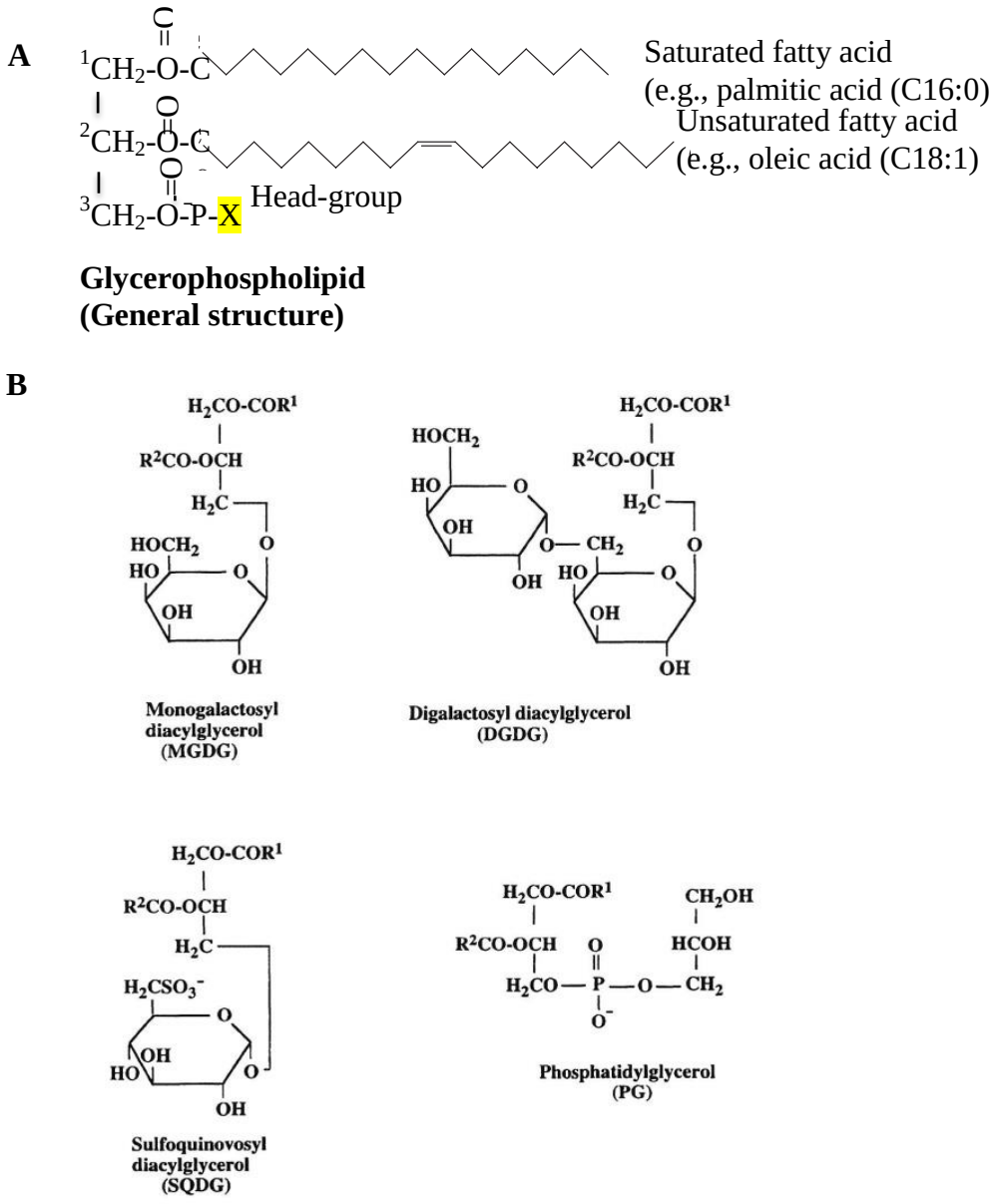

Figure 1.4 Simplified common glycerophospholipids (GPLs) which are diacylglycerol linked to head-group alcohol (X) through a phosphodiester bond and attached two fatty acyl groups (Nelson and Cox, 2008) (A). The structures of the major glycerolipids in thylakoid membranes are MGDG, DGDG, SQDG and PG from plant and cyanobacteria (B).

Table 1.2 Membrane lipids found in cyanobacteria and other species

Organisms	**Types of membrane lipid**	**Total lipid content (%)**	**References**
Sprirulina platensis	MGDG	43.6 %	(Xue *et al.*, 2002)
	DGDG	37.6 %	
	SQDG	52.5%	
Gloeobacter violaceus sp. PCC 7421	MGDG	51%	(Selstam and Campbell, 1996)
	DGDG	24%	
	PG	18%	
	Phosphatidic acid	4%	
Synechocystis sp. PCC 6803	MGDG	62%	(Wada and Murata, 1998)
	DGDG	14%	
	SQDG	18%	
	PG	6%	

*monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) sulfoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG)

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1.2 Abiotic stress

Abiotic stress is the most harmful stress generated by non-living factors influenced on living organisms which is concerned to their growth and productivity. The most severe abiotic stresses are such as pH strength, temperature, light, salinity and drought. Generally, plant and cyanobacteria perform tolerance or sensitivity response to abiotic stress. Lipid content and its composition also stress-inducible functioned in living cells. The membrane lipids of major importance of organism were found that responded to environmental stresses (Olie and Potts, 1986, Ritter and Yopp, 1993).

Salt stress is one of abiotic stresses, which affects many physiological processes, such as respiration rate, ion toxicity, changes in plant growth, lipid metabolism and membrane permeability (Allakhverdiev *et al.*, 2001, Gupta *et al.*, 2002, Ranjbarfordoei *et al.*, 2002, Muranaka *et al.*, 2002a, Muranaka *et al.*, 2002b, Murphy *et al.*, 2003, Parida and Das, 2005). The hyperosmotic stress-induced and the salt stress-induced inactivation of the photosynthetic machinery, particularly the oxygen evolving machinery of the photosystem II complex, were found in *Synechococcus* sp. PCC 7942 (Allakhverdiev *et al.*, 2001), as well as the distribution membrane bound enzymes and membrane fluidity, particularly in plant and cyanobacteria (Allakhverdiev *et al.*, 1999). The defensive response to salt stress encompasses many mechanisms helping organisms to gain more tolerance to salt stress, such as osmolyte accumulations of glycine betaine, proline, polyamines, and the increase of unsaturated fatty acid composition within cell membranes (Incharoensakdi and Wutipraditkul, 1999, Jantaro *et al.*, 2003, Takagi *et al.*, 2006).

Temperature stress is an environmental factor that limits growth and induces

inactivation of photosynthesis of plants and cyanobacteria. The physical properties of low temperatures are biological changes of membrane lipids depending on the levels of unsaturated fatty acid composition. In cyanobacteria, the unsaturated fatty acid occurs into de novo synthesis of fatty acid during low temperature (Wada and Murata, 1990, Gombos *et al.*, 1992). However, lower temperature enhanced highest yields of eicosapentaenoic acid (EPA) and polyunsaturated fatty acids (PUFAs) amounts of 4.9% and 2.6% per unit dry mass, respectively, in *Phaeodactylum tricornutum* (Bohlin) when temperature was shifted from 25 to 10 °C for 12 h (Jiang and Gao, 2004). In *Synechocystis* sp. PCC 6803, the mutant that had lower level of enolyl-(acyl-carrier-protein) reductase encoded by *fabl* gene (*slr1051*) that a key component of type II fatty acid synthase system were observed. At 40 °C, the *fabI* mutant cells were unable to grow compared to wild type. On the other hand, saturated fatty acids in thylakoid membrane were not changed in both strains at 25 °C transferred to 38 °C for 24 h (Nanjo *et al.*, 2010). On the other hand, *Spirulina platensis* UTEX 1928 was affected by changing in growth temperature from 25 to 38 °C. It was found that *Spirulina* showed increases on growth rate and total lipid content up to 7.2 % (Tedesco and Duerr, 1989). However, the *desA−/desD−* mutant, that lacked two genes of Δ12 and Δ6 desaturases and *Synechocystis* wild type cells were incubated under cold stress at 22 °C and continuous light, the PUFAs induced the accumulation of PUFAs in membrane (Mironov *et al.*, 2012).

Nutrient starvation affected on lipid concentration, especially nitrogen deficiency. When increasing the incubation time of nitrogen starvation at 0, 14, 33, 40, 57, 64, 82 and 88 hours, the result showed that the total lipids were increased at 57 to 88 hours of about 8.2 % of cell dry weight but the composition ratio of unsaturated

and saturated fatty acid content was not changed under nitrogen starvation in *Spirulina platensis* UTEX 1928 (Tedesco and Duerr, 1989). On the other hand, marine cyanobacterium *Oscillatoria willei* BDU 130511 cells were grown under nitrogen deprivation stress which could decrease total lipid content (26.08 %g/DW) and fatty acids, such as pentadecanoic acid (C15:0), oleic acid (C18:1 cis), linoleic acid (C18:2 cis), behenic acid (C22:0) eicosapentaenoic acid (C20:5) and eicosenoicacid (C20:1). For lauric acid and α-linolenic acid, which increased under nitrogen-starved conditions by 14.53% and 40.55 %g/DW, respectively (Saha *et al.*, 2003).


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1.2 *Synechocystis* sp. PCC 6803

Formerly grouped with the blue-green algae, the cyanobacteria are living photoautotrophic prokaryotes and have higher oxygenic photosynthesis capturing sunlight for energy using chlorophyll *a* and various accessory pigments (Stewart, 1980, Sinha, 1996a, Sinha and Häder, 1996b). However, the pigments are embodied in phycobilisomes, which found in rows on the outer surface of cyanobacterial thylakoids (Douglas, 1994). All cyanobacteria contain chlorophyll *a* and phycocyanin. They can be classified as the Procaryota, Division of Cyanophyta and as gram-negative microorganism, which means they have an outer membrane, a peptidoglycan layer and a plasma membrane (Figure 1.5A and 1.5 B). In addition to this double outer membrane system, there is an internal thylakoid membrane system for photosynthesis. Among prokaryotes, cyanobacteria are the only organisms to engage in oxygen photosynthesis that is responsible for the majority of the oxygen in the atmosphere. Cyanobacteria can grow in many different regions throughout the world such as lakes, ponds, springs, wetlands, streams, and rivers (Schafer, 1989). However, the phototrophic metabolism of cyanobacteria could generate renewable biofuels and chemicals via sunlight and the greenhouse gas CO2 (Kaneko *et al.*, 1996). Additionally, biochemical similarities between the plant chloroplasts and *Synechocystis* sp. PCC 6803 are promising for studying the molecular mechanisms underlying stress responses and stress adaptation in higher plants (Los *et al.*, 2010).

One model of cyanobacterial species is the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. It is a unicellular non-nitrogen (N_2)-fixing cyanobacterium, which grows ubiquitously in fresh water. Importantly, it had a complete genome sequenced since 1996 (Kaneko *et al.*, 1996). More importantly, this

strain is naturally transformable and effectively performs double homologous recombination (Zhang and Rock, 2008). Then, *Synechocystis* sp. PCC 6803 is one of the most popular organisms for genetic engineering and physiological studies serving as a model of higher plant (Ikeuchi and Tabata, 2001) (Figure 1.7). However, *Synechocystis* sp. PCC 6803 is popularly studied in five topics consisted of rate-limiting factors for cell cultivation, molecular tools for genetic modifications, high-throughput system biology for genome wide analysis, metabolic modeling for physiological prediction and rational metabolic engineering, and applications in producing diverse chemicals (Yu *et al.*, 2013). In the database, namely Cyanobase, the circular genome was deduced to be 3,573,470 bp long (Figure 1.6). Its genome has been sequenced and many systems biology and molecular biology tools are available to study. *Synechocystis* sp. PCC 6803 was also used as a model for studying the mechanisms of the stress response and long-term adaptation (Jantaro *et al.*, 2003). Biomass yield or growth rate depends on nutrient availability (such as CO_2, nitrogen, and phosphorus) and cultivation conditions (such as light irradiance, temperature, pH, *etc.*) (Kim *et al.*, 2011).

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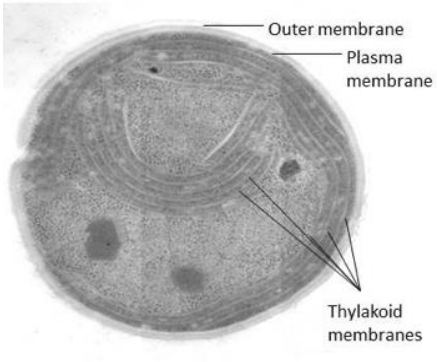
1.4 Objectives of this research

1.To overexpress *plsX* gene in cyanobacterium *Synechocystis* sp. PCC 6803

2.To investigate their fatty acid and lipid levels under environmental stress in *plsX*-overexpressing strain compared to wild type under normal and stress conditions

A



B

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Figure 1.5 Ultrastructure image (A) and cell cultures both in liquid medium and agar medium (B) of *Synechocystis* sp. PCC 6803.

(Source: https://newunderthesunblog.wordpress.com/the-basics/cyanobacteris/)

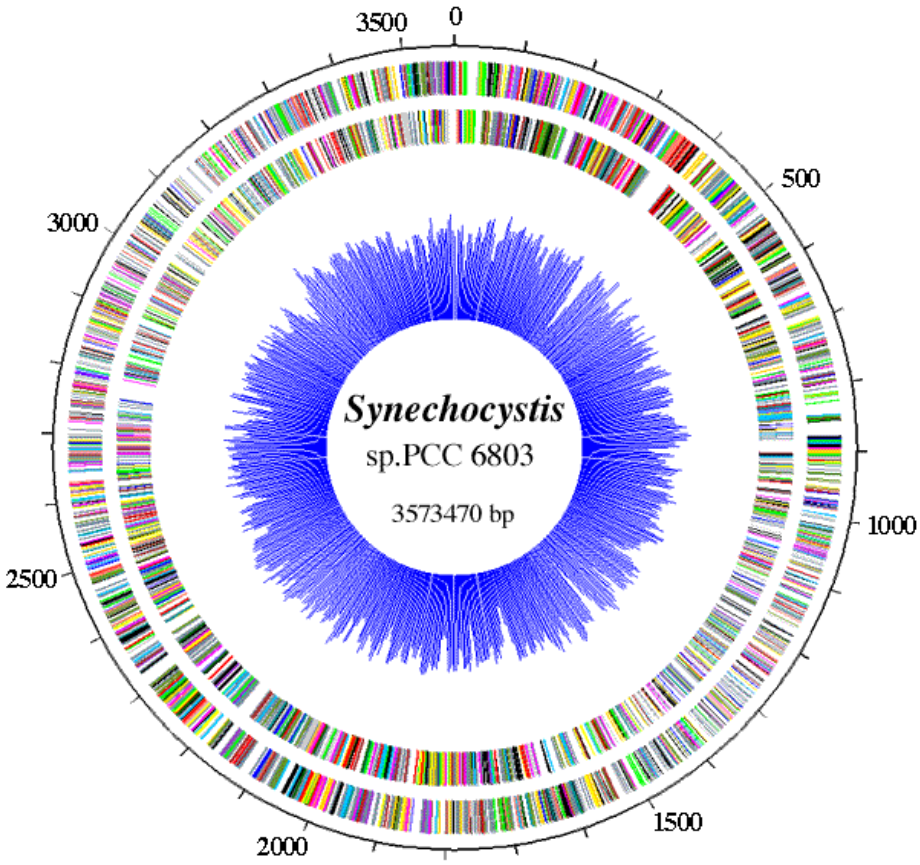

Figure 1.6 The cellular genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 according to Cyanobase.

(Source:http://genome.microbedb.jp/cyanobase/Synechocystis/map/Chr)

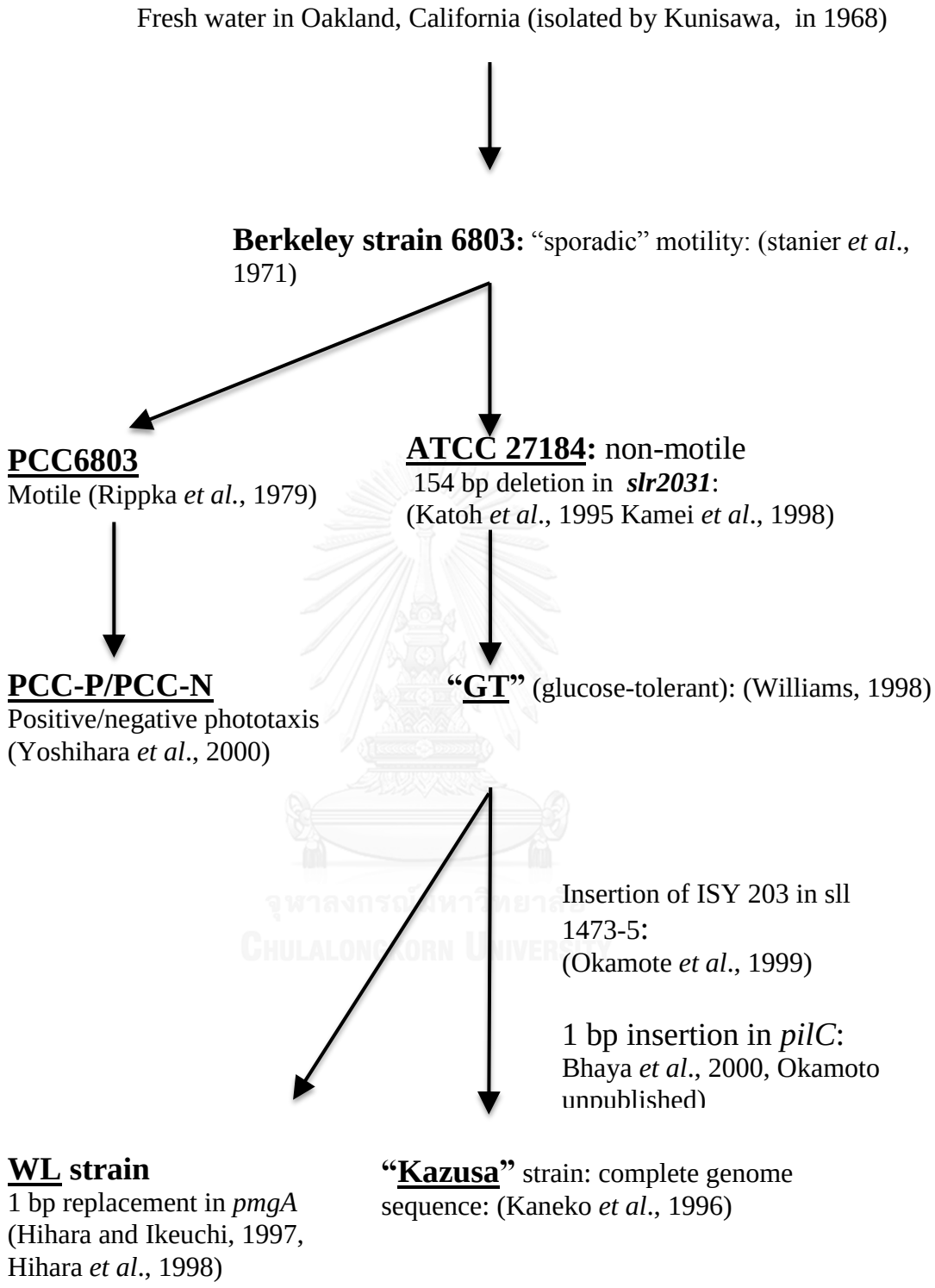

Figure 1.7 Strain history of *Synechocystis* sp. PCC6803 (Ikeuchi and Tabata, 2001).

