การเปลี่ยนแปลงปริมาณพอลิเอมีนภายใต้ภาวะเครียดออสโมติกและรังสีอัลตราไวโอเลต
ในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาว อภิรดี โพธิพงศา

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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CHANGES OF POLYAMINES CONTENT UNDER OSMOTIC AND UV-RADIATION STRESSES IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

Miss Apiradee Pothipongsa

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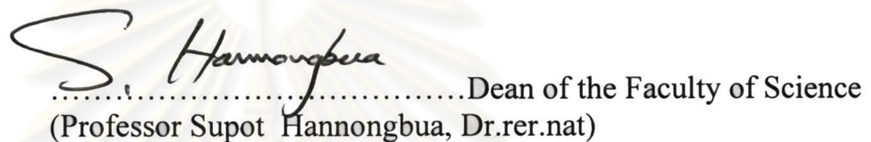

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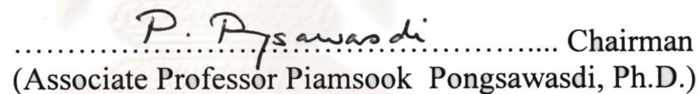

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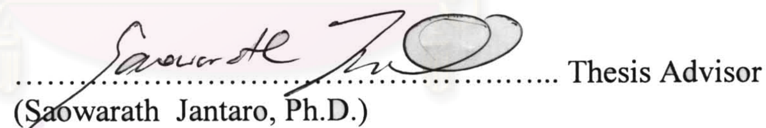

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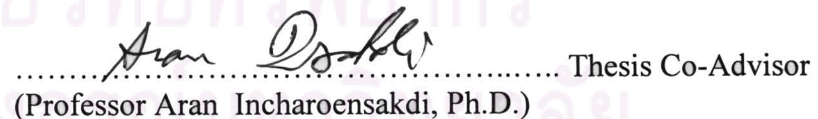

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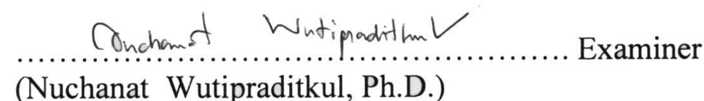

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อภิรดี โพธิพงศา : การเปลี่ยนแปลงปริมาณพอลิเอมีนภายใต้ภาวะเครียดออสโมติกและรังสีอัลตราไวโอเลตในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803. (Changes of polyamines content under osmotic and UV-radiation stresses in cyanobacterium *Synechocystis* sp. PCC 6803) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร. เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร. อรัญ อินเจริญศักดิ์, 133 หน้า.

จากการศึกษาถึงผลกระทบของเกลือโซเดียมคลอไรด์และซอร์บิทอลในอาหารเลี้ยงเชื้อ (BG-11) ร่วมกับรังสีอัลตราไวโอเลต ชนิดรังสียูวี-เอ (365 นาโนเมตร, 11-13 วัตต์/ตารางเมตร), รังสียูวี-บี (302 นาโนเมตร, 1.2-1.3 วัตต์/ตารางเมตร) และ รังสียูวี-ซี (254 นาโนเมตร, 1.0-1.3 วัตต์/ตารางเมตร) เป็นเวลา 3 ชั่วโมง ต่อการเจริญเติบโตของเซลล์ ปริมาณรงควัตถุภายในเซลล์ (คลอโรฟิลล์-เอ และแคโรทีนอยด์) และปริมาณพอลิเอมีนในไซยาโนแบคทีเรียแบบเซลล์เดี่ยว *Synechocystis* sp. PCC 6803 พบว่าภายใต้แสงปกติ อาหารเลี้ยงเชื้อที่มีเกลือโซเดียมคลอไรด์เข้มข้น 650 มิลลิโมลาร์ และซอร์บิทอลเข้มข้น 500 มิลลิโมลาร์ นั้นไม่มีผลต่อการเจริญเติบโต และการเปลี่ยนแปลงปริมาณคลอโรฟิลล์และปริมาณแคโรทีนอยด์ของเซลล์ อย่างไรก็ตามเซลล์ที่ถูกกดดันเนื่องจากเกลือและซอร์บิทอลมีปริมาณคลอโรฟิลล์ และแคโรทีนอยด์สูงกว่าในอาหารเลี้ยงเชื้อปกติ สำหรับการเจริญเติบโตของเซลล์ภายใต้ความเครียดเนื่องจากรังสียูวี นั้นมีการตอบสนองที่แตกต่างกัน การเจริญเติบโตของเซลล์ลดลงเล็กน้อยเมื่อได้รับรังสียูวี-เอ แต่จะลดลงมากขึ้นภายใต้ภาวะเครียดร่วมของเกลือหรือซอร์บิทอลที่ความเข้มข้นสูงร่วมกับรังสียูวี-เอ และรังสียูวี-บี นอกจากนี้พบว่ารังสียูวี-ซี ร่วมกับซอร์บิทอลมีผลต่อการเจริญเติบโตของเซลล์เช่นกัน สำหรับภาวะเครียดของยูวี-ไอออนิก และยูวี-ออสโมติก ไม่ส่งผลต่อการเปลี่ยนแปลงปริมาณคลอโรฟิลล์ ในขณะที่ปริมาณแคโรทีนอยด์มีแนวโน้มเพิ่มขึ้นภายใต้ภาวะเครียดจากรังสียูวี-เอ, รังสียูวี-เอและไอออนิก, และรังสียูวี-บีและออสโมติก ภายในเวลา 3 ชั่วโมง สำหรับพอลิเอมีนมีการสะสมมากในรูปแบบอิสระ โดยเฉพาะพอลิเอมีนชนิดสเปอร์มีดีน จากการศึกษาพบว่าปริมาณพอลิเอมีนเพิ่มขึ้นอย่างมีนัยสำคัญภายหลังได้รับรังสียูวี-บี หรือรังสียูวี-ซี เป็นเวลา 1 ชั่วโมง ขณะที่ปริมาณพอลิเอมีนลดลงหลังได้รับรังสียูวี-ออสโมติกเป็นเวลานาน 3 ชั่วโมง

นอกจากนี้งานวิจัยได้ทำการศึกษายีนอาร์จีนีน ดีคาร์บอกซิเลส (*adc1* และ *adc2*) สำหรับการสังเคราะห์พิวเทรสซีน ในระดับยีนและโปรตีน ด้วยเทคนิค RT-PCR และ Western blot พบว่ามีปริมาณ *adc1* mRNA สูงกว่า *adc2* mRNA ภายใต้ความเครียดจากเกลือ ส่วนความเครียดจากออสโมติกนั้นไม่มีผลต่อปริมาณ *adc1* และ *adc2* mRNA สำหรับการแสดงออกระดับโปรตีนนั้น โปรตีน ADC1 มีปริมาณเพิ่มขึ้นเล็กน้อย ภายใต้ความเครียดจากไอออนิก, ยูวี-เอและไอออนิก, ยูวี-บีและไอออนิก แต่ในทางกลับกัน ภาวะออสโมติก, ออสโมติก-ยูวี ไม่มีผลต่อระดับยีนและระดับโปรตีนอย่างมีนัยสำคัญ สำหรับความสัมพันธ์ในระดับยีนและโปรตีน พบว่า ภายในระยะเวลา 1 ชั่วโมงของภาวะเครียดเนื่องจากเกลือ, ออสโมติก, ยูวี-ซี, เกลือและยูวี-เอ, เกลือและยูวี-บี, เกลือและยูวี-ซี, ออสโมติกและยูวี-ซี มีความสอดคล้องกันทั้งระดับยีนและโปรตีน ADC ในเซลล์ *Synechocystis*

ภาควิชา..............ชีวเคมี..............ลายมือชื่อนิสิต.........อภิรดี.......โพธิพงศา..............................

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APIRADEE POTHIPONGSA : CHANGES OF POLYAMINES CONTENT UNDER OSMOTIC AND UV-RADIATION STRESSES IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. THESIS ADVISOR : SAOWARATH JANTARO, Ph.D., THESIS CO-ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., 133 pp.

Effect of salt and sorbitol at various concentrations and combined with UV radiations, including UV-A (365 nm; 11-13 w/m^2), UV-B (302 nm; 1.2-1.3 w/m^2) and UV-C (254 nm; 1.0-1.3 w/m^2), for 3 hours on cell growth, intracellular pigments (chlorophyll a and carotenoids) and polyamine contents were investigated in a unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Long-term stress (6-7 days) of *Synechocystis* cells grown in media up to 650 mM NaCl or 500 mM sorbitol did not severely affect growth, chlorophyll-a and carotenoids content of cells under normal growth light. Salt and sorbitol stresses stimulated the chlorophyll a and carotenoids accumulation. However, the response of cell growths showed different patterns after exposing to UV radiations. Under UV-A alone, growth was slightly decreased but was greatly decreased after combining with high concentration of salt or sorbitol. UV-B affected growth after co-treating with NaCl or sorbitol and UV-C showed the effect only when co-stress with sorbitol. The levels of chlorophyll a were constant under the combined stresses of both UV-ionic and UV-osmotic whereas those levels of carotenoids were slightly increased during the last 3 hours under UV-A both alone and in combination with salt stress and UV-B plus sorbitol treatments when compared to untreated cells. PCA-soluble polyamines were found as major forms and spermidine was present dominantly in all conditions. Short-term stress (1 hour) of UV radiations significantly increased their polyamine contents, especially salt stress combined with either UV-B or UV-C while long-time stress (3 hours) of UV radiations decreased polyamines content, especially under osmotic treatments.

Two different genes (*adc*1 and *adc*2) encoding ADC, the first enzyme for putrescine synthesis. The transcription and protein levels of ADC were analyzed by RT-PCR and Western blot, respectively. The results showed that *adc1* mRNA level was up-regulated higher than *adc2* mRNA level under salt stress while osmotic stress seemed to have no effect on *adc1* and *adc2* mRNA levels. For the protein levels, ADC1 was slightly increased under high concentrations of salt, salt combined with UV-A or UV-B. However, osmotic stress did not have much effect on both transcription and protein levels. In addition, osmotic stress seemed to abolish a tight correlation of both levels when combined osmotic stress with UV radiation. Both transcription and protein levels of ADC were connected by salt and osmotic, as well as salt treatment combined with UV-A and UV-B. On the other hand, UV-C alone or combined with salt or sorbitol had influence on the transcription and protein levels in *Synechocystis* cells under short-term stress (1 hour).

Department : .....Biochemistry... Student's Signature....Apiradee Pothipongsa.........
Field of Study: ...Biochemistry... Advisor's Signature.....................................
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LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
bp	Base pair
BSA	Bovine serum albumin
^{o}C	Degree Celsius
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
g	Gram
HEPES	Hydroxyethyl piperazineethanesulfonic acid
kb	Kilo base
kDa	Kilo Dalton
h	hour
L	Liter
mM	Millimolar
min	Minute
µg	Microgram
µl	Microliter

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ml	Milliliter
mM	Millimolar
M	Molar
nm	nanometer
OD	Optical density
PCR	Polymerase Chain Reaction
Put	Putrescine
PMSF	Phenylmethylsulfonyl fluoride
RT-PCR	Reverse transcription- Polymerase Chain Reaction
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
Spd	Spermidine
Spm	Spermine
TAE	Tris-acetate electrophoresis
TEMED	Tetramethylethylenediamine
TES	Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
UV	Ultraviolet

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CHAPTER I

INTRODUCTION

1.1 Environment stresses

1.1.1 Ultraviolet radiation

An essential factor of environment stresses contributing to organism is ultraviolet (UV) radiation. Due to depletion of the stratospheric ozone layer results in increased levels of incident solar UV radiation at the Earth's surface (Lubin and Jensen, 1995; Madronich *et al*., 1998). The stratospheric ozone layer efficiently which filters out most of the detrimental, shortwave UV radiation, shorter than 280 nm was destroyed (Robberecht, 1989). A small decrease of ozone layers may cause a large relative increase in biologically effective UV radiation (Madronich, 1992, 1993). In general, each 1% reduction in ozone causes an increase of 1.3-1.8% in UV-B radiation reaching the biosphere (McFarland and Kaye, 1992).

Ultraviolet radiation is a part of the non-ionizing region of the electromagnetic spectrum (Figure 1.1) which comprises approximately 8-9% of the total solar radiation (Frederick, 1993). UV is traditionally divided into three wavelength ranges (Figure 1.2) including UV-C (200-280 nm) is extremely harmful to organisms, but not relevant under natural conditions of solar irradiation; high energy, UV-B (280-320 nm) is of particular interest because this wavelength represents only approximately 1.5% of the total spectrum, but can induce a variety of damaging effects in plants; lower energy and UV-A (320-400 nm) represents approximately 6.3% of the incoming solar radiation and is the less hazardous part of UV radiation (Hollósy, 2002; Barta *et al*., 2004).

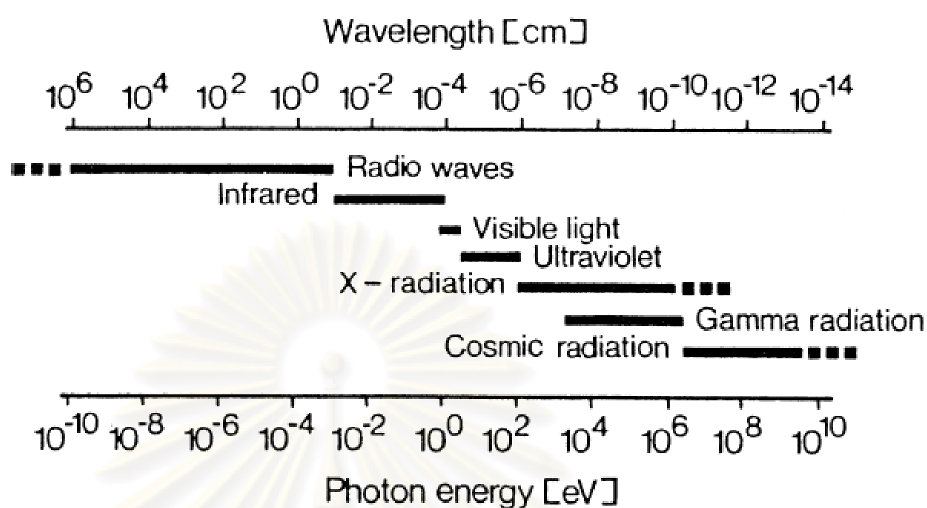

Figure 1.1 The electromagnetic spectrum. (Kovács and Keresztes, 2002)

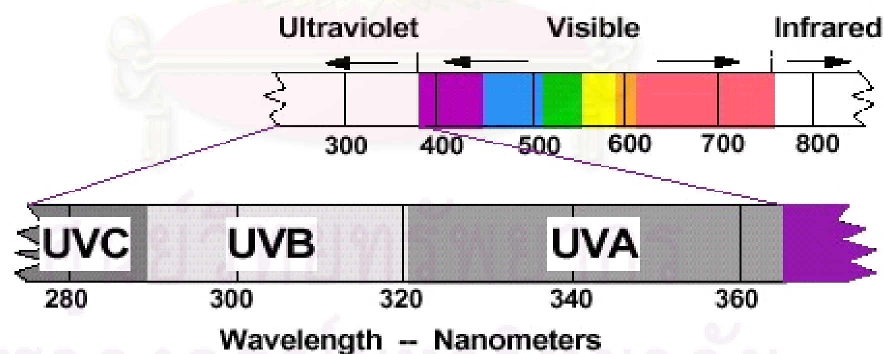

Figure 1.2 The three wavelength range of ultraviolet radiation**,** namely, UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm), respectively. (Source: http://www.vcharkarn.com/uploads/63/63591.jpg).

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UV radiation can induce deleterious and specific effects on living organisms (Worrest, 1982; Caldwell *et al*., 2003). UV radiation can affect principally many biological processes (Fiscus and Booker, 1995) because it is readily absorbed by important biomolecules (nucleic acids, proteins and lipids) that are essential for genetic, biochemical and physiological functions within cells (Melis *et al.*, 1992; Frohnmeyer and Staiger, 2003). For non-photosynthetic organisms, DNA absorbs ~50% of the incident UV-B radiation and is the primary target of photodamage. However, in oxygenic photosynthetic organisms, such as plants, algae, and cyanobacteria, chlorophyll and other pigments may contribute significantly to shield DNA from ultraviolet radiation (He and Häder, 2002). It has been calculated that the light-harvesting proteins (phycobiliproteins and chlorophyll proteins) account for >99% of the UV-B absorption (Lao and Glazer, 1996). The negative effects of UV radiation were shown to impair various physiological and biochemical processes including growth, photosynthesis (Kumar *et al*., 2003;Wu *et al*., 2005; Rinalducci *et al*., 2006; Gao and Ma, 2008), pigmentation (Cakirlar *et al*., 2008), nitrogen metabolism (Sinha *et al*., 1996). However, the various organism response of UV action spectra are limited by a given dose of radiation (Barta *et al*., 2004). Also, under biotic and abiotic stimuli including UV radiation, generation of reactive oxygen species (ROS) including superoxide (O_2^-), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), enhances and can overcome the enzymatic and nonenzymatic detoxification mechanisms, giving rise to oxidative stress (He and Häder, 2002; Obermüller *et al*., 2005) which react very rapidly with DNA, lipids and proteins causing cellular damage (Zacchini and Agazio, 2004). As well as, UV radiation increased the levels of lipid peroxidation in *Physcia semipinnata* (Unal *et*

al., 2008), *Capsicum annuum* (Mahdavian *et al*., 2008) and *Pisum sativum* (Agrawal and Mishra, 2009). Also, adaptation strategies of some cyanobacteria and eukaryotic algae try to avoid toxicity of UV radiation by evolving a variety mechanisms (Fedina *et al*., 2005) including production of UV-absorbing substances, such as scytonemin (Garcia-Pichel and Castenholz, 1991) and mycosporine-like amino acids (MAAs) (Obermüller *et al*., 2005; Zhang and Wu, 2007). To escape from UV radiation was a migration into habitats with reduced light exposure such as sinking and floating behaviour by a combination of gas vacuoles and ballast (Rajagopal *et al.*, 2005), a production of quenching agents such as carotenoids (Obermüller *et al*., 2005; Gao and Ma, 2008) a production of antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), and glutathione reductase (GR, EC 1.6.4.2) (Gao and Zhang, 2008). Furthermore, specific role of polyamines in preventing photooxidative damages is reported (Løvaas, 1997). The antioxidative effect of polyamines was due to a combination of their anion- and cation-binding properties involving a radical scavenging function (Bors *et al*., 1989), and a capability to inhibit both lipid peroxidation (Kramer *et al.,* 1991; Unal *et al*., 2008) and metal-catalysed oxidative reactions (Tadolini, 1988). Moreover, polyamine catabolism produces hydrogen peroxide that could enter the stress signal transduction chain promoting an activation of an antioxidative defence response (Agazio and Zacchini, 2001).

1.1.2 Salinity

One of the major environmental factors limiting the worldwide productivity and distribution of cereal crops is water stress resulting from drought and salinity (Lee *et al.,* 2001) which found major in arid and semi-arid regions of soil or water (Ashraf and Harris, 2004). High salinity causes both hyperionic and hyperosmotic effects and the consequence of these can be plant demise (Niu *et al*., 1995; Leshem *et al*., 2007). The deleterious effects of salinity are associated with the induction of water stress induced by the increase of osmotic potential, the increase of ions or other plant toxins in the soil, the increase of ions in the plant tissues (Howard and Mendelssohn, 1999) and a combination of these factors (Ashraf, 1994). All of these cause adverse pleiotropic effects on plant growth and development at physiological, biochemical and molecular levels (Munns, 2002; Mansour, 2000; Jantaro *et al*., 2003; Demetriou *et al*., 2007; He *et al*., 2008).

Salt stress can also trigger various interacting events including the reduction of initial growth, inhibition of cell division and expansion, acceleration of cells death (Yeo, 1998), the inhibition of enzyme activities in metabolic pathways and the decomposition of protein and membrane structures (Tsugane *et al*. 1999). Moreover, salt stress generated the active oxygen species (ROS) are thought to play an important role in inhibiting plant growth, photosynthetic pigments (Thompson, 1987; Lee *et al*., 2001; Mahdavian *et al*., 2008).

However, stress adaptation effectors are categorized as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport, and transducers of long-distance response coordination (Asada, 1999; Morgan and Drew, 1997; Niu *et al*., 1995; Leung and Giraudat, 1998). One response of cells against salt

stress, to changes in the external osmotic potential is the accumulation of metabolites that act as "compatible" solutes which do not inhibit normal metabolic reactions (Ford, 1984; Yancey *et al*., 1982). With protection of structures and osmotic balance supporting continued water influx (or reduced efflux) accepted functions of low molecular weight osmolytes such as glycine betaine (Incharoensakdi and Wutipraditkul, 1999), proline (He *et al*., 2008; Mahdavian *et al*., 2008), ectoine (Louis and Galinski, 1997) and plant growth regulator polyamines (Das *et al*., 1995; Bouchereau *et al*., 1999; Jantaro *et al*., 2003; Sanchez *et al*., 2005; Demetriou *et al*., 2007). Furthermore, many research found polyamines act as scavenging of reactive oxygen species (Drolet *et al*., 1986; Bors *et al*., 1989; Løvaas and Carlin, 1991; Li and Wang, 2004; He *et al*., 2008). However, polyamine accumulation is a non-specific response to salt stress (Ashraf and Harris, 2004).

1.2 Polyamine

1.2.1 Physiology

Polyamines (PAs) are small aliphatic polycations that are widely present in living organisms (Wang and Liu, 2009). The most common ones are the diamine putrescine (Put; 1,4-diaminobutane), triamine spermidine (Spd; 1,8-diamino-4-azaoctane) and tetraamine spermine (Spm; 1,12-diamino-4,9-diazododecane) shown in Figure 1.3.

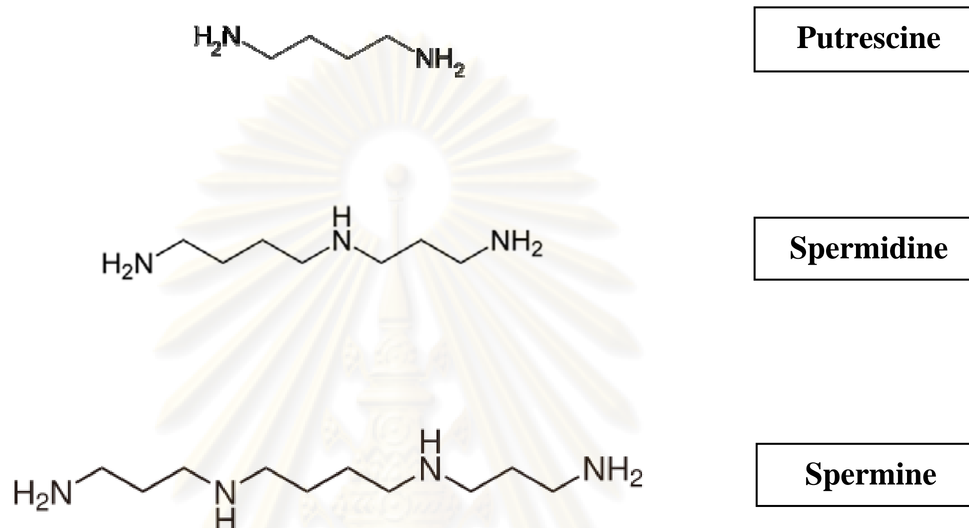

Figure 1.3 The most common polyamines are putrescine (Put; $NH_2(CH_2)_4NH_2$), spermidine (Spd; $NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine (Spm; NH_2 $(CH_2)_3$ NH $(CH_2)_4NH(CH_2)_3NH_2$) (Source: http://en.wikipedia.org/wiki).

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Many other di- and polyamines are usually present in plants and microorganisms, such as the diamines 1,3-diaminopropane and cadaverine (1,5-diaminopentane). Strikingly, unusual polyamines have also been found in bacteria, algae, fungi, animals and higher plants (Niitsu and Samejima, 1993). In the extreme thermophilic bacteria *Thermus thermophilus* at least 14 polyamines, among which some linear and branched pentamines, hexamines and heptamines, have been isolated (Table 1). Caldopentamine was accumulated in detectable content especially in bacterial cells grown at extremely high temperatures (80°C or more) (Oshima, 1989). These unusual polyamines, typical and abundant in thermophilic bacteria, can be found widespread. They were detected previously in some Leguminoseae, such as *Canavalia gladiata* and *Vicia radiata* (Matsuzaki *et al*., 1990**;** Hamana *et al*., 1991), leading to hypothesize a putative role of these molecules in growth and differentiation processes (Bagni and Tassoni, 2001). Moreover, many polyamine analogs were discovered in the algae (Hamana and Matsuzaki, 1982; Maiss *et al*., 1982).

Polyamines, low molecular weight compounds, are positively charged at physiological pH and ubiquitous in nature. By their the positive charge, polyamines are known to bind to negatively charged molecules, e.g. nucleic acids, acidic phospholipids and various types of proteins (Cohen, 1998). In higher plants, polyamines were occurred dominantly in free form, bound electrostatically to negatively charged molecules, and conjugated to small molecules and proteins (Martin-Tanguy, 1997). Polyamines are required for normal development of prokaryotes and eukaryotes (Tabor and Tabor, 1984). For biotic stresses, polyamine levels were changed in plant responding to pathogen infection (Walters, 2000). Moreover, many reports suggest that polyamines play a critical role in a range of

Table 1.1 Common and uncommon natural occurring aliphatic polyamines*

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

*Bagni and Tassoni, 2001

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developmental processes including root growth, somatic embryogenesis, floral initiation, and the development of flowers and fruits (Evans and Malmberg, 1989; Takahashi *et al*., 2003; Ziosi *et al*., 2006). These compounds were related to plant growth regulator or hormonal second messengers, avoiding senescence (Galston, 1983) and stabilization of nucleic acids and membranes (Thomas and Thomas, 2001). Polyamines have also been implicated in plant responses to abiotic stress such as potassium deficiency, osmotic shock, drought, salt stress (Watson and Malmberg, 1996; Evans and Malmberg, 1989; Tassoni *et al*., 2008). Moreover, enhanced UV-B radiation may cause the accumulation of polyamines in plants (Kramer and Mirecke, 1992; An *et al*., 2004). It was also revealed that polyamines might play an important role in the protection mechanisms of plants during exposure to UV-B radiation in cucumber (Kramer *et al.,* 1991; An *et al*., 2004) which increase under enhanced UV-B radiation (Predieri *et al*., 1993). On the other hand, the reduction of free polyamines was found in *Phaseolus vulgaris* under UV-B radiation (Smith *et al*., 2001). Thus, polyamines might act as scavengers of active oxygen species and stabilize membranes under different environmental stress conditions (Bouchereau *et al*., 1999) and UV-A radiation (Unal *et al*., 2008). The amount of the various polyamines, was reported that, depends on environmental and stress conditions. This variation in the amount of polyamines under different conditions has suggested an adaptive and protective role for these compounds (Smith, 1985). Furthermore, the control of polyamine levels is regulated in a very fast, sensitive and precise manner. This control can be achieved at four different strategies; *de novo* biosynthesis, degradation (oxidative deamination), conjugation and transport (Bouchereau *et al*.,

1999; Urdiales *et al*., 2001). However, the polyamine contents under combination stresses of UV and salt or sorbitol have never been reported.

1.2.2 Polyamine biosynthesis

In higher plants and bacteria, the first step in polyamine biosynthesis (Figure 1.4) is the formation of putrescine. Putrescine is synthesized directly from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17) and indirectly from arginine by arginine decarboxylase (ADC; EC 4.1.1.19) via two important intermediates are involved; agmatine and N-carbamoylputrescine, respectively, which is subsequently converted to putrescine. In mammalian cells and fungi, only the ODC reaction leads to putrescine formation (Walters, 2000). According to *Arabidopsis* has not only no ODC activity but also no ODC gene (Hanfrey *et al*., 2001). Moreover, the two different genes coding for ADC (*adc1* and *adc2*) in *Arabidopsis thaliana* have been identified under potassium deficiency stress. The several reports showed that *adc*2 is induced upon osmotic stress (Soyka and Heyer, 1999) and salt stress (Bagni *et al*., 2006). In animals and plants have been reported that ODC is located in both the cytoplasm and nucleus (Voigt *et al*., 2000). As the ADC protein which widely appears in plants is localized in chloroplasts associated with the thylakoid membrane of oat (Borrell *et al*., 1995) that shown in Figure 1.5.