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave	Model HA-30, Hirayama Manufacturing Corporation, Japan
Centrifuge	Mikro 220 R Hettich, Germany
	5417C Eppendorf, Germany
Gel documentation	Syngene® Gel Documentation, USA
Gel Electrophoresis System	Gibthai, Thailand
Fume Hood	Science technology protection laboratory hood, USA
Laminar flow	Boss Tech Scientific Instruments, Thailand
Microscope	Olympus BX51, USA
Microwave	Sharp, Thailand
Oxygraph system	Clark-type oxygen electrode, Hansatech Instruments, England, USA
PCR apparatus	Master cycler gradient eppendorf, Germany
PH meter	Metter Toledo, USA
Power supply	Power PAC 1000 BIO-RAD, USA
Spectrophotometer	Biomate 3 Thermo Scientific, USA
Vortex	Model 232 Touch mixer Fischer Scientific, USA
Water bath	Hangzhou Bioer Technology, China

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2.1.2 Chemicals

Agarose	Invitrogen, USA
Ammonium ferric citrate	Ajax Finechem, Australia
Ammonium persulfate (APS)	Merck, Germany
Boric acid	Scharlau Chemie S.A., Spain
Bromophenol blue	Sigma, USA
Calcium chloride dehydrate	Ajax Finechem, Australia
Citric acid	Ajax Finechem, Australia
Chloroform	Merck, Germany
Coomassie blue G-250	Fluka, USA
Copper (II) sulfate	Carlo Erba Reagents, France
Dimethylformamide	Lab Scan, Ireland
Dithiothreitol (DTT)	Sigma, USA
EDTA	Ajax Finechem, Australia
Ethanol	Burdick & Jackson®, Australia
Ethidium bromide	Sigma, USA
Glycerol	Ajax Finechem, Australia
Glycine	Ajax Finechem, Australia
HEPES	Calbiochem®, Germany
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate heptahydrate	Ajax Finechem, Australia
Mercaptoethanol	Sigma, USA

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Methanol	Burdick & Jackson®, Australia
Phenol	Merck, Germany
Potassium sodium tartrate	Merck, Germany
Sodium acetate	Ajax Finechem, Australia
Sodium chloride	Ajax Finechem, Australia
Sodium hydroxide	Ajax Finechem, Australia
Sodium nitrate	Ajax Finechem, Australia
Tris (hydroxymethyl)-aminomethane	USB Corporation, USA
Trizol® reagent	Invitrogen, USA

2.1.3 Kits

GeneRuler™ 1 kb DNA Ladder	Fermentas, Canada
Plasmid extraction kit	Invitrogen, USA
RNase-Free DNase	Fermentas, Canada
SuperScrip™ III First-strand Synthesis System	Invitrogen, USA
Tag DNA polymerase	Invitrogen, USA

2.1.4 Enzyme and restriction enzymes

*Pst*I	Fermentas, Canada
*Spe*I	Fermentas, Canada
T4 DNA ligase	Fermentas, Canada

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2.1.5 Expression vector

The expression vector was used in this study, namely pEERM vector which provided by Professor Peter Lİnblad from the Photochemistry and Molecular Science, Department of Chemistry – Ångström Laboratory, Uppsala University, Sweden. This vector contains a strong promoter (P_{psbA}), a selective chloramphenicol antibiotic cassette gene and multiple cloning sites: *Xba*I, *Spe*I and *Pst*I. The size of pEERM is around 3.6 kb (Appendix B).

2.1.6 Organisms

Synechocystis sp. PCC 6803 wild type strain was obtained from the Photochemistry and Molecular Science, Department of Chemistry - Ångström Laboratory, Uppsala University, Sweden.

Escherichia coli (DH5α) strain was used as a host for plasmid propagation.

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2.1.7 Primers

Table 2.1 Sequences of the primers used in this study

Target gene	**Name**	**Primers**	**Length in pairs (bp)**
PlsX	Forward-*PlsX*	5'-TAGAGAACTAGTATGGCTGTAACGCGG-3'	1,047
PlsX	Reverse-*PlsX*	5'-TAGAGACTGCAGCTAGATATTCTGTAATTCCTC-3'	
Cm	Forward-*Cm*	5'-GAGTTGATCGGGCACGTAAG-3'	899
Cm	Reverse-*Cm*	5'-CTCGAGGCTTGGATTCTCAC-3'	
Up-psbA2	Forward-*psbA2*	5'-TGCCTGTCAGCAAAACAACTT-3'	3,330
Down-psbA2	Reverse - *psbA2*	5'-CGAGGGCAATCATCAATTCCG-3'	

Table 2.2 Sequences of the primers for RT-PCR

Target gene	**Name**	**Primers**	**Length in pairs (bp)**
16s	Forward-*16s*	5'-AGTTCTGACGGTACCTGATGA -3'	521
16s	Reverse-*16s*	5'- GTCAAGCCTTGGTAAGGTTAT-3'	
PlsX	Forward-*PlsX*	5'-AAGGGGTGGTGGAAATGGAA -3'	488
PlsX	Reverse-*PlsX*	5'- AAGTAGGTCCCTTCCTTCGG-3'	
AccA	Forward-*AccA*	5'-ATGCACGGCGATCGAGGAGGT-3'	428
AccA	Reverse-*AccA*	5'-TGGAGTAGCCACGGTGTACAC-3'	

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2.2 Methods

2.2.1 The construction and transformation of *plsX*-overexpressing strain of *Synechocystis* sp. PCC 6803

2.2.1.1 The genomic DNA extraction

Synechocystis sp. PCC 6803 cells were grown in 50 ml of normal BG_{11} medium (Appendix A). The growth condition was performed at 30 °C, under continuous white light of 40-50 μmole photon $m^{-2}s^{-1}$ intensity. After that, cells were harvested by centrifugation at 6,000 rpm (4,025 × *g*), 4°C for 10 minutes. The obtained pellet was resuspended in 1 × TE pH 7.5 buffer (0.4 ml) in a tube with the addition of acid washed-glass beads (0.2 g). Cell disruption was performed by vortexing for 2-3 times until cells were completed broken and transferred the supernatant fraction into a new tube. Then, extracted chromosomal DNA was obtained by phenol-chloroform method (Sambrook *et al.*, 1989). Each tube was added of 1 volume of the phenol: chloroform: isoamyl alcohol mixture of sample and mixed that solution by shaking for 2-3 times. The sample tube was centrifuged at 12,000 rpm (21,009 × *g*), 25°C for 10 minutes and transferred the upper aqueous phase containing DNA into a new tube. The addition of 1 volume of chloroform: isoamyl alcohol mixture was done and centrifuged again at 12,000 rpm (21,009 × *g*), 25°C for 10 minutes. After that, 0.1 volume of 3M NaOAC and 2.5 volume of absolute ethanol were added and mixed that solution by shaking for 2-3 times. Later, that reaction tube was incubated at -20°C for 2 hours and centrifuged at 12,000 rpm (21,009 × *g*), 4°C for 10 minutes. In order to get DNA pellet, the supernatant was discarded and washed pellets by 70% ice-cold ethanol. Next step, the DNA sample tube was centrifuged at

12,000 rpm (21,009 × *g*), 4°C for 10 minutes. That washed DNA pellets were air-dried for 10 minutes in order to remove ethanol vapor and dissolved those DNA pellets with 20 µl of 1xTE buffer.

2.2.1.2 The construction of recombinant plasmid

2.2.1.2.1 *PlsX* gene information and primer design

PlsX gene was searched for its information from the genome database of cyanobacteria (Cyanobase, http://kazuza.or.jp/cyanobase/). That gene is identified as Slr1510 and encodes fatty acid/phospholipid synthesis protein PlsX. It has the nucleotide sequence of **1,047** bp (Figure 2.1) and translated sequence of **348** aa. Then, the primer was designed using Primer 3 program.

```
1       TAGAGAACTA GTATGGCTGT AACGCGGGCG AAAATTGCTT TAGACGCAAT GGGCGGTGAC
61      TATGCCCCCG AAGAGATTGT TATCGGTGCT ATCCGGGCGA GTCAGGAATT AGATGTAGAT
121     ATTTTCCTTG TCGGCGATCG CCAGGCCATT GAGGATTGTT TAAACCGCCA TCCCCATCAG
181     GGCATTAACC TTACCATTGT CGATGCGGAA GGGGTGGTGG AAATGGAAGA AGATGCGGTG
241     GTGGTGCGCC GTAAGCCCAA GGCGTCCATT AATGTGGCGA TGAATTTGGT CAAAGAAAAA
301     CAAGCCGATG CGGTGGTGTC CGCTGGCCAT TCGGGAGCGG CCATGGCGGC GGCCCTACTG
361     CGGCTGGGCC GTTTAAAAGG CATTGACCGG CCGGCGATCG GCACCTTATT TCCCACAATG
421     GTTCCCGGCA AATCGGTAAT TGTGCTAGAC GTGGGGGCCA ATGTGGATTG CAAGCCTAAA
481     TACCTGGAAC AGTTTGCCCT CATGGGAACG GTGTACAGCC AATATGTGTT GGGGGTTGAT
541     AGCCCCAAGG TTGGTCTGCT GAACATTGGT GAAGAGTCTA ATAAAGGTAA CACCCTCGCC
601     CTGCAAACCC ATGAATTGTT GCAGAGTAAT CCGGAAATTC CCTTTGTGGG CAACGCCGAA
661     GGAAGGGACG TACTTTCCGG TAATTTTGAT GTGATTGTTT GTGATGGTTT TGTCGGCAAT
721     ATTGTGCTTA AATTTGCCGA AGCGGTGGGG GAAATTTTAC TGAGCATTGT TAAAGAAGAA
781     CTACCCAGGG GCTGGCGGGG CAAATTGGGG GCCATTATTT TAGCCCCCAA CCTCAAACGC
841     ATTAAGCAAC GGGTGGACCA TGCCGAACAT GGAGGAGCCT TACTATTCGG GGTCGATGGG
901     GTTTGTGTAA TTAGCCATGG TAGTTCCCGC AGTGGCTCCA TTTTTAACGC CATTCGCCTA
961     GCTAAGGAGG CGATCGATAA TCAGGTTTCT GTGCGCATTA ACAGTTCCAC TTCCCTATTA
1021    ATGGAACGAC AAAAAACAGA GGAATTACAG AATATCTAGG ACGTCAGAGA T
```

Figure 2.1 The nucleotide sequence of *plsX* gene. Bold letters in color represent the designed primers including a forward primer location (red color) and a reverse primer location (blue color).

2.2.1.2.2 Gene cloning to the overexpression vector

Synechocystis sp. PCC 6803 genomic DNA prepared form 2.2.1.1 was used as the DNA template for *PlsX* gene fragment amplification. The *plsX* was amplified by PCR method (Anjos *et al*., 2006) using a primer pair of *PlsX_F1* and *PlsX_R1* containing *Spe*I and *Pst*I digestion sequences. The *plsX* gene fragment was amplified using PCR method. The PCR condition included various steps; initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of three steps consisting of denaturation 94 °C for 30 seconds, annealing using primer of *PlsX_F1* and *PlsX_R1* at 60 °C for 30 seconds, extension at 72 °C for 1 minute and the last step was a final extension at 72 °C for 5 minutes. The PCR products were checked by 1.5% gel electrophoresis, later visualized by ethidium bromide staining under Gel document instrument. After that, amplified 1,047 bp DNA fragment was digested with *Spe*I and *Pst*I enzymes and then DNA fragment was ligated using T4 DNA ligase into the multiple cloning sites of pEERM vector.Then, the sample tube was incubated at 22 °C for 1-2 hours and inactivated the reaction at 65°C for 10 minutes. After obtaining the recombinant plasmid, it was transformed firstly into competent *E.coli* DH5α cells by heat shock method for screening. Each reaction was added 10 µl of sample into competent cells and mixed the tubes gently by shaking with hand for 3-5 times. Later, the reaction tube was incubated on ice for 30 minutes and heat-shocked at 37°C for 2 minutes, then hold on ice for 3 minutes. 900 µl of LB medium was added (Appendix C) into the test tube and incubated at 37°C on the shaker for 1 hour. The positive clones were selected on LB medium containing 30 µg/ml chloramphenicol and incubated at 37 °C for 16 hours before extracting plasmid using Presto™ Mini Plasmid

kit. In order to verify the recombinant plasmid, the sample was checked by double digesting with *Spe*I and *Pst*I restriction enzymes, then monitored the DNA fragments by agarose gel electrophoresis. Later, the recombinant plasmid was also confirmed by DNA sequencing.

2.2.1.3 Transformation into *Synechocystis* sp. PCC 6803 cells

Synechocystis sp. PCC 6803 cell culture (50 ml) was carried out in normal BG_{11} medium. The growth condition was set at 30 °C, under continuous white light of 40-50 μmole photon $m^{-2}s^{-1}$ intensity. After that, cells were harvested by centrifugation at 6,000 rpm (4,025 × *g*), 4°C for 10 minutes. The obtained pellets were resuspended in 5 ml of BG_{11} medium and transferred those cells into a new tube with 500 μl volume of cell suspension. The recombinant plasmid was transformed into those prepared *Synechocystis* sp. PCC 6803 cells by natural transformation method. The tube was added 17 μl of the recombinant plasmid solution and incubated at 30 °C for 6 hours under continuous white light. Next, those cells were grown in BG_{11} medium containing 10, 20 and 30 μg/ml chloramphenicol and incubated at 30 °C for 2-3 weeks under normal growth condition, respectively. After that, the genomic DNA was extracted and used as a template for checking both size and location of constructed *plsX*-overexpressing strain by PCR method . The different primer pairs consisted of *PlsX_F1/PlsX_R1, Cm_F1/Cm_R1, PlsX_F1/Cm_R1*, and then DNA fragments were used in PCR reaction and further checked by agarose gel electrophoresis.

2.2.2 *Synechocystis* cell culture

Synechocystis cells of both wild type and *plsX*-overexpressing strains were grown in normal BG_{11} medium. The growth condition was performed at 30 °C, under continuous white light of 40-50 μmole photon $m^{-2}s^{-1}$ intensity. For the study of NaCl stress, cell culture with mid-logarithmic phase of growth was harvested by centrifuging at 6,000 rpm (4,025 × *g*), 25°C for 10 minutes and transferred cell pellets to new BG_{11} medium containing various NaCl concentrations of 50, 100, 150, 350 and 650 mM, respectively (Figure 2.2 B). After that, growth of cells were monitored by measuring the optical density at 730 nm by spectrophotometer. Cell density was monitored for their growth at interval times of 0, 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15 days, respectively. On the other hand, cells were grown in normal BG_{11} medium until reaching mid-logarithmic phase and transferred to new BG_{11} medium containing nitrogen deficiency (N, without $NaNO_3$ and ferrous ammonium citrate). Moreover, the supplement with 0.4% (w/v) acetate (or 6.7 mM concentration) was also performed. Cells were grown and subsequently collected at interval times of 0, 1, 3, 5 and 7 days, respectively (Figure 2.2). The experiments were repeated for three times.

2.2.3 Determination of intracellular pigments

One ml of cell culture was harvested by centrifuging at 10,000 rpm (17,507 *x g*) at 25 °C for 10 minutes and discarded supernatant. The obtained pellet was further carried out by extracting with *N*,*N*-dimethylformamide (DMF) and incubated the reaction in the dark for 10 minutes. Then, centrifugation again at 10,000 rpm

(17,507 *x g*), 25 °C for 10 minutes. After that, the supernatant was transferred into a new tube. The DMF extract solution was then measured the absorbance at 461, 625 and 664 nm, respectively. The calculation of chlorophyll *a* and carotenoid contents was done as following equations:

Chlorophyll *a* (μg/cell)

= $[(12.1x\ OD_{664}) - (0.17 \times OD_{625})]$/total cells* (Moran, 1982)

Carotenoid (μg/cell)

= $[(OD_{461} - (0.046 \times OD_{664})) \times 4]$/total cells* (Chamovitz *et al.*, 1993)

Total cells* (cells/ml)

= $(OD_{730} / 0.25) \times 10^8$

2.2.4 Measurement of oxygen evolution

Cell culture containing 10 μg of chlorophyll *a* was harvested by centrifuging at 6,000 rpm (4,025 × *g*), 4 °C, for 10 minutes. The obtained pellet was resuspended in 1 ml of 1 × TE pH 7.5 buffer. The oxygen evolution was measured on a yield of oxygen during light reaction of photosynthesis ($2H_2O$ + light -> $4H^+ + 4e^- + O_2$) by the Clark-type Oxygen electrode at 25°C. The unit of oxygen evolutions was μmol/mg chlorophyll *a*/ h

2.2.5 Extraction of total RNA and reverse transcription-polymerase chain reaction (RT-PCR)

2.2.5.1 Extraction of total RNA

PlsX-overexpressing cell culture (50 ml) was harvested by centrifuging at 6,000 rpm (4,025 × *g*), 4°C for 10 minutes. The obtained pellets were resuspended in 1 ml of Trizol® reagent mixing with 0.2 g of acid washed-glass beads. Cell disruption was done by a vortex-type mixer for 3-5 times until cells were completely broken. After that centrifuged the reaction tube at 12,000 rpm (21,009 × *g*), 4 °C for 10 minutes and transferred the supernatant into a new tube. Then, the addition of 0.2 ml of chloroform was done and mixed that solution by shaking for 15 second. Later, that reaction tube was incubated at room temperature for 5 minutes. The sample tube was then centrifuged at 12,000 rpm (21,009 × *g*), 4 °C for 15 minutes and transferred the upper aqueous phase containing RNA into a new tube. The addition of 0.25 ml of isopropanol was carried out and mixed before incubating at room temperature for 10 minutes. In order to get RNA pellet, the tube was centrifuged at 12,000 rpm (21,009 × *g*), 4 °C for 15 minutes. RNA was precipitated as gel-like pellets. The supernatant was discarded and washed pellets by 1 ml of 75% ice-cold ethanol. Next step, the RNA sample tube was centrifuged at 12,000 rpm (21,009 × *g*), 4°C for 10 minutes. That washed RNA pellets were air-dried on ice for 10 minutes in order to remove ethanol vapor and dissolved those RNA pellets with 20 µl of DEPC-treated water. Consequently, the RNA concentration was measured on the absorbance at 260 nm by spectrophotometer and calculated following formula:

RNA concentration (µg/µl) = OD_{260} × 0.04 µg/µl of RNA × dilution factor

2.2.5.2 Reverse transcription-polymerase chain reaction (RT-PCR)

The extracted RNA was used as a template for cDNA by SuperScript ™ III Frist-Strand Synthesis System kit. The cDNA synthesis was performed in the first step containing 1 µl of total RNA, 1 µl of 50 ng/µl random hexamers and 1 µl of 10 mM dNTP mixed with adjusted final volume by DEPC-treated water of 10 µl. Then, the reaction was incubated at 65 °C for 5 minutes and further placed on ice for 1 minute. In the second step, cDNA synthesis mix was performed using PCR reaction mixture containing 2 µl of 10 x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl RNaseOUT ™ and 1 µl Superscript ™ III RT. The reaction was incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes and terminated the reaction at 85°C for 5 minutes. Next step, cDNA was amplified by PCR method. The PCR condition included various steps; initial denaturation at 94 °C for 3 minutes, followed by 14, 26 and 32 cycles of three steps consisted of denaturation 94 °C for 30 seconds, annealing of *16s, plsX* and *accA* primers at 60 °C for 30 seconds, extension at 72 °C for 1 minute and the last step was a final extension at 94 °C for 5 minutes. The PCR products were checked using 1.5 % gel electrophoresis, visualized by ethidium bromide staining and band intensity was quantified using Gel Analyzer 2010a program.

2.2.6 Determinations of fatty acid and lipid contents

2.2.6.1 Total lipid screening

2.2.6.1.1 Sudan black B staining (Wei *et al.*, 2011)

Cell culture (0.1 ml) with the optical density at 730 nm in a range of 0.8 was spread and smeared on the slide. Later, the slide was air-dried and dropped Sudan black B solution (Appendix D) onto the slide for 10 minutes, then washed slide with distilled water. After that, the addition of safranin O solution was done and incubated for 1 minute and washed slide again by distilled water. Then, stained cells were visualized under the light microscope.