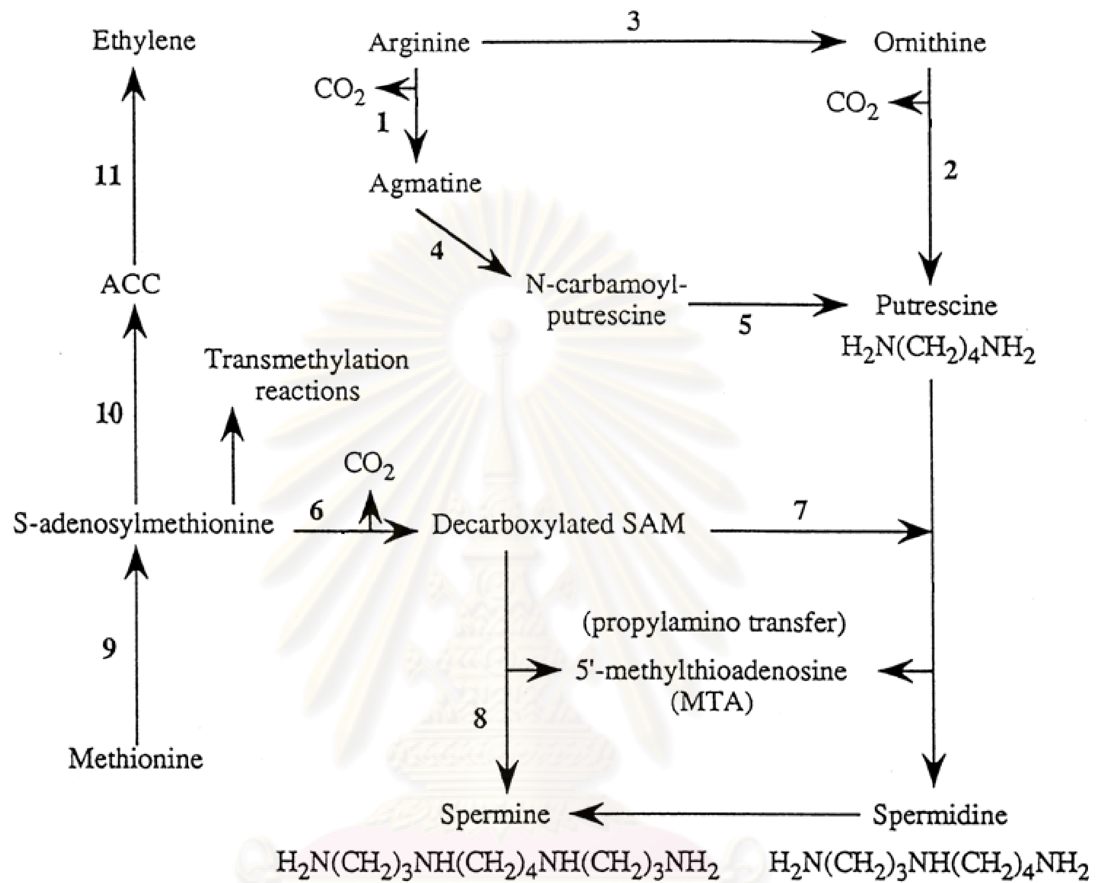

Figure 1.4 Pathways of biosynthesis of the major plant polyamines (Putrescine, Spermidine and Spermine), relationships with ethylene biosynthesis. 1, Arginine decarboxylase (ADC); 2, Ornithine decarboxylase (ODC); 3, Arginase; 4, Agmatine iminohydrolase; 5, *N*-carbamoyl putrescine amidohydrolase; 6, SAM decarboxylase (SAM DC); 7, Spermidine synthase; 8, Spermine synthase; 9, SAM synthase; 10, ACC synthase; 11, ACC oxydase. (modified from Bouchereau *et al*., 1999).

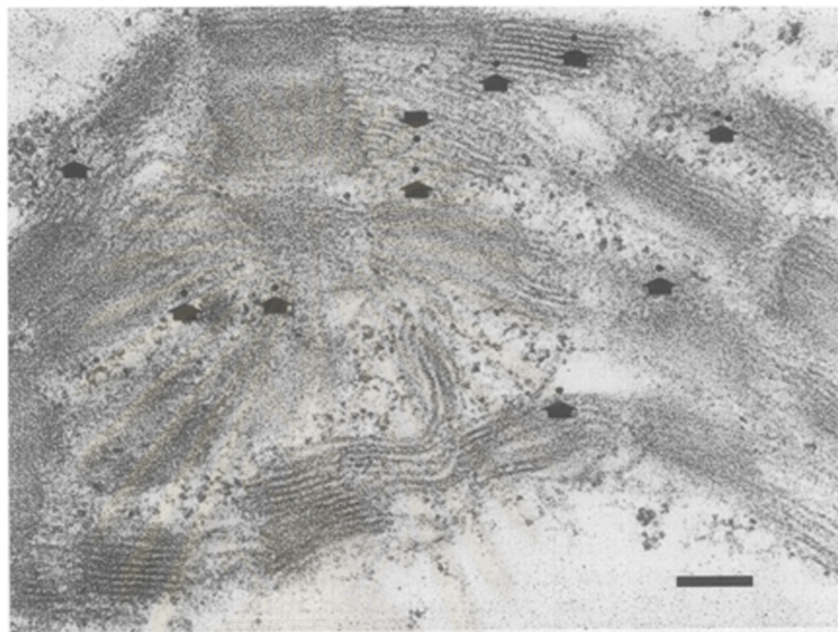

Figure 1.5 Electron micrograph of arginine decarboxylase protein localization. The osmotically stressed oat leaves were investigated by immunogold labelling. Gold particles localizing ADC antigen are indicated by arrows. Scale bar represents 200 nm. (Borrell *et al*., 1995)

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The polyamines spermidine and spermine are formed by the subsequent addition of an aminopropyl moiety to putrescine and spermidine, respectively. These reactions are catalysed by the aminopropyltransferase enzymes, spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively. The aminopropyl moiety is formed by the decarboxylation of S-adenosylmethionine (AdoMet or SAM) in a reaction catalysed by the enzyme AdoMet decarboxylase (AdoMetDC or SAMDC; EC 4.1.1.50) (Walters, 2003).

1.2.3 Polyamine degradation

The polyamines are oxidatively deaminated by the action of amine oxidases. These enzymes include the copper containing diamine oxidases (DAO; EC 1.4.3.6), which preferentially oxidize diamines, and the polyamine oxidases (PAO; EC 1.5.3.3.) which contain flavoprotein. Then, PAOs oxidize their substrates, spermidine and spermine (Cohen, 1998). The action of DAO on putrescine yields pyrroline, hydrogen peroxide and ammonia while PAO action on spermidine and spermine yields pyrroline and 1,5-diabicylcononane, respectively, as well as diaminopropane (DAP) and hydrogen peroxide (Figure 1.6). Diaminopropane can be metabolised to β-alanine, while pyrroline can be converted to γ-aminobutyric acid (GABA) by pyrroline dehydrogenase (PDH). GABA can then be transaminated and oxidized to form succinic acid, following by entering the Krebs cycle (Flores and Filner, 1985). Also, this pathway ensures that the carbon and nitrogen resulting from putrescine is recycled (Walters, 2000). For degradation of spermidine by PAO yields Δ1-pyrroline and 1,3-diaminopropane, while spermine oxidation yields 1,3-aminopropylpyrroline, along with diaminopropane and hydrogen peroxide (Bagni and Tassoni, 2001).

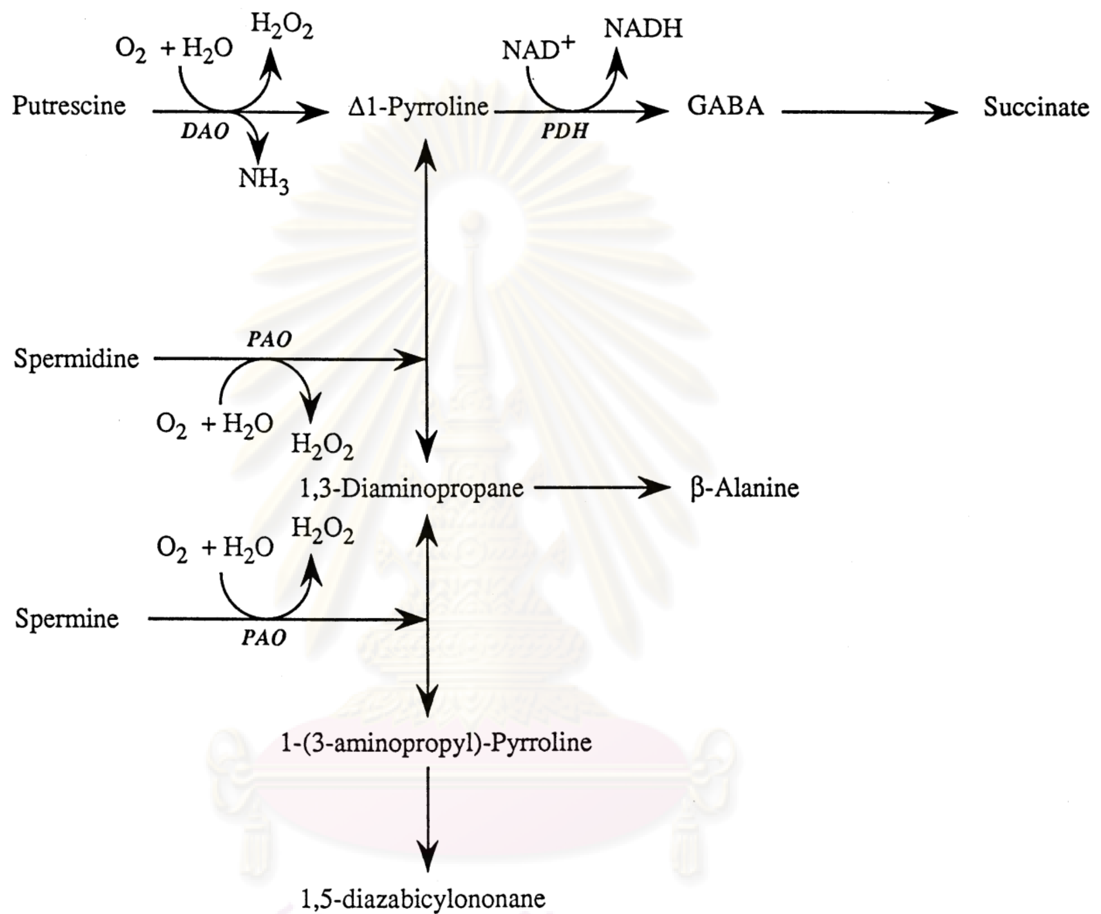

Figure 1.6. Schematic representation of polyamine degradation. Amine oxidases. DAO, diamine oxidase; GABA, 7-aminobutyric acid; PAO, polyamine oxidase; PDH, pyrroline dehydrogenase. (Bouchereau *et al*., 1999).

1.2.4 Polyamine conjugation

In nature, polyamines often occur as free molecular bases, but they can also be associated with small molecules like phenolic acids (conjugated forms) and also to various macromolecules like proteins (bound forms). The most common amine conjugated, i.e. polyamines and aromatic amines conjugates, covalently linked to hydroxycinnamic acids have also been shown to occur at high levels in plants and are thought to be correlated with developmental phenomena (Martin-Tanguy, 1985).

This conjugation occurs as water-soluble or water-insoluble forms. In the former, the single amine group of an aliphatic amine is linked with a phenolic cinnamic acid. The water-insoluble forms can be divided into two classes. In the first, each terminal amine group of an aliphatic amine is bound to cinnanic acid, while in the second class the amine group of aromatic amine is linked to cinnamic acid. These amine conjugates are found in roots but do not normally in shoots. In the roots of tobacco, increases in water-soluble and water-insoluble conjugates have been shown before flowering (Martin-Tanguy *et al*., 1990) and occur in shoot apices upon floral initiation (Havelange *et al*., 1996). The synthesis of polyamine conjugates has been elucidated in tobacco callus (Negrel, 1989). They are conjugated by the formation of an amide linkage, using esters of Co-A for provision of the activated carboxyl groups such as cinnamoylputrescine (Martin-Tanguy *et al*., 1985), which are formed by non-specific putrescine caffeoyl-CoA transferase. Therefore, the synthesis of the water-insoluble polyamine such as di-*p*-coumaroylputrescine, di-*p*-coumaroylspermidine, di-feruloylputrescine and di-feruloylspermidine has not been elucidated (Martin-Tanguy, 1997). Furthermore, posttranslational covalent linkage of

polyamines to protein is catalyzed by a class of enzymes known as transglutaminases (EC; 2.3.2.13) (Margosiak *et al*., 1990).

1.2.5 Polyamine transport

The transport of polyamines across the plasmalema of plant cells is energy-dependent and it is now clear that calcium is involved in the uptake mechanism. Indeed, calcium-activated putrescine uptake can be markedly reduced by treatments which reduce calmodulin action or the activities of protein kinases or phosphatase (Antognoni *et al*., 1995). Polyamine-specific carriers are widely distributed in prokaryotes and eukaryotes and can replenish polyamine pools upon inhibition of the biosynthetic enzymes (Seiler *et al*., 1996). The transport of polyamines classified as ABC (ATP binding cassette)-type transporters (Higgins, 1992) have been well characterized in bacteria, yeast, parasite, animal (Igarashi and Kashiwagi, 1999; Tassoni *et al*., 2002; Rinehart and Chen, 1984) and cyanobacterium *Synechocystis* sp. PCC 6803 (Raksajit *et al*., 2006). For the putrescine and spermidine transport into *Synechocystis* cells found that pH dependent with highest activity at pH 7.0 and 8.0, respectively. Moreover, the transport of putrescine and spermidine are energy-dependent (Raksajit *et al*., 2006; 2009).

1.3 *Synechocystis* sp. PCC 6803

Living organisms have the capability to adapt to sudden changes in their environment. Since most of these stimuli (temperature up- and downshift, UV-B light, etc.) have detrimental effects on cells, they had to develop adequate protective systems (Glatz *et al*., 1999). Among prokaryotes, cyanobacteria are the only organisms to engage in oxygenic photosynthesis, and there is evidence to suggest that they are the progenitor(s) of plant plastids. Their phylogenetic position in the bacterial kingdom is still obscure, although recent analysis of ancient genes has indicated a genetic relationship with Gram-positive bacteria (Xiong *et al*. 2000). Cyanobacteria can be classified as the Procaryota, Division of cyanophyta and Cyanophyseae class. The bacteria and cyanobacteria lack mitochondria, chloroplast, true vacuoles, endoplasmic reticular. There is no membrane bounded chloroplast; in cyanobacteria the photosynthetic lamellae are usually distributed in the peripheral cytoplasm. Cells of cyanobacteria are surrounded by the cytoplasmic membrane, the cell wall which contains an outer membrane and a peptidoglycan layer, and in many case a glycocalyx layer (Figure 1.7). The outer membrane functions more as a passive molecular sieve, whereas the cytoplasmic membrane serves as a true selective permeability barrier (Gantt, 1994).

Synechocystis sp. PCC 6803 is an unicellular, non-nitrogen (N_2)-fixing cyanobacterium and a ubiquitous inhabitant of fresh water. They divide by binary fission at two or three successive planes. Based on their GC contents, many cultured strains of *Synechocystis* can be classified into three groups; the marine group, the low GC group and the high GC group (Holt *et al*., 1998). Strain PCC 6803 belongs to the latter group, whose members, including PCC 6714, have been mostly isolated from

freshwater. They also have the propensity to utilize glucose (Rippka *et al*.,1979). A phylogenetic tree based on 16S rRNA sequences suggests that high GC content species are more closely related to *Microcystis aeruginosa*, which is a unicellular spherical cyanobacterium with gas vesicles, than to other *Synechocystis* groups (Honda *et al*., 1999). There are four culture substrains of *Synechocystis* ('PCC', 'ATCC', 'GT' (glucose-tolerant) and 'Kazusa'), all of which were derived from the Berkeley strain 6803, which was isolated from freshwater in California by R. Kunisawa (Stanier *et al*. 1971) shown in Figure 1.8.

The complete nucleotide sequence of *Synechocystis* sp. PCC 6803 was determined in 1996. This was the first photoautotrophic organism to be fully sequenced. Sequencing was carried out using a clone-by-clone strategy based on the physical map of the genome, resulting in a highly accurate sequence. The circular genome was originally deduced to be 3,573,470 bp long (Figure 1.9). The average GC content is 47.7% (Kaneko *et al*. 1996). Consequently, *Synechocystis* has been one of the most popular organisms for genetic and physiological studies of photosynthesis for two major reasons; it is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982) and grows heterotrophically at the expense of glucose (Rippka *et al.,* 1979). Moreover, *Synechocystis* cells has several features that make this strain particularly suitable for studying stress response at the molecular level (Glatz *et al*., 1999). There is unequivocal evidence that in higher plant cells exposed to heat stress, the photosynthetic apparatus is irreversibly damaged prior to impairment of other cellular functions (Berry and Bjorkman, 1980). The general assembly of photosynthetic membranes in cyanobacteria is similar to that of higher plant, therefore

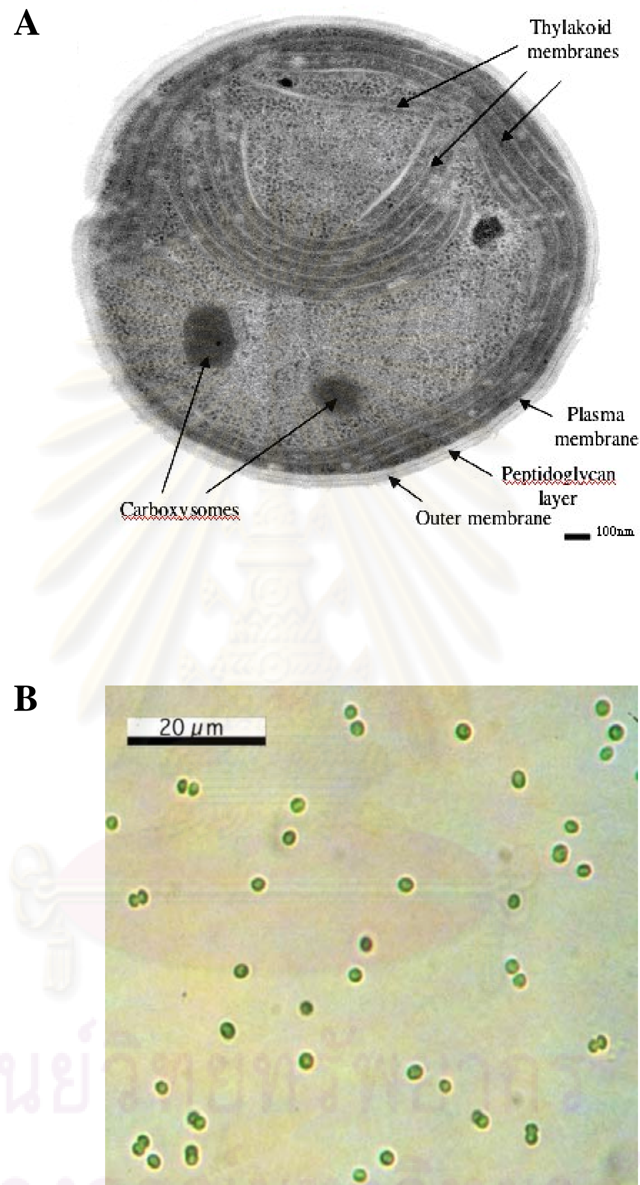

Figure 1.7 Ultrastructure of a cyanobacterial cell. [Schematic representation of a thin section of a cyanobacteria cell (A) and *Synechocystis* cells (B)].

(Source: http://www.danforthcenter.org/imf/pakrasi/Image2.jpg, **Error! Hyperlink reference not valid.**, respectively).

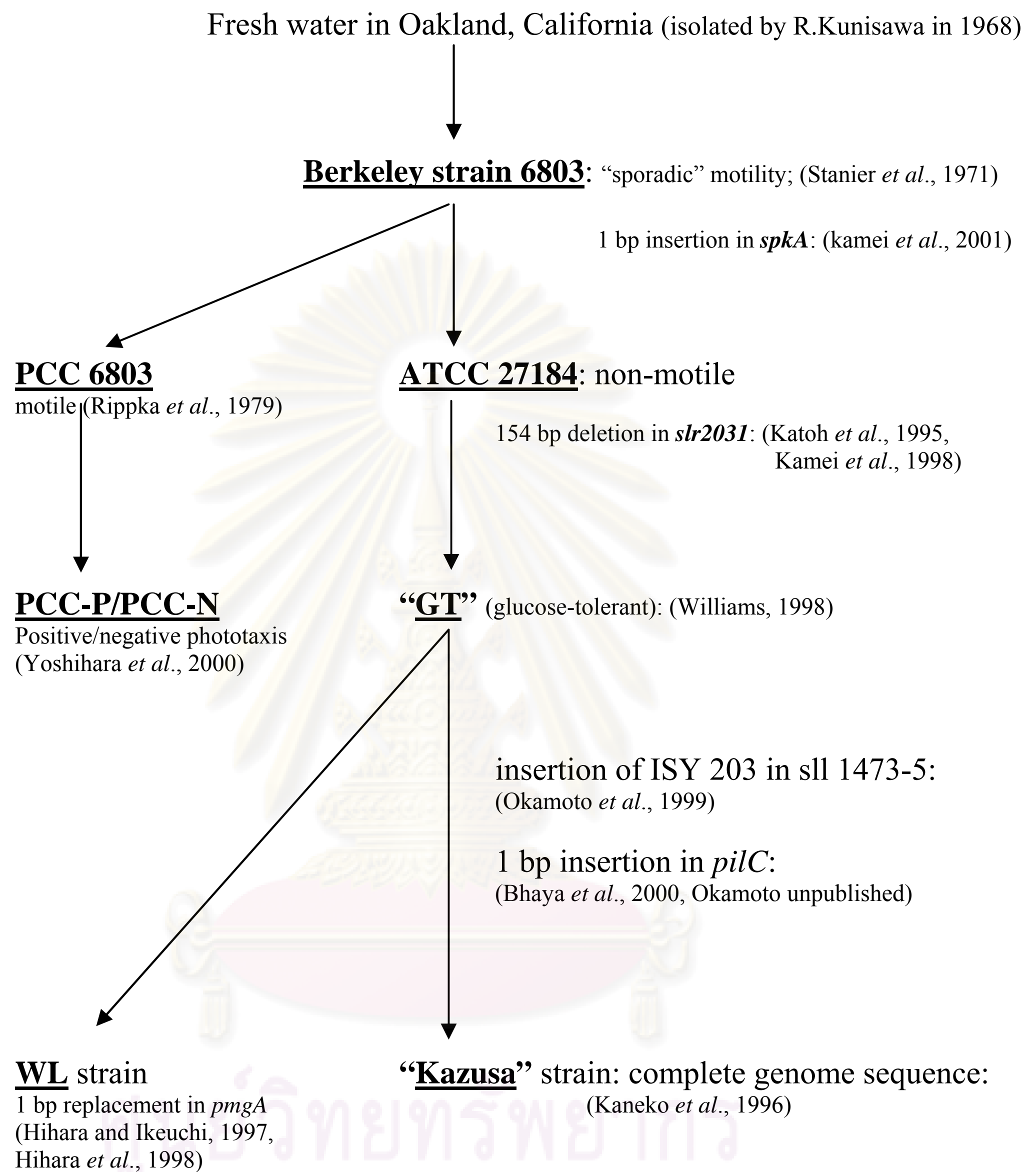

Figure 1.8 Strain history of *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001).

Synechocystis 6803 might serve as a powerful model for studying the molecular mechanisms of stress response and long-term adaptation (Lehel *et al*., 1993; Jantaro *et al*., 2003).

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

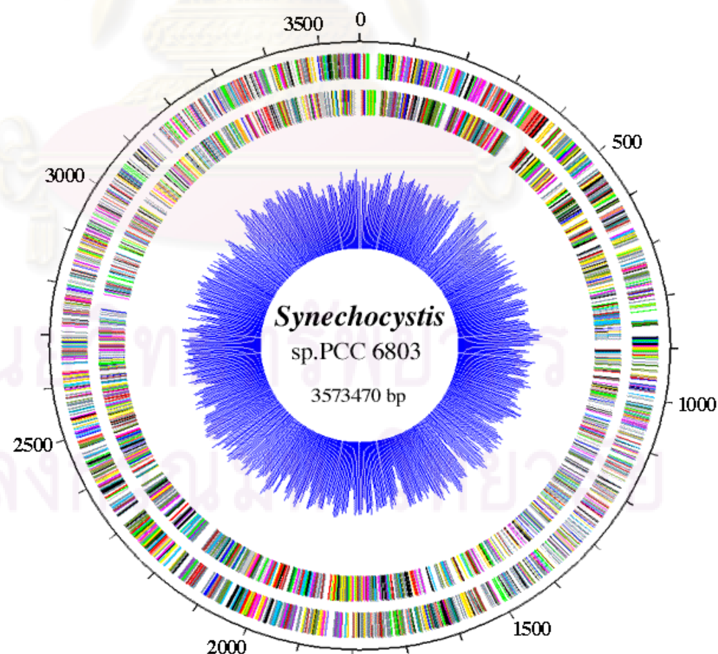

Figure 1.9 The cellular genome of *Synechocystis* sp. PCC 6803 according to Cyanobase (Source: www.kazusa.or.jp/cyano/Synechocystis).

1.4 OBJECTIVE OF THIS RESEARCH

1. To determine the effects of salinity and UV radiation on cell growth, intracellular pigments and polyamines content in *Synechocystis* sp. PCC 6803.
2. To study the transcription of arginine decarboxylase (*adc1* and *adc2*) genes and the translation of ADC protein under salinity and UV radiation in *Synechocystis* sp. PCC 6803.

CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Equipments

Balances	METTLER PJ360 DeltaRange® GWB, USA
Centrifuge	HERMLE Z233 MK, USA
C-18 column	4.6 x 150 mm inertsil®ODS-3 5 µm i.d.,
Electrophoresis Unit	BIO-RAD PROTEIN® II xi Cell, USA
Gel documentation	Syngene® Gel Documentation
HPLC	Hewlett Packard series 1050, Japan
Laminar flow	BVT-124 International Scientific Supply, Thailand
Light source unit	Prekeo S250 Zeiss IKON, Japan
PCR apparatus	PERKIN ELMER DNA Thermal Cycler, Japan
pH meter	ORION model 420A, USA
Power supply	BIO-RAD POWER PAC 1000, USA
Spectrophotometer	Jenway UV/VIS 6400, USA
Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.2 Chemicals

Acetic acid	Lab Scan, Poland
Acrylamide	Scharlau Chemie S.A., Spain

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Agarose	Invitrogen, USA
Ammonium ferric citrate	Ajax Finechem, Australia
Ammonium persulfate (APS)	Merck, Germany
Benzoyl chloride	Sigma, USA
Calcium chloride	Ajax Finechem, Australia
Citric acid	Ajax Finechem, Australia
Chloroform	Merck, Germany
Coomassie blue R-250	Sigma, USA
Diethyl ether	Lab Scan, Poland
Dimethylformamide	Lab Scan, Poland
Dipotassium phosphate	Ajax Finechem, Australia
Dithiothreitol (DTT)	Sigma, USA
EDTA	Merck, Germany
Ethanol	Merck, Germany
Ethidium bromide	Sigma, USA
Glycerol	Ajax Finechem, Australia
Glycine	Ajax Finechem, Australia
Hexanediamine	Sigma, USA
HEPES	USB Corporation, USA
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate	Ajax Finechem, Australia
Mercaptoethanol	Sigma, USA

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Methanol	Lab Scan, Poland
Methylene-bis-acrylamide	Amersham Bioscience, Sweden
Perchloric acid	Merck, Germany
Pyridoxal-5-phosphate	Sigma, USA
Phenol	Merck, Germany
PMSF	Sigma, USA
Putrescine	Sigma, USA
Sodium acetate	Ajax Finechem, Australia
Sodium bicarbonate	Ajax Finechem, Australia
Sodium chloride	Ajax Finechem, Australia
Sodium dithiosulfate	Ajax Finechem, Australia
Sodium dodecyl sulfate	Ajax Finechem, Australia
Sodium hydroxide	Ajax Finechem, Australia
Sodium nitrate	Ajax Finechem, Australia
Sodium phosphate	Ajax Finechem, Australia
Sodium thiosulfate	Ajax Finechem, Australia
Sorbitol	Ajax Finechem, Australia
Spermidine	Sigma, USA
Spermine	Fluka, USA
Sucrose	Ajax Finechem, Australia
TEMED	BIO-RAD, USA
TES	Sigma, USA
Tris (hydroxymethyl)-aminomethane	USB Corporation, USA
Triton X-100	Packard, USA

Tween-20 BIO-RAD, USA

Urea Ajax Finechem, Australia

2.3 Kits and supplies

Nylon membrane filter 0.45 µm, Sartorius, Germany

Immobilon-P membrane Millipore Coorperation, USA

1 kb DNA Ladder Invitrogen, USA

PCR amplification kit Invitrogen, USA

SuperScript^{TM} III First-Strand Synthesis System Invitrogen, USA

Prestained Protein Marker Fermentas, Canada

RNase-Free DNase Invitrogen, USA

2.4 Primers

Table 2.1 Sequences of the primers for RT-PCR

Target gene	Name	Primers	Length in pairs
16s rRNA	forward-*16s*	5'-AGTTCTGACGGTACCTGATGA-3'	521
	reverse-*16s*	5'-GTCAAGCCTTGGTAAGGTTCT-3'	
adc1	forward-*adc1*	5'-ATATTACCTGCGACAGTGATGG-3'	315
	reverse-*adc1*	5'-GATCAAGGCTAACTCCGTATGAC-3'	
adc2	forward-*adc2*	5'-ATATTACCTGCGACAGTGATGG-3'	457
	reverse-*adc2*	5'-TTAGCTGGTGTGGATGCCT-3'	

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2.5 Organism

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

Methods

2.6 Culture conditions

Axenic cells of *Synechocystis* sp. PCC 6803 were grown in BG-11 medium (Appendix A) with continuously bubbling by filtered air, under normal growth light (40-50 μmol photon/m^2 s^{-1}) at 32°C. Cell growth of the culture was measured for the optical density at 730 nm with Spectronic spectrometer. For ionic stress conditions, NaCl was added in various concentrations of 25, 125, 350 and 650 mM in BG-11 media, respectively, whereas BG-11 media containing 50, 250 and 500 mM sorbitol, respectively, were used as osmotic stress conditions. The culture with mid-logarithmic stage was diluted to the optical density at 730 nm of 0.5 and exposed under UV radiations; UV-A (365 nm; 11-13 w/m^2), UV-B (302 nm; 1.2-1.3 w/m^2) and UV-C (254 nm; 1.0-1.3 w/m^2), respectively. The UV-stressed cells were harvested for further analysis. The radiation intensity was measured by a UVX radiometer (UVP, Inc., Upland, CA).

2.7 Determination of intracellular pigments

The UV-stressed cells were collected in order to determine for the intracellular pigments at intervals of 0, 30, 60, 90, 120, 150 and 180 min. One ml of

cell culture was extracted by *N,N*-dimethylformamide with vortexing vigorously. After centrifugation at 8,000 rpm for 5 min to remove debris, the optical density of supernatant was measured at 461, 625 and 664 nm, respectively. The contents of chlorophyll a and carotenoids were calculated according to following equations (Jantaro, *et al*., 2006) :

chlorophyll a content (μg/cell)

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

carotenoid content (μg/cell)

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

total cells* (cell/ml)

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

2.8 Polyamine biosynthesis analysis

2.8.1 Extraction and determination of polyamines

Synechocystis cells under UV radiation at 1 and 3 hours were harvested and extracted by 5% cold $HClO_4$. After the extraction by 5% perchloric acid for 1 hour on an ice bath, the samples were centrifuged at 8,000 rpm for 10 min. The supernatant and pellet fractions (represented as free and bound forms of polyamines, respectively) were derivatized by benzoylation reaction. The derivatized-polyamines were then analyzed by high performance liquid chromatography (HPLC) (Flores and Galston, 1982), using 1,6-hexanediamine as an internal standard. One ml of 2 M NaOH was added into 500 μl of 5% $HClO_4$ extract firstly following by mixing with 10 μl of benzoyl chloride. The mixture was vigorously vortexed and incubated for 20 min at room temperature. To stop the reaction, 2 ml of saturated NaCl was added.

The benzoyl-polyamines were then separated by solvent fractionation with 2 ml of cold diethyl ether. Two ml of the ether phase with benzoyl-polyamines was taken to a new tube and evaporated to dryness, followed by redissolving in 1 ml of methanol. Samples were filtered through a 0.45µm cellulose acetate membrane filter. Authentic polyamine standards were prepared similarly as the stressed cells. Derivatized polyamines were analyzed by high performance liquid chromatography (HPLC) with inertsil®ODS-3 C-18 reverse phase column (5 µm; 4.6 x 150 mm) using UV-Vis detector at 254 nm. The mobile phase was a gradient of 60 – 100% methanol : water. The flow rate was 0.5 ml/min. Chromatogram and standard curve of polyamines shown in the Appendix B

2.8.2 Extraction of total RNA

One hundred ml of *Synechocystis* cells grown at the mid-logarithmic phase were harvested by centrifugation at 5000 rpm, 4°C for 15 min. The pellet was immediately frozen in liquid nitrogen. The total RNA was extracted by the hot phenol method (Mohamed and Jansson, 1989). Cells were resuspended in 1 ml resuspension buffer (Appendix C) on ice.and centrifugated at 12,000 rpm, 4°C for 5 min. Pellet was resuspened in 250 µl resuspension buffer and added 75 µl of 250 mM EDTA, pH 8.0 before incubating on ice for 5 min. The 375 µl lysis buffer (Appendix C) was added and incubated at 65°C for 3 min. After that, hot phenol (65°C) was added into the reaction mixture and incubated at 65°C for 3 min. Then, the mixture was cooling down on a freeze aluminium block for 1 min. Centrifugation at 12,000 rpm for 5 min was done at room temperature. Repeatedly, the upper phase was reextracted with the hot phenol. Subsequently, the mixture was extracted once with

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an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The extracted mixture was gentle mixed and centrifuged at 12,000 rpm for 5 min at room temperature. After that, the RNA was precipitated by adding 1/10 volume of 3 M sodium acetate buffer, pH 6.0 and 2.5 volume of cold ethanol (-20°C). After incubation at -20°C for 30 min, the tube was centrifuged at 12,000 rpm (4°C) for 10 min. The resulting pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. Then, the dried pellet was resuspened in 50 µl of RNA storage buffer (Appendex C). The total RNA was kept at freezer (-80°C) until used. To determine concentration and purity of RNA, sample was diluted with RNA storage buffer and checked by measuring the optical density at 260 nm and ran 0.8% agarose gel in 1x TAE buffer (Appendex E).

2.8.3 Preparation of total RNA

Firstly, RNA samples from 2.9.2 were treated with RNase-free DNase. The reaction mixture was contained 50 µg of RNA, 5 µl of 10x buffer, 5 µl of RNase-free DNase and adjusted the final volume to 50 µl with Milli-Q water. After that, the samples were added 5 µl more of RNase-free DNase and incubated at 37°C for 2 hours. Then, the mixture was added 200 µl of 40 mM Tris-HCl, pH 8.0 and extracted once with 250 µl of phenol/chloroform/isoamyl alcohol (25:24:1) mixture. After centrifugation at 12,000 rpm (room temperature) for 5 min, the upper phase was added 1/10 volume of 3 M sodium acetate buffer, pH 5.2 and 0.6 volume of isopropanol and incubated at -20°C for 30 min. The extract was then centrifuged at 12,000 rpm, 4°C for 10 min to separate supernatant and pellet. The resulting pellet was washed with 70% ethanol and mixed gently by inversion and centrifuged at

12,000 rpm, 4°C for 2 min. Finally, the dried pellet was resuspened with 25 µl of RNA-storage buffer. The solution was checked for RNA concentration by measuring at 260 nm and ran 0.8% agarose gel in 1x TAE buffer.

2.8.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA of *Synechocystis* cells obtained from 2.8.3 was used as template to generate cDNA by SuperScript™ III First-Strand Synthesis System. Each reaction was added 5 µg of total RNA which treated with RNase-free DNase, 0.8 µl of 25 µM of 3'-primer, namely, *adc1*, *adc2* and 16s rRNA genes which designed from the Cyanobase sequence (Figure 2.1) and dissolved with Milli-Q water to 10 µl. After incubation the reaction at 65°C for 5 min, the 10 µl of cDNA Synthesis Mix (containing 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ and 1µl of SuperScript™ III Reverse transcriptase) was added to the mixture and incubated the tube at 50°C for 50 min. Terminate the reactions of cDNA synthesis at 85°C for 5 min. The one microlitter of RNaseH was added and incubated again at 37°C for 20 min to remove RNA. The cDNA synthesis reaction can be used for PCR amplification. PCRs were performed, the initial denaturation at 95°C for 5 min was done, followed by 29 cycles of denaturation at 95°C for 1 min, annealing step of *adc1*, *adc2*, *16s* rRNA genes were performed at 50.7°C, 64°C and 56°C, respectively, for 1 min and extention at 72°C for 1 min, followed by final extention at 72°C for 5 min. The PCR products were analyzed by electrophoresis of 0.8% agarose gel in 1x TAE buffer. Quantification was carried out using Syngene® Gel Documentation.

```
adc1            ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
adc2            ATGGA AGGGC AGTCA ATCGA ACTAG AACTA AGTGT CATGG CAATG CCGGA GTTAA TCGAC 60

adc1            -ATGG GGGAA GAACC TGTGC CGGCG GATAA AGCAT TAGGC AAGAA ATTCA AGAAA A---- 55
adc2            AGTAC TGAAG CAGGC CATAC CGCCG GGGTG AAAAC TGATT CCAAT CCCCA GGCGA TCGCC 120
                  *    * *   *  *   * * ** ** *     *  *  *              *      **  *  *

adc1            -AAAA TGCCT CCTGG AGCAT TGAAG AAAGC GAAGC TCTGT ACCGG GTTGA GGCCT GGGGG 114
adc2            CAAGA CCGTC GTTGG ACCAT TGACG ACAGC GAAAA CCTCT ACCGC ATCAC TGGTT GGGGG 180
                 ** *         *** * *** *** * * *** ***    ** * ****   *     *  * *****

adc1            GCACC TTATT TTGCC ATTAA TGCCG CTGGT AACAT AACCG TCTCT CCCAA CGGCG ATCGG 174
adc2            GAACC TTACT TTTCC ATTAA TGCGG CCGGC CATGT GACCG TTTCT CCCCA GGCTG ACCAT 240
                * *** *** * ** ** ***** *** * * **   *  *  **** * *** *** *  *  * * *

adc1            GGCGG TTCGT TAGAT TTGTT GGAAC TGGTG GAAGC CCTGC GGCAA AGAAA GCTCG GCTTA 234
adc2            GGGGG AGCGT TGGAT TTGTA CGAAC TGGTT AAGGG TTTGA GGCAA AGAAA TATTG GCTTG 300
                ** **   *** * *** ****   **** ****   * *    **  ***** *****   * * ****

adc1            CCCCT ATTAA TTCGT TTTTC CGATA TTTTG GCCGA TCGCC TAGAG CGATT GAATA GTTGT 294
adc2            CCTTT ACTGT TGCGC TTTTC TGATA TTCTG GCTGA CCGCA TCAAT CGCCT CAATG CGGCC 360
                **  * * *   * **  *****  **** ** ** ** **  ***  *  *  **  *  ***

adc1            TTTGC CAAGG CGATC GCCCG TTACA ATTAC CCCAA CACCT ATCAG GCGGT TTATC CGGTC 354
adc2            TTTGC CCGGG GCATT GCCCG TTACC GCTAT CCCAA CACCT ACCGG GGGGT TTATC CCATT 420
                ***** *  **   **  ***** ****    **  ***** ***** * * * * *** ***** *  *

adc1            AAATG TAACC AGCAA CGACA TCTGG TGGAA GCCCT GGTTC GCTTT GGGCA AACTT CCCAG 414
adc2            AAGTG CAACC AGCAT CGCCA CATTG TGGAA TCCCT GGTGC GCTAC GGCAC TCCCT ATAAT 480
                ** **  **** **** ** **   * * *****  **** *** * ***   **       * *    *

adc1            TGTGG ATTGG AGGCA GGTTC CAAAC CGGAA TTGAT GATTG CCCTC GCAAC TCTAC CACCT 474
adc2            TTTGG CTTGG AAGCC GGTTC TAAAC CGGAG TTGAT GATTG CCCTG GCTAT GCTCC AACCC 540
                * ***  **** * **  *****  **** ****  ***** ***** ****  ** *   ** *  ***

adc1            CCCTT AGACC ---GT CAGGA CAAGC ATACC AAGCC CCTAA TCATT TGTAA TGGCT ACAAA 531
adc2            CAGGA GAACC CAGAG CCGGA TCAAC AAAAT CAGCC TTTAC TAATT TGTAA TGGTT ATAAA 600
                *       ***       * ***   * * * *    ****   **  * *** ***** *** * * ***

adc1            GACCA GGATT ATCTA GAAAC AGCTC TGTTA GCCAA ACGCT TAGGC CATCG TCCCA TCATC 591
adc2            GACCG GGAAT ATATT GAAAC CGCCT TGCTA GCCCG TCGTC TGGGG CATCG GCCGA TTATT 660
                ****  *** * ** *  *****  **   ** ** ***    **   * **  *****  ** * * **

adc1            ATCAT TGAAC AACTA CGGGA ACTGG AATGG GTACT ACACA TCTCT CAGCA GT-TA AACAT 650
adc2            GTGGT GGAGC AGGTA GCGGA GGTGG CCTTG GCCAT CGAAA TTTC- CAGCA ATCTG GGCAT 719
                 *  *  ** * *  **   ***   ***   * * *   *   * * * **  *****  * *    ***

adc1            TAAAC CCATG TTGGG GGTAC GGGCC CGGTT AAGTT GTCAG TCGCT CAAAT CCTCG GAAAT 710
adc2            TAAGC CAATT TTGGG GGTAC GGGCC AAACT GAGTA CCCAG GGCAT GGGCC GTTGG GGCAT 779
                *** * * **  ***** ***** *****     *  ***    ***     *         * * *  **

adc1            TTCCT CTGGC AACGG CGATC GGGCT AAGCT TGGTC TGACC ATGCC GGACA TTGTG ACCGT 770
adc2            TTCCA CTGGC ----- -GATC GGGCT AAATT TGGTT TAACC ATCCC GGAAA TGTTG ACGGC 833
                ****  *****        **** ***** **  * ****  * *** ** ** *** * *  ** ** *

adc1            AATTC ATCGT CTAGA GGAAA ATAAT TGTCT CGATT GCCTG AAAAT GCTTC ATTTT CATCT 830
adc2            GATCG AGCAA CTGCG CCGAG CTGAT ATGTT GGACA GTCTG CAATT GCTCC ATTTT CACAT 893
                 **   * *   **        *   * **     *  **   * ***  ** * *** * ***** **  *

adc1            GGGAA CGCAA GTTTC GGACA TTGCT CTGAT TAAAG AAGCT ATGCG GGAAG CCAGC CAACT 890
adc2            CGGTT CCCAG ATCTC TTCCA TCTCT GTGAT CAAAG AAGCG ATGAC GGAAG CCAGC CAAAT 953
                 **   * **   * **    ** *  **  ****  **** ****  ***   ***** ***** *** *

adc1            CTATG TGGAA TTGGT CAAGC TGGGG GCAAA AATGC GCTAC CTCAA TGTGG GGGGC GGTTT 950
adc2            TTTTG TCCAG TTGGC CAAGC TGGGG GCCAA TATGC GCTAC CTGGA TGTGG GGGGC GGACT 1013
                 * ** *  *  ****  ***** ***** ** **  **** ***** **  * ***** ***** **  *

adc1            GGCGG TGGAT TATGA CGGTT CCAAG ACCAA CTATC CCGCC TCGAA AAATT ACAAC ATGCA 1010
adc2            AGGGG TGGAC TACGA CGGTT CCAAA ACCAA TTTTT ACGCT TCCAA AAATT ACAAC ATCCA 1073
                 * ** ****  ** ** ***** ****  *****  * *   ***  ** ** ***** ***** ** **

adc1            AAACT ACGCC AACGA CATTG TGGCG GCCAT TCAAG ATGCC TGTGA GCTTG GGCAG GTTTC 1070
adc2            GAATT ACGTT AATGA TGTGA TTTCG GCAGT GCAGG ATGCC TGTGT GGCCG CTGAA GTGCC 1133
                 ** * ***   ** **   *    *  ** **  *  ** * ***** ****  *   *     *  **  *
```

```
adc1            CCCTC CCATT CTTGT GAGCG AAAGT GGGCG GGCAA TTATG GCCCA TCAGT CGGTG CTAGT 1130
adc2            CTGTC CTGTG CTGAT TAGTG AAAGC GGCCG GGCGA TCGCC AGCCA TCAGT CAGTA CTCAT 1193
                *  ** *  *  **  *  ** * ****  ** ** *** * *       *** ***** * **  **  *

adc1            TTTTG ATGTG CTAGG CAGCA ACCAA ACAGG CTTCA GTGAA CCCCA TCCCC CCGAT GAAAA 1190
adc2            TTTTG ATGTT GTGGC CACCA ATGAC ATTAA TCCCC CCTTG CCTAA GGTGA AGGGC AAAGA 1253
                ***** ****   * *  ** ** *  *  *        *          **  *         *     ** *

adc1            TGCCC ATCCC CTGCT AAAAA ATCTC TGGGA ATGTT ACGAA ACAAT TACAG CGGAA CAATA 1250
adc2            ---CC ATGCC ATTTT GCGTA ATTTG ATGGA AACCT GGGAA ACCAT TACGG TGGAT AATTA 1310
                   ** ** **  *  *     * ** *     *** *   *   *** ** ** *** *  ***    * **

adc1            CCAGG AGCAA TACCA TGATG CTCTG CAATT AAAGA CGGAG GCTAG TAGTC TTTTT AACTT 1310
adc2            CCAAG AGGCG TACCA TGATG TGGAA CAGTT TAAAA CTGAA GCCAT TAGTT TATTT AACTT 1370
                *** * **    ***** *****       ** **  ** * * **  ** *  ****  * *** *****

adc1            CGGCT ATTTA AGTCT GACGG AACGG GGACA AGCAG AGCAA ATTCA CTGGG CTTGT TGTCG 1370
adc2            TGGTT ATTTA GGTCT GAAAG AAAGG GCTAA AGCAG AGGAG CTTTA TTGGG CTTGT TGCCG 1430
                 ** * *****  **** **  * ** ** *   * ***** ** *   ** *  **** ***** ** **

adc1            TAAAA TTTTT GAAAT CACCA GACAA CTAGA GTATA TTCCC GAAGA TTTTC AAGCG CTGGA 1430
adc2            CAAGA TTTTA CAAAT TTGCC GTCAG CAAGA ATACG TCCCC GATGA TTTGG AAAAT TTGGA 1490
                 ** * ****   ****    *  * **  * ***  **   * *** ** ** ***   **      ****

adc1            TAAAA TAATG ACCGA TATTT ATTAC GTTAA CTTAT CGGTT TTCCA GTCAG CACCG GAATC 1490
adc2            AGTTA ACTTG GCTTC TATTT ACTAC GCCAA TATGT CGGTG TTTCA GTCGG CCCCG GATTC 1550
                    *    **  *    ***** * *** *  **   * * ****  ** ** *** * * *** ** **

adc1            CTGGT CTTTA GATCA ACTTT TTCCC ATTTT GCCCA TTCAC CATCT CAATG AGAAA CCTAG 1550
adc2            CTGGG CGATC GATCA ACTTT TCCCG ATTAT GCCCA TCCAC CGTTT GGATG AAGAA CCCAC 1610
                ****  *  *  ***** ***** * **  *** * ***** * *** * * *   *** *  ** ** *

adc1            TCAAA GGGTG ATTTT AGCCG ATTTA ACCTG TGACA GTGAT GGTAA AATTG ACCGT TTTAT 1610
adc2            CCAGC GGGGC ATTCT GGCGG ATATT ACCTG CGACA GTGAT GGCAA AATTG ACCAA TTTAT 1670
                 **   ***   *** *  ** * ** *  *****  **** ***** ** ** ***** ***   *****

adc1            TGACC TGTGG GATGT CAAGT CATAC CTAGA AGTTC ACCCC CTAGA AAATG -ACGG CAATC 1669
adc2            TGACC TGCGG GATGT CAAAT CAGTA TTGGA ATTGC ATCCT TTAAT AGAAG TGCAT CAGCC 1730
                ***** ** ** ***** *** * **     * ** * * * * **   **   * * *   *   **  *

adc1            ----- ----- ----- ----- ---CT TACTA TTTAG GTATG TTTTT AGTCG GTGCT TACCA 1706
adc2            AGGGA CTCCC CCCAG GGTGG AACCC TATTA TTTGG GCATG TTTTT GGTGG GGGCT TACCA 1790
                                           *  ** ** *** * * *** *****  ** * * *** *****

adc1            AGAAA TTATG GGCAA TTTAC ATAAT TTATT TGGTG ACATT AATGT AGTTC ACATT GCCAC 1766
adc2            AGAAA TTATG GGTAA TTTAC ATAAT TTATT TGGGG ACATC AATGT GGTGC ATATT CAGAT 1850
                ***** ***** ** ** ***** ***** ***** *** * ****  *****  ** * * ***    *

adc1            TACTC CCCAA GGTTA TCAGA TTGAA TCGGT GGTGC GGGGA GATAC CATGA CGGAG GTTTT 1826
adc2            GAATC CGAAA GGTTA CCAAA TTGAA CATTT AGTGA GGGGG GATAC CATCG CCGAA GTGTT 1910
                 * ** *  ** *****  ** * *****     *  ***  ****  ***** ***   * **  ** **

adc1            GGGTT ATGTT CAGTA CGATT CTGAT GATTT ACTCG AAGGC CTGCG GCGTC ATACG GAGTT 1886
adc2            GGGCT ATGTG CAGTA CGATC CCGAA GATTT GCTGG AAAAT ATGCG CCGCT ACTGT GAACA 1970
                *** * ****  ***** ****  * **  *****  ** * **     ****  **   *     **

adc1            AGCCT TGAGC AATGG ACAAA TTACC CTGGA GGAAT CTCGG CGCTT ATTGG AAGAT TATGA 1946
adc2            GGCCA TGGAA GATAA ACGTA TGAGC TTGGA GGAAG CCCAA TTATT GCTGG AAAAT TACGA 2030
                 ***  **     **  **  * * * *  **** ****  * *      **   *** ** ** ** **

adc1            GCAAA GTTTA CGGCG CTACA CCTAT CT-AA GTTGA ----- ----- ----- ----- --- 1980
adc2            GCGCA GTTTG TTGCA ATACA CCTAC CTCAA GCCCA CTTCA GGCAT CCACA CCAGC TAA 2088
                **  * ****    **   **** ****  ** ** *   *
```

Figure 2.1 Alignment of arginine decarboxylase nucleotide sequences (*adc1* and *adc2* genes) of *Synechocystis* sp. PCC 6803 from Cyanobase (Source: http://bacteria.kazusa.or.jp/cyanobase/). Star symbols represented the homology area (65% similarity). The area designing for the forward (red font) and reverse primers of *adc1* (pink font) and *adc2* (blue font), respectively.

2.8.5 Protein extraction

Synechocystis cells, grown 7-8 days with OD_{730} ~ 0.6, were harvested (about 100 ml of culture) by centrifugation at 5000 rpm, 4°C for 15 min. The pellet was diluted with extraction buffer (Appendix D) in ratio of 1:2 (cells:buffer, w/v) (Primikirios and Roubelakis-Angelakis, 1999). After pre-cooled glass beads, the glass beads were added into the tube in ratio of 1:1 (glass beads:solution, w/v) and vortexed the mixture for 20 sec per time, about 7-8 times (after vortexing kept on ice). After centrifugation, the solutions of crude protein were transferred to new tube. The 5 ml of extraction buffer was added into the tube and repeated the vortexing step for 3 times and centrifugation at 5,000 rpm, 4°C for 30 min. The supernatant was collected in new tube and kept in -20°C until used.

2.8.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Total proteins from *Synechocystis* cells were extracted following in 2.8.5. To analyse the extracted proteins, 10 μg of total proteins in sample buffer (Appendix .F) with the ratio of 4:1 of total proteins: sample buffer were denatured by boiling for 5 min. SDS-polyacrylamide gel with 12% separating gel and 5% stacking gel (Appendix F) was prepared. After that, the polymerized SDS-polyacrylamide gel was placed into electrophoresis chamber and samples were loaded into each well of SDS-gel electrophoresis was performed at a constant current of 20 mA per gel for 40 min and stained the gel with staining solution containing coomassie blue (Appendix F).

2.8.7 Western blot analysis

The 7 x 9 cm SDS-polyacrylamide gels following in 2.9.6 were transferred to immobilon™ PVDF transfer membrane by blotting solution (Appendix G). After activation the polyvinylidene fluoride (PVDF) membrane with 100% methanol for 30 min, blotting was done at 100 mA for 4 hours. After that membrane were washed with 1XTBS (Appendix G) and blocked with blocking solution (Appendix G) for 1 hour at room temperature. The membranes were washed with TBS-T (Appendix G) and follow with incubated with first antibody (diluted 1:3000) in PBS containing 3% skim milk (w/v) and 0.05% Tween-20 for 4 h at room temperature and later with second antibodies with anti-Horseradish peroxidase – Goat antimouse, HRP-GAM (diluted 1:3000) at 4°C, overnight in PBS containing 3% skim milk (w/v) and 0.05% Tween-20. Peroxidase activity was visualized by staining with the substrate mixture, consisting of 0.025% DAB, 0.01% H_2O_2 and 0.001% $CoCl_2$ in PBS. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

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CHAPTER III

RESULTS

3.1 Effect of salinity stress on growth of *Synechocystis* sp. PCC 6803

3.1.1 Effect of ionic stress

3.1.1.1 The growth of *Synechocystis* cells

Axenic cells of *Synechocystis* grown in BG-11 media adding various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl, respectively, for 20 days were determined for the cell growth. The results show that the mid-logarithmic stage of cell growth (OD_{730} ~ 0.7) in NaCl-containing medium, represented as an ionic condition, was found at 6-7 days. The growth of cells under ionic stress (Figure 3.1a) was increased with the concentration of NaCl up to 650 mM. However, cell growth at 650 mM NaCl condition was lower than that of the control and those of low NaCl concentrations (25, 125 and 350 mM NaCl, respectively).

3.1.1.2 The content of intracellular pigments

Synechocystis cells grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl, respectively, were determined for the intracellular pigments of chlorophyll a and carotenoids content under normal growth light for 20 days. The results are shown in Figure 3.1b that the chlorophyll a content of cells grown in those concentrations started about 0.68 µg/10^8 cells. After 6- day of cell cultures, they tended to increase. However, cells grown in normal BG-11 and BG-11 plus moderate salt concentrations of 25, 125 and 350 mM gave higher chlorophyll a content than cells grown in the high salt concentration of 650 mM. On the other hand, the carotenoids content (Figure 3.1c) showed the pattern which was as similar as that of

chlorophyll a. The carotenoids content of day 0-cultivation was about 0.20 μg/10^8 cell/ml which was 3.4-fold lower than chlorophyll a content. However, cells grown in various salt concentrations for 20 days showed an increase in carotenoids content. Only the conditions of 350 and 650 mM NaCl affected the carotenoids content when compared to those of lower concentrations of 0, 25 and 125 mM NaCl.

3.1.2 Effect of osmotic stress

3.1.2.1 The growth of *Synechocystis* cells

Axenic cells of *Synechocystis* grown in BG-11 media adding various sorbitol concentrations for 20 days were determined for the cell growth. The results show that the mid-logarithmic stage of cell growth (OD_{730} ~ 0.7) in sorbitol-containing media (0, 50, 250 and 500 mM sorbitol, respectively), represented as osmotic condition, was found at 6-7 days. For osmotic stress, cell growth showed similar pattern as that in ionic stress condition. However, *Synechocystis* cells which were grown under the 500 mM sorbitol-containing medium showed the slowest growth rate when compared to those under low sorbitol concentrations of 0, 50 and 250 mM (Figure 3.2a).