2.2.6.1.2 Nile red staining (Chen *et al.*, 2009)

Cell culture (0.1 ml) with the optical density at 730 nm in a range of 0.8-1.0 was stained with 30 μg/ml of Nile red solution containing 0.9% (w/v) NaCl and incubated in the dark for 12 hours. Then, cells were smeared on the slide and visualized under the fluorescent microscope.

2.2.6.2 Determination of unsaturated lipid content

Total unsaturated lipid content was determined by sulfo-phospho-vanillin (SPV) method (Cheng *et al.*, 2011). Firstly, *Synechocystis* cell culture (5 ml) was centrifuged at 10,000 rpm (17,507 x g), 25°C for 10 minutes. The obtained pellet was then resuspended in 1 ml of conc. sulfuric acid solution and boiled at 100°C for

20 minutes. The addition of vanillin-phosphoric acid reagent (Appendix E) was carried out for color development. Its absorbance at 540 nm was measured by spectrophotometer after 10 minutes of incubation. Standard cannula oil was prepared in same procedure as sample (Appendix E). The unit of product was % gram lipid per dry cell weight (DCW) (w/w).

2.2.6.3 Determination of total lipid content

Total lipid determination was carried out by acid-dichromate oxidation method (Fales, 1971). *Synechocystis* cell culture (5 ml) was harvested and centrifuged at 10,000 rpm (17,507 × *g*), 25°C for 10 minutes. After that, cell pellets were collected and resuspended in 2 ml conc. sulfuric acid and then, added 2 ml of potassium dichromate solution (Appendix F) into each sample tube. The reaction tube was mixed by a vortex-type mixer and further boiled at 100°C for 30 minutes. After that, there was allowed the tube to stand at room temperature for 3 minutes. Then, the addition of 2.0 ml of distilled water was done, mixed gently and cooled to room temperature in a water bath. Total lipid content was measured on the absorbance of reaction mixture at 600 nm by spectrophotometer. Standard used was γ-linolenic acid (18:3), which prepared in the same procedure as sample (Appendix G). The unit of product was % gram lipid per dry cell weight (DCW) (w/w).

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2.2.6.4 Analysis of fatty acid composition

Firstly, both wild-type and *plsX*-overexpressing cells of about 800 - 1,000 ml volume with optical density of about 0.8, were harvested by centrifuging at 10,000 rpm (17,507 x g), 25°C, for 10 minutes. Harvested samples were hydrolyzed and further derivatized into the corresponding fatty acid methyl esters (FAMEs). FAMEs are moderately a polar and sufficiently volatized to be determined by GC/MS instrument. The determination of fatty acid composition was performed at The Halals Science Center, Chulalongkorn University.

2.2.7 Sequence alignment and phylogenetic tree of *plsX* gene

The amino acid sequences of *plsX* gene in various cyanobacteria were searched (Table 3.1) and obtained from the cyanobacteria database, namely Cyanobase (http://genome.kazusa.or.jp/caynobase). Moreover, other amino acid sequences of bacteria and plant were searched and obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Multiple alignment and phylogenetic tree of full-length of *plsX* genes were performed using ClustalW 2.0 and Phylogeny.fr program (Dereeper *et al*., 2008). The phylogenetic tree was used bootstrapping value as 100 for analysis.

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CHAPTER III

RESULTS

3.1 The construction and transformation of *plsX*-overexpressing strain of *Synechocystis* sp. PCC 6803

The PCR method was performed using *Synechocystis* sp. PCC 6803 genomic DNA as the template DNA. The PCR product showed a band of the *plsX* gene fragment of about 1,047 bp (Figure 3.1). After that, the *plsX* gene fragment was ligated into pEERM vector between *Spe*I and *Pst*I restriction sites. The recombinant plasmid containing *plsX* gene was extracted from *E.coli* DH5α and confirmed by double digestion with *Spe*I and *Pst*I. The result showed the correct sizes of digested fragments of about 3,595 bp of pEERM vector and 1,047 bp of *plsX* gene, respectively (Figure 3.2). After that, the recombinant plasmid containing *plsX* gene was transformed into *Synechocystis* sp. PCC 6803 cells by natural transformation method. The confirmation of *plsX*-overexpressing strain was carried out by different primer pairs including chloramphenicol gene fragment amplified by Cm_F1/Cm_R1 of about 899 bp, *plsX* gene fragment amplified by PlsX_F1/PlsX_R1 of about 1,047 bp, *plsX* to chloramphenicol gene fragment amplified by PlsX _F1/Cm_R1 of about 1,946 bp and a gene fragment of *Up-psbA2* gene to *down-psbA2* gene amplified by Up-psbA2_F/Down-psbA2_R of about 3,330 bp (Figure 3.3).

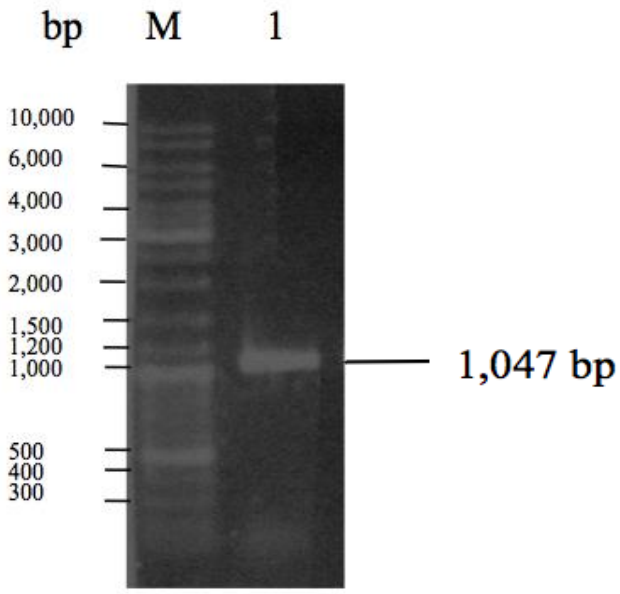

Figure 3.1 Agarose gel electrophoresis of the amplified DNA fragment. The PCR product was run on 0.8% agarose gel.

Lane M DNA Marker Ladder 1 Kb

Lane 1 PCR product of *plsX* gene fragment

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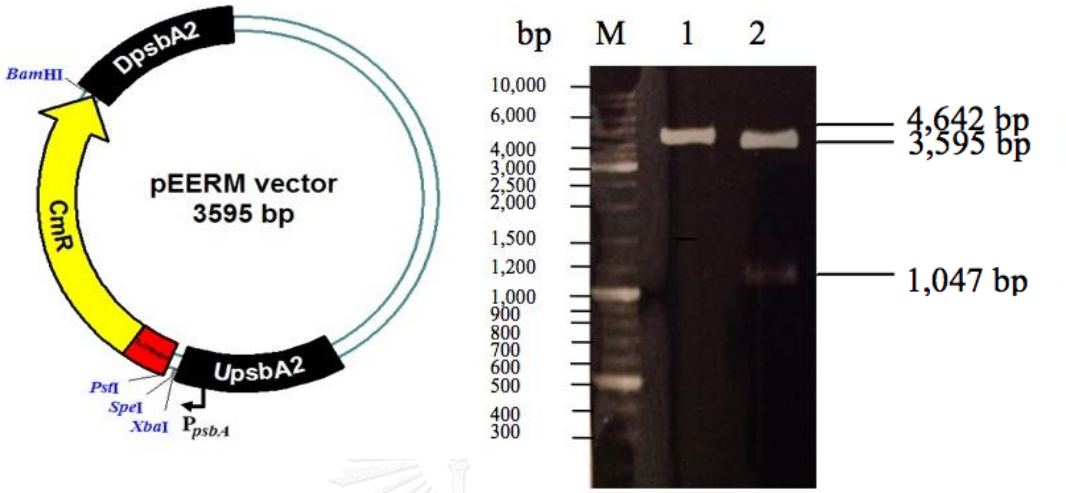

Figure 3.2 Agarose gel electrophoresis of the recombinant plasmid after digesting by *Spe*I and *Pst*I restriction enzymes. The PCR products were run on 0.8% agarose gel. *PlsX* gene fragment was 1047 bp size.

Lane M DNA Marker Ladder 1 Kb

Lane 1 Recombinant plasmid

Lane 2 DNA fragments digested with *Spe*I and *Pst*I enzymes

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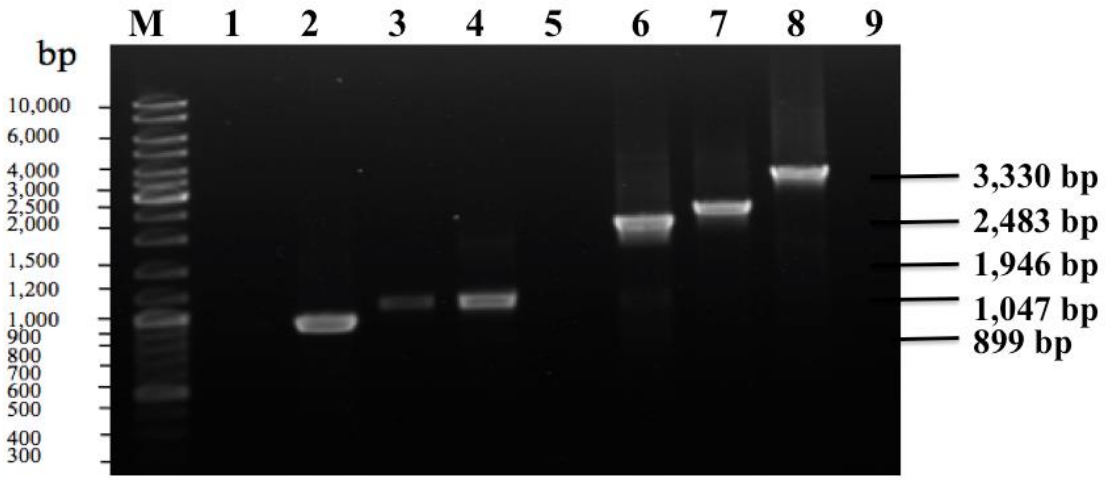

Figure 3.3 Agarose gel electrophoresis of the constructed *plsX*-overexpressing strain (OX) and wild type (WT). The PCR products were separated on 0.8% agarose gel.

Lane M DNA Marker Ladder 1 Kb

Lane 1 Chloramphenicol gene fragment amplified by Cm_F1/Cm_R1 of WT

Lane 2 Chloramphenicol gene fragment amplified by Cm_F1/Cm_R1 of OX

Lane 3 *PlsX* gene fragment amplified by PlsX_F1/PlsX_R1 of WT

Lane 4 *PlsX* gene fragment amplified by PlsX_F1/PlsX_R1 of OX

Lane 5 *PlsX* gene to chloramphenicol gene fragment amplified by PlsX_F1/Cm_R1 of WT

Lane 6 *PlsX* gene to chloramphenical gene fragment amplified by PlsX_F1/Cm_R1 of OX

Lane 7 *Up-psbA2* gene to *down-psbA2* gene fragment amplified by UpsbA2_F/Down-psbA2_R of WT

Lane 8 *Up-psbA2* gene to *down-psbA2* gene fragment amplified by Up-psbA2_F/Down-psbA2_R of OX

Lane 9 Negative control

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3.2 Effects of salt stress and nutrient modification on *Synechocystis* sp. PCC 6803 wild type and *plsX*-overexpressing cells

3.2.1 Growths of *Synechocystis* sp. PCC 6803 wild type and *PlsX*-overexpressing strains under various NaCl concentrations and nutrient modification

Synechocystis sp. PCC 6803 wild type (WT) and *plsX*-overexpressing (OX_plsX) strains were grown in BG_{11} medium containing various NaCl concentrations of 50, 100, 150, 350 and 650 mM, respectively. After that, cell growth was determined for 15 days. The result showed in Figure 3.4A that *Synechocystis* WT cell growth was significantly decreased under 650 mM NaCl condition starting at 5 days of cultivation, compared with normal condition whereas other conditions of 50, 100, 150 and 350 mM NaCl concentration showed no apparent changes. The *plsX*-overexpressing strain showed lower growth under NaCl stress when compared to WT (Figure 3.4 B).

Synechocystis sp. PCC 6803 wild type and *plsX*-overexpressing strains were grown in BG_{11} medium under adaption in nitrogen deficiency and 0.4 % acetate supplementation. Cell growth was determined for 7 days. Result is shown in Figure 3.5 that *Synechocystis* WT cell growth under nitrogen deficiency was obviously decreased after cell growth for 5 day of cultivation compared with normal condition. However, cell growth was significantly increased under 0.4% acetate supplementation starting at 3 days of cultivation. For *plsX*-overexpressing strain, the nitrogen deficiency effectively reduced cell growth whereas the acetate supplementation increased cell growth.

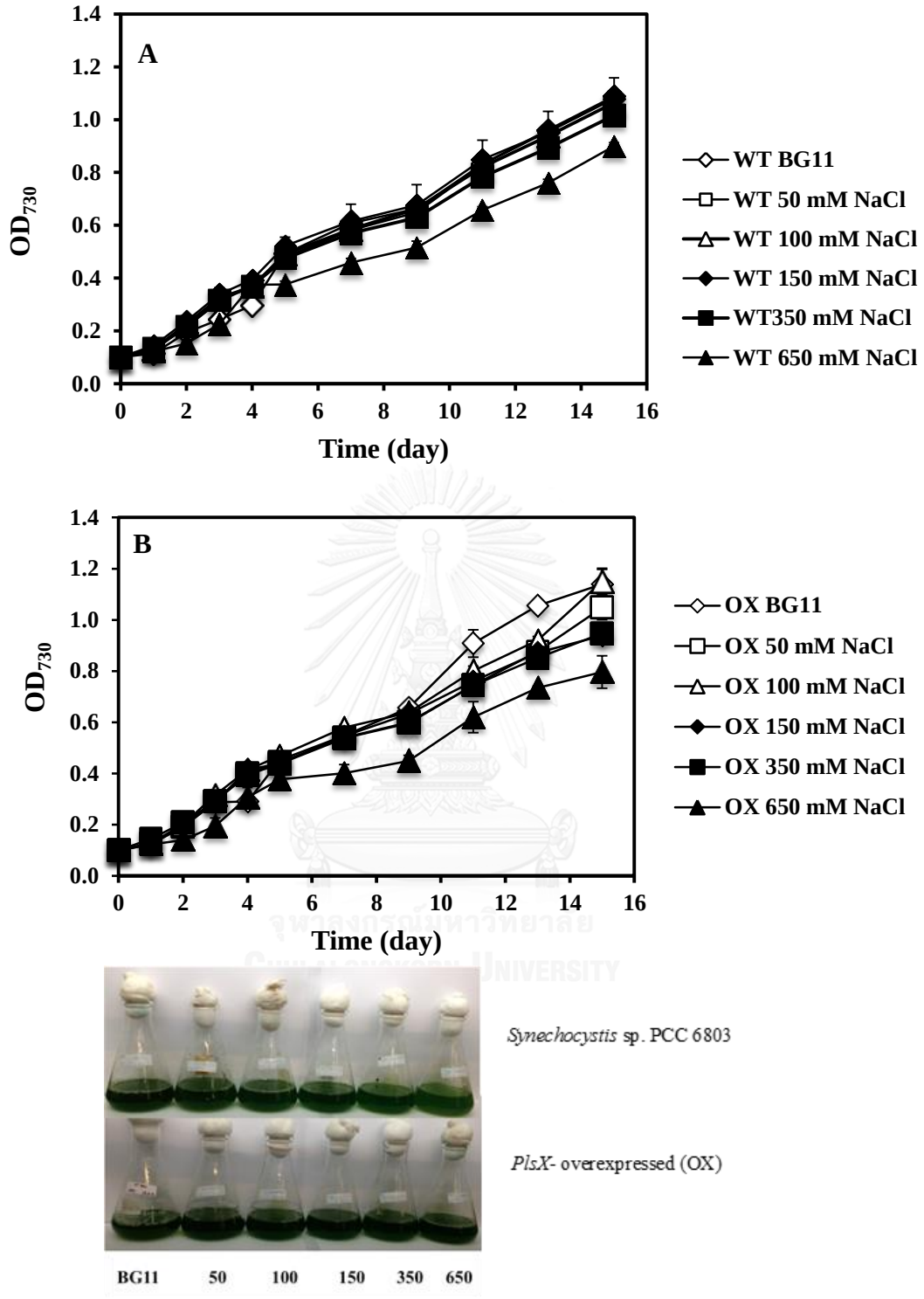


Figure 3.4 Growth curve of *Synechocystis* sp. PCC 6803 wild type (WT) (A) and *plsX*- overexpressing strain (OX) (B). Cells were grown in BG_{11} media containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. Data represents mean ± S.D., n=3.

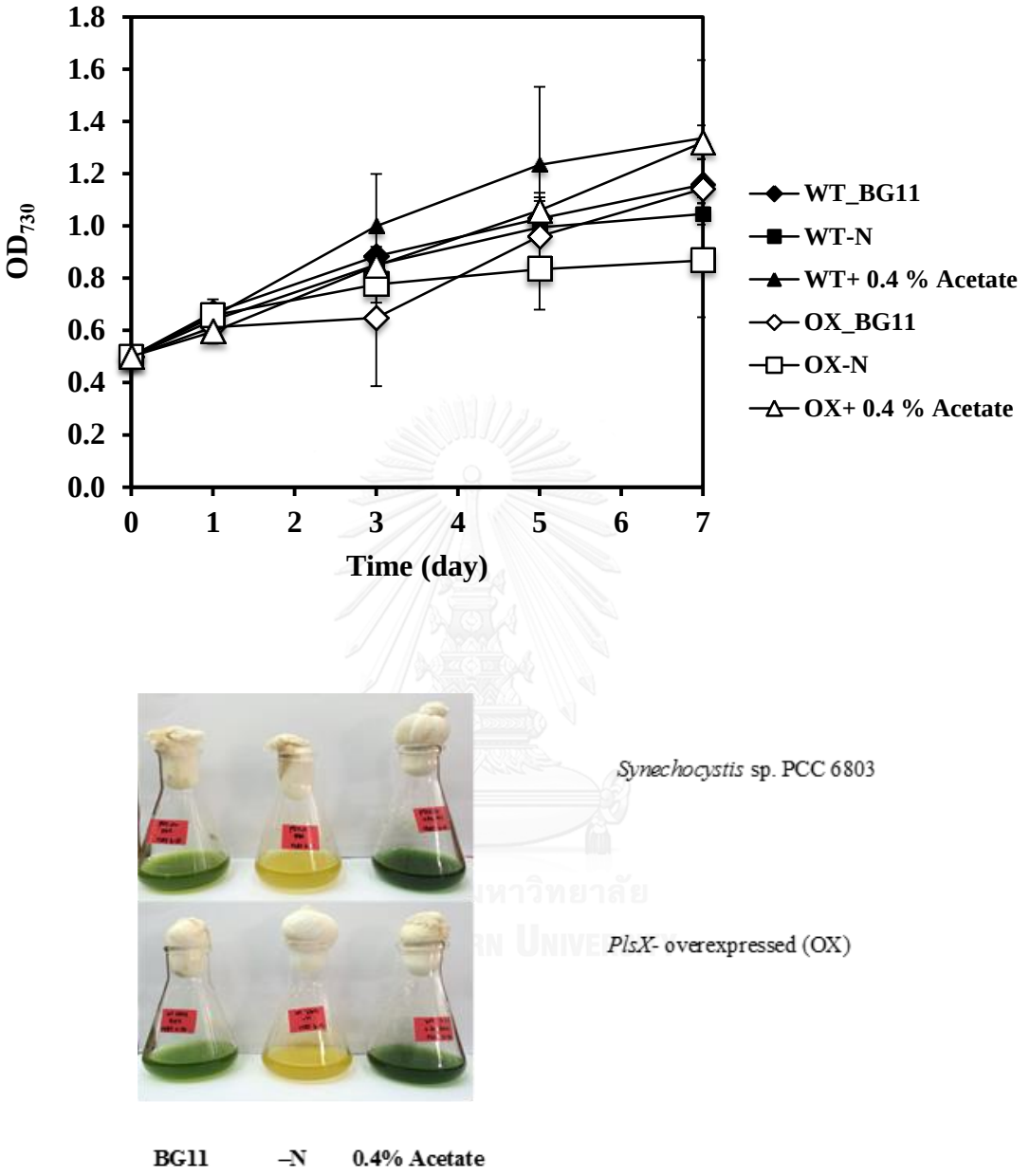


Figure 3.5 Growth curve of *Synechocystis* sp. PCC 6803 wild type and *plsX*-overexpressing (OX) strains. Cells were grown in BG_{11} media under nutrient modification including nitrogen deficiency and 0.4% acetate supplementation. Data represents mean ± S.D., n=3.