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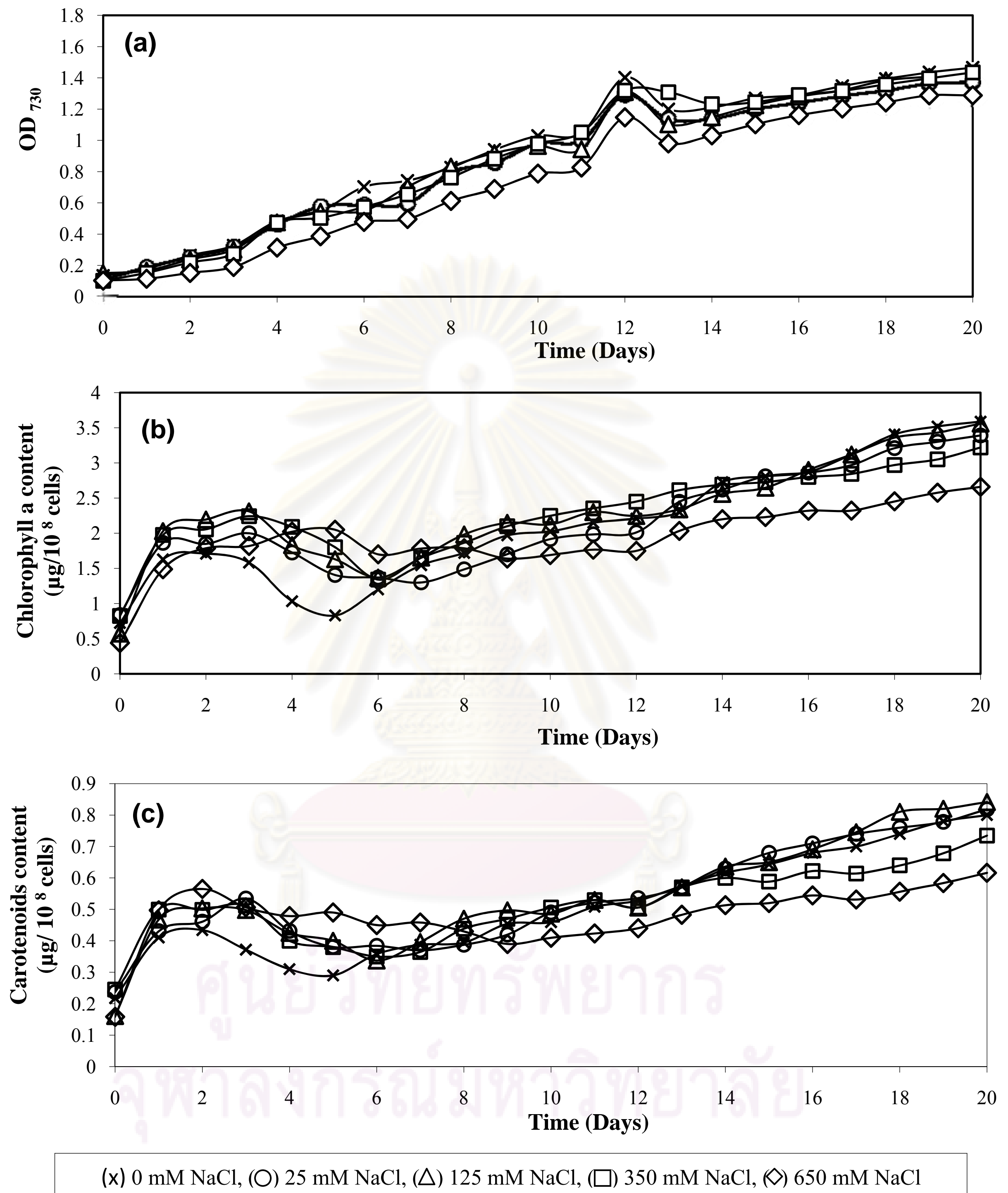

Figure 3.1 Effect of NaCl concentrations on *Synechocystis* cells grown under normal growth light for 20 days. Cells were grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl. Growth curve (a), chlorophyll a (b) and carotenoids content (c).

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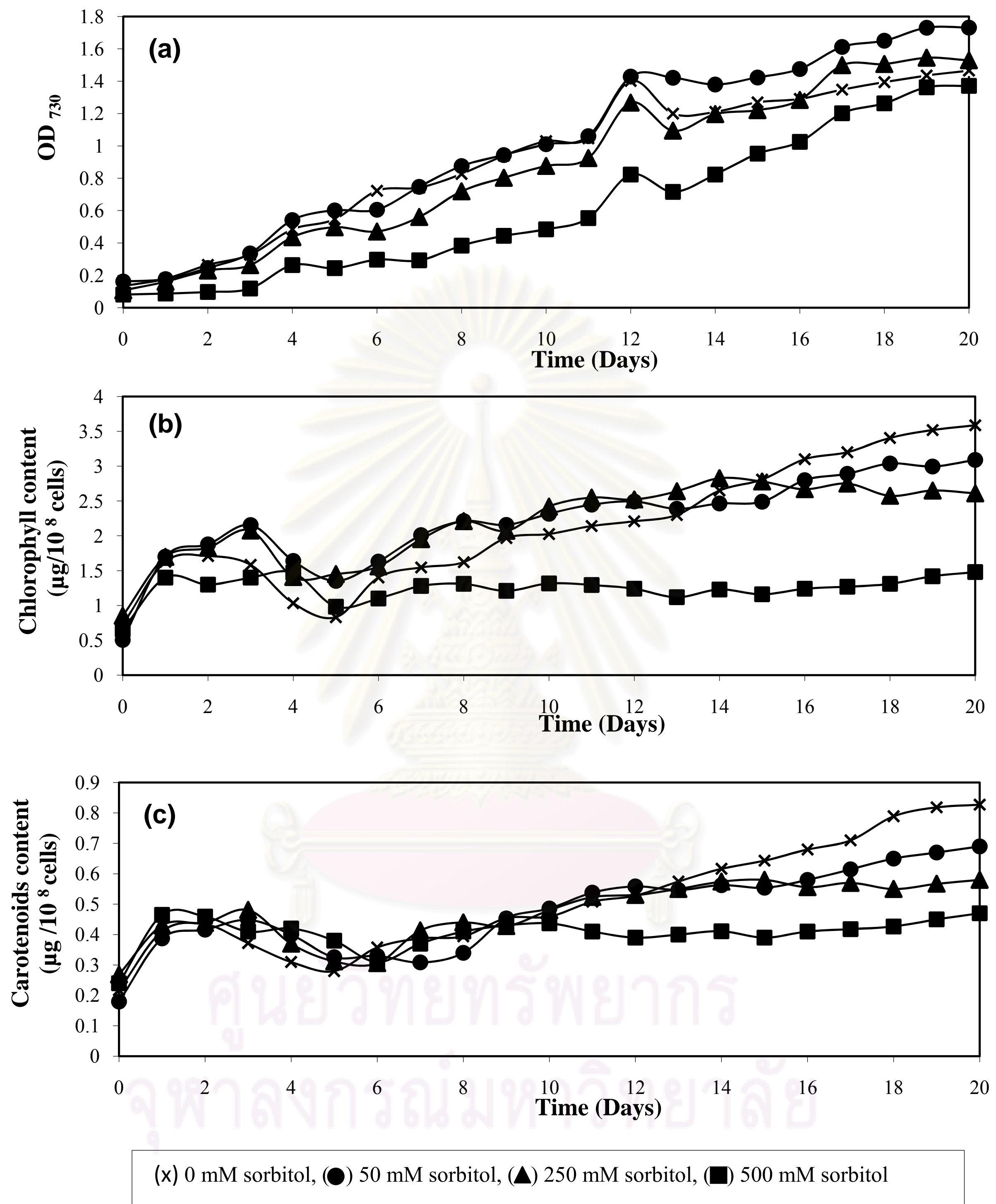

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Figure 3.2 Effect of sorbitol concentrations on *Synechocystis* cells grown under normal growth light for 20 days. Cells were grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol. Growth curve (a), chlorophyll a (b) and carotenoids content (c)

3.1.2.2 The content of intracellular pigments

Synechocystis cells grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, were determined for the intracellular pigments of chlorophyll a and carotenoids content under normal growth light for 20 days. The results showed that the chlorophyll a content (Figure 3.2b) started about 0.68 μg/10^8 cells at day 1. After 6 day-cultivation, the chlorophyll a content tended to increase. Cells grown in normal BG-11 and BG-11 plus sorbitol concentrations of 50 and 250 and 250 mM sorbitol gave higher pigment contents higher than cells grown in 500 mM sorbitol condition. For the carotenoids content of cells (Figure 3.2c) showed similar trend as that of chlorophyll a. On the other hand, carotenoids content was about 0.23 μg/10^8cells at starting culture. The high sorbitol concentration (500 mM) had influenced on carotenoids content at day 20-culture, which showed the stable/decreasing tendency whereas the others showed increasing-pattern.

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3.2 Combined effects of salinity and UV-radiation on growth and intracellular pigments of *Synechocystis* cells

3.2.1. Salt stress under normal growth light

3.2.1.1 The growth of *Synechocystis* cells

The mid-logarithmic stage of *Synechocystis* cells grown in BG-11 media plus various NaCl concentrations were diluted to the optical density at 730 nm of 0.5 and followed by determining cell growth within 3 hours. The growth of cells grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl under normal growth light for 3 hours tended to increase (F igure 3.3a). Under normal growth light conditions were used as their control when compared with those exposed to UV radiation. However, cells grown in BG-11 without salt showed higher growth rate than these of cells grown in the media plus NaCl.

3.2.1.2 The content of intracellular pigments

One ml of cultures was collected and extracted for the intracellular pigments. It was clear that the chlorophyll a content (Figure 3.3b) of cells grown in BG-11 plus various NaCl concentrations gave higher levels than that of cells grown in normal BG-11 under normal growth light. These contents showed no apparent change within 3 hours of treatment. For carotenoids content (Figure 3.3c), they were somewhat less changed except the 125 mM NaCl-treated cells which showed gradually decreased. All salt stresses (650, 350, 125, 25 mM NaCl, respectively) stimulated the accumulation of intracellular pigments which was higher than that of cells grown in medium without salt.

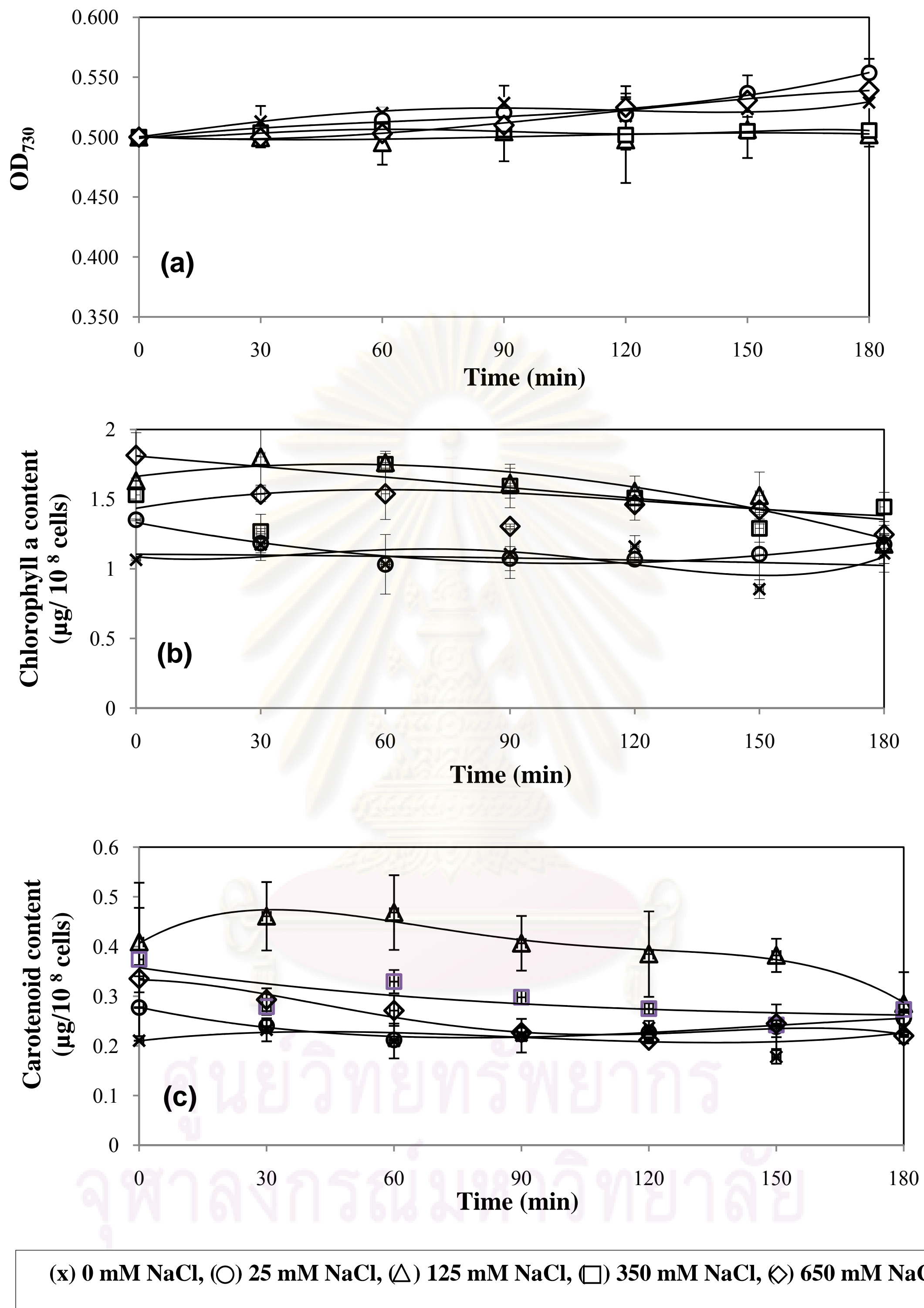

Figure 3.3 Effect of NaCl concentrations on *Synechocystis* cells grown under normal growth light for 3 hours. Cells were grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.2 Salt and UV-A stresses

3.2.2.1 The growth of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 25, 125, 350 and 650 mM NaCl concentrations were determined for the growth of cells within 3 hours under UV-A treatment. The results show that the growth of cells (Figure 3.4a) was not changed significantly. However, at 650 mM NaCl, growth was obviously decreased when compared to control without salt addition. Moreover, the result indicates that the growth of UV-A treated cells alone (Figure 3.4a) was slightly decreased when compared to the control under normal growth light condition.

3.2.2.2 The content of intracellular pigments

Under UV-A radiation, the chlorophyll a content (Figure 3.4b) of cells grown in BG-11 plus various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl, respectively, showed the constant levels within 3 hours-treatment. For carotenoids content under either UV-A radiation alone or combination with salt stress (Figure 3.4c) showed the constantly levels and tended to increase slightly at last 3 hours.

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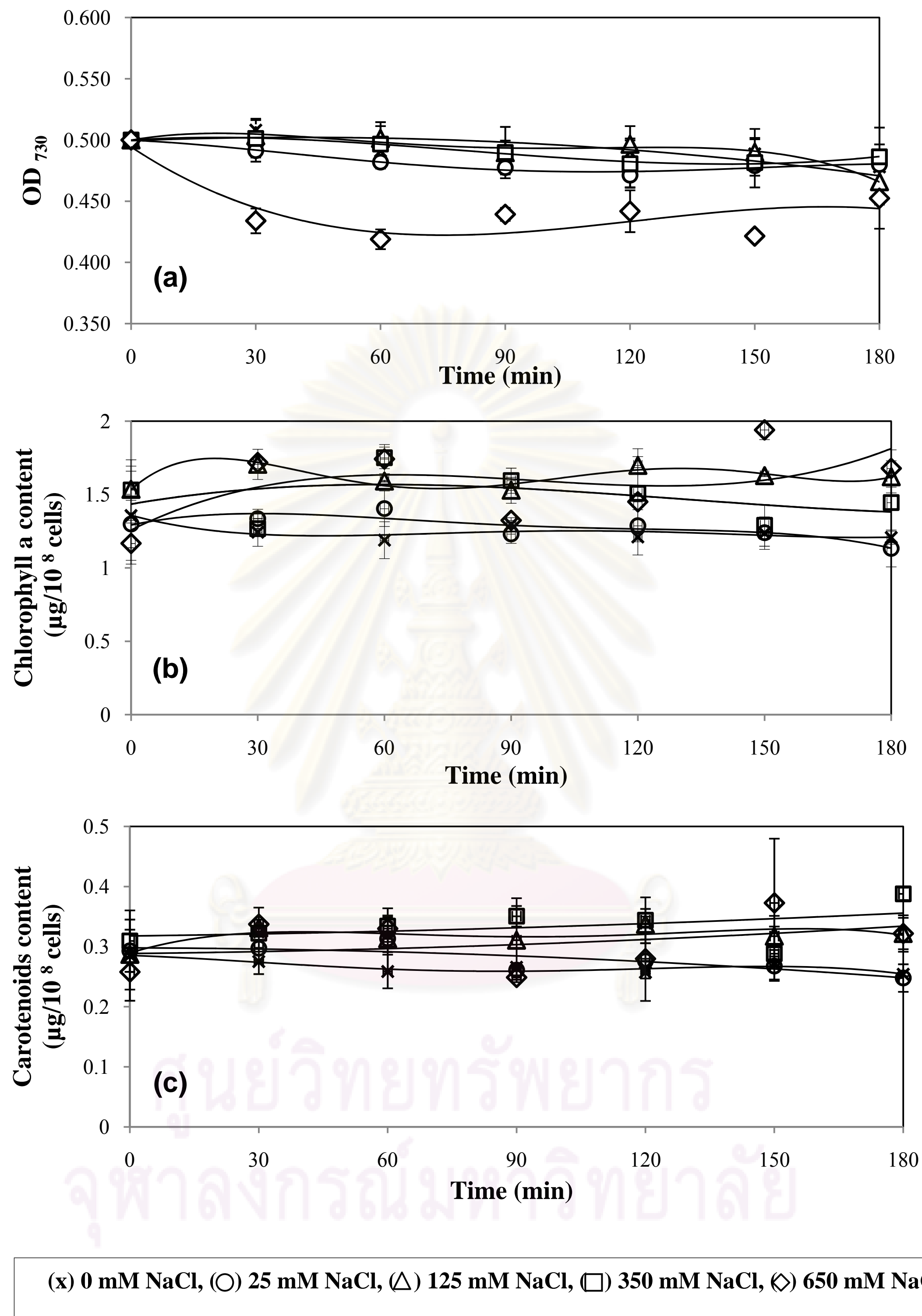

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Figure 3.4 Effect of NaCl concentrations on *Synechocystis* cells grown under UV-A radiation for 3 hours. Cells were grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.3 Salt and UV-B stresses

3.2.3.1 The growth of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 25, 125, 350 and 650 mM NaCl, respectively, were determined for the growth of cells within 3 hours under UV-B radiation. The results show that the growth of cells (Figure 3.5a) tended to decrease obviously in media plus 0, 350 and 650 mM NaCl conditions, respectively, whereas growth of cells grown in media containing 25 and 125 mM NaCl, respectively, were slightly increased. However, they showed the increase within 3 hours under control of normal growth light condition (Figure 3.3a).

3.2.3.2 The content of intracellular pigments

Under UV-B radiation, the chlorophyll a content (Figure 3.5b) of cells grown in BG-11 plus various NaCl concentrations showed the constant levels. However, cells in high salt conditions of 650 and 350 mM NaCl, respectively, exposed under UV-B for 3 hours showed the chlorophyll a content higher than those in control (without NaCl addition). For carotenoids content under UV-B radiation (Figure 3.5c) also gave constant levels. *Synechocystis* cells grown in 350 and 650 mM NaCl conditions showed the carotenoids content higher than those in the medium without NaCl addition.

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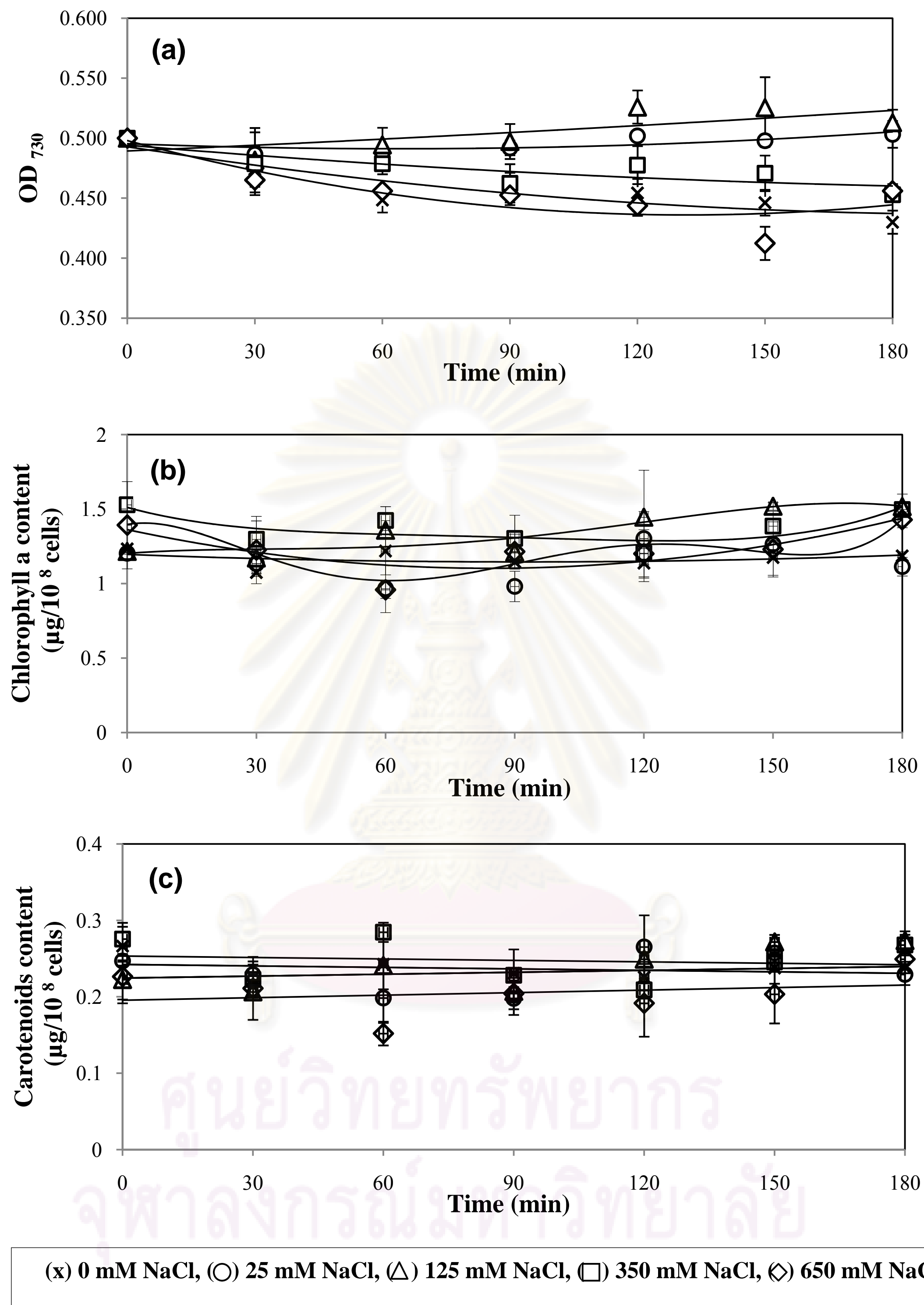

Figure 3.5 Effect of NaCl concentrations on *Synechocystis* cells grown under UV-B radiation for 3 hours. Cells were grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.4 Salt and UV-C stresses

3.2.4.1 The growth of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 25, 125, 350 and 650 mM NaCl, respectively, were determined for the growth of cells within 3 hours under UV-C radiation. The results show that their cells growth were not different significantly in various media containing 0, 25, 125, 350 and 650 mM NaCl, respectively (Figure 3.6a). Interestingly, these results demonstrated differently from those pattern of cells grown under normal growth light (Figure 3.3a).

3.2.4.2 The content of intracellular pigments

Under UV-C radiation, the chlorophyll a content (Figure 3.6b) of cells were decreased under the condition of 125, 350 and 650 mM NaCl stressed media, respectively whereas these contents in low NaCl concentration (25 mM) and medium without salt showed no apparent changes on their levels within 3 hours under UV-C exposure. For the carotenoids content under UV-C radiation (Figure 3.6c) were slightly decreased. *Synechocystis* cells grown in media plus 125, 350 and 650 mM NaCl, respectively, gave the carotenoids content higher than those cells grown in the medium without NaCl addition.

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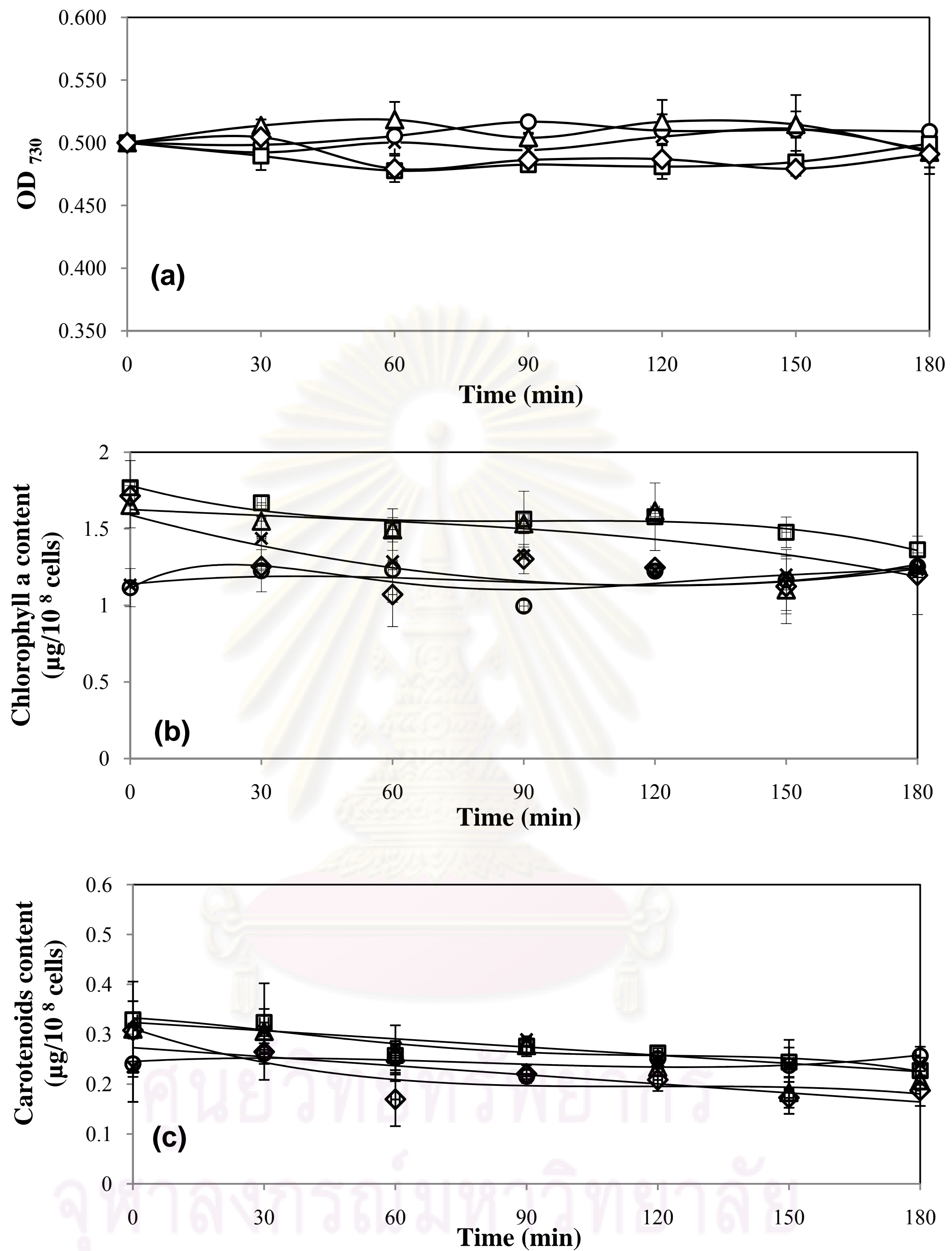

(x) 0 mM NaCl, (◯) 25 mM NaCl, (△) 125 mM NaCl, (□) 350 mM NaCl, (◇) 650 mM NaCl

Figure 3.6 Effect of NaCl concentrations on *Synechocystis* cells grown under UV-C radiation for 3 hours. Cells were grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.5 Sorbitol stress under normal growth light

3.2.5.1 The growth of *Synechocystis* cells

The mid-logarithmic stage of *Synechocystis* cells grown in BG-11 media plus various sorbitol concentrations were diluted to the optical density at 730 nm of 0.5 and followed by determining the growth of cells within 3 hours. The cells grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, tended to increase their growth under normal growth light for 3 hours (Figure 3.7a). These normal growth light conditions were used as controls when compared with those exposed to UV-radiation. However, cells grown in BG-11 without sorbitol showed no significant changes to cells grown under sorbitol condition.

3.2.5.2 The content of intracellular pigments

The content of intracellular pigments, namely chlorophyll a and carotenoids, were determined. For the chlorophyll a content of cells which grown in BG-11 media plus 50, 250 and 500 mM sorbitol, respectively, under normal growth light (Figure 3.7b) shown chlorophyll a content higher mM sorbitol (normal BG-11) significantly. However, these contents were constant within 3 hours of treatment. For carotenoids content (Figure 3.7c), they were as similar result as those patterns of chlorophyll a content under the same condition. However, content of carotenoids were 5-fold lower than chlorophyll a content. Although cells in media plus various sorbitol concentrations gave higher contents than that grown in medium without sorbitol, all these cells still possessed the constant levels of pigments within 3 hours under normal growth light.

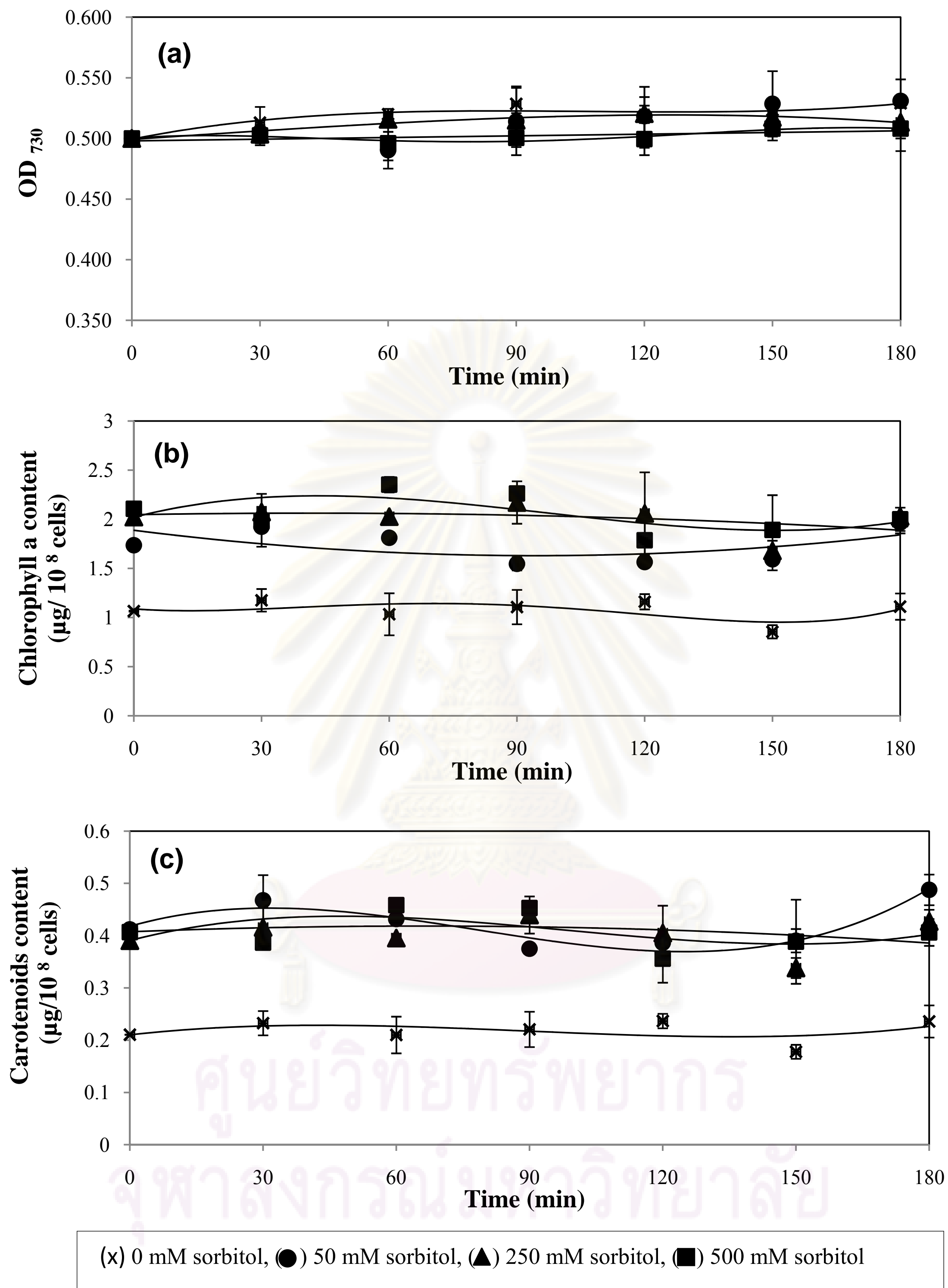

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Figure 3.7 Effect of sorbitol concentrations on *Synechocystis* cells grown under normal growth light for 3 hours. Cells were grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.6 Sorbitol and UV-A stresses

3.2.6.1 The growth of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 50, 250 and 500 mM sorbitol, respectively, were determined for the growths of cells under UV-A radiation for 3 hours. From the results, the growth of cells grown in BG-11 media containing various sorbitol concentrations combined with UV-A radiation were decreased, especially the cells grown in 500 mM sorbitol condition within 3 hours-treatment (Figure 3.8a). These conditions showed the decrease on their growth rate of cells when compared with control (under normal growth light; Figure 3.7a).

3.2.6.2 The content of intracellular pigments

The pigments of *Synechocystis* cells under UV-A radiation gave the chlorophyll a content in constant levels (Figure 3.8b) under the condition of cells grown in BG-11 plus various sorbitol concentrations, namely 0, 50, 250 and 500 mM sorbitol, respectively. Interestingly, under combination stresses of sorbitol and UV-A were observed that the increase of chlorophyll a content depended on the increase of sorbitol concentration in media obviously when compared to cells in medium without sorbitol. In addition, the carotenoids content of cells showed that they were similar to the pattern of chlorophyll a content. The cell cultures grown in media adding sorbitol gave contents higher than those cells grown in normal BG-11 without sorbitol (Figure 3.8c) within 3 hours of UV-A exposure.

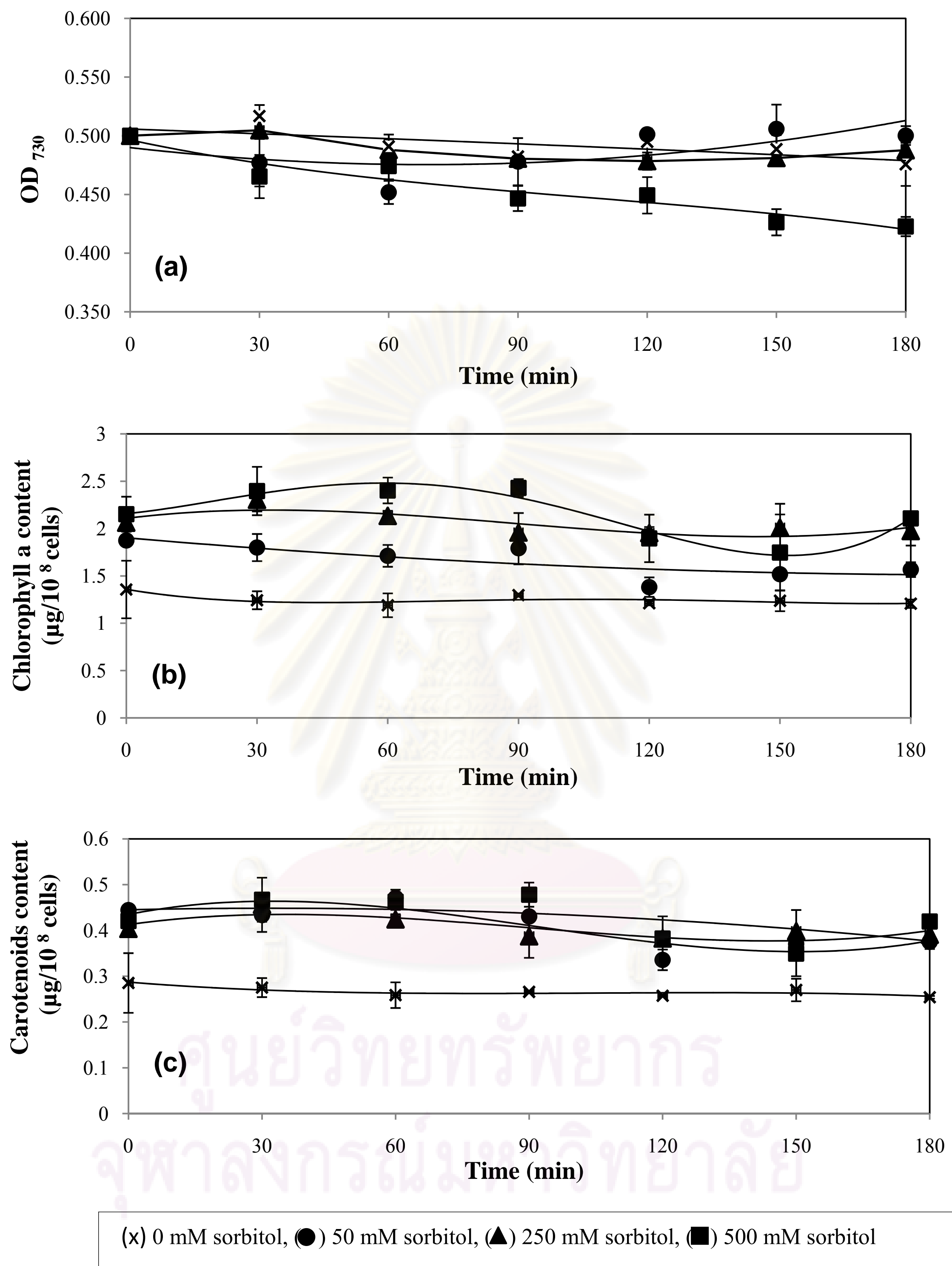

Figure 3.8 Effect of sorbitol concentrations on *Synechocystis* cells grown under UV-A radiation for 3 hours. Cells were grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.7 Sorbitol and UV-B stresses

3.2.7.1 The growths of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 50, 250 and 500 mM sorbitol, respectively were determined for the growths of cells under UV-B radiation for 3 hours. As the results, UV-B radiation had affected on the growths of cells grown in BG-11 media containing various sorbitol concentrations. The tendency of cell growth was decreased after exposing under UV-B for 3 hours (Figure 3.9a). These combined conditions tended to decrease the growth of cells when compared to control (under normal growth light; Figure 3.7a).

3.2.7.2 The content of intracellular pigments

The intracellular pigments of *Synechocystis* cells under UV-B radiation showed that the chlorophyll a content (Figure 3.9b) of cells grown in BG-11 plus various sorbitol concentrations, namely 0, 50, 250 and 500 mM sorbitol, respectively, gave constant levels. However, after UV-B exposure for 3 hours, the chlorophyll a levels of cells grown in normal BG-11 (without sorbitol) were lower than these grown in BG-11 plus various sorbitol concentrations. In addition, the carotenoids content of cells were accumulated in the similar tendency as chlorophyll a content in the same condition. Their contents gave higher than those pigments from cells grown in normal BG-11 without sorbitol (Figure 3.9c) within 3 hours of UV-B radiation.

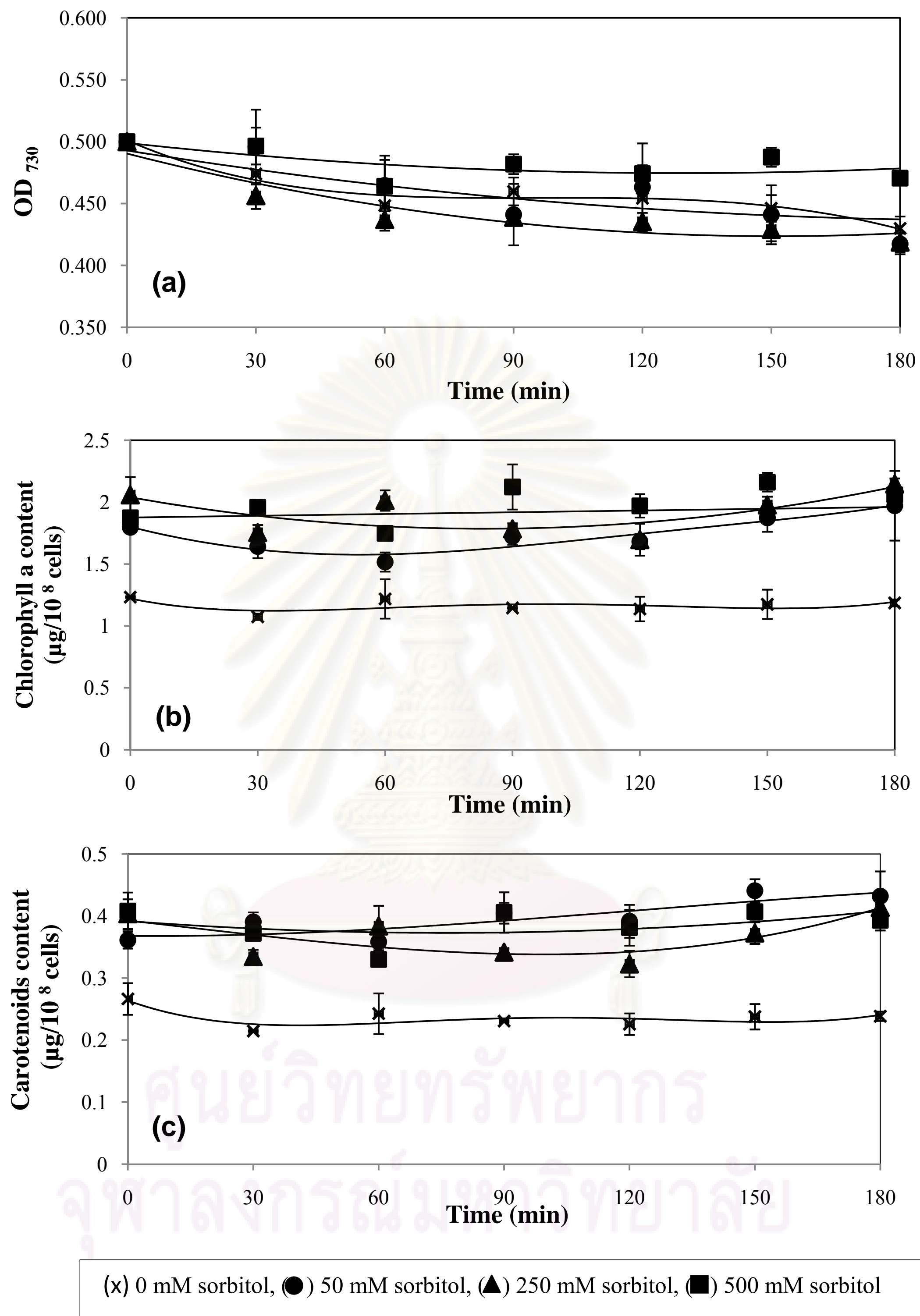

Figure 3.9 Effect of sorbitol concentrations on *Synechocystis* cells grown under UV-B radiation for 3 hours. Cells were grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.2.8 Sorbitol and UV-C stresses

3.2.8.1 The growths of *Synechocystis* cells

The *Synechocystis* cells grown in BG-11 media plus 0, 50, 250 and 500 mM sorbitol, respectively, were determined for the growths of cells under UV-C radiation for 3 hours. The growth of cells grown in BG-11 media containing various sorbitol concentrations combined with UV-C radiation were decreased, especially cells grown in 500 mM sorbitol condition within 3 hours of stresses (Figure 3.10a). On the other hand, cells grown in normal BG-11 medium without sorbitol were not affected by UV-C radiation due to the growth rate still showed the constant amount.

3.2.8.2 The content of intracellular pigments

The intracellular pigments of *Synechocystis* cells under UV-C radiation of chlorophyll a content (Figure 3.10b) gave the constant levels in cells grown in BG-11 plus various sorbitol concentrations, namely 0, 50, 250 and 500 mM sorbitol, respectively. However after UV-C exposure for 3 hours, the chlorophyll a levels of cells grown in normal BG-11 (without sorbitol) showed lower amount than those in cells from BG-11 plus various sorbitol conditions. Additionally, the carotenoids content of cells were similar to the tendency of chlorophyll a content namely, all cultures grown in media containing sorbitol gave chlorophyll a content higher than those cells grown in normal BG-11 without sorbitol (Figure 3.10c) within 3 hours-treatment. Both chlorophyll a and carotenoids content under UV-C radiation demonstrated their contents higher than those under normal growth light in significance.

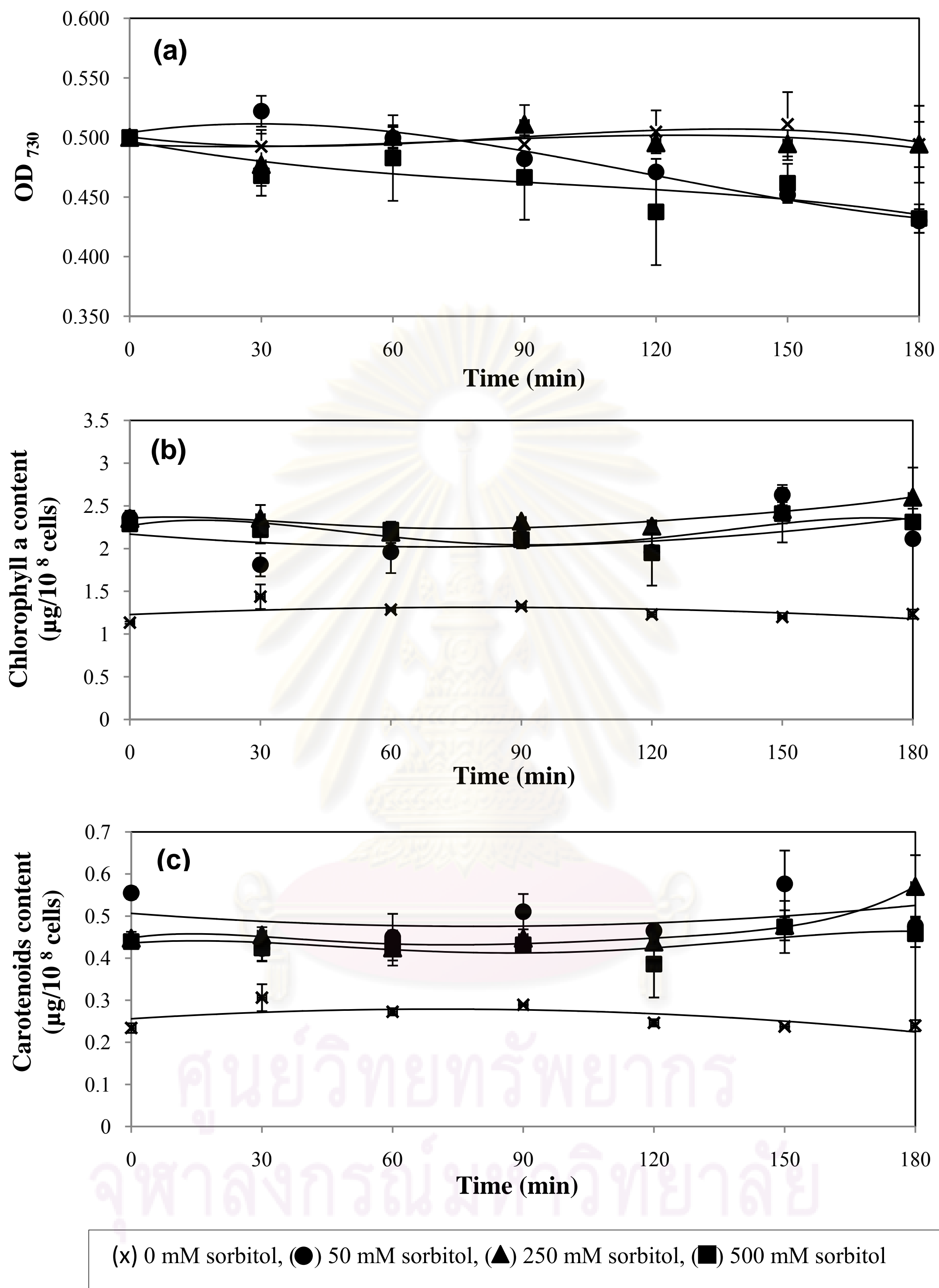

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Figure 3.10 Effect of sorbitol concentrations on *Synechocystis* cells grown under UV-C radiation for 3 hours. Cells were grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol. Growth curve (a), chlorophyll a (b) and carotenoids content (c). The data represent means ± SD., n = 3.

3.3 Polyamine biosynthesis of *Synechocystis* cells under salinity and UV radiations

3.3.1 Polyamines content under salt and normal growth light

Polyamines content of *Synechocystis* cells at mid-logarithmic stage in BG-11 containing various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl exposed under normal growth light (shown in Figure 3.11). The polyamines content at short-term stress (Figure 3.11a) were higher in PCA-soluble fraction (the major form of free form) than in PCA-insoluble fraction (minor form). For PCA-soluble polyamines, the results show that the highest level among three kinds of them was spermidine, especially from the cells grown in BG-11 plus 650 and 350 mM NaCl, respectively, whereas putrescine and spermine contents showed very small amounts. On the other hand, PCA-insoluble polyamines were not changed significantly. The total polyamines including PCA-soluble and PCA-insoluble polyamines were increased upon the increase of NaCl concentrations. However, results from long term stress of 3 hours (Figure 3.11b) show the decrease of the polyamines content when compared with short term stress (1 hour).

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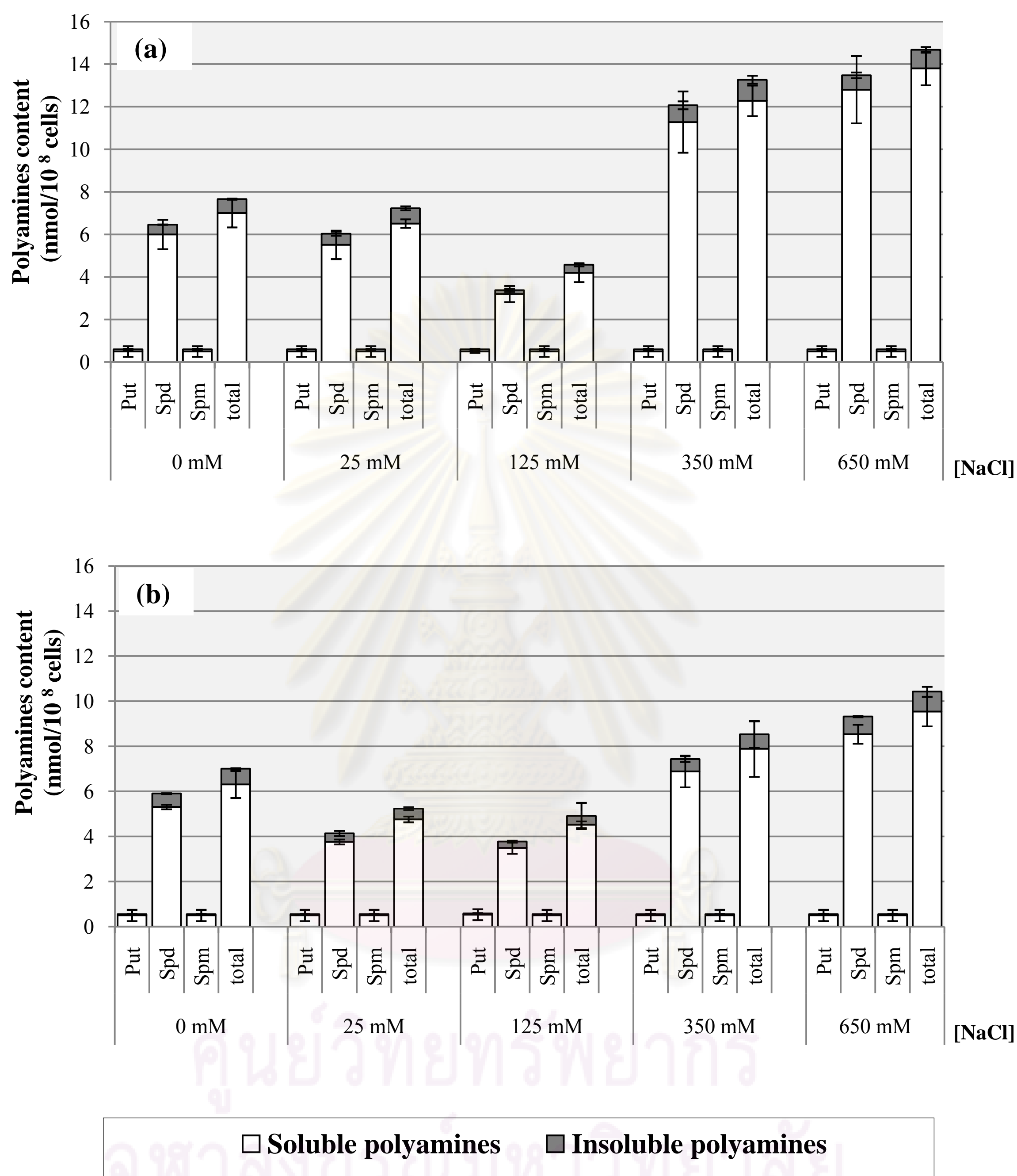

Figure 3.11 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl, respectively, under normal growth light for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.2 Polyamines content under salt and UV-A stresses

Polyamines content of *Synechocystis* cells at mid-logarithmic stage in BG-11 containing various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl exposed under UV-A for 3 hours (shown in Figure 3.12). The polyamines content at short-term (Figure 3.12a) were found higher in PCA-soluble fraction than in PCA-insoluble fraction. For PCA-soluble polyamines, the results show that the highest level among three kinds of them was spermidine, especially from the cells in BG-11 plus 350 mM NaCl whereas putrescine and spermine contents showed very small amounts. On the other hand, for PCA-insoluble polyamines, spermidine showed the highest content of polyamines from cells grown in all media condition, especially BG-11 plus 650 mM NaCl. Moreover, the total polyamines including PCA-soluble and PCA-insoluble polyamines were increased under UV-A, especially cells grown in BG-11 plus 350 mM NaCl (for 1 hour). However, at long term stress (Figure 3.12b), the major form was PCA-soluble polyamines whereas the PCA-insoluble polyamines occurred as minor forms. Spermidine was found in higher level than putrescine and spermine. Interestingly, polyamines of cells exposed to long term stress (3 hours) of UV-A were increased, higher than those of cells at 1-hour stress, especially in BG-11 plus 350 and 650 mM NaCl. Furthermore, the contents of PCA-insoluble polyamines at 3-hours treament were increased significantly when compared to cells grown under normal growth light (Figure 3.11), especially in BG-11 plus 350 and 650 mM NaCl.

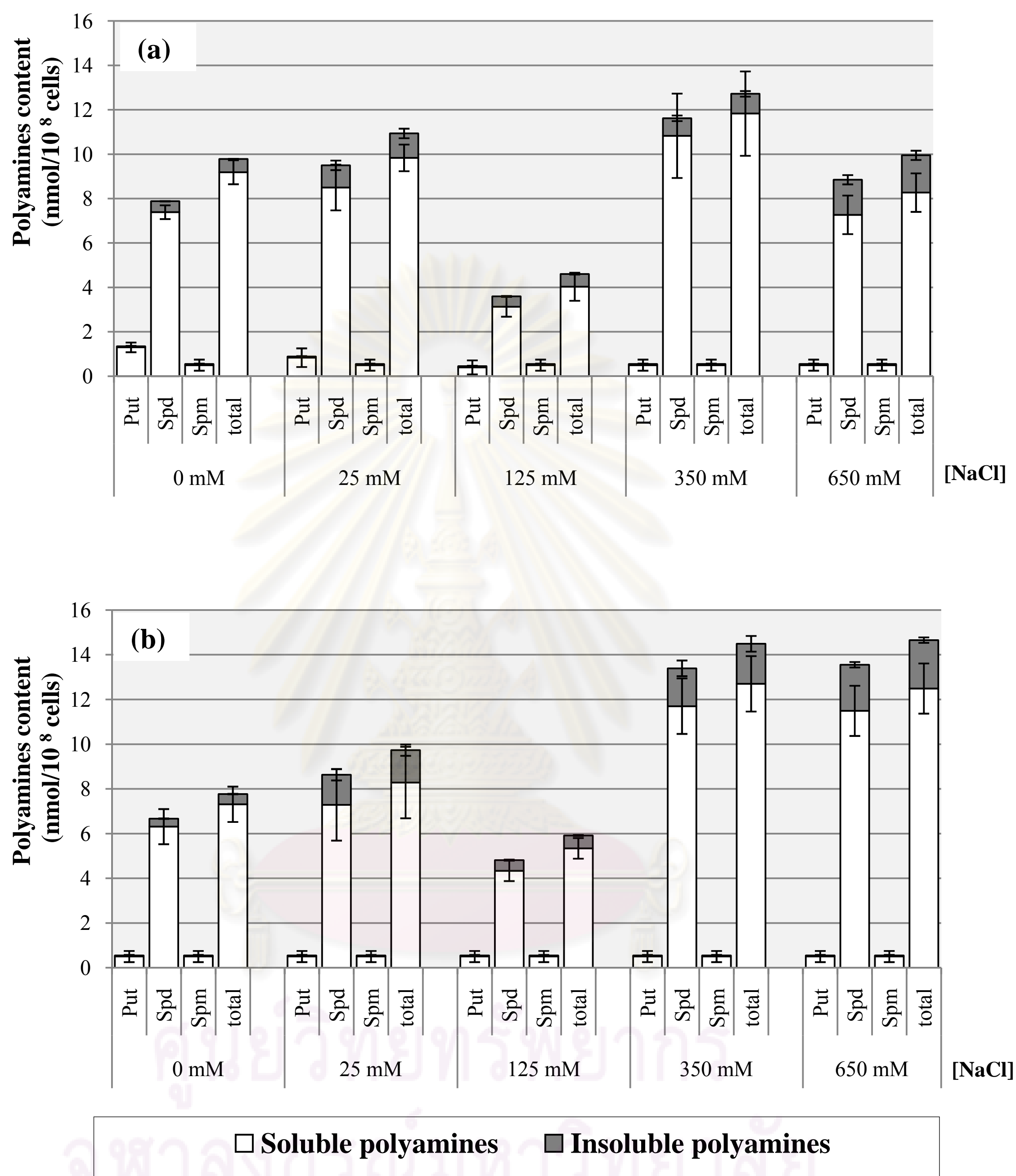

Figure 3.12 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl, respectively, under UV-A radiation for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.3 Polyamines content under salt and UV-B stresses

Polyamines content of *Synechocystis* cells at mid-logarithmic stage in BG-11 containing various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl exposed under UV-B for 3 hours (shown in Figure 3.13). The polyamines content at short-term stress (Figure 3.13a) were higher in PCA-soluble fraction than in PCA-insoluble polyamines. For PCA-soluble polyamines, the results show that the highest level among three kinds of them was spermidine whereas putrescine and spermine contents showed very small amounts. The polyamines content were increased obviously from cells grown in all conditions under UV-B stress, especially in BG-11 plus 350 mM NaCl. It was 5.58 fold higher than control group (under normal growth light). Moreover, the results from long-term stress under UV-B (Figure 3.13b) showed that cells grown in BG-11 plus 350 mM NaCl showed the induction of soluble-polyamine content of 1.53 and 8.8 fold of those from cells after exposing to UV-B for short-term stress and to normal growth light, respectively. However, UV-B stress induced the contents of PCA-insoluble spermidine slightly when compared to short-term exposure of UV-B. On the other hand, putrescine and spermine contents were accumulated in small amounts. Also, the total polyamines including PCA-soluble and PCA-insoluble fractions were increased under UV-B exposure, especially from cells grown in BG-11 plus 350 mM NaCl both for 1 and 3 hours-treatments.