3.2.2 The contents of intracellular pigments under various NaCl concentrations and nutrient modification

Chlorophyll *a* and carotenoid contents of *Synechocystis* wild type cells (Figure 3.6 A and B) were slightly decreased after 9 day-culture under 650 mM NaCl compared with normal condition. Interestingly, the 150 mM NaCl condition induced the accumulation of both chlorophyll *a* and carotenoids whereas no significant change was observed under 50 and 100 mM NaCl conditions compared to normal control. Moreover, the *plsX*-overexpressing strain (Figure 3.7 A and B) demonstrated that chlorophyll *a* was insignificantly decreased after 9 day-culture under 650 mM NaCl and increased significantly after 9 day. On the other hand, the 50, 100, 150 and 350 NaCl conditions induced the accumulation of carotenoid content, which slightly increased after 9 day-culture compared with normal condition.

On the other hand, chlorophyll *a* and carotenoid contents of *Synechocystis* wild type cells were slightly decreased after 3 day-culture under nitrogen deficiency compared to normal condition. The chlorophyll *a* content was significantly increased under 0.4% acetate supplementation after 5 day-culture compared to normal condition. For carotenoid content, it was significantly decreased under 0.4% acetate supplementation after 5 day-culture. Moreover, the *plsX*-overexpressing strain showed significant decreases on chlorophyll *a* and carotenoid contents under nitrogen deficiency compared to normal condition. On the other hand, 0.4% acetate supplementation significantly increased the accumulation of both pigments as shown in Figure 3.8 A and B.

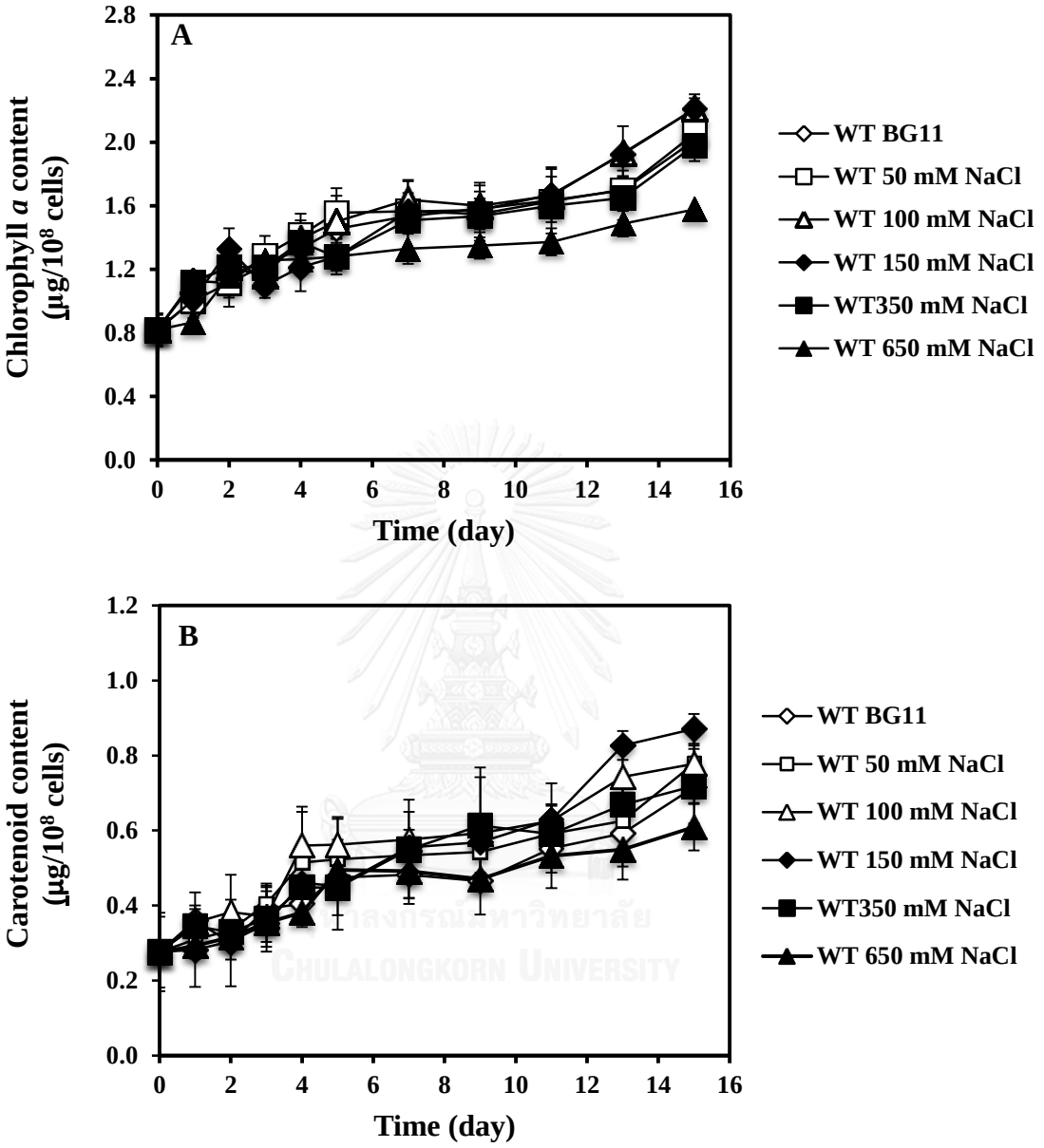


Figure 3.6 The effect of salt stress on chlorophyll *a* (A) and carotenoid (B) contents in *Synechocystis* sp. PCC 6803 wild type (WT). Cells were grown in BG_{11} media containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. Data represents mean ± S.D., n=3.

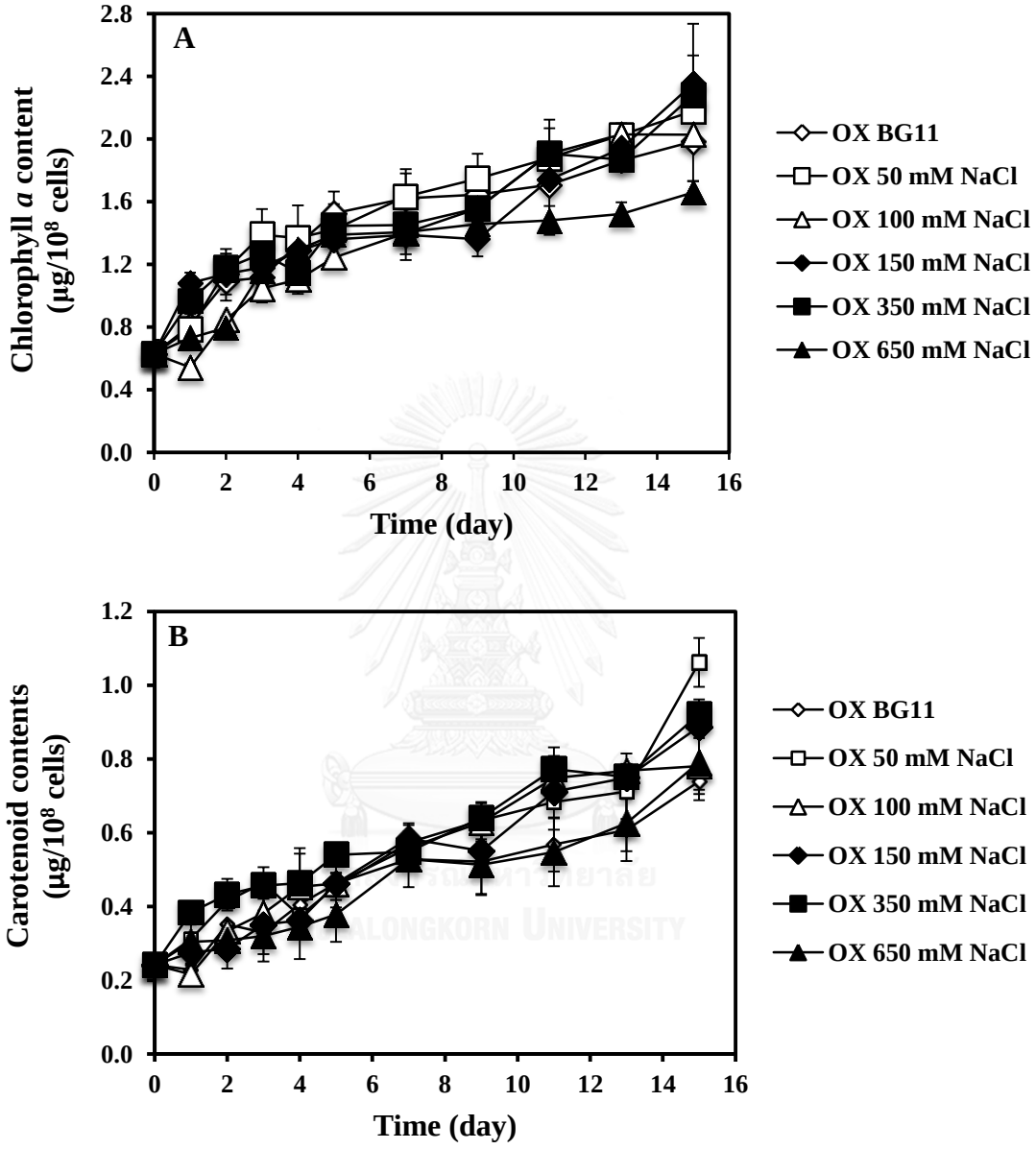


Figure 3.7 The effect of salt stress on chlorophyll *a* (A) and carotenoid (B) contents of *plsX*- overexpressing (OX) cells. Cells were grown in BG_{11} media containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. Data represent mean ± S.D., n=3

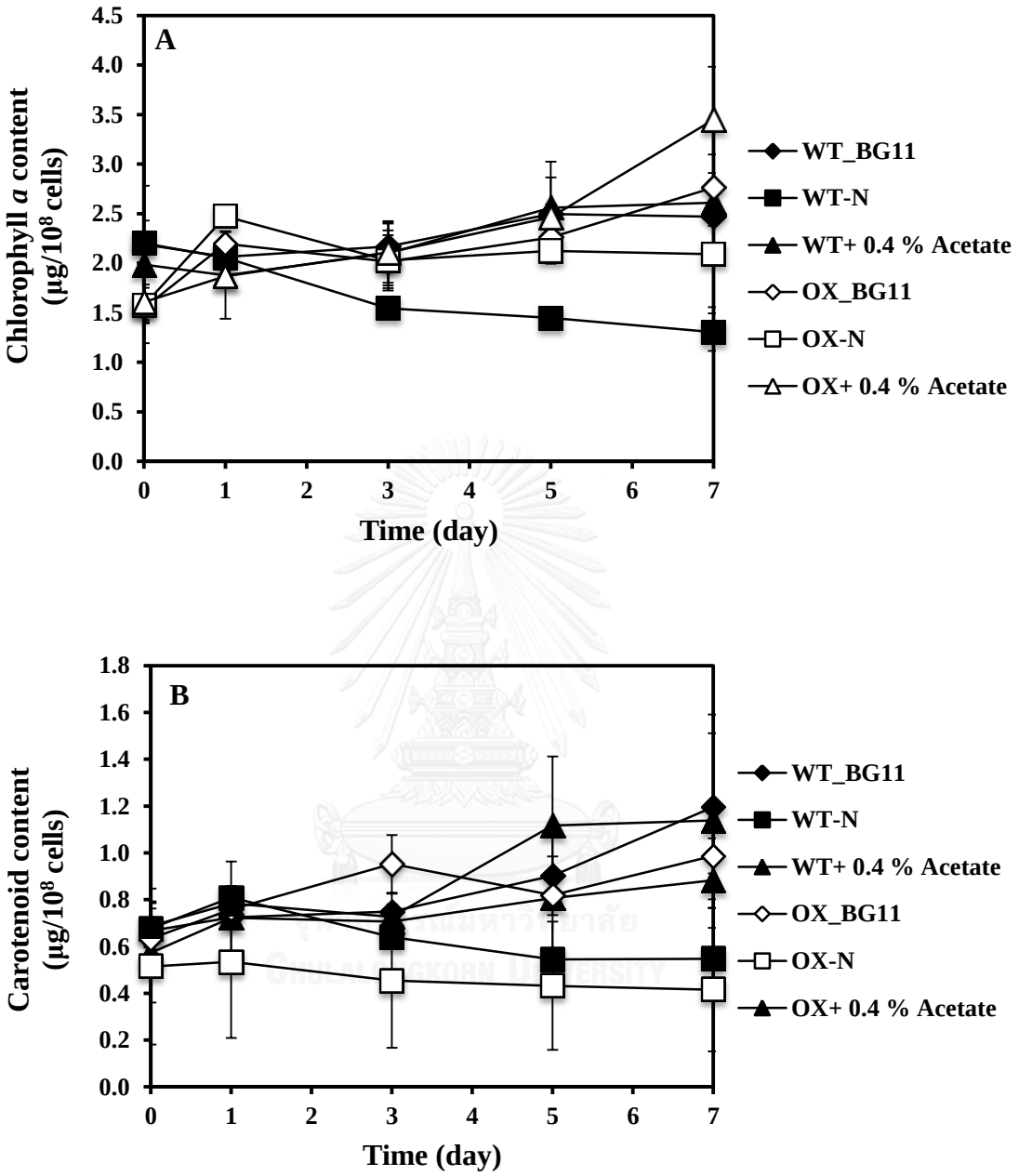


Figure 3.8 The chlorophyll *a* (A) and carotenoid (B) contents of *Synechocystis* wild type (WT) and *plsX*- overexpressing (OX) cells in nutrient modified media. Cells were grown in BG_{11} media under nitrogen deficiency (-N) and 0.4% acetate supplementation. Data represent mean ± S.D., n=3.

3.2.3 Effects of salt stress and nutrient modification on oxygen evolution rate of *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains

The oxygen evolution was measured for a yield of oxygen during light reaction of photosynthesis by Clark-type Oxygen electrode at 25°C. The oxygen evolution represents the photosynthetic efficiency of cyanobacterial cells. For salt stress, the photosynthetic efficiency of *Synechocystis* wild type cells was slightly decreased under all NaCl conditions including 100, 150, 350 and 650 mM NaCl, respectively (Figure 3.9A). On the other hand, the *plsX*-overexpressing strain showed lower level of oxygen evolution rate under normal BG_{11}, 50 and 150 mM NaCl conditions compared to wild type. The less efficiency on photosynthesis was observed in *PlsX*- overexpressing cells rather than wild type cells in all conditions.

For nutrient modification, wild type cells showed the increased level of oxygen evolution rate under normal BG_{11} conditions compared to that of overexpressing strain at 0, 1 and 3 day-culture, respectively. However, the photosynthetic efficiency of *Synechocystis* wild type cells was significantly decreased under nitrogen deficiency after 7 day-treatment (Figure 3.9B). Moreover, a significant increase of oxygen evolution rate was increased under 0.4% acetate supplementation after 5 day-treatment compared to normal condition. On the other hand, the *plsX*-overexpressing strain also showed a significant decrease under nitrogen deficiency at 7 day-treatment. However, the *plsX*-overexpressing strain had higher oxygen evolution rate when compared to normal BG_{11} control under 0.4% acetate supplementation.

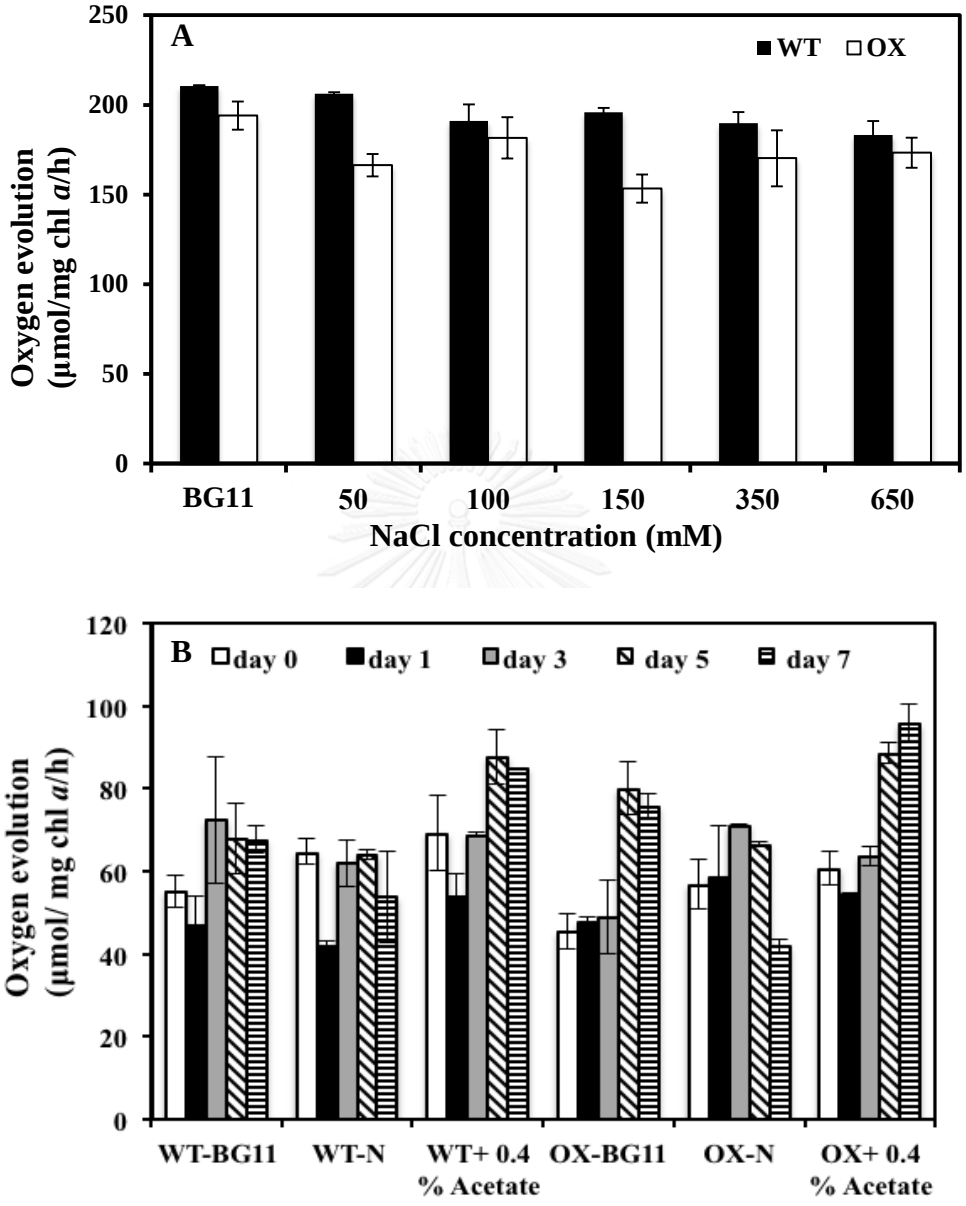


Figure 3.9 Oxygen evolution of *Synechocystis* sp. PCC 6803 (WT) and *plsX*-overexpressed strain (OX) cells grown in BG_{11} media containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively (A) and nitrogen deficiency and 0.4% acetate supplementation (B). Data represents mean ± S.D., n=3.

3.3 Effects of salt stress and nutrient modification on mRNA levels in *Synechocystis* sp. PCC 6803 wild type and *plsX*-overexpressing strains

The transcript levels and the ratio of band intensity (gene/*16s* rRNA) were shown in Figure 3.10 and 3.11. The *plsX* mRNA levels of *plsX*-overexpressing cells under 0, 50, 100 and 150 mM NaCl conditions were constant whereas the 350 and 650 mM NaCl conditions showed apparent decrease on *plsX* mRNA levels. Interestingly, the overexpressed strain obviously showed higher *plsX* transcript amount than WT. The *plsX* transcripts of WT cells were decreased significantly by all conditions. On the other hand, the *AccA* gene encoding acetyl-CoA carboxylase alpha subunit, a key enzyme catalyzing the conversion of acetyl-CoA to malonyl-CoA, was also detected. The transcript levels of *AccA* gene of *plsX*- overexpressing cells were constant under 50, 100 and 150 mM NaCl conditions whereas 0, 350 and 650 mM NaCl conditions significantly decreased. Moreover, wt cells showed down regulated on *AccA* transcript levels under 50, 350, 650 mM NaCl conditions accept 50 mM NaCl conditions no apparent change compared to BG_{11} normal condition.

The *PlsX* mRNA levels of *plsX*-overexpressing cells under nutrient modifications including nitrogen deficiency and 0.4% acetate supplementation showed no apparent change on *plsX* mRNA level compared to BG_{11} normal condition. Furthermore, *plsX*-overexpressing cell contained higher *PlsX* mRNA levels than WT. of about 1 to 2.5 fold. The *plsX* transcrips of WT cells were decreased by all conditions. On the other hand, *AccA* gene of overexpressing cells was decreased under BG_{11} normal and nitrogen deficiency condition whereas 0.4% acetate supplementation showed apparent decrease on *AccA* mRNA levels. Interestingly, WT

cells showed increased *AccA* transcript levels by all condition compared to overexpressing cells.

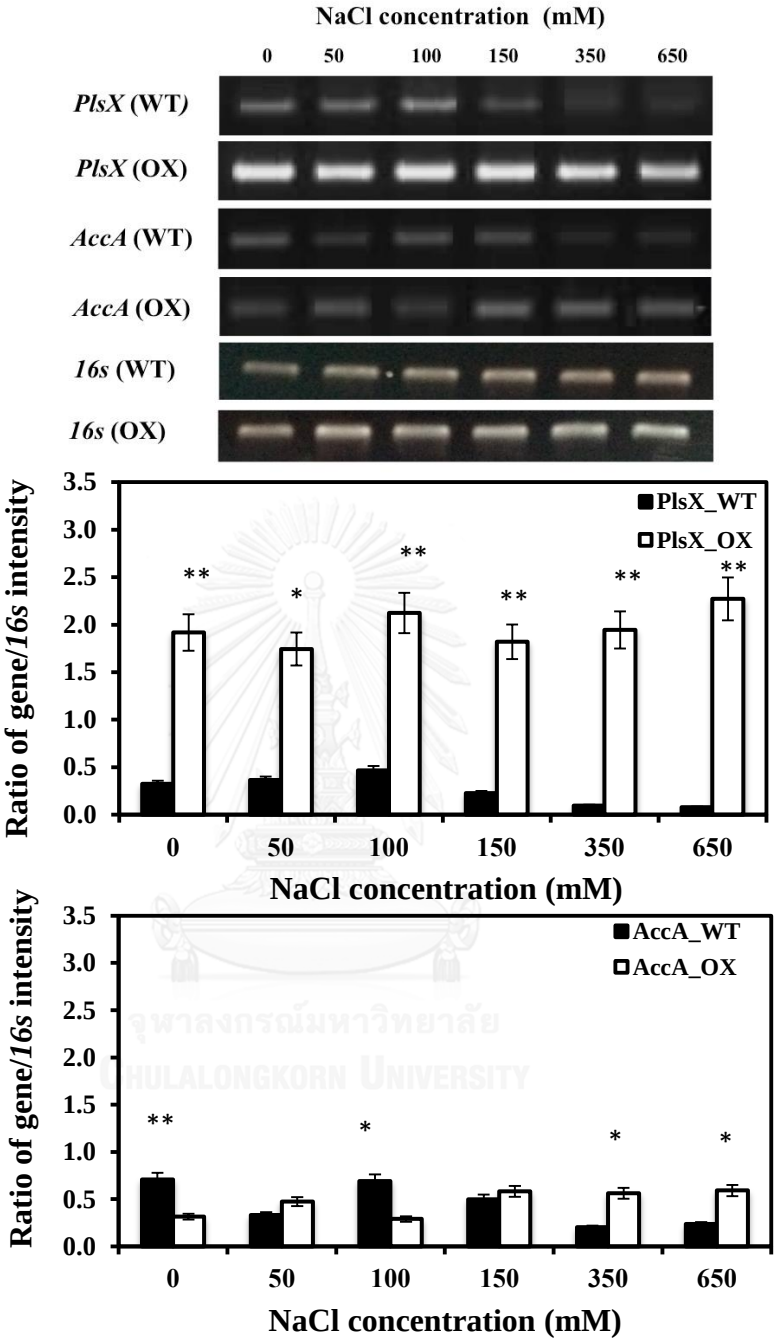


Figure 3.10 Transcript levels of *PlsX* and *AccA* genes in *Synechocystis* sp. PCC 6803 (WT) and *plsX*- overexpressed strain (OX) cells grown in BG_{11} medium containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. RT-PCR products were checked by agarose gel electrophoresis with 0.8% agarose gel. The visualization was performed using Gel Analyzer 2010a program.

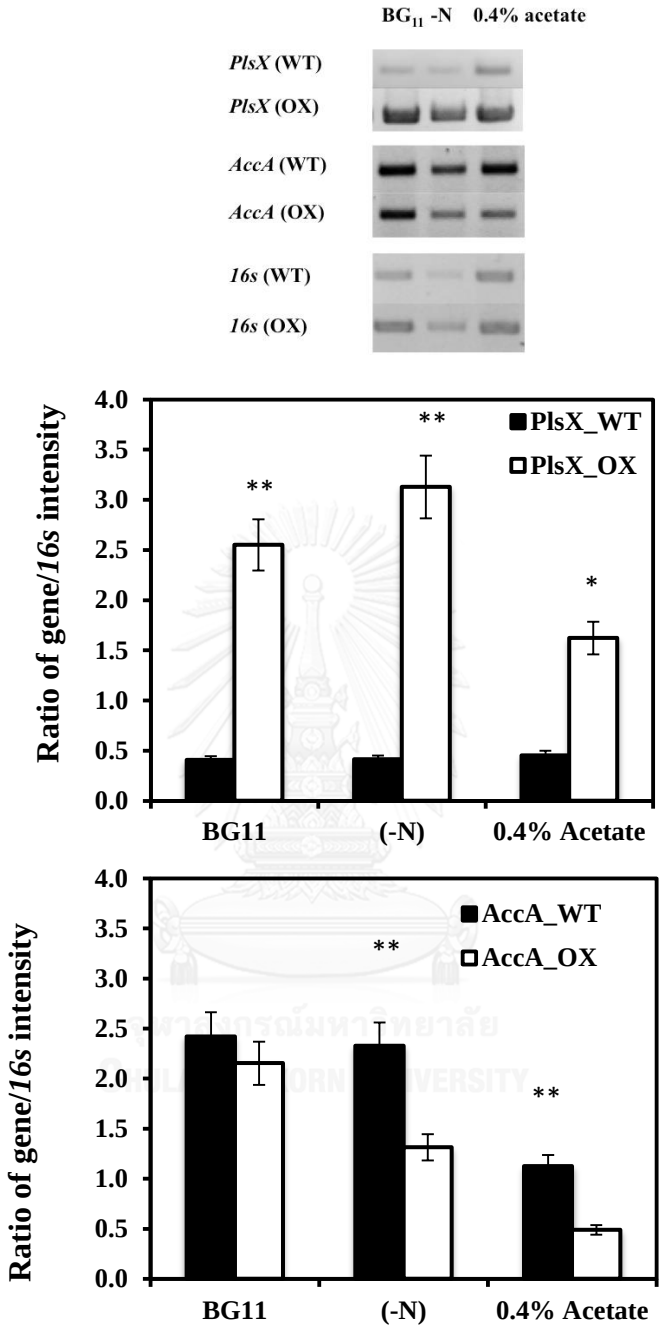


Figure 3.11 Transcript levels of *PlsX* and *AccA* genes in *Synechocystis* sp. PCC 6803 (WT) and *plsX*- overexpressed strain (OX) cells grown in BG_{11} media under nutrient modifications including nitrogen deficiency and 0.4% acetate supplementation. RT-PCR products were checked by agarose gel electrophoresis with 0.8% agarose gel. The visualization was performed using Gel Analyzer 2010a program. *$P \leq 0.05$, **$P \leq 0.01$.

3.4 Determinations of lipid content and fatty acid composition in *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains

3.4.1 Total lipid screening

The total lipid screening was monitoring by both Sudan black B staining and Nile red staining. The Sudan black B stained mainly polar lipid, such as membrane lipid. The result showed in Figure 3.12. The *plsX*- overexpressing cells under 50, 100, 150, 350 and 650 mM NaCl conditions showed darker color of membrane lipid when compared to WT. On the other hand, the Nile red staining shown in Figure 3.13 which stained mainly showed some granules induced by NaCl stress when compared to WT. Interestingly, *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains did not accumulate much storage lipids under normal BG_{11} and NaCl stress in all concentrations.

Under nutrient modified conditions, cells were also stained by Nile red solution (Figure 3.14). The Nile red staining showed highest amounts of granules induced by nitrogen deficiency of both wild type and *plsX*- overexpressing strains. On the other hand, *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains moderately accumulated their storage lipids under an addition of 0.4 % acetate when compared to normal condition and nitrogen deprived condition

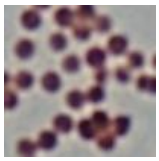
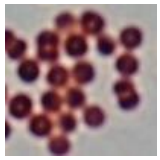
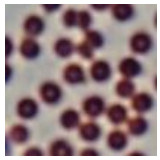
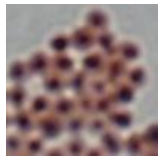
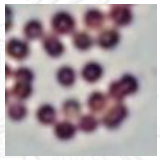
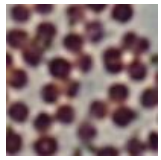
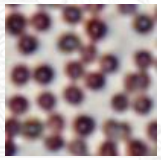
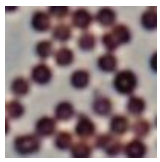
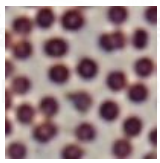
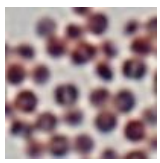
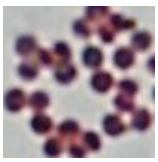
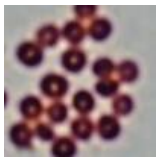
NaCl concentration (mM)	WT 6803	OX
0		
50		
100		
150		
350		
650		

Figure 3.12 The Sudan black B staining of membrane lipids in *Synechocystis* sp. PCC 6803 wild type (WT 6803) and *plsX*-overexpressed strain (OX) cells grown in BG_{11} medium containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. The stained cells were visualized under light microscope with a magnification of 100X.

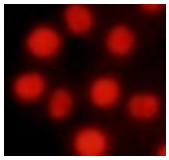
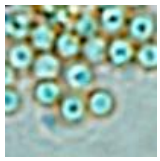
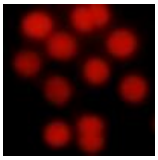
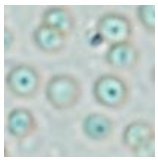
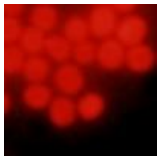
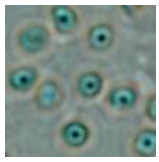
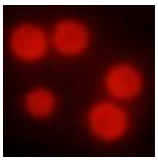
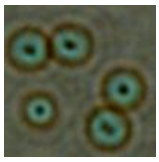
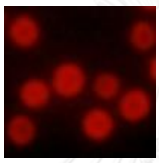
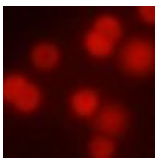
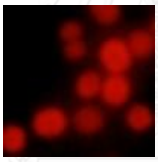
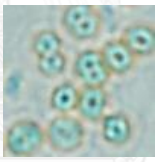
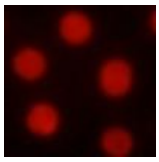
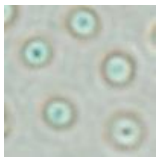
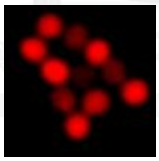
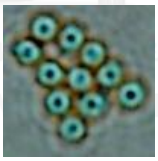
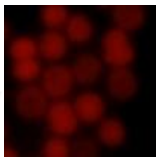
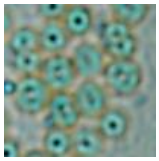
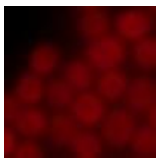
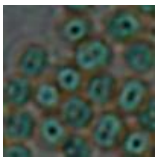
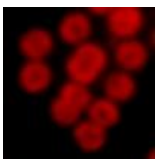
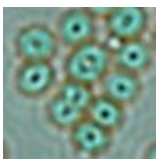
NaCl concentration (mM)	WT 6803		OX	
0				
50				
100				
150				
350				
650				

Figure 3.13 The Nile red staining of neutral lipids in *Synechocystis* sp. PCC 6803 wild type (WT) and *plsX*-overexpressed strain (OX) cells grown in BG_{11} medium containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. The stained cells were visualized under light microscope with a magnification of 100X.

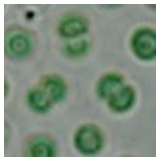
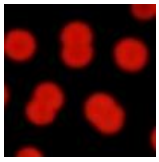
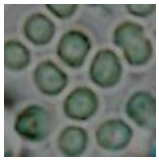
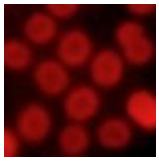
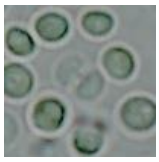
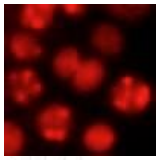
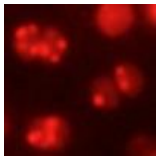
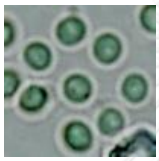
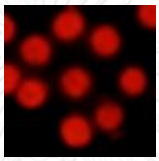
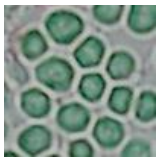
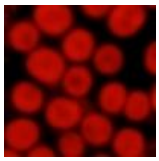
Stress conditions	WT 6803		OX	
BG_{11}				
Nitrogen deficiency				
0.4 % acetate				

Figure 3.14 The Nile red staining of neutral lipid in *Synechocystis* sp. PCC 6803 wild type (WT) and *plsX*-overexpressed strain (OX) cells grown in BG_{11} medium under nutrient modification. The stained cells were visualized under light microscope with a magnification of 100X.

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3.4.2 Determination of unsaturated lipid and lipid contents in *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains

The total amount of intracellular lipid content in log-phase growing *Synechocystis* cell normally existed under normal condition in a range of about 10-15 %w/DCW whereas unsaturated lipid content was in a range of about 0.6-0.8 %w/DCW (Figure 3.15A and B). The total lipid content was then 16-19 fold-higher than unsaturated lipid content. The *plsX*-overexpressing strain obviously accumulated higher amounts of both total lipid and unsaturated lipid contents with 20% and 1% w/DCW, respectively. The higher amount of total lipid content in OX strain was observed in 1.3 fold-increase when compared to that of WT. Likewise, the 1-fold increase of unsaturated lipid content in OX strain was demonstrated when compared to that of WT.

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3.4.2.1 Unsaturated lipid contents under salt stress and nutrient modified conditions

The total unsaturated lipid contents under normal condition and NaCl stress were showed in Figure 3.15A. It was found in wild type that NaCl stress at 50, 100, and 150 mM NaCl concentration conditions significantly induced the unsaturated lipid contents whereas 650 mM NaCl condition significantly decreased the accumulation of unsaturated lipid content. On the other hand, 50, 100 and 150 mM NaCl conditions could induce higher amount of unsaturated lipid contents in *plsX*-overexpressing strain when compared with that under normal BG_{11} condition. The

high NaCl concentration, particularly at 350 and 650 mM NaCl, significantly decreased unsaturated lipid content. However, unsaturated lipid in *plsX*-overexpressing was accumulated higher content than wild type in all conditions, especially BG_{11} control.

Results of total unsaturated lipid contents under nitrogen deficiency and 0.4% acetate supplementation were shown in Figure 3.16B and 3.16C. The total unsaturated lipid content of WT cells under nitrogen deficiency was induced after 1, 3, 5 and 7 day-treatments. The maximum level of unsaturated lipid content of WT under nitrogen-deprived condition was about 0.9 %w/DCW at day 3 and day 5 of treatments. Moreover, 0.4% acetate supplementation accumulated higher amount of unsaturated lipid contents in WT cells at day 5- treatment with the maximum amount of about 1.2 %w/DCW compared to normal BG_{11} control. On the other hand, the OX cells treated under nitrogen deficiency gave a significant increase at day 5 of treatment with the highest amount of about 2.3 %w/DCW when compared to OX cells treated under normal BG_{11} control. Interestingly, the highest accumulation of unsaturated lipid in OX strain was observed of about 3.4 %w/DCW after adding 0.4% acetate within 5 days. The unsaturated lipid in day 7-treatment of OX strain still showed the high amount at 3 % w/DCW under 0.4% acetate addition.

3.4.2.2 Total lipid contents under salt stress and nutrient modified conditions

The total lipid contents under normal condition and NaCl stress were showed in Figure 3.15B. It was found in wild type that NaCl stress at 50, 100 and 150

mM NaCl concentration conditions significantly induced the lipid contents whereas 350 and 650 mM NaCl did not accumulation of lipid content. On the other hand, 100 and 150 mM NaCl conditions could induce higher amount of lipid contents in *plsX*-overexpressing strain when compared with that under normal BG_{11} condition. The high NaCl stress, particularly at 650 mM NaCl, significantly decreased lipid content. However, *plsX*-overexpressing accumulated higher lipid content than wild type at 100 and 350 mM NaCl conditons amount of 20-30 %w/DCW.

Log-phase cells were used to further treatments with nutrient modified conditions consisting of nitrogen deficiency and 0.4% acetate supplementation (Figure 3.17B and 3.17C). At the start day 0 of treatment, OX strain had a slight increase of total lipids compared to WT. After nitrogen-deprived condition, the total lipid contents of both WT and OX cells showed a significant decrease at day 0 and day 1 of treatments whereas a significant increase was observed at day 3, 5 and day 7 of treatments. However, the total lipid levels of OX strains was induced highly of about 12 %w/DCW within 3 day-treatment under nitrogen deficiency and the highest level of total lipids was observed at day 7-treatment with 13 %w/DCW. On the other hand, the total lipid accumulation of WT cells were increased along day 1 to day 7 of acetate treatment with the highest level of about 16 %w/DCW at day 5-treatment whereas the OX cells treated by 0.4% acetate supplementation were accumulated the highest lipid content after 3 day-culture with about 18 %w/DCW when compared with that under normal BG_{11} condition.