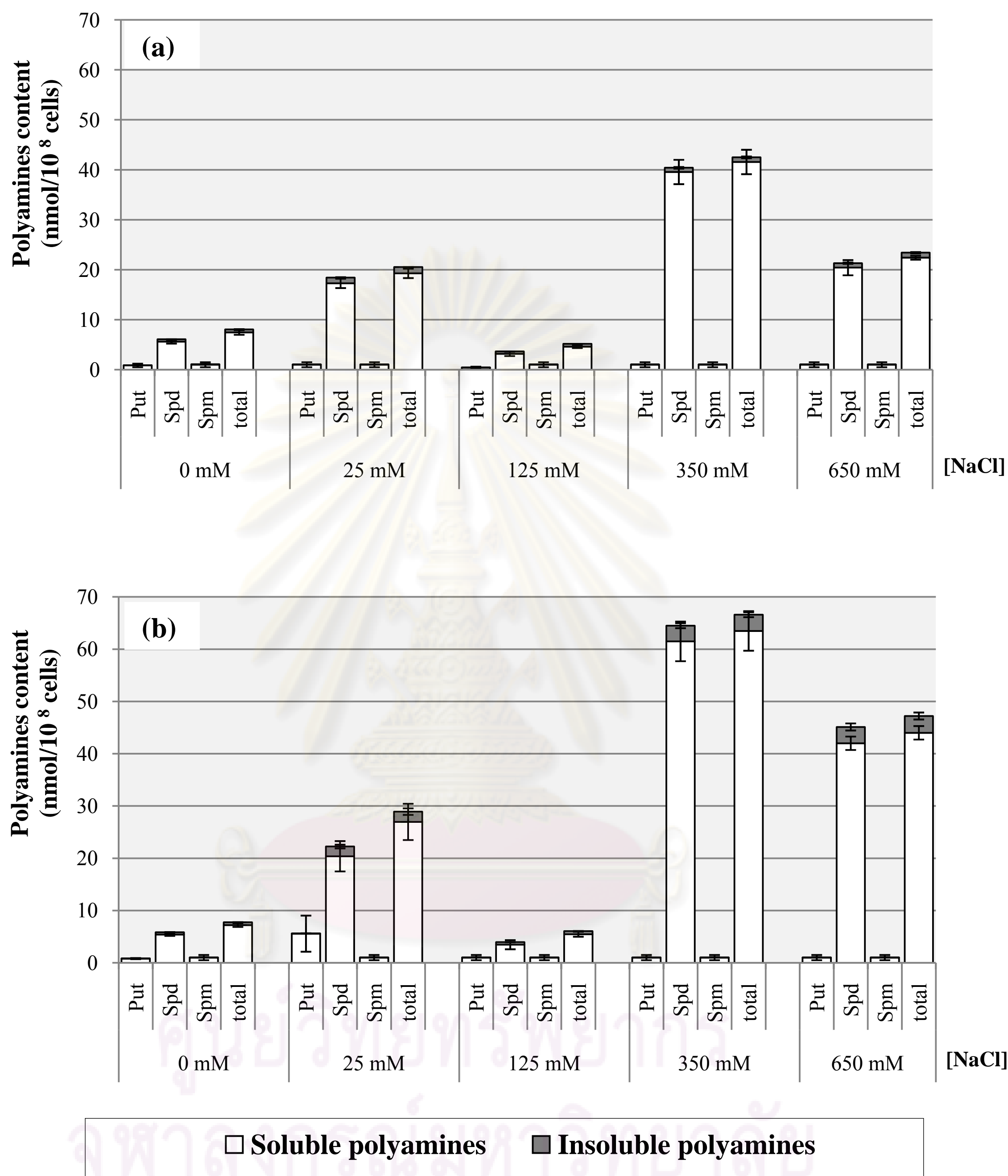

Figure 3.13 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl, respectively, under UV-B radiation for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.4 Polyamines content under salt and UV-C stresses

Polyamines content of *Synechocystis* cells at mid-logarithmic stage in BG-11 containing various NaCl concentrations of 0, 25, 125, 350 and 650 mM NaCl exposed under UV-C for 3 hours (shown in Figure 3.14). The polyamines content at short-term stress (Figure 3.14a) were found in PCA-soluble fraction (the major form of free form) higher than minor form of PCA-insoluble fraction. For PCA-soluble polyamines, the results show that the highest level among three kinds of them was spermidine whereas putrescine and spermine contents showed very small amounts. The polyamines content of cells grown in BG-11 plus 350 mM NaCl were increased obviously at 3.74 fold of control group (under normal growth light). While PCA-insoluble fraction, only spermidine was increased at cells grown in BG-11 plus 350 mM NaCl. Moreover, the results from long-term stress under UV-C radiation (Figure 3.14b) show that PCA-soluble spermidine was higher than putrescine and spermine. Cells grown in BG-11 plus 350 mM NaCl accumulated the soluble polyamine content of 1.29- and 3.73-fold of those from cells after exposing to UV-C for short-term stress and to normal growth light, respectively. However, PCA-insoluble polyamines in BG-11 plus 350 mM NaCl and UV-C conditions with for 3 hours-treatment were increased on spermidine content slightly when compared to those of cells in other media conditions whereas putrescine and spermine contents showed in small amounts. Also, the total polyamines including PCA-soluble and PCA-insoluble polyamines were increased under UV-C radiation, especially in BG-11 plus 350 mM NaCl-treated cells for 1 and 3 hours-treatments.

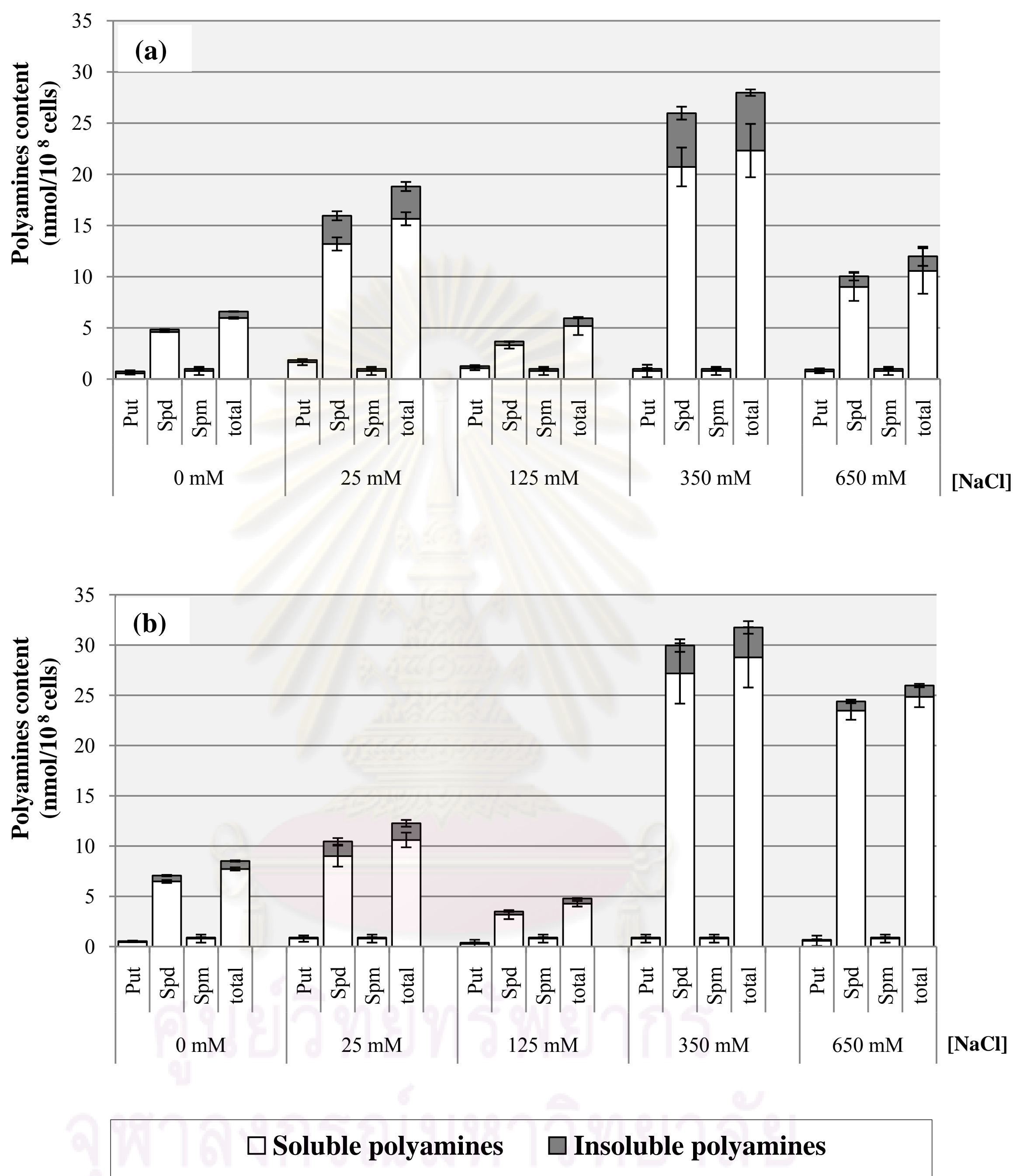

Figure 3.14 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 25, 125, 350 and 650 mM NaCl, respectively, under UV-C radiation for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

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3.3.5 Polyamines content under sorbitol and normal growth light

Polyamines content of *Synechocystis* cells grown in BG-11 various sorbitol concentrations, namely 0, 50, 250 and 500 mM sorbitol, respectively, under normal growth light shown in Figure 3.15. The polyamines content at short-term stress (Figure 3.15a) were found in PCA-soluble fraction which was the major form whereas their minor form was occurred in PCA-insoluble fraction. For PCA-soluble polyamines, the results show that the highest levels of polyamines, especially from the cells grown in BG-11 plus 250 mM sorbitol were spermidine and putrescine whereas spermine gave very small amounts. For PCA-insoluble polyamines, they were not changed significantly. Obviously, the total polyamines including both soluble and insoluble fractions were increased under 250 mM sorbitol condition within 3 hours of treatment. Surprisingly, putrescine in soluble form was stimulated obviously at 3 hours-treatment under 250 mM NaCl condition. The results under this normal growth light were used as the control when compared to cells after exposed to UV-radiation.

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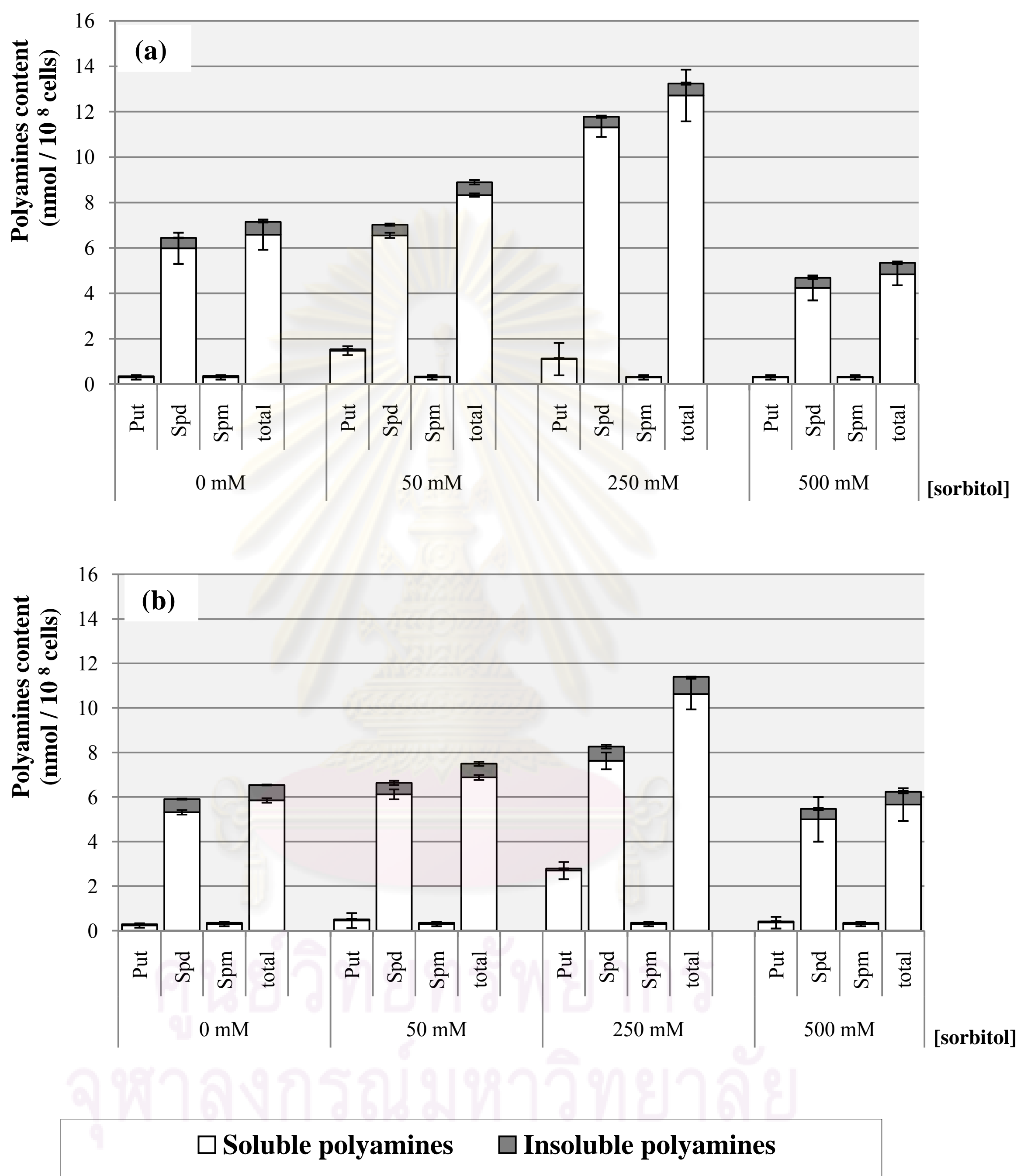

Figure 3.15 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, under normal growth light for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.6 Polyamines content under sorbitol and UV-A stresses

Polyamines content of *Synechocystis* cells grown in BG-11 various sorbitol concentrations, namely, 0, 50, 250 and 500 mM sorbitol, respectively, under UV-A radiation for 3 hours shown in Figure 3.16. The polyamines content at short-term stress of one hour under UV-A and sorbitol stresses (Figure 3.16a) were found in PCA-soluble fraction as the major form whereas their minor form was in insoluble fractions. For PCA-soluble polyamines, spermidine was up-regulated dominantly whereas putrescine and spermine were found in trace amounts. On the other hand, only PCA-insoluble spermidine gave induced contents in all conditions, though somewhat less than PCA-soluble fractions. The total polyamines including PCA-soluble and PCA-insoluble fractions were slightly increased under sorbitol stress of 250 mM. However, these amounts were less than those of control without sorbitol. Results in Figure 3.16b show that PCA-soluble polyamines of cells grown under BG-11 plus 250 and 500 mM sorbitol for long term stress (3 hours) of UV-A were decreased when compared to cells grown in normal BG-11 without sorbitol. Furthermore, the contents of PCA-insoluble polyamines were unchanged significantly in all media conditions.

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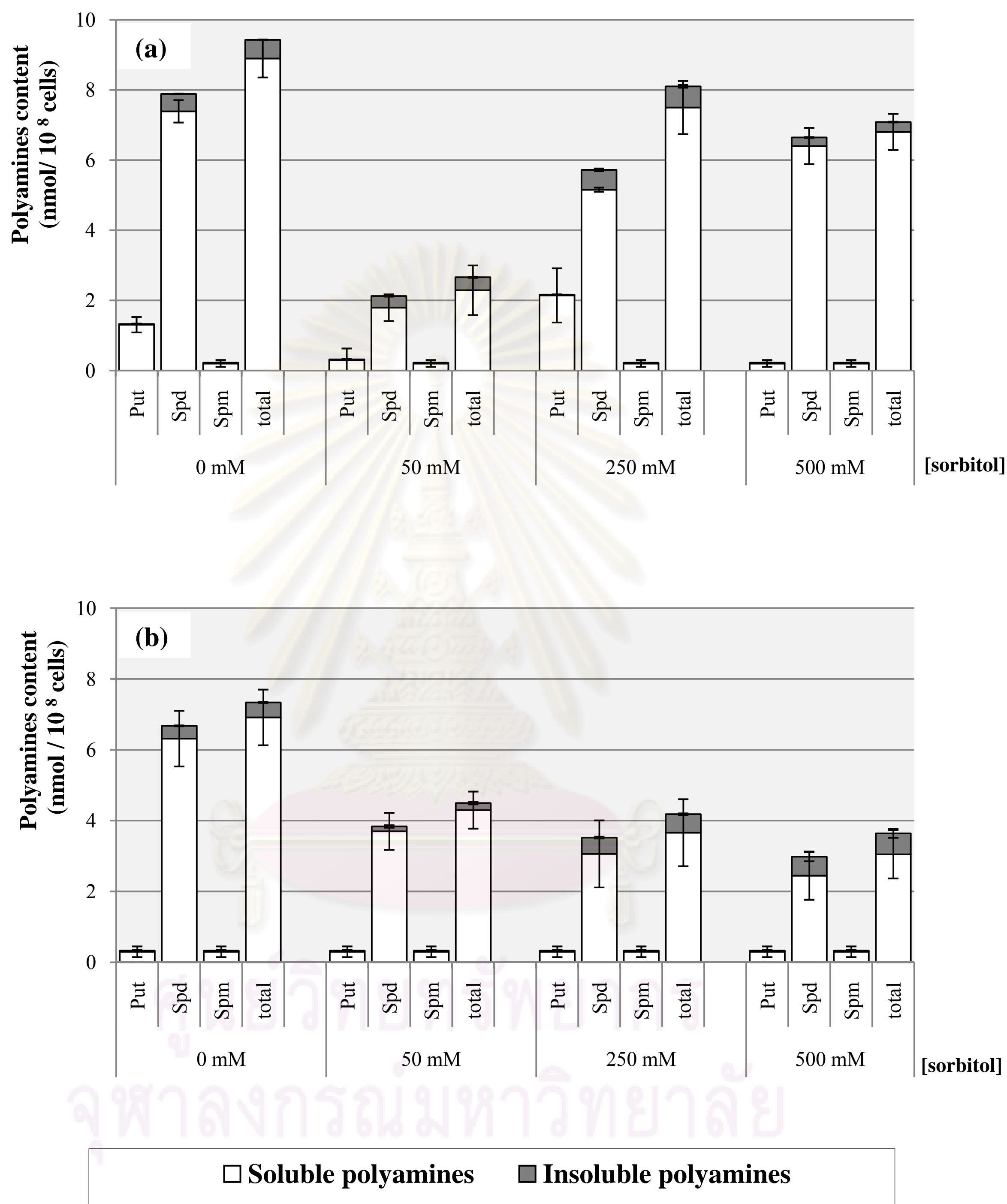

Figure 3.16 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, under UV-A radiation for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.7 Polyamines content under sorbitol and UV-B stresses

Polyamines content of *Synechocystis* cells grown in BG-11 various sorbitol concentrations, namely, 0, 50, 250 and 500 mM sorbitol, respectively, under UV-B radiation within 3 hours shown in Figure 3.17. The polyamines content at short-term of one hour under UV-B combined with sorbitol stresses (Figure 3.17a) were found in PCA-soluble as the major form whereas minor form was found in PCA-insoluble fractions. For PCA-soluble polyamines, high level of spermidine was observed whereas putrescine and spermine were accumulated in small levels. The PCA-insoluble polyamines had the similar pattern to PCA-soluble polyamines. Although the total polyamines including PCA-soluble and PCA-insoluble fractions were up-regulated at 500 mM sorbitol condition when compared to these cells grown in normal BG-11 without sorbitol under UV-B stress for 1 hour. For longer term stress of 3 hours (Figure 3.17b), soluble polyamines under 250 and 500 mM sorbitol conditions were decreased significantly when compared to cells grown in normal BG-11 without sorbitol. Furthermore, cells grown in BG-11 plus 250 mM sorbitol were slightly increased the PCA-insoluble polyamines under UV-B radiation for 3 hours.

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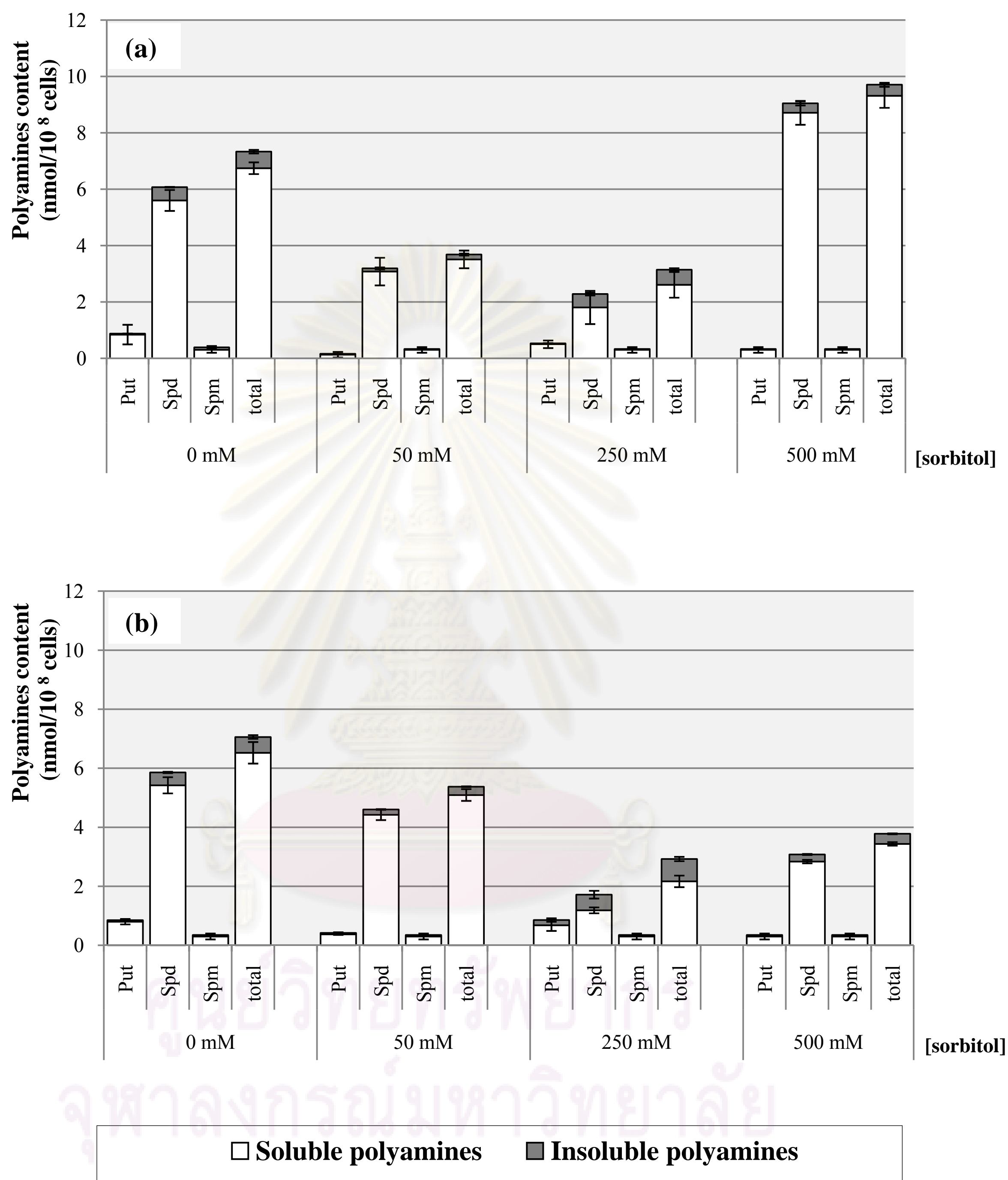

Figure 3.17 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, under UV-B radiation for 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.3.8 Polyamines content under sorbitol and UV-C stresses

Polyamines content of *Synechocystis* cells grown in the conditions which were varied sorbitol concentrations, namely, 0, 50, 250 and 500 mM sorbitol, respectively, into BG-11 medium under UV-C radiation for 3 hours (Figure 3.18). The polyamines content at short-term stress of one hour under UV-C and sorbitol stresses (Figure 3.18a) were found in PCA-soluble fraction in majority whereas PCA-insoluble polyamines were found in minority. For PCA-soluble polyamines, spermidine and putrescine were up-regulated whereas spermine was accumulated in small amounts under BG-11 plus 250 mM sorbitol condition. On the other hand, PCA-insoluble polyamines were induces in trace level in all conditions. Total polyamines including PCA-soluble and PCA-insoluble fractions of cells grown in BG-11 plus 250 mM sorbitol were increased significantly when compared to cells grown in normal BG-11 without sorbitol under UV-B stress for 1 hour. At 3 hours of long term stress (Figure 3.18b) total polyamines were down-regulated under BG-11 plus 250 and 500 mM sorbitol conditions. The PCA-soluble polyamines of cells under 50, 250 and 500 mM sorbitol conditions were significantly decreased when compared to cells grown in normal BG-11 without sorbitol.

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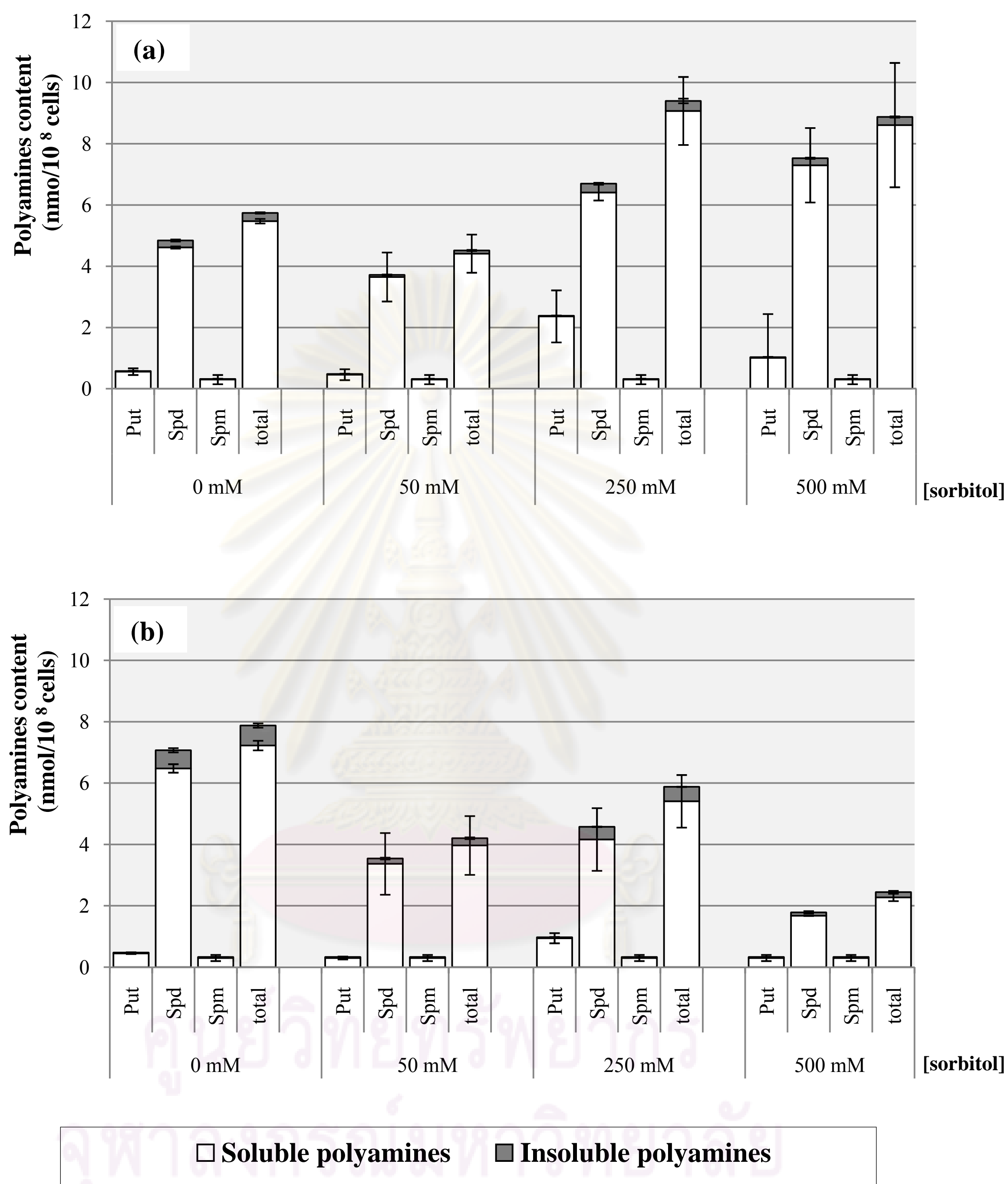

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Figure 3.18 Polyamines content of *Synechocystis* sp. PCC 6803 grown in BG-11 media containing 0, 50, 250 and 500 mM sorbitol, respectively, under UV-C radiation at 1 hour (a) and 3 hours (b) treatments. (Put; Putrescine, Spd; Spermidine, Spm; Spermine, Total; Total-Polyamines). The data represent means ± SD., n = 3.

3.4 Combination effects of salinity and UV-radiation on *adc* mRNA levels

3.4.1 Effects of salinity under normal growth light

Total RNAs of *Synechocystis* cells grown in normal BG-11, BG-11 plus 350 mM NaCl and 500 mM sorbitol, respectively were used as a template for RT-PCR amplification. The relative amount of arginine decarboxylase (*adc*) mRNA was shown in Figure 3.19. From the results, *adc1* mRNA level of 350 mM NaCl-treated cells was higher than that of cells grown in normal BG-11. Whereas *adc2* mRNA level of cells under same salt-treatment showed a slight increase when compared to control (Figure 3.19). From the results of cells under salt stress, *adc1* mRNA was increased under high salt concentration. For sorbitol stress, *adc1* and *adc2* mRNA levels were constant when compared to the control. However, both salt and sorbitol treatments showed the levels of *adc2* mRNA higher than *adc1* mRNA. The results for *16s* rRNA in samples derived from the same amount of total RNA was also included which represented as an internal standard.

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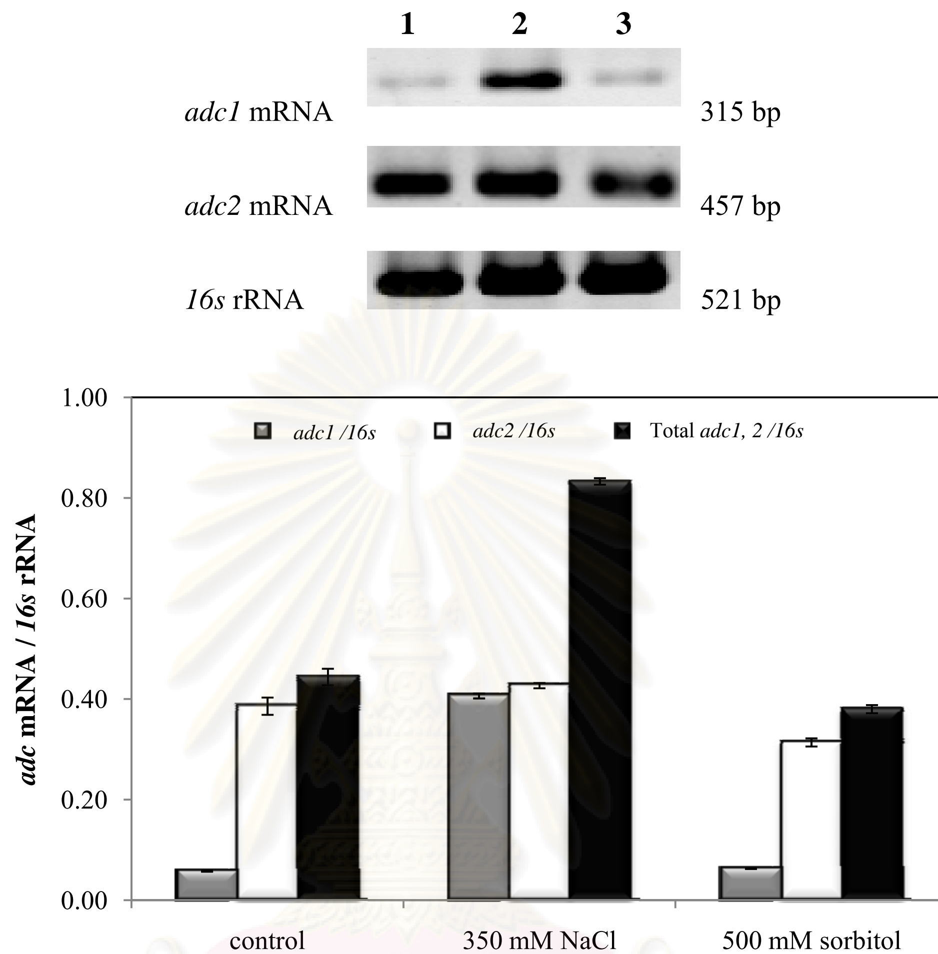

Figure 3.19 RT-PCR expression analysis of arginine decarboxylase (*adc1* and *adc2*) mRNA levels in *Synechocystis* sp. PCC 6803 exposed under normal growth light for 1 hour. Cells were grown in salinity treatment, namely, normal BG-11 media, control (lane 1), BG-11 containing 350 mM NaCl (lane 2) and 500 mM sorbitol (lane 3) respectively. The relative abundance of *16s* rRNA is also shown in lower row. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

3.4.2 Effects of salinity and UV-A stresses

Total RNAs of *Synechocystis* cells which grown in normal BG-11, BG-11 plus 350 mM NaCl and 500 mM sorbitol, respectively, under UV-A exposure for 1 hour were used as a template for RT-PCR amplification. The relative amount of arginine decarboxylase (*adc*) mRNA was shown in Figure 3.20. The transcription levels of *adc1* mRNA under salt stress were increased upon increasing of salt concentration. However, UV-A radiation induced *adc1* mRNA levels when compared to both controls under UV-A exposure and under normal growth light (Figure 3.19). Moreover, combined stresses of salt and UV-A to cells grown in 350 mM NaCl-condition affected the level of *adc1* mRNA decreasingly when compared to that of cells grown under normal growth light. While *adc1* mRNA level of 500 mM sorbitol-treated cells under short-term of UV-A exposure was higher constant when compared to control, but cells grown under normal growth light. For *adc2* mRNA level of cells under UV-A exposure was increased at high concentration of salt and sorbitol when compared with control. Interestingly, both cells grown in salt- and sorbitol-treatments showed the levels of *adc2* gene higher than *adc1,* as well as those of cells grown in normal growth light. The results for *16s* rRNA in samples derived from the same amount of total RNA was also included which represented as an internal standard.

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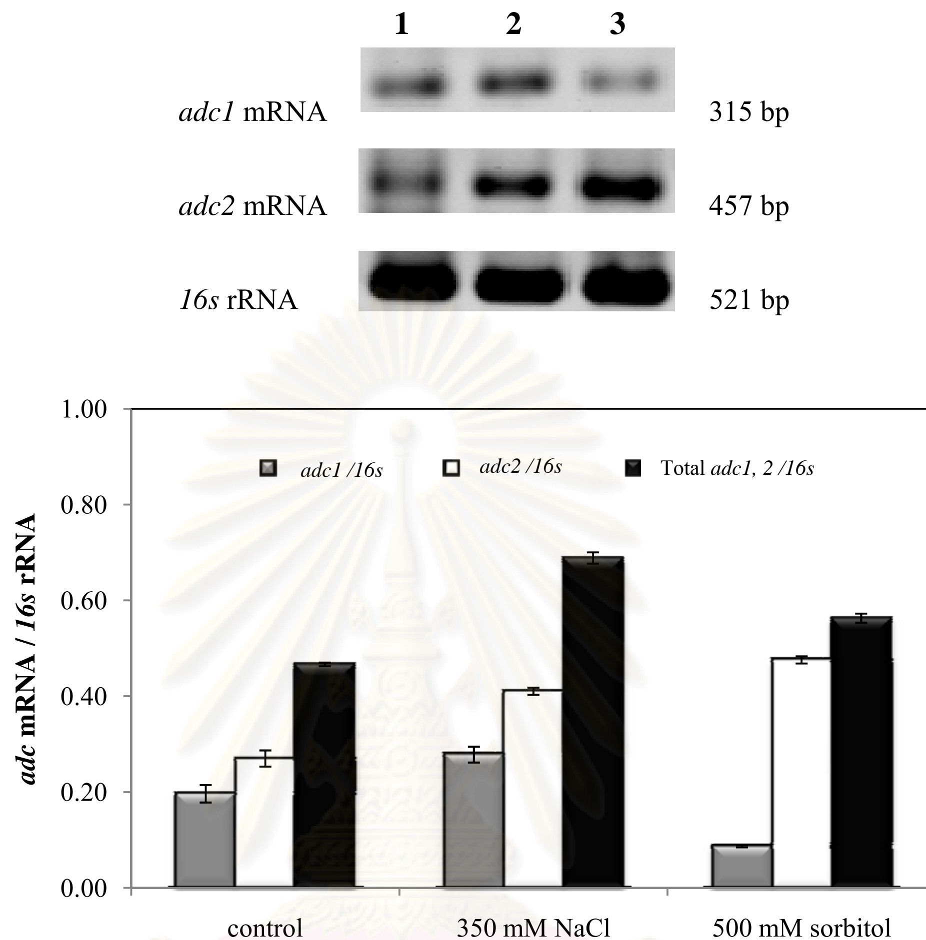

Figure 3.20 RT-PCR expression analysis of arginine decarboxylase (*adc1* and *adc2*) mRNA levels in *Synechocystis* sp. PCC 6803 exposed under UV-A exposure for 1 hour. Cells were grown in salinity treatment, namely, normal BG-11 media, control (lane 1), BG-11 containing 350 mM NaCl (lane 2) and 500 mM sorbitol (lane 3) respectively. The relative abundance of *16s* rRNA is also shown in lower row. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

3.4.3 Effects of salinity and UV-B stresses

Total RNAs of *Synechocystis* cells under UV-B exposure for 1 hour which cells grown in normal BG-11, BG-11 plus 350 mM NaCl and 500 mM sorbitol, respectively, were reverse-transcribed. The relative amount of arginine decarboxylase (*adc*) mRNA was shown in Figure 3.21. The amount of *adc1* mRNA under salt stress was increased apparently at high salt concentration of 350 mM NaCl whereas it was slightly increased under 500 mM-sorbitol treatment. Expression level of *adc2* mRNA was increased under both salt (350 mM NaCl) and sorbitol (500 mM) stresses when compared to those of cells grown in BG-11 media without salt and sorbitol addition (control). Interestingly, the levels of *adc2* mRNA were higher than *adc1* mRNA levels under normal BG-11 (control) and BG-11 plus 500 mM sorbitol condition. Moreover, cells treated with 350 mM NaCl and UV-B radiation showed no difference on expressions of *adc1* and *adc2* mRNA levels. Moreover, they were higher than that of control. For *16s* rRNA of cells were represented as an internal standard.

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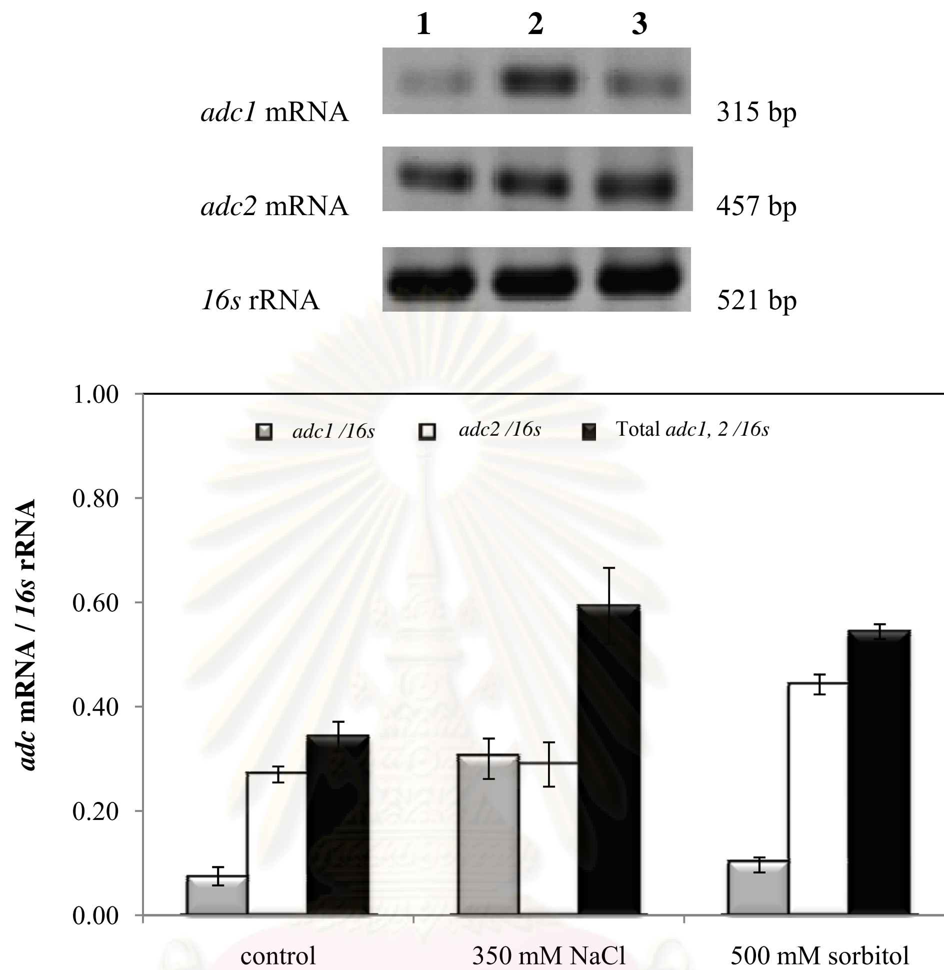

Figure 3.21 RT-PCR expression analysis of arginine decarboxylase (*adc1* and *adc2*) mRNA levels in *Synechocystis* sp. PCC 6803 exposed under UV-B exposure for 1 hour. Cells were grown in salinity treatment, namely, normal BG-11 media, control (lane 1), BG-11 containing 350 mM NaCl (lane 2) and 500 mM sorbitol (lane 3) respectively. The relative abundance of *16s* rRNA is also shown in lower row. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

3.4.4 Effects of salinity and UV-C stresses

Total RNAs of *Synechocystis* cells which grown in normal BG-11, BG-11 plus 350 mM NaCl and 500 mM sorbitol, respectively, under UV-C exposure for 1 hour were reverse transcribed. The relative amount of arginine decarboxylase (*adc*) mRNA was shown in Figure 3.22. The amount of *adc1* mRNA was apparently increased after treating with 350 mM NaCl and UV-C radiation. However, cell grown in BG-11 plus 500 mM sorbitol treatment was not different from the *adc1* mRNA level of control. On the other hand, *adc2* mRNA amount was lower than *adc1* mRNA level under 350 mM NaCl condition combined with UV-C. The results for *16s* rRNA in samples derived from the same amount of total RNA was also included which represented an internal standard.

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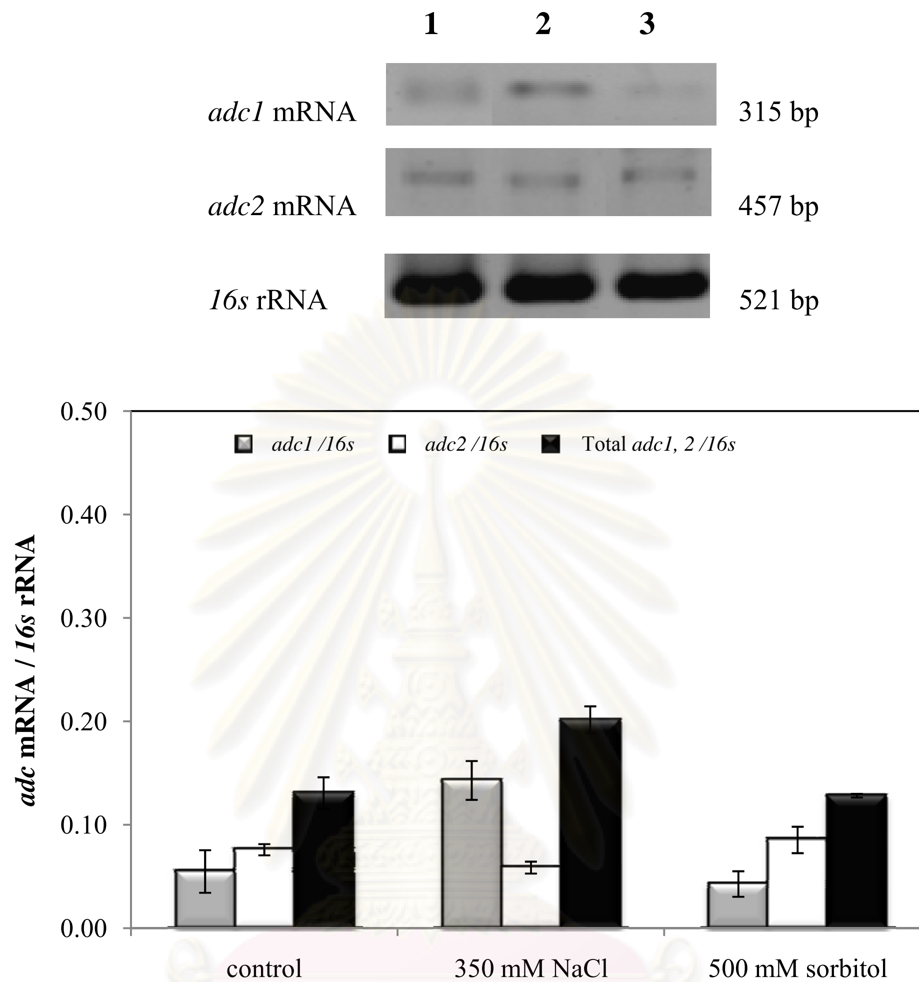

Figure 3.22 RT-PCR expression analysis of arginine decarboxylase (*adc1* and *adc2*) mRNA levels in *Synechocystis* sp. PCC 6803 exposed under UV-C exposure for 1 hour. Cells were grown in salinity treatment, namely, normal BG-11 media, control (lane 1), BG-11 containing 350 mM NaCl (lane 2) and 500 mM sorbitol (lane 3) respectively. The relative abundance of *16s* rRNA is also shown in lower row. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation.

3.5 Combination effects of salinity and UV-radiation on ADC protein

3.5.1 Effects of salinity under normal growth light

Total protein of *Synechocystis* cells under salinity treatment were extracted and observed by SDS-PAGE analysis (Figure 3.23A). The arginine decarboxylase (ADC) protein was analyzed by Western blot (Figure 3.23B). After western blot incubation with the antibody, it was immunodetected and found a single protein band with an apparent molecular mass of about 55 kDa. However, ADC proteins of cells were moderately increased under salt stress whereas highly increased under sorbitol stress, namely, BG-11 plus 500 mM sorbitol (Figure 3.23C).

3.5.2 Effects of salinity and UV-A stresses

Total protein of *Synechocystis* cells under combined stresses of salinity and UV-A radiation were extracted and performed by SDS-PAGE analysis (Figure 3.24A). The arginine decarboxylase (ADC) protein was analyzed by Western blot analysis (Figure 3.24B). After western blot incubation with the antibody, it was immunodetected and found a single protein band with an apparent molecular mass of about 55 kDa. The ADC protein levels of cells were slightly increased under 25 and 350 mM NaCl conditions whereas highly increased under sorbitol stress of 500 mM sorbitol (Figure 3.24C).

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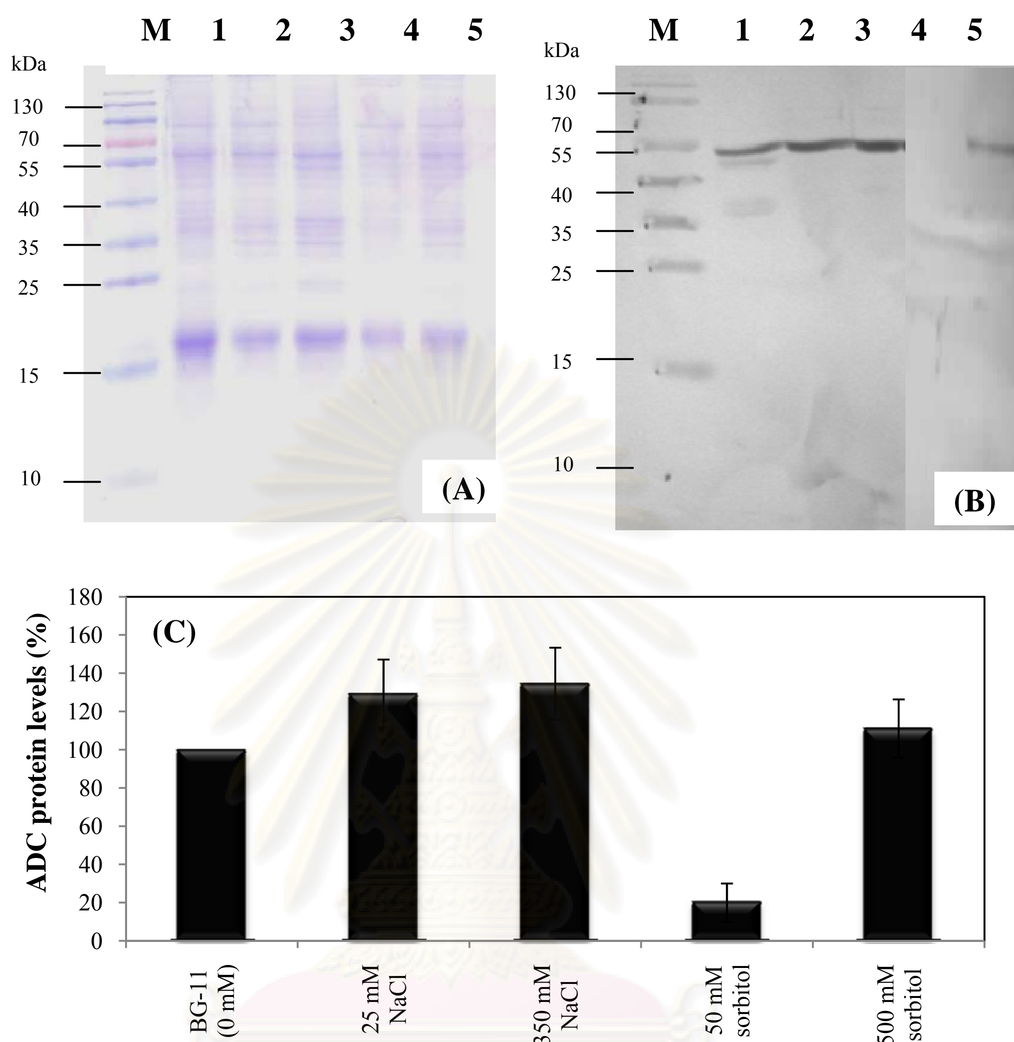

Figure 3.23 Western blot analysis of Arginine decarboxylase (ADC) under salt and sorbitol stresses from *Synechocystis* cells under normal growth light (B). Total protein extract (10 µg) was loaded onto the SDS-gel (A). After SDS-PAGE analysis. The gel was electrophoretically transferred onto PVDF membrane and probe for 4 h at room temperature with a 1:3000 dilution of antiserum against MBP-ADC. Lane M, standard marker ; Lane 1, control ; Lane 2, 25 mM NaCl ; Lane 3, 350 mM NaCl ; Lane 4, 50 mM sorbitol ; Lane 5, 500 mM sorbitol. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation (C). (100% ratio of 13,932)

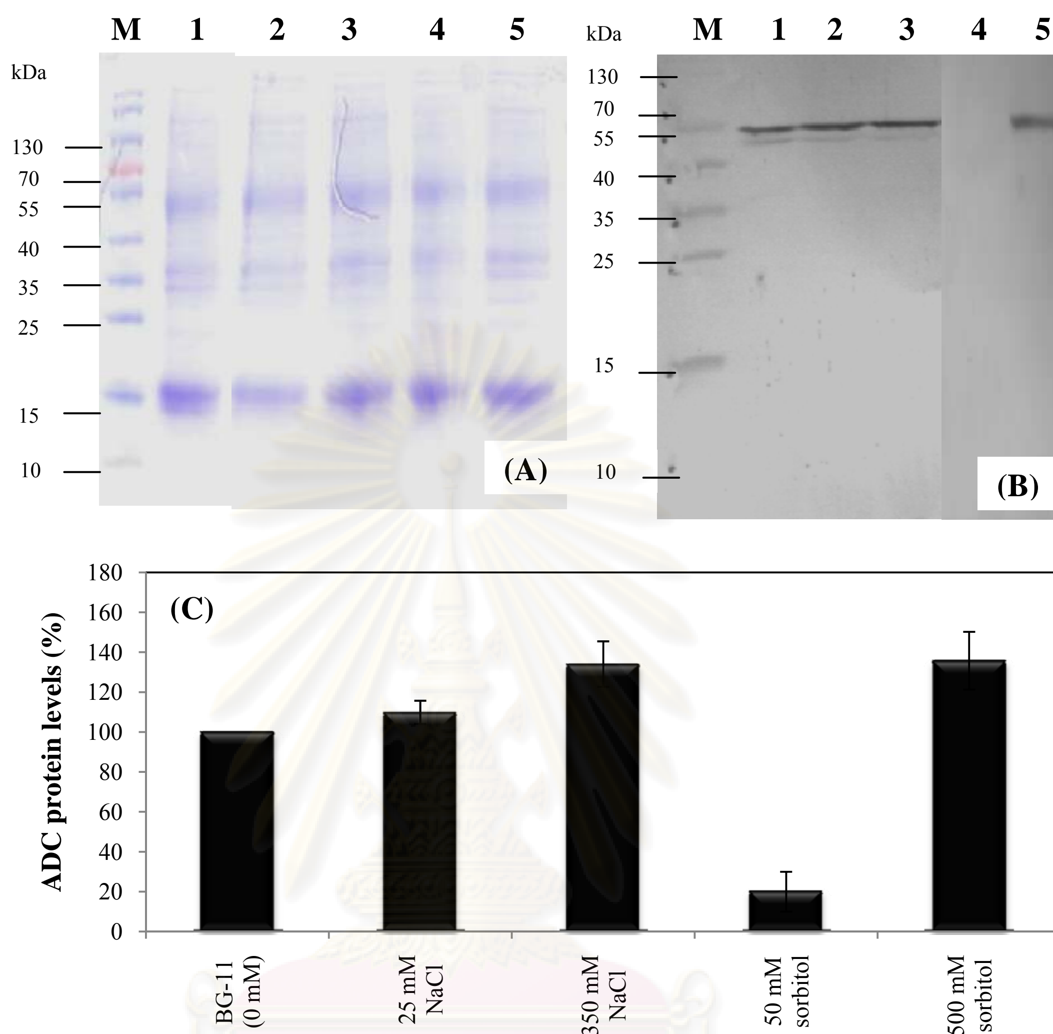

Figure 3.24 Western blot analysis of Arginine decarboxylase (ADC) under salt, sorbitol and UV-A stresses from *Synechocystis* cells (B). Total protein extract (10 μg) was loaded onto the SDS-gel (A). After SDS-PAGE analysis. The gel was electrophoretically transferred onto PVDF membrane and probe for 4 h at room temperature with a 1:3000 dilution of antiserum against MBP-ADC. Lane M, standard marker ; Lane 1, control ; Lane 2, 25 mM NaCl ; Lane 3, 350 mM NaCl ; Lane 4, 50 mM sorbitol ; Lane 5, 500 mM sorbitol. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation (C). (100% ratio of 15,979).

3.5.3 Effects of salinity and UV-B stresses

Total protein of *Synechocystis* cells under combined stresses of salinity and UV-B radiation were extracted and performed by SDS-PAGE analysis (Figure 3.25A). The arginine decarboxylase (ADC) protein was analyzed by Western blot analysis (Figure 3.25B). After western blot incubation with the antibody, it was immunodetected and found a single protein band with an apparent molecular mass of about 55 kDa. However, ADC protein of cells was significantly increased under 350 mM NaCl stress and 500 mM sorbitol stress. However, ADC protein of 500 mM sorbitol-treated cells was not consistent, it showed in trace amount (Figure 3.25C).