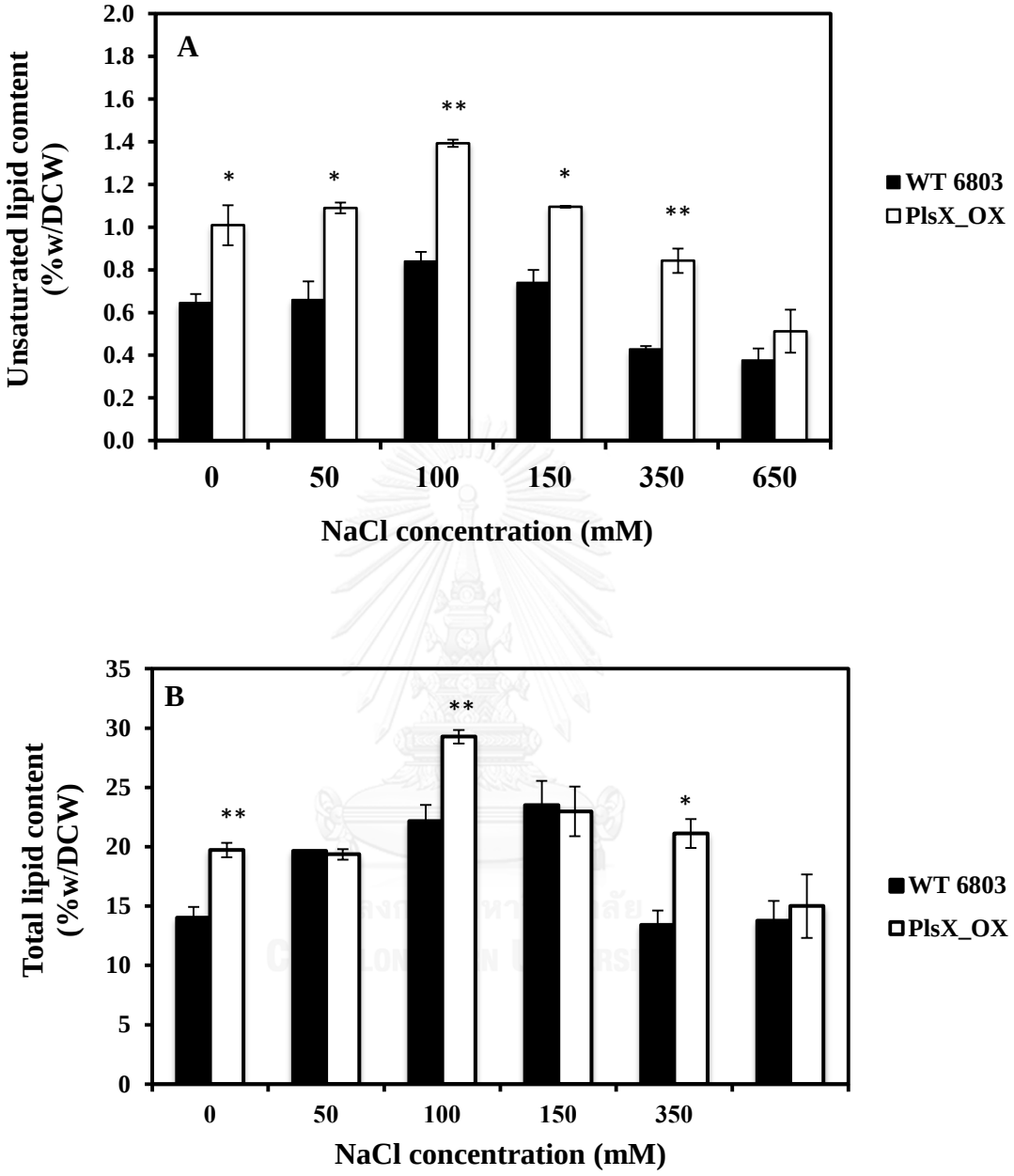

Figure 3.15 Total unsaturated lipid (A) and total lipid (B) contents in *Synechocystis* sp. PCC 6803 wild type (WT) and *plsX*- overexpressed strain (OX) cells grown in BG_{11} medium containing 0, 50, 100, 150, 350 and 650 mM NaCl, respectively. Data represent mean ± S.D., n=6 and significant levels of $*P \leq 0.05$ and $**P \leq 0.01$.

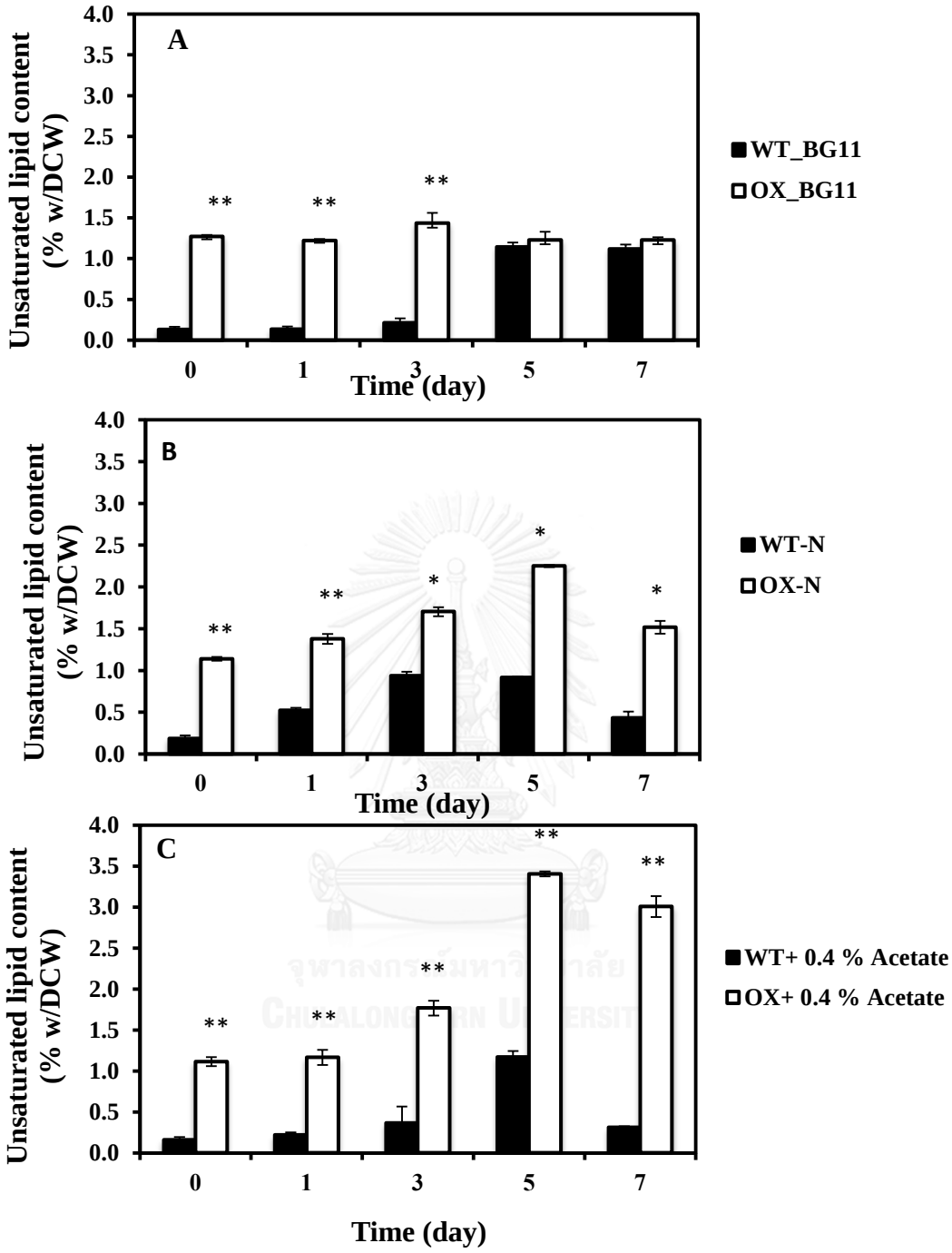

Figure 3.16 Total unsaturated lipid in *Synechocystis* sp. PCC 6803 wild type (WT) and *plsX*- overexpressing (OX) cells grown in BG_{11} medium under various nutrient modifications including BG_{11} medium (A), nitrogen deficiency (-N) (B) and 0.4% acetate supplementation (C). Data represent mean ± S.D., n=3, and significant differences are represented by * $P \leq 0.05$ and ** $P \leq 0.01$.

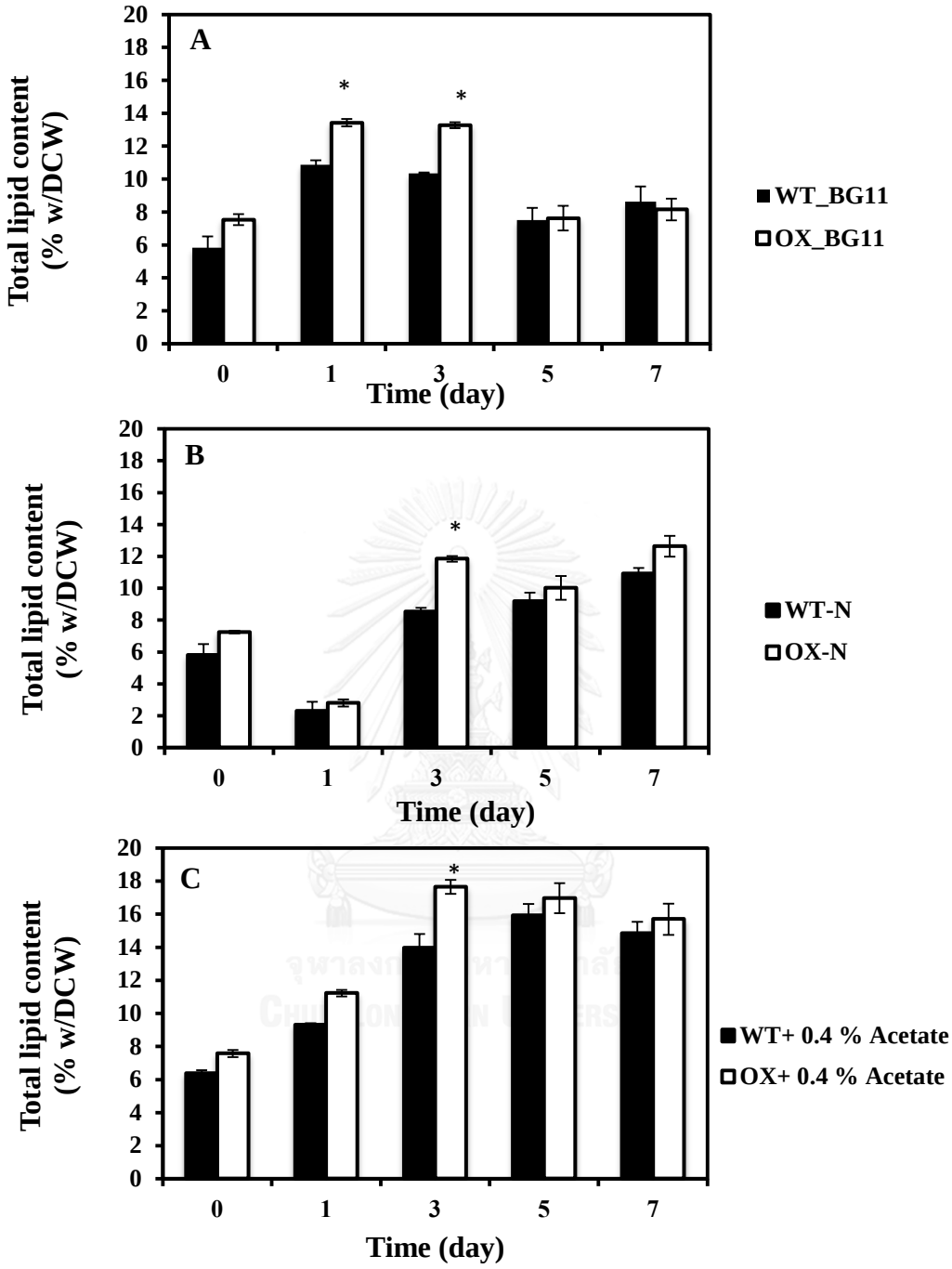

Figure 3.17 Total lipid contents in *Synechocystis* sp. PCC 6803 wild type (WT) and *plsX*- overexpressing (OX) cells grown in BG_{11} medium under various nutrient modifications including BG_{11} medium (A), nitrogen deficiency (-N) (B) and 0.4% acetate supplementation (C). Data represent mean ± S.D., n=3, and significant differences are represented by * $P \leq 0.05$ and ** $P \leq 0.01$.

3.4.3 Fatty acid composition (%) of *Synechocystis* sp. PCC 6803 wild type and *plsX*- overexpressing strains

Percentage of fatty acid composition using GC-MS detection is shown in Figure 3.18 and 3.19. In present study, the most abundance of saturated fatty acid was palmitic acid (C16:0) whereas major unsaturated fatty acids were palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3). GC-MS analysis on WT cell under normal condition showed the identified proportional percentage of saturated fatty acid(s): total unsaturated fatty acids of about 40:27. The unidentified fatty acids was also found to about 33%. However, when the low concentration of 50 mM NaCl was treated, the higher proportional percentage of saturated fatty acid, particularly palmitic acid (C16:0), was observed whereas the composition percentage of unsaturated fatty acids was not significantly changed (28%). Interestingly, this increase of palmitic acid was mainly derived from the unidentified fatty acids. Similarly, the moderate concentration at 150 mM NaCl affected to changes in fatty compositions including saturated and unsaturated fatty acids (56% saturated fatty acid and 26% unsaturated fatty acid). On the other hand, the low-moderate concentration at 100 mM NaCl did not affect much on proportional percentage of both saturated and unsaturated fatty acids when compared to WT. Surprisingly, this 100 mM NaCl condition effectively enhanced the production of saturated fatty acid, stearic acid (C 18:0) when compared among all conditions studied. On the other hand, the 650 mM NaCl condition enabled to enhance the proportional percentage of unsaturated fatty acids by increasing linolenic acid (C18:2) and palmitoleic acid (C16:1). The unidentified fatty acid composition of each

condition was ranging from 32-34% except under 50 and 150 mM NaCl conditions with 15% and 18%, respectively.

For the *plsX*- overexpressing (OX) strain (Figure3.19) under normal condition, the identified proportional composition of saturated fatty acid(s): unsaturated fatty acids was 43:28 % which was not significantly different from that of WT (40:27%). The main saturated fatty acid was also palmitic acid (C16:0). Interestingly, the moderate concentration at 150 mM NaCl obviously affected to fatty acid proportion of OX strain by changing from 43:28% to 66:34% of saturated fatty acid(s): unsaturated fatty acids composition. Palmitic acid (C16:0) was highly induced by 150 mM NaCl. On the other hand, a low concentration at 50 mM NaCl could increase the proportional accumulation of palmitic acid (C16:0) from 43%, under normal condition, to 59%. The proportional increase of palmitic acid (C16:0) was derived from both the unidentified fatty acid and unsaturated fatty acids. Nevertheless, the low-moderate concentration at 100 mM NaCl did not affect to proportional composition of fatty acids when compared to those of WT cells. Likewise, this 100 mM NaCl condition was only one condition, which enabled OX cells to accumulate the stearic acid (C18:0). Interestingly, the high concentration at 650 mM NaCl obviously affected the proportional composition in OX cells, especially decreasing the identified unsaturated fatty acids. However, it was interesting that this 650 mM NaCl condition highly enhanced a bulk of unidentified fatty acids up to 47%.

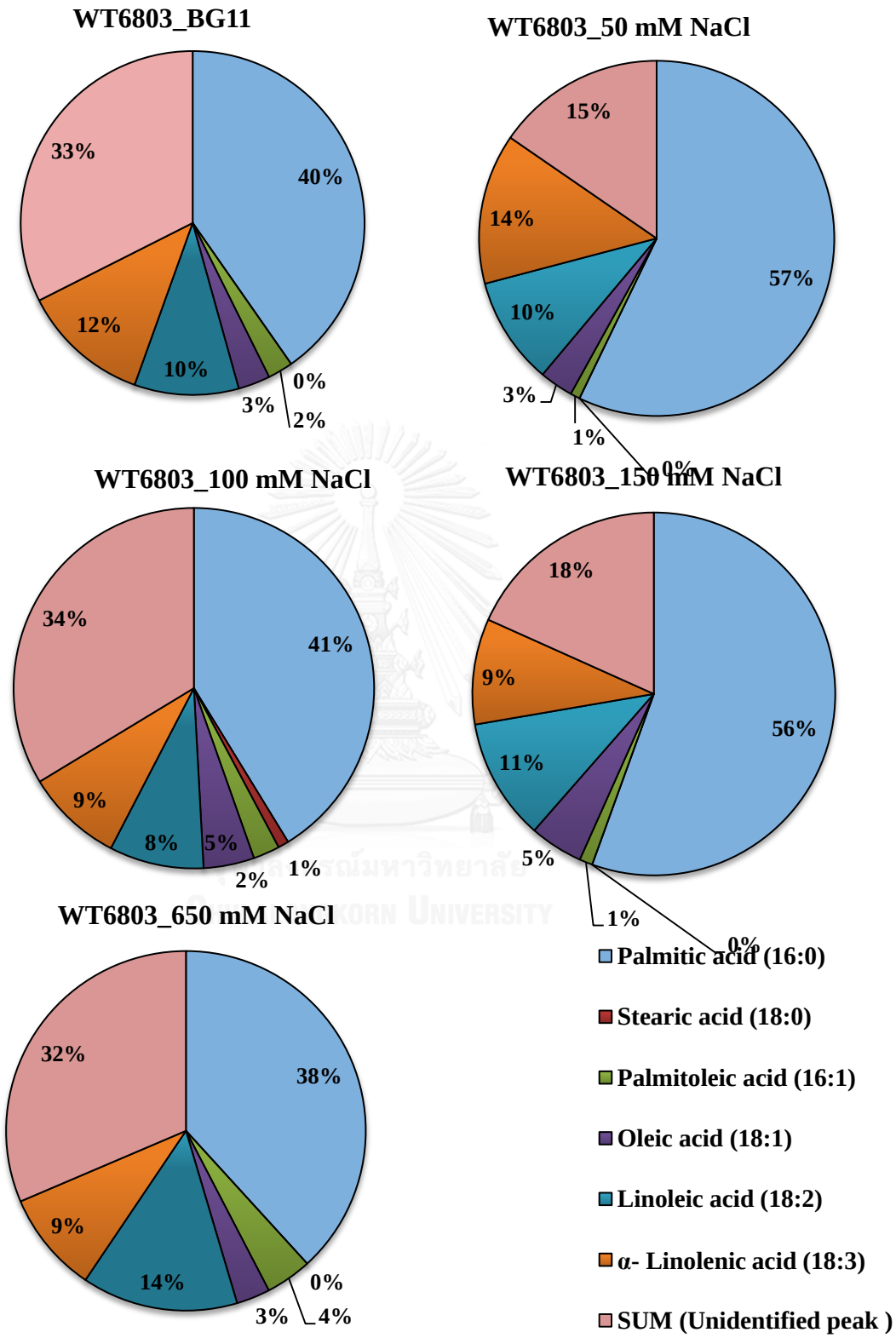

Figure 3.18 Fatty acid composition (%) of *Synechocystis* sp. PCC 6803 (WT) cells grown in BG_{11} medium containing 0, 50,100, 150 and 650 mM NaCl, respectively.

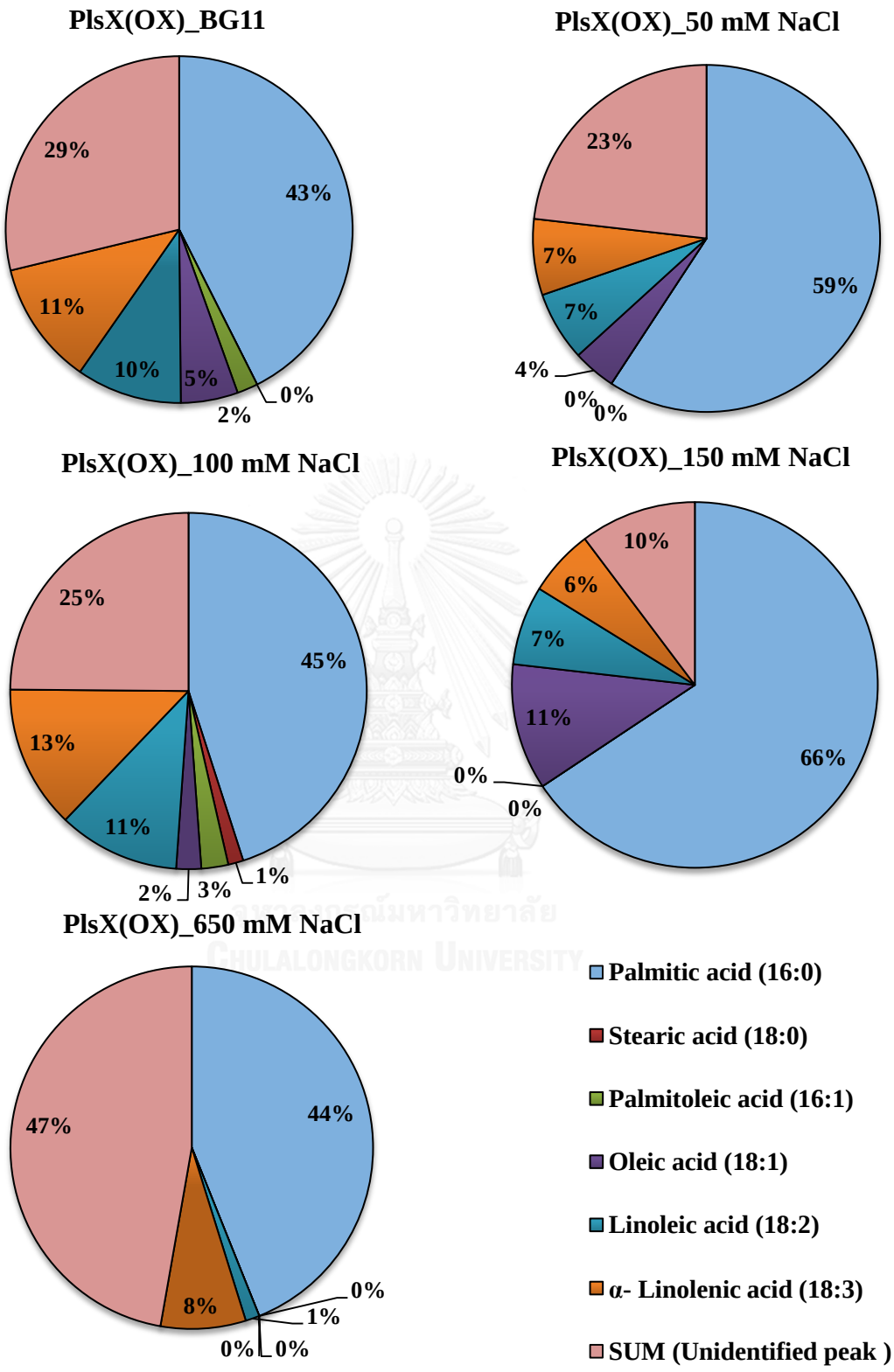

Figure 3.19 Fatty acid composition (%) of *plsX*- overexpressed (OX) cells grown in BG_{11} medium containing 0, 50, 100,150 and 650 mM NaCl, respectively.

3.5 Sequence analysis of fatty acid/phospholipid synthesis protein PlsX

The result of multiple alignment and phylogenetic tree from *Synechocystis* sp. PCC 6803 and other species were performed using ClustalW2. Recently, the database of Cyanobase has reported that *PlsX* gene encodes a protein related to fatty acid/phospholipid synthesis, which has been unknown for its function. The amino acid multiple alignment of *Synechocystis* PlsX with known other species was shown in Figure 3.20. The percentages of identity of *Synechocystis* sp. PCC 6803 PlsX protein to putative PlsX protein of unicellular cyanobacterium *Synechococcus* sp. PCC 7002 and filamentous *Anabaena* sp. PCC 7120 were highly conserved with 72.7 and 65.77%, respectively. However, the identity percentages when aligned with *Arabidopsis thaliana* gi|332193327 encoded glycerol-3-phosphate acyltransferase, *Escherichia coli* gi|585334175 gene encoded phosphate acyltransferase, and *Bacillus subtilis* gi|449028196 gene encoded phosphate acyltransferase plsX were lower conserved about 16.09, 36.72, and 42.42% respectively. Moreover, the similarity percentages of *Arabidopsis thaliana*, *Escherichia coli*, *Bacillus subtilis*, *Anabaena* sp. PCC 7120 and *Synechococcus* sp. PCC 7002 were conserved about 34.8, 48.8, 63.0, 80.8 and 81.1% respectively. Likewise, the phylogenetic tree of PlsX was also shown in Figure 3.21. In cyanobacteria database, PlsX protein showed not yet its annotation. However, PlsX gene in some bacteria strains are annotated that encoding phosphate acyltransferase whereas it encodes glycerol-3-phosphate acyltransferase in higher plants. The amino acid sequence of PlsX encoded by slr1510 of S*ynechocystis* sp. PCC 6803 belonged to the same clade with PlsX protein of unicellular cyanobacteria including *Synechococcus* sp. PCC 7002 (SYNPCC7002_A0526), *Microcystis*

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aeruginosa NIES-843 (MAE19480), *Cyanothece* sp. PCC 8801 (PCC8801_1806) and *Cyanothece* sp. PCC 7424 (PCC7424).

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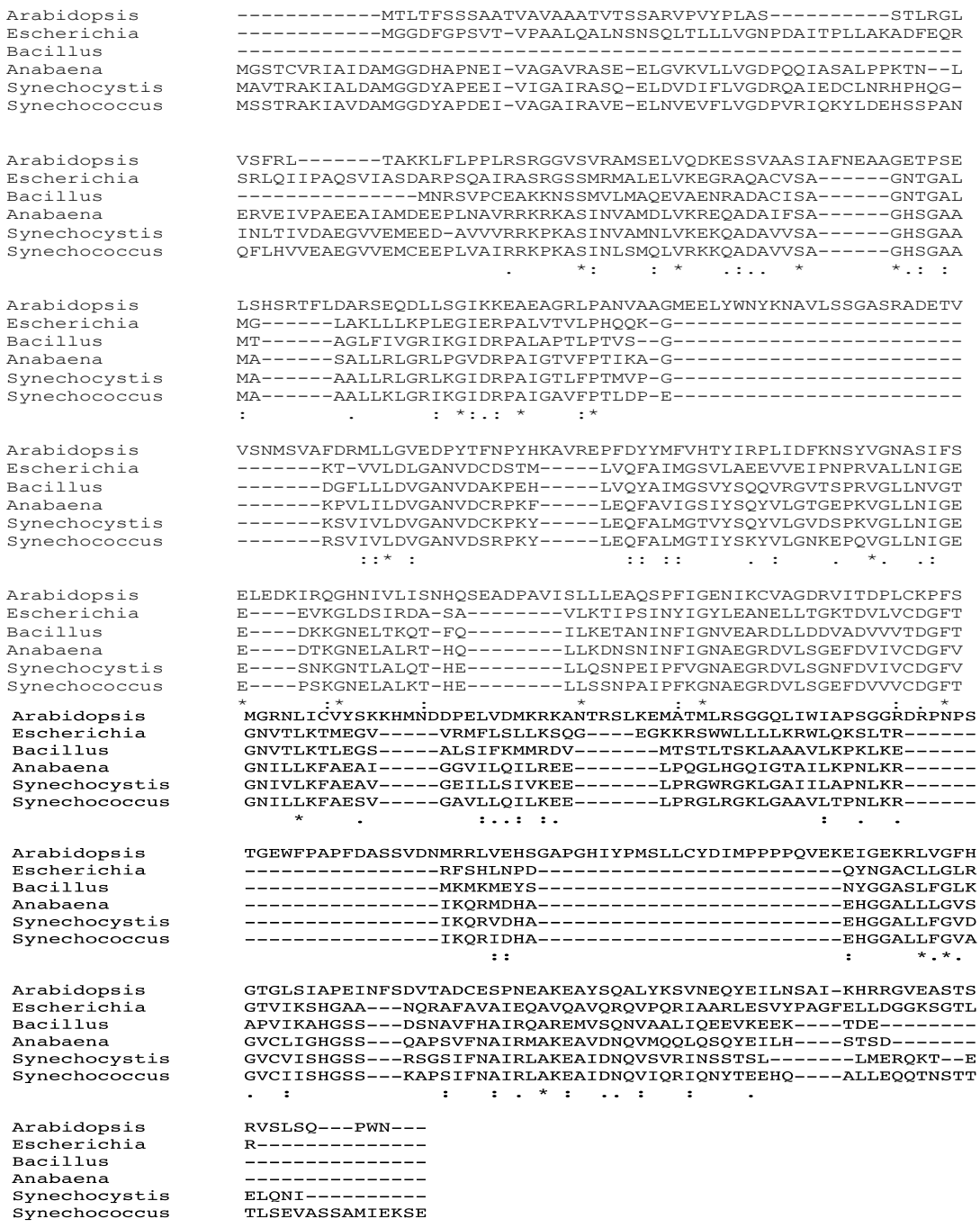

Figure 3.20 ClustalW2 amino acid alignment of fatty acid/phospholipid synthesis protein PlsX in *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002 and *Anabaena* sp. PCC 7120 from cyanobase and other species including *Arabidopsis thaliana* (a gene encoding glycerol-3-phosphate acyltransferase), *Escherichia coli* and *Bacillus subtilis* (each gene encoding phosphate acyltransferase).

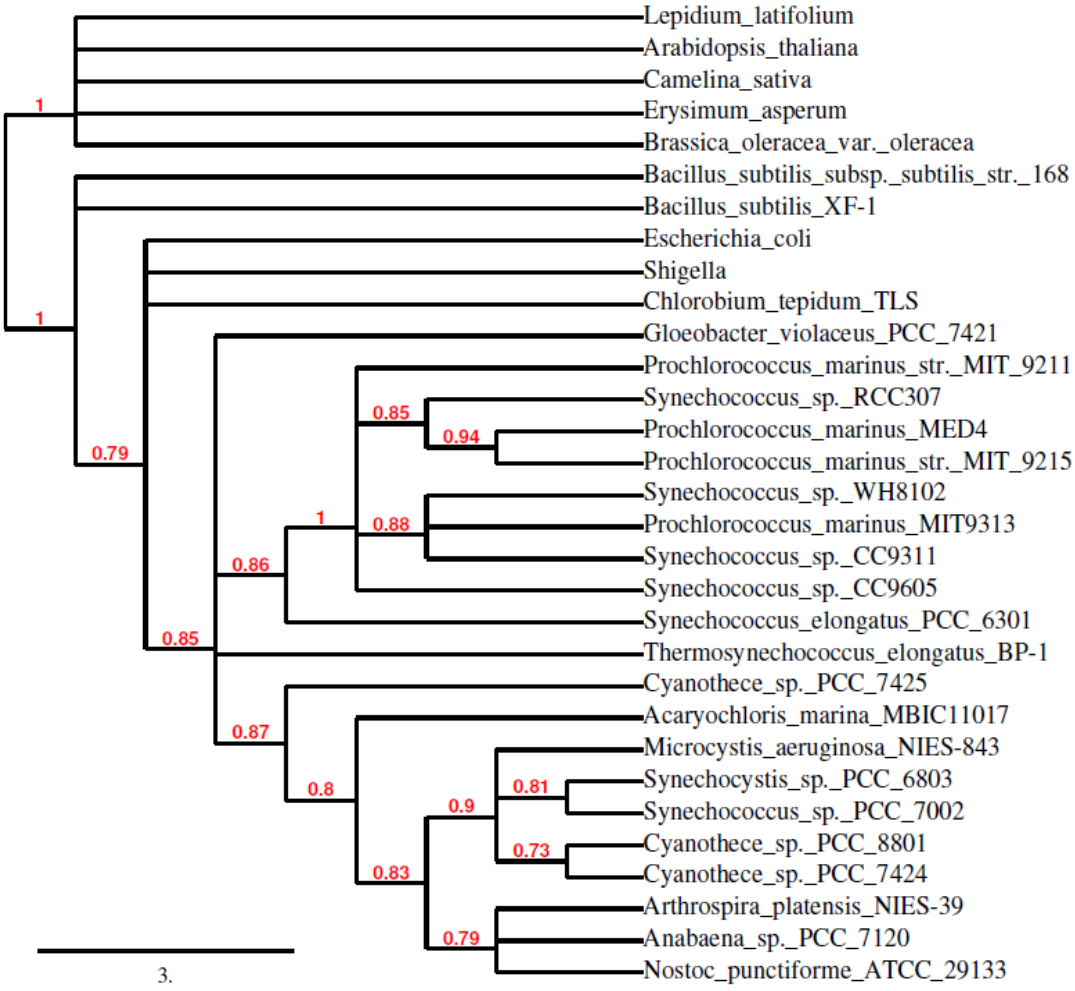

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Figure 3.21 Phylogenetic tree analysis. The amino acid sequence of fatty acid/phospholipid synthesis protein PlsX in cyanobacteria from cyanobase and other known species from NCBI database.

Table 3.1 Accession number, gene indexing names, organisms and sources in cyanobacteria and other species including bacteria and plant

Accession no.	Gene indexing name	Organisms	Source
Cyanobacteria			**Cyanobase**
Slr1510	Fatty acid/phospholipid synthesis protein PlsX	*Synechocystis* sp. PCC 6803	
Alr0238	Fatty acid/phospholipid synthesis protein PlsX	*Anabaena* sp. PCC 7120	
Tlr0844	Fatty acid/phospholipid synthesis protein PlsX	*Thermosynechococcus elongatus* BP-1	
Gll0800	Fatty acid/phospholipid synthesis protein PlsX	*Gloeobacter violaceus* PCC 7421	
MAE19480	Fatty acid/phospholipid synthesis protein PlsX	*Microcystis aeruginosa* NIES-843	
PMM0135	Fatty acid/phospholipid synthesis protein PlsX	*Prochlorococcus marinus* MED4	
PMT1995	Fatty acid/phospholipid synthesis protein PlsX	*Prochlorococcus marinus* MIT9313	
SYNW2247	Fatty acid/phospholipid synthesis protein PlsX	*Synechococcus* sp. WH8102	
syc0103_c	Fatty acid/phospholipid synthesis protein PlsX	*Synechococcus elongatus* PCC 6301	
sync_2598	Fatty acid/phospholipid synthesis protein PlsX	*Synechococcus* sp. CC9311	
SYNPCC7002_A0526	Fatty acid/phospholipid synthesis protein PlsX	*Synechococcus* sp. PCC 7002	
SynRCC307_0249	Fatty acid/phospholipid synthesis protein PlsX	*Synechococcus* sp. RCC307	

Accession no.	Gene indexing name	Organisms	Source
AM1_0652	Fatty acid/phospholipid synthesis protein PlsX	*Acaryochloris marina* MBIC11017	
P9215_01521	Fatty acid/phospholipid synthesis protein PlsX	*Prochlorococcus marinus* str. MIT 9215	
P9211_01491	Fatty acid/phospholipid synthesis protein PlsX	*Prochlorococcus marinus* str. MIT 9211	
Npun_F0098	Fatty acid/phospholipid synthesis protein PlsX	*Nostoc punctiforme* ATCC 29133	
CT2113	Fatty acid/phospholipid synthesis protein PlsX	*Chlorobium tepidum* TLS	
PCC7424	Fatty acid/phospholipid synthesis protein PlsX	*Cyanothece* sp. PCC 7424	
Cyan7425_2501	Fatty acid/phospholipid synthesis protein PlsX	*Cyanothece* sp. PCC 7425	
PCC8801_1806	Fatty acid/phospholipid synthesis protein PlsX	*Cyanothece* sp. PCC 8801	
NIES39_N01180	Fatty acid/phospholipid synthesis protein PlsX	*Arthrospira platensis* NIES-39	
Bacteria			NCBI
WP_024215825	phosphate acyltransferase	*Escherichia coli*	
WP_000197594	phosphate acyltransferase plsX	*Shigella*	
AAC44305	phosphate acyltransferase plsX	*Bacillus subtilis* XF-11	
AGE63435	PlsX	*Bacillus subtilis* subsp. subtilis str. 168	

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Accession no.	Gene indexing name	Organisms	Source
Plant			NCBI
NP_174499	glycerol-3-phosphate acyltransferase	*Arabidopsis thaliana*	
AEO93268	glycerol-3-phosphate acyltransferase	*Lepidium latifolium*	
XP_010499766	glycerol-3-phosphate acyltransferase	*Camelina sativa*	
XP_013600050	glycerol-3-phosphate acyltransferase	*Brassica oleracea*	

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CHAPTER IV

DISCUSSION

In this work, we successfully constructed *plsX*-overexpressing strain of *Synechocystis* sp. PCC 6803. The log-phase growing cells were further treated in BG_{11} media containing various NaCl concentrations of 50, 100, 150, 350 and 650 mM, respectively. Cell growth was decreased by 650 mM NaCl condition starting at 5 days of cultivation compared to normal BG_{11} condition whereas other conditions of 50, 100, 150 and 350 mM NaCl concentrations showed no apparent changes in both wild type and *plsX*-overexpressing strains (Figure 3.4). Previously, reported that 0.5 M NaCl or 0.5 M sorbitol conditions generated salt stress and hyperosmotic stress, respectively, which decreased the growth rate to about 50% in *Synechocystis* sp. PCC 6803 (Mironov *et al.*, 2012). The high salt concentration had negatively influenced on physiological processes of cyanobacteria (Sudhir and Murthy, 2004). Salt stress inhibited protein synthesis and the energy produced by photosynthesis, which is the system that the most susceptible to such environmental stress (Allakhverdiev *et al.*, 2001). In nutrient modifications including nitrogen deficiency (-N) and 0.4% acetate supplementation conditions (Figure 3.5), cell growth was determined for along 6-8 days-treatment with cell density at OD_{730} started of about 0.5-0.6. Cell growth of wild type and *plsX*-overexpressing strains showed under nitrogen deficiency (-N) was obviously decreased after 5 day of cultivation compared with normal condition. Saha and co-workers (2003) reported that nitrogen limitation affected on growth reduction of *Oscillatoria willei* BDU 130511 about 17% of dry biomass compared to its growth

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in nitrogen-supplemented medium. However, the 0.4% acetate supplementation significantly increased cell growth within 3 days of cultivation of both wild type and *plsX*-overexpressing strains. Previously, 1% acetate was reported that could increase cell growth of *Chlorella vulgaris* within 7 days of cultivation (Liang *et al.*, 2009).

On the other hand, intracellular pigments including chlorophyll *a* and carotenoid contents (Figures 3.7, 3.8 and 3.9) showed significant decreases under both 650 mM NaCl condition and nitrogen starvation whereas 0.4% acetate supplementation increased intracellular pigments of both wild type and *PlsX*-overexpressing strains. It was coincident with a previous report that salt stress at 1 M NaCl condition led a negative effect to decrease the ratio of chlorophyll:carotenoids in *Dunaliella salina* and *Dunaliella bardawil* (Gomez *et al.*, 2003). Similarly, nitrogen starvation affected on pigment reductions of *Oscillatoria willei* BDU 130511 which the chlorophyll *a* and carotenoids were reduced by 31.81% and 18.38% of dry biomass (Saha *et al.*, 2003). On the other hand, the oxygen evolution rate that represents photosynthetic efficiency of living cells showed a decrease under salt stress. It was coincident to support salt stress effect, Marin and co-workers (2004) reported that salt stress effectively inhibited PSII-mediated oxygen evolution activity of *Synechocystis* sp. PCC 6803.

In this work, we also provide evidences that demonstrate the transcription level abundance of gene related in fatty acid biosynthesis pathway in the wild type and *plsX*-overexpressing strains. Interestingly, it was found that *plsX* mRNA was up-regulated under all conditions including normal BG_{11}, NaCl condition, BG_{11} without N and 0.4% acetate supplementation conditions in *plsX*-overexpressing strain when compared to wild type. The salt stress effect itself did not induce the *PlsX* transcript

level. The high salt stress at 350 and 650 mM NaCl concentrations effectively reduced the *PlsX* transcript level. Previously, there was a report in Δ*plsX* mutant showed that the depleted *plsX* of *Bacillus* had lower amount of total lipid and fatty acid which indicating that absence of PlsX, the syntheses of both fatty acid and phospholipid was inhibited (Paoletti *et al.*, 2007). On the other hand, *AccA* gene, namely sll0728 which encodes acetyl-CoA carboxylase alpha subunit catalyzing a rate limiting step of the conversion of acetyl-CoA into malonyl-CoA, was up-regulated under normal BG_{11}, 50 and 100 mM NaCl concentrations whereas there were no changes on *AccA* mRNA levels under 150 mM, 350 mM, 650 mM NaCl. The transcript level of *AccA* naturally showed higher amount than *PlsX* transcript level whereas its lowered amount was observed apparently in *plsX*-overexpressing strain.

We also demonstrated the screening of total lipid was performed by Sudan Black B and Nile Red staining (Figure 3.11). Results showed that cells did not accumulate much storage lipids under normal BG_{11} medium, NaCl condition and 0.4% acetate supplementation while nitrogen deficiency (-N) induced storage lipids in granule with WT and *plsX*-overexpressing strains. Thomson and co-workers (2010) suggested that storage lipids of most bacterial including poly(hydroxyalkanoates) (PHAs), triacylglycerols (TAGs) and wax esters (WEs) were produced as a carbon and energy storage accumulated in cytoplasmic granules.

On the other hand, it was found in wild type that NaCl stress at 50, 100, 150 and 350 mM NaCl concentration conditions significantly induced the unsaturated lipid contents whereas 650 mM NaCl condition significantly inhibited the accumulation of unsaturated lipid content. Previously, it was reported that salt stress at 0.5 NaCl concentrations increased the composition content of unsaturated fatty

acids in membrane because NaCl itself might activate membrane-bound enzymes and changes in membrane fluidity (Allakhverdiev *et al.*, 1999). In *Synechocystis*, the unsaturation of fatty acids in the thylakoid membranes is important for the tolerance of photosynthetic machinery to salt stress. Especially, unsaturation of fatty acids could reverse the suppressed activity and synthesis of the Na+/H+ antiporter system due to salt stress (Allakhverdiev *et al.*, 1999). In addition, nutrient modifications including nitrogen deficiency (-N) and 0.4% acetate supplementation, induced the accumulation of unsaturated lipid content of about 2.0-2.5 %W/DCW. Previously, there was reported that nitrogen starvation affected on the content of fatty acids including lauric acid and γ-linolenic of about 14.53% and 40.55% respectively in *O. willei* BDU 130511 (Saha *et al.*, 2003).

The effect of NaCl condition on lipid content, which at 100 mM NaCl condition, showed the highest amount about 20-30 %w/DCW in both wild type and *PlsX*-overexpressing strains. Coincidently, Takagi and co-workers (2006) also reported that stress conditions at 0.5 to 1 M NaCl concentrations apparently increased intracellular lipid content (67%) in *Dunaliella* cells. Thus, this lipid modification might involve potentially in the protection against salt stress due to the fact that photosynthetic organisms exposed to salt stress had the higher level of desaturated fatty acids of membrane lipids (Allakhverdiev *et al.*, 2001). Furthermore, nitrogen deficiency (-N) and 0.4% acetate supplementation induced the accumulation of total lipid up to 2-4 %w/DCW. For another previous report, it was in contrast that nitrogen starvation decreased in total lipid content of about 26.08 %mg/g DW in *O. willei* BDU 130511 (Saha *et al.*, 2003). The lipid content of freshwater green algae *Chlorella vulgaris* was significantly increaseed of about 40% in low nitrogen

containing medium (Illman *et al.*, 2000). However, other previous work reported suggested that microalgae cells used ATP and NADPH for growth and generated biomass from photosynthesis. When ADP and $NADP^+$ as electron acceptor molecules for photosynthesis were depleted due to the lack of nutrient (nitrogen, phosphorus), cells were increased their fatty acid accumulation as TAG form, which effectively restored $NADP^+$ for growth-limiting condition by fatty acid biosynthesis (Sharma *et al.*, 2012). Moreover, it was also reported the combined condition effects of 1% acetate without NO_3 and 1% acetate with NO_3 which could induce lipid content of about 31 and 36 % within 12 day-cultivation of *Chlorella vulgaris* (Liang *et al.*, 2009).

On the other hand, the percentage of fatty acid composition using GC-MS detection is shown in Figures 3.16 and 3.17. In present study, the most abundance of saturated fatty acid was palmitic acid (C16:0) whereas major unsaturated fatty acids were palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3). GC-MS analysis showed that palmitoleic acid (C16:1) and linoleic acid (C18:2) were increased apparently by salt stress induction compared to those under normal control of wild type (Figure 3.16). However, in *plsX*-overexpressing showed that palmitic acid (C16:0) were increased apparently by salt stress induction of about 40-60 %. Interestingly, the 100 mM NaCl condition could induce the accumulation of stearic acid (18:0) (Figure 3.17). It was consistent with a recent finding that salt stress induced more unsaturated fatty acid composition on membrane lipids in *Synechococcus* sp. (Allakhverdiev *et al.*, 2001). For another previous report, it was found in temperature change that fatty acid composition of lipids in *Synechocystis* sp. PCC 6803 cells after grown at high temperature of 34 °C

was increased (Wada and Murata, 1998). On the other hand, the pervious reported that fatty acid contents in thylakoid membranes in *Synechocystis* sp. PCC 6803 cells after grown at high temperature of 38 °C were increased including palmitic acid (C16:0) up to 61.3 %, oleic acid (C18:1) with 10.0 % increase and linoleic acid (C18:2) with 13.6 % increase, respectively (Nanjo *et al.*, 2010).

The multiple alignment and phylogenetic tree were performed in Figure 3.18 and 3.19). Since in most cyanobacteria, reported in Cyanobase, have demonstrated that PlsX is the fatty acid/phospholipid synthesis protein and lack of its annotation. We then employed the bioinformatics to gain more understanding of PlsX in *Synechocystis* sp. PCC 6803. The amino acid multiple alignment of *plsX* gene showed percentages of identity of *Synechocystis* sp. PCC 6803 PlsX compared to *Synechococcus* sp. PCC 7002 PlsX was 72.70 which was highly conserved among cyanobacteria. On the other hand, higher plant has glycerol-3-phosphate acyltransferase (GPAT) which catalyzes the synthesis of lysophosphatidic acid from glycerol-3-phosphate and long-chain acyl-CoA. The result of amino acid alighment with *Arabidopsis thaliana* GPAT was low conserved at 16.09%. The annotated PlsX in some bacteria such as *Escherichia coli* and *Bacillus subtilis* was identified as phosphate acyltransferase enzyme. Previously, suggested that the structure of plsX protein of *B. subsilis* represented as phosphotransferase enzyme. The identities between *Synechocystis* PlsX and PlsX amino acid sequences of those two bacterial strains showed 36.72 and 42.42%, respectively (Xu *et al.*, 2005). However, other previous work reported that *plsX* gene compared between *Streptococcus pneumoniae* and *Escherichia coli* model result showed 33.2% identity and 55.0% similarity. Moreover, the plsX gene of *Streptococcus pneumoniae* was classified as a

phosphate:acyl-ACP acyltransferase because this enzyme catalyzed the acyl-phosphate for Acyl-ACP (Lu and Rock, 2006).

We also demonstrated the phylogenetic tree of PlsX protein compared among cyanobacteria and some other species (Table 3.1 and Figure 3.20). The phylogenetic profiling tool showed that *Synechocystis* PlsX belonged to the same clade with unicellular cysnobacteria including *Synechococcus* sp. PCC 7002 (SYNPCC7002_A0526), *Microcystis aeruginosa* NIES-843 (MAE19480), *Cyanothece* sp. PCC 8801 (PCC8801_1806) and *Cyanothece* sp. PCC 7472 (PCC7472). The higher plant GPATs and bacterial PlsXs were located in long distance of tree from *Synechocystis* PlsX (about 0.002 – 0.08). From this result, it was indicated that PlsX protein in *Synechocystis* sp. PCC6803 might be classified in the group of acyltransferase enzyme, which further needed more experiments to prove its function. Lu and co-workers (2006) suggested that Achaea and eukaryote did not have a recognizable PlsX/Y pathway whereas the most bacteria were also contained PlsX/Y pathway, such as *P. aeruginosa* (Jacobs *et al.*, 2003). On the other hand, there was proved that *B. subtilis* lacking plsB, and lacking both the plsX and plsY genes gained less fatty acids and phospholipids which indicated that those PlsB, PlsX and PlsY proteins were essential for phospholipid production (Kobayashi *et al.*, 2003).

CHAPTER V

CONCLUSION

In this study, we successfully constructed the *plsX*- overexpressing strain of *Synechocystis* sp. PCC 6803 (OX_plsX strain). Cell growth and intracellular pigment contents of OX_plsX strain were not different from those of wild type, as well as their oxygen evolution rate. Moreover, both total lipid and unsaturated lipid contents of OX_plsX strain were higher than those of wild type. On the other hand, it was found that sodium chloride at 100 mM concentration could induce the accumulation of total lipid in OX_plsX strain higher than wild type, up to about 1.3 fold whereas unsaturated lipid content of OX_plsX strain was higher wild type in all salt stress studied. The fatty acid composition from GC-MS analysis mainly consisted of saturated and unsaturated fatty acids including palmitic acid (C16:0), stearic acid (C18:0), pailmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3). We found that salt stress effectively influenced the intracellular composition ratio of saturated and unsaturated fatty acids. On the other hand, acetate supplementation enabled OX_plsX strain to accumulate more total lipids (of about 1.3 fold) and unsaturated lipids (of about 2.9 fold) when compared to wild type. Amino acid sequence alignment and phylogenetic tree analysis might suggest that *Synechocystis* plsX belong to acyltransferase group. Altogether, plsX gene had a crucial role in fatty acid synthesis since the overexpression of *plsX* gene enhanced the lipid accumulation in *Synechocystis* cells. Also, the acetate

supplementation could induce the lipid production in both wild type and plsX-overexpressing strains

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APPENDIX

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APPENDIX A

Normal medium (BG_{11}) volume 1 liter (Rippka *et al.*, 1979)

Nutrients	Stock solution (1 L)	BG_{11} medium	
		Liquid medium	Solid medium
$MgSO_4.7H_2O$	75 g	1 ml	1 ml
$CaCl_2.2H_2O$	36 g	1 ml	1 ml
KH_2PO_4	30 g	1 ml	1 ml
EDTA	1 g	1 ml	1 ml
$NaCO_3$	20 g	1 ml	1 ml
Citric acid	6 g	1 ml	1 ml
Ferric ammonium citrate	6 g	1 ml	1 ml
Trace element	detailed below	1 ml	1 ml
$NaNO_3$	150 g	10 ml	10 ml
1M HEPES-NaOH	238.3 g	10 ml	10 ml
30% $Na_2S_2O.5H_2O$	300 g	-	10 ml
Bacto-agar	-	-	15 g
Distilled water	-	1000 ml	1000 ml

**Adjusted pH to approximately 7.5

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

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Nitrogen modification from normal medium (BG_{11})

Without $NaNO_3$ from BG_{11} medium

Ferric ammonium citrate was replaced by $FeSO_4$ 6 g/liter

0.4% acetate supplementation

Added Na-acetate 5.5 g/liter

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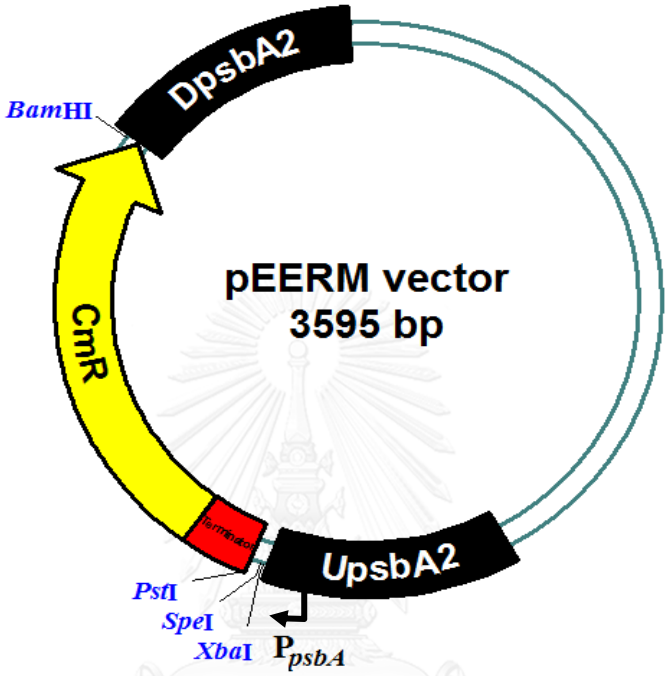
APPENDIX B

- pEERM vector



pEERM vector was used for overexpression gene in *Synechocystis* PCC 6803 which created for using integration into *Synechocystis* genome at photosyntem II, D1 protein region (*psbA2*). The physical map of pEERM vector containing strong promoter (P_{psbA}), selective chloramphenicol antibiotic resistance cassette gene and multiple cloning site; XbaI, SpeI and PstI. The size of pEERM vector is 3,595 bp.

APPENDIX C

LB Medium (1 liter)

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

Added distilled water to a total volume of 1 liter. The medium was sterilized by autoclaving at 120 °C for 15 min.

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APPENDIX D

-Sudan black B solution

Sudan black B 3 g

Added 70 % ethanol to a total volume of 100 ml.

-Safranin O solution

Safranin O 5 g

Added distilled water to a total volume of 100 ml.

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APPENDIX E

-Vanillin –phosphoric acid reagent for total unsaturated lipid content

Vanillin 100 ml

Vanillin 20 g

Added distilled water to a total volume of 100 ml.

17% phosphoric acid 100 ml

Phosphoric acid 20 ml

Added distilled water to a total volume of 100 ml.

The mixture volume contained 0.2 mg vanillin per ml 17 % phosphoric acid.

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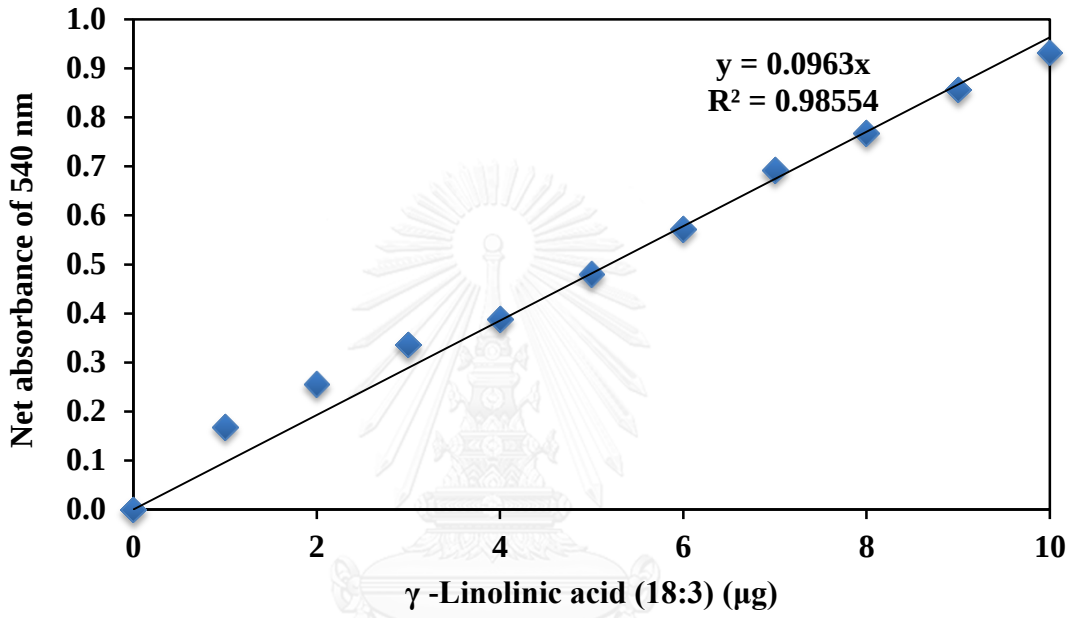
APPENDIX F

- Standard curve for total unsaturated lipid content using γ -Linolinic acid (18:3) as a standard



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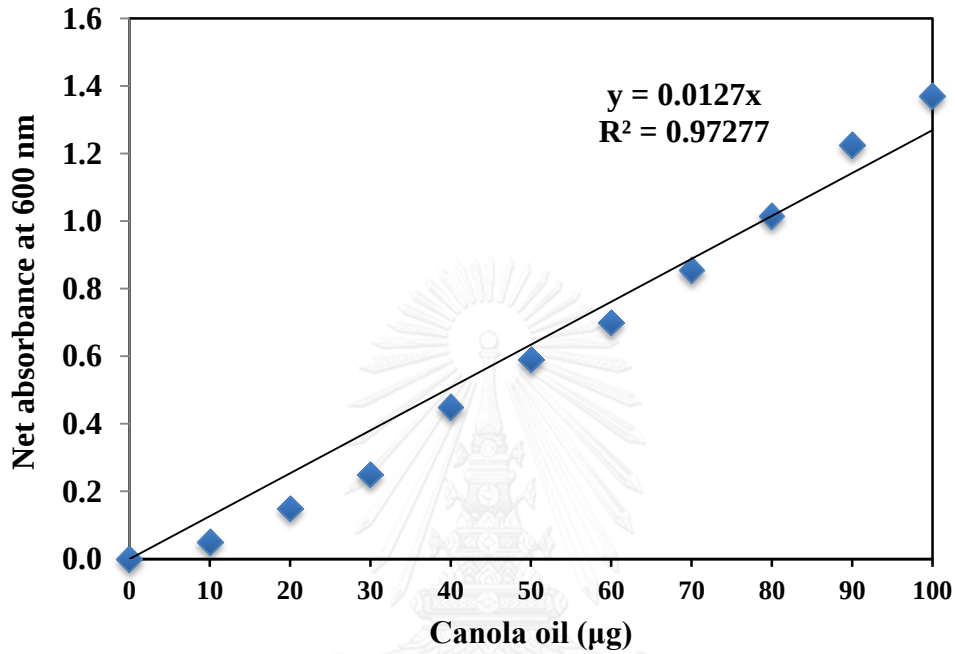
APPENDIX G

- Standard curve for total lipid content using canola oil as standard



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VITA

Miss Nutchaya Songruk was born on September 11, 1989 in Nakhon Si Thammarat province, Thailand. She graduated with a Bachelor of Science degree in Biomedical Science, Faculty of Science from Rangsit University, Thailand in 2011. She has further studies for Master of Science degree in Biochemistry, Faculty of Science, Chulalongkorn University since 2012.

Proceeding

Nutchaya Songruk, Aran Incharoensakdi, Saowarath Jantaro (2015). Effect of salt stress on fatty acid and lipid levels in cyanobacterium Synechocystis sp. PCC 6803. Burapha University International Conference 2015 (BUU2015). 10-12, July 2015, Bangsaen, Chonburi, Thailand.

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