3.5.4 Effects of salinity and UV-C stresses

Total protein of *Synechocystis* cells under combined stresses of salinity and UV-C radiation were extracted and ran by SDS-PAGE analysis (Figure 3.26A). The arginine decarboxylase (ADC) protein was analyzed by Western blot analysis (Figure 3.26B). After incubation of western blot membrane with the antibody, it was found a single protein band with an apparent molecular mass of about 55 kDa. The treatments with 350 mM NaCl and 50 mM sorbitol-treated cells obviously induced the accumulation of ADC proteins when compared with untreated cells (control). However, ADC protein of 500 mM sorbitol-treated cells was not consistent, it showed in less amount. Although, under these stresses were as similar pattern of ADC protein as that under salinity and UV-B treatments, protein levels of salinity and UV-C treatments were higher than those of salinity and UV-B treatments (Figure 3.26C).

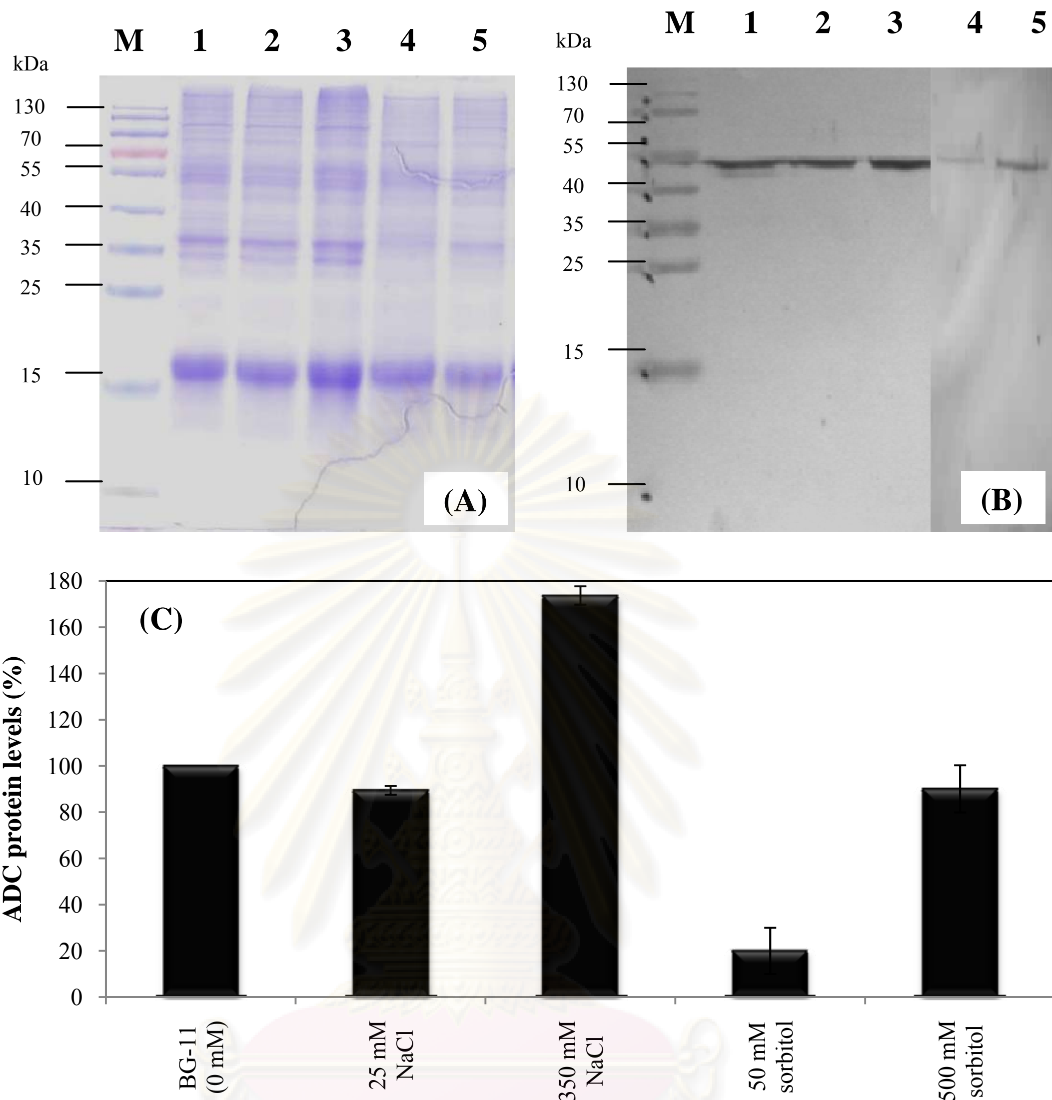

Figure 3.25 Western blot analysis of Arginine decarboxylase (ADC) under salt, sorbitol and UV-B stresses from *Synechocystis* cells (B). Total protein extract (10 µg) was loaded onto the SDS-gel (A). After SDS-PAGE analysis. The gel was electrophoretically transferred onto PVDF membrane and probe for 4 h at room temperature with a 1:3000 dilution of antiserum against MBP-ADC. Lane M, standard marker ; Lane 1, control ; Lane 2, 25 mM NaCl ; Lane 3, 350 mM NaCl ; Lane 4, 50 mM sorbitol ; Lane 5, 500 mM sorbitol. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation (C). (100% ratio of 16,055)

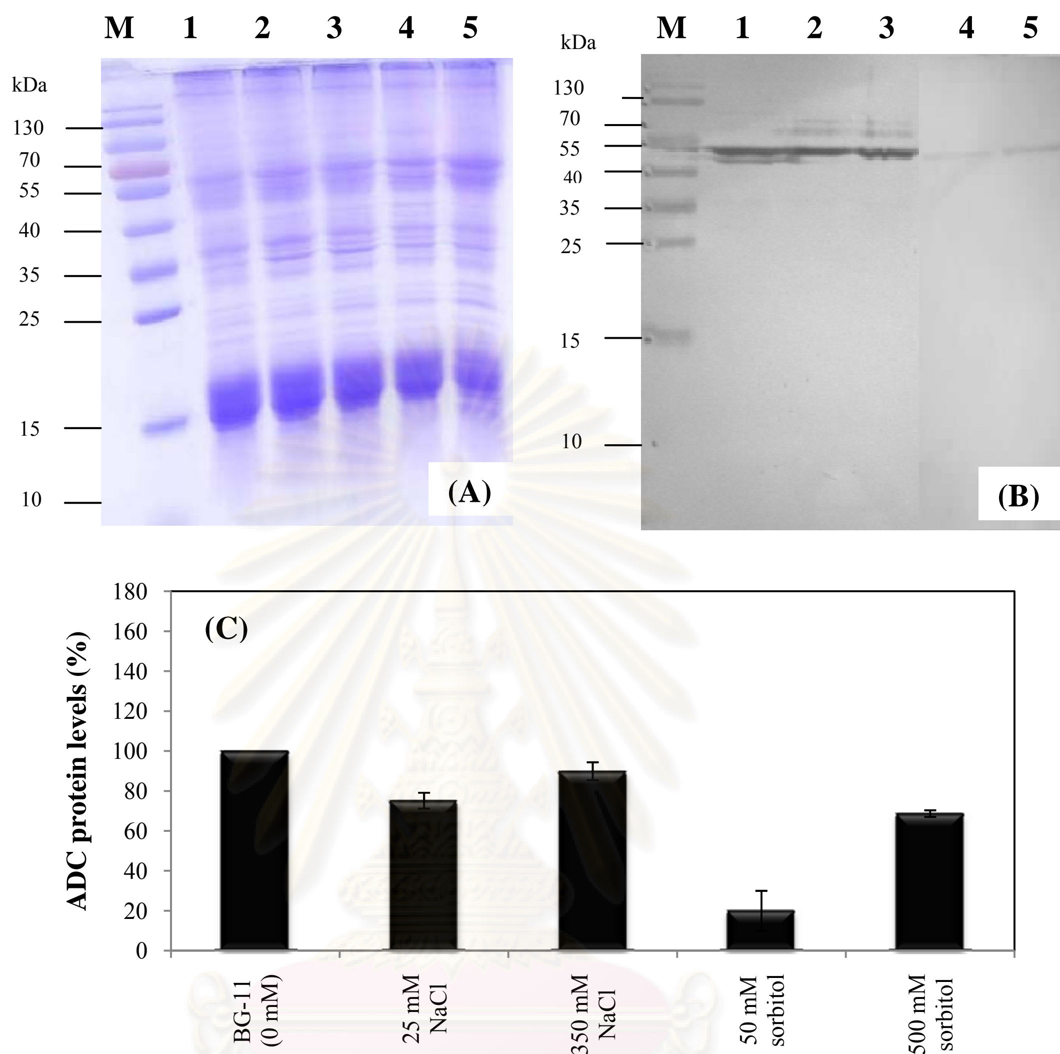

Figure 3.26 Western blot analysis of Arginine decarboxylase (ADC) under salt, sorbitol and UV-C stresses from *Synechocystis* cells (B). Total protein extract (10 µg) was loaded onto the SDS-gel (A). After SDS-PAGE analysis. The gel was electrophoretically transferred onto PVDF membrane and probe for 4 h at room temperature with a 1:3000 dilution of antiserum against MBP-ADC. Lane M, standard marker ; Lane 1, control ; Lane 2, 25 mM NaCl ; Lane 3, 350 mM NaCl ; Lane 4, 50 mM sorbitol ; Lane 5, 500 mM sorbitol. Quantification was carried out using GeneSnap program from Syngene® Gel Documentation (C). (100% ratio of 3,876)

CHAPTER IV

DISCUSSION

4.1 Effects of salinity and UV radiation on growth and content of intracellular pigments

We have demonstrated that 6-7 days culture of *Synechocystis* sp. PCC 6803 under salinity treatments was at mid-logarithmic phase. These results represented a long term stress of cells on growth and content of intracellular pigments, chlorophyll a and carotenoids under ionic and osmotic stresses (Figure 3.1 and 3.2, respectively). A previous study by Jantaro *et al*. (2003) showed that *Synechocystis* cells had increased growth rate up to 550 mM NaCl as well as the growth of cells under untreated condition for long-term stress. While under osmotic stress, the growth of cells grown in sorbitol treatment was decreased upon increasing of sorbitol concentrations. However, no growth was observed at 700 mM sorbitol or higher. In our study, the growth of cells was decreased slightly at high concentration of NaCl and sorbitol, especially 500 mM sorbitol, as well as on their pigment contents. These results suggest that long term stress of *Synechocystis* cells up to 650 mM NaCl or 500 mM sorbitol did not affect severely on growth, chlorophyll a and carotenoid contents of cells under normal growth light. It was reported recently that salt stress led to a decrease in the total chlorophyll content in *Hordeum vulgare* whereas the carotenoids content were not affected by NaCl treatment (Cakirlar *et al*., 2008). Moreover, there is an interesting evidence that salt stress could induce the antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase in the rice (*Oryza sativa* L.) (Lee *et al*., 2001).

We have demonstrated that the UV treatment affected growth and intracellular pigments, compared to those cells under normal growth light (control) (Figures 3.3 and 3.7). Our results showed that the growth of cells under UV-A alone was slightly decreased but was obviously affected after combining with high concentration of salt (Figure 3.4a) or sorbitol (Figure 3.8a). UV-B radiation affected the growth of cells when treated with NaCl (Figure 3.5a) or sorbitol (Figure 3.9a). However, only synergistic effect of sorbitol and UV-C (Figure 3.10a) decreased the growth of cells rather than UV-C plus salt stress. Similar finding was reported by Shinkle *et al.* (2004) that the growth of cells showed different responses to three UV wavebands of short wavelength UV-C, long wavelength UV-B and UV-A. This study it was shown that the content of chlorophyll was no significantly affected by UV radiation (Figures 3.4b-3.6b and 3.8b-3.10b, respectively).

Photosynthesis is dependent on the light harvesting properties of the chlorophylls (Gao *et al*., 2004). Our results showed that salt and sorbitol treatments stimulated chlorophyll a accumulation higher than cells grown in normal BG-11 (Figures 3.3b and 3.7b). However, these contents did not change markedly within 3 hours of treatments under normal growth light. Similarly, UV treatments did not affect the chlorophyll content of *Synechocystis* cells after exposing to UV-A (Figures 3.4b and 3.8b), UV-B (Figure 3.5b and 3.9b) and UV-C (Figure 3.6b and 3.10b), respectively, within 3 hours of treatments. Many studies reported that UV radiation had an effect the content of pigments, such as the reduction of chlorophyll content in *Capsicum annuum* L. under UV-B and UV-C (at 27 min/day for 14 days) but was not significantly decreased under UV-A radiation (Mahdavian *et al*., 2008). Moreover, salt stress as well as UV-B led to a decrease of the total chlorophyll content in

Lactuca sativa (Caldwell and Britz, 2006), *Hordeum vulgare* (Cakirlar *et al*., 2008) and *Pisum sativum* L. (Agrawal and Mishra, 2009). The reduction of chlorophylls by UV-B may be expected to result in lower levels of biomass accumulation and, hence, be a useful indicator of UV-B sensitivity (Smith *et al*., 2002). Then, we could say that *Synechocystis* 6803 could maintain its chlorophyll accumulation under 3 hours of UV-stresses. On the other hand, many reports showed that the light-harvesting protein pigments in cyanobacteria are readily affected by UV-B as a consequence of reducing their photosynthetic and metabolic activity (Aráoz and Häder, 1997) and also indicated that the photosynthesis of cyanobacterium *Spirulina platensis* was inhibited by UV-B radiation for at least 4 hours (Wu *et al*., 2005). The reduction of the chlorophyll content has a negative effect on plant photosynthetic efficiency by UV radiation (Cakirlar *et al*., 2008; Mahdavian *et al*., 2008). The high levels of UV-B may reduce phycobilisomes related fluorescence and PSII activity in *Arthrospira* (Rajagopal *et al*., 2005). Damage to D1 protein is known to be responsible for the inhibited activity of PSII (Sass *et al*., 1997). UV-B irradiation alone and with salt significantly decreased the Fv/Fm of four Barley (*Hordeum vulgare* L.) cultivars studied whereas salt stress alone had no effect (Cakirlar *et al*., 2008).

Moreover, we determined the carotenoids content of cells for 3 hours. The results showed that the content of carotenoids under salt and osmotic conditions were higher than those of cells grown in normal BG-11 (Figures 3.3c and 3.7c). This is similar to a previous report that carotenoids are not affected by NaCl treatment (Cakirlar *et al*., 2008). After *Synechocystis* was exposed to UV radiations, the level of carotenoids showed different patterns, under UV-A both alone and combination with salt stress. The carotenoids levels were constant and slightly increased during the last

3 hours (Figure 3.4c). This result is consistent with the previous report by Mahdavian *et al*. (2008) who found that the carotenoids content of UV-A treated plants was no significant changes when compared to control (under normal growth light). Moreover, carotenoids content in our study showed a slight enhancement under UV-B plus sorbitol treatments (Figure 3.9c). Coincidently, the previous report demonstrated that UV-B irradiation caused an increase by 5–20% of carotenoids content of all cultivars (Cakirlar *et al*., 2008) and this may be effective in protecting the photosynthetic apparatus against UV-B (Rakhimberdieva *et al*., 2004). On the other hand, lutein was the major carotenoid in the lettuce samples with less amounts of β-carotene and neoxanthin (Caldwell and Britz, 2006). It was reviewed previously in higher plants that photosynthetic pigments such as chlorophylls in plant and phycobilins in cyanobacteria can act as photosensitizers and produce ROS under UV or visible light excess by reaction of chromophore triplet states with molecular oxygen (Rinalducci *et al*., 2006). The efficacy of carotenoids in protecting the photosystems and acting as scavengers of ROS, thus protecting chlorophylls against photooxidative damage by interaction with triplet form of ROS is likely due to their function as efficient quenchers of high energy short wave radiation (Krinsky, 1979; Mahdavian *et al*., 2008).

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4.2 Effects of salinity and UV radiation on polyamine biosynthesis

In the present study, we found PCA-soluble polyamines as the major form. Several work showed the evidence that the free molecular bases of polyamines (PAs) were commonly found in nature (Bouchereau *et al.*, 1999; Wang and Liu, 2009). High accumulation of free PAs in callus exposed to stresses can be considered to

directly participate in stress defense via membrane rigidification or free radical scavenging (Shen *et al*., 2000) and also possible via activation of some antioxidant systems by indirect role (Wang and Liu, 2009). In this study, polyamines were accumulated by salt and osmotic stresses, especially in 650 mM NaCl (Figure 3.11) and 250 mM sorbitol (Figure 3.15) treated-cells, respectively, under normal growth light. The cellular levels of polyamines in *Synechocystis* cells were induced by long-term osmotic stress and to a less extent by salt stress (Jantaro *et al*., 2003). In our study, it was found that PCA-soluble spermidine was present as the major type of polyamines in all conditions. Many reports recently showed that the free spermidine content was observed among high salinized conditions (Jantaro *et al*., 2003; Maiale *et al*., 2004). Free spermidine was the most abundant polyamine levels against salt concentration (Tassoni *et al*., 2008). Moreover, several reports indicated that high titers of spermidine and/or spermine, but not putrescine, are correlated with the response of plants to long-term salinization (Sanchez *et al*., 2005; Jiménez-Bremont *et al*., 2007). For other common polyamines, namely putrescine and spermine, it was hardly detected both in PCA-soluble and insoluble forms of polyamines. The previous report by Jantaro *et al.* (2003) revealed that putrescine appeared to be unaffected by osmotic stress. Moreover, the PCA-insoluble conjugated polyamines were detected only in trace amounts (Tassoni *et al*., 2008). Additionally, spermine was found least abundant (Wang and Liu, 2009) and not synthesized in most prokaryotes (Pegg, 1983) and also do not represent a salt tolerance trait under salt stress (Maiale *et al.,* 2004). Several reports have supported a protective role of spermidine and spermine against salinity. From previous study, it has been suggested that the main role of polyamines is to maintain a cation-anion balance in a long term

of salt treatment in tomato leaves (Santa-Cruz *et al.,* 1997). In *Synechocystis* sp. PCC 6803, a similar role of polyamines is also likely in view of the fact that intracellular Na^+ would be maintained at a low level by the functional Na^+/H^+ antiporter under salt stress (Hamada *et al*., 2001), thereby making anions especially Cl^- in excess.

We have demonstrated the cellular polyamines levels in *Synechocystis* cells after treating with salt or sorbitol treatments and exposed to UV radiation. The report presented by An *et al.* (2004) showed that polyamine synthesis could be induced by a lower dose of UV-B radiation but such long-time treatment (7 h per day for 25 days). Their results are in agreement with our study that cells after exposing to UV radiation for 1 hour showed significant increased in their polyamine contents, especially under salt stress combined with either UV-B or UV-C (Figures 3.13a and 3.14a), respectively. However, long-time treatment of UV radiation decreased the polyamine accumulation, especially under osmotic treatments (Figures 3.16b, 3.17b and 3.18b). This result was similar to a decrease of these contents at long-time treatment of UV-B radiation in cucumber leaves (An *et al*., 2004). On the other hand, ionic stress slightly increased polyamines within 1 hour-stress (Figures 3.12b, 3.13b and 3.14b). Coincidently, UV-B radiation treatment caused increases in the contents of putrescine, spermine, and spermidine in cucumber (An *et al.,* 2004). Changes in polyamine contents have also been reported in response to different stresses (Mansour *et al*., 2000). An interesting report indicates that polyamine accumulation is a non-specific response to salt stress (Ashraf and Harris, 2004).

The polyamines including putrescine, spermidine and spermine were found associating with light harvesting complex (LCH) and the photosystem II (PSII) in higher plants (Kotzabasis *et al*., 1993). Many results have confirmed that the increase

in intracellular polyamine contents played an important role in growth and developmental processes of plants and in stress resistance (An and Wang, 1995). Polyamines, especially the thylakoid associated polyamines, play a decisive role in protecting photosynthetic apparatus and resist to UV-B treatment (Lütz *et al*., 2005; Unal *et al*., 2008).

In this study the transcription level of arginine decarboxylase was also investigated. The results showed that the increase of *adc* mRNA of *Synechocystis* sp. PCC 6803 was highest among cells treated with high concentration of NaCl (350 mM) and sorbitol (500 mM). Salt stress caused the increase in *adc* mRNA more profoundly than osmotic stress (Jantaro *et al*., 2003). One recent research also mentioned that salt stress efficiently activated ADC expression in *Citrus sinensis* (Wang and Liu, 2009). The expression of two different genes encoding ADC (*adc*1 and *adc*2) has not been reported previously in *Synechocystis*. Then, our results demonstrated for the first time the effects of either salt or sorbitol stress combined with UV radiation on *adc*1 and *adc*2 mRNA levels in *Synechocystis* cells by RT-PCR approach. The *adc1* mRNA level was more up-regulated than *adc2* mRNA level under short-term NaCl stress (Figure 3.19). Therefore, the result suggests that *adc1* was an inducible gene whereas *adc2* was a constitutive gene, under salt stress (350 mM NaCl). Osmotic stress seemed to have no effect on *adc1* and *adc2* mRNA levels. On the other hand, many reports showed that the S-adenosylmethionine decarboxylase 1 (*samdc1*) gene was expressed at higher levels than *samdc2* in *Arabidopsis*. Furthermore, in *Arabidopsis***,** spermidine synthase 2 gene (*spds2*) was more expressed than spermidine synthase 1 (*spds1*) (Soyka and Heyer, 1999; Tassoni

et al., 2008). All of these findings indicated that the ADC pathway was tightly connected to the salt stress response (Liu, *et al.*, 2006).

Furthermore, we have investigated the level of ADC1 protein under salt and sorbitol stresses. The ADC1 protein was slightly increased under high concentration of salt and sorbitol (Figure 3.23). The total polyamine contents showed a similar pattern at transcriptional level, as well as translational level under UV-B combined with salt stress. The results indicated that salt stress connected tightly on transcription and translation levels under normal growth light (Figures 3.19 and 3.23). Moreover, salt stress combined with UV-A (Figures 3.20 and 3.24) and combined with UV-B (Figures 3.21 and 3.25) also showed those level correlations. Osmotic stress did not affect highly on both transcription and translation levels under normal growth light whereas it seemed abolish a tight correlation of both levels when combined osmotic stress with UV radiation (Figures 3.19 and 3.23). However, ADC protein of cells treated with UV-C plus salinity was down-regulated when compared to control (without either salt or sorbitol) (Figure 3.26). However, the apparent change in the pattern of *adc1* transcription level was more than ADC1 protein level. These results indicate that UV-C alone or combined with salt or sorbitol had influence on the transcription and translation levels in *Synechocystis* cells under short-term stress (1 hour). In this research, the size of ADC protein was 55 kDa in *Synechocystis*. In higher plants such as oat, ADC was originally reported to be cleaved into a 42 kDa N-terminal and a 24 kDa C-terminal part that are held together with a disulfide bond (Malmberg *et al.* 1992). Similarly, a 42 kDa part of a processed form of *A. thaliana* ADC has been detected *in vivo* (Watson and Malmberg, 1996). However, *Arabidopsis* bears an open reading frame which encodes a 76 kDa of protein, a

monoclonal antibody produced against its product recognized a 42 kDa protein in Western blot (Watson and Malmberg, 1996). On the other hand, ADC enzyme of *Brassica campestris* (a species in the same family as *Arabidopsis*) was found to be homotetramer, with a subunit molecular mass of 60 kDa (Das *et al.*, 1995) and the *adc* of *Brassica juncea* encodes a 76 kDa protein (Mo and Pua, 1998). Jantaro *et al*. (2006) suggested that *Synechocystis* ADCs are post-translationally regulated.

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CHAPTER V

CONCLUSION

Based on the results, the following specific conclusion were drawn :

1. The long-term stress (6-7 days) of *Synechocystis* cells under up to 650 mM NaCl or 500 mM sorbitol condition did not affect severely on growth, chlorophyll a and carotenoid contents of cells under normal growth light.

2. The growth of *Synechocystis* cells showed different responses after exposing to UV radiations for 3 hours.

3. For salt and sorbitol stressed-cells, the increase in chlorophyll a and carotenoids level was higher than cells grown in normal BG-11.

4. The content of chlorophyll was not significantly affected by UV radiations.

5. The content of carotenoids showed slightly increases at last 3 hours under UV-A both alone and combination with salt stress, and UV-B plus sorbitol treatments.

6. PCA-soluble polyamines were found as major forms and PCA-soluble spermidine was present dominantly in all conditions, rather than putrescine and spermine.

7. Polyamines were up-regulated by salt and osmotic stresses, especially in 650 mM NaCl and 250 mM sorbitol treated-cells, respectively, under normal growth light.

8. Short-term stress (1 hour) of UV radiations significantly increased the polyamine contents, especially under salt stress combined with either UV-B or UV-C exposure.

9. Long-time stress (3 hours) of UV radiations decreased the intracellular polyamine accumulation, especially under osmotic treatments.

10. The *adc1* mRNA level was more up-regulated by salt stress than *adc2* mRNA level. Therefore, *adc1* was an inducible gene whereas *adc2* was a constitutive gene under 350 mM NaCl condition.

11. Osmotic stress did un-affect on *adc1* and *adc2* mRNA levels obviously.

12. The ADC1 protein was slightly increased under high concentrations of salt and sorbitol.

13. The relationship of total polyamine contents was consistent to ADC transcription and protein levels under salt stress combined UV-B.

14. Both transcriptional and protein levels were connected by salt and osmotic stresses, salt treatment combined with UV-A and UV-B.

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15. UV-C alone or combined with salt or sorbitol stress had influenced on the ADC transcriptional and translational levels in *Synechocystis* cells, at least for short-term stress (1 hour).

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APPENDICES

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APPENDIX A

BG-11 medium (1,000 ml)

	Solid medium	Liquid medium
H_2O	947 ml	967 ml
Bacto-agar	15 g	-
100x BG-FPC*	10 ml	10 ml
189 mM Na_2CO_3	1 ml	1 ml
175 mM K_2HPO_4	1 ml	1 ml
6 mg/ml Ammonium ferric citrate	1 ml	1 ml
1 M TES	10 ml	-
30% $Na_2S_2O_3$ x $5H_2O$	10 ml	-
1 M Hepes-NaOH, pH 7.5	20 ml	20 ml

100x BG-FPC* (100 ml)	
$NaNO_3$	14.96 g
$MgSO_4.7H_2O$	0.75 g
$CaCl_2.2H_2O$	0.36 g
Citric acid	0.065 g
0.5 M Na-EDTA	55.4 µl
After autoclaved, add 10 ml of 1,000x Trace metal mix**	

1,000x Trace metal mix** (1,000 ml)	
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.221 g
$Na_2MoO_4.2H_2O$	0.390 g
$CuSO_4.5H_2O$	0.080 g
$Co(NO_3)_2.6H_2O$	0.049 g
Sterile filtrate, store at 4 ^{o}C	

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APPENDIX B

Chromatogram of standard curve of polyamines

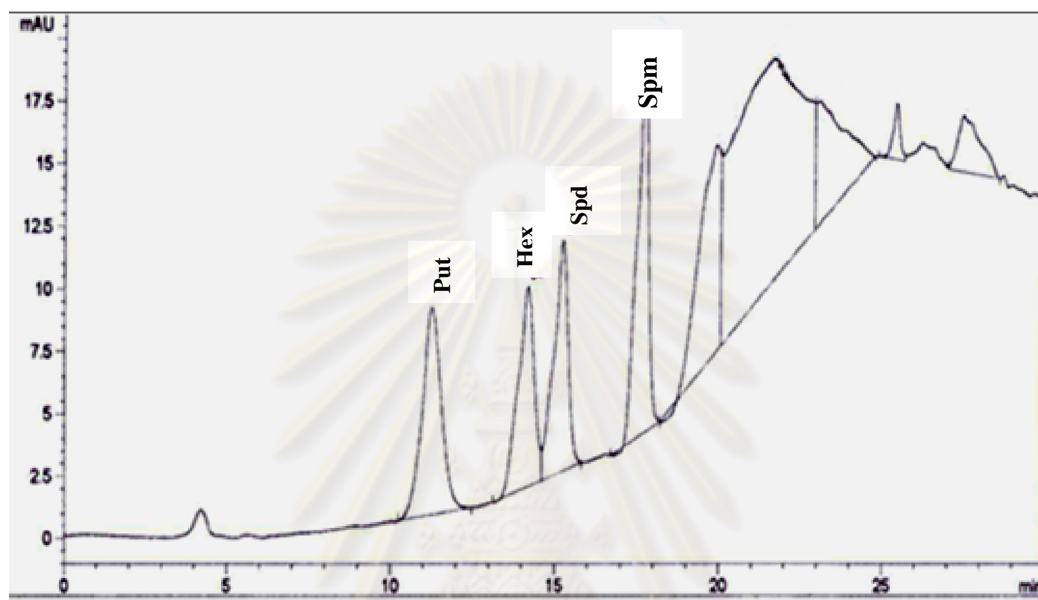

Figure B.1 Chromatogram of standard polyamines (Put = Putrescine, Spd = Spermidine, Spm = Spermine, respectively) and internal standard (Hex = 1,6-Hexanediamine) at each polyamines contents of 1.875 nmol.

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Standard curve of polyamines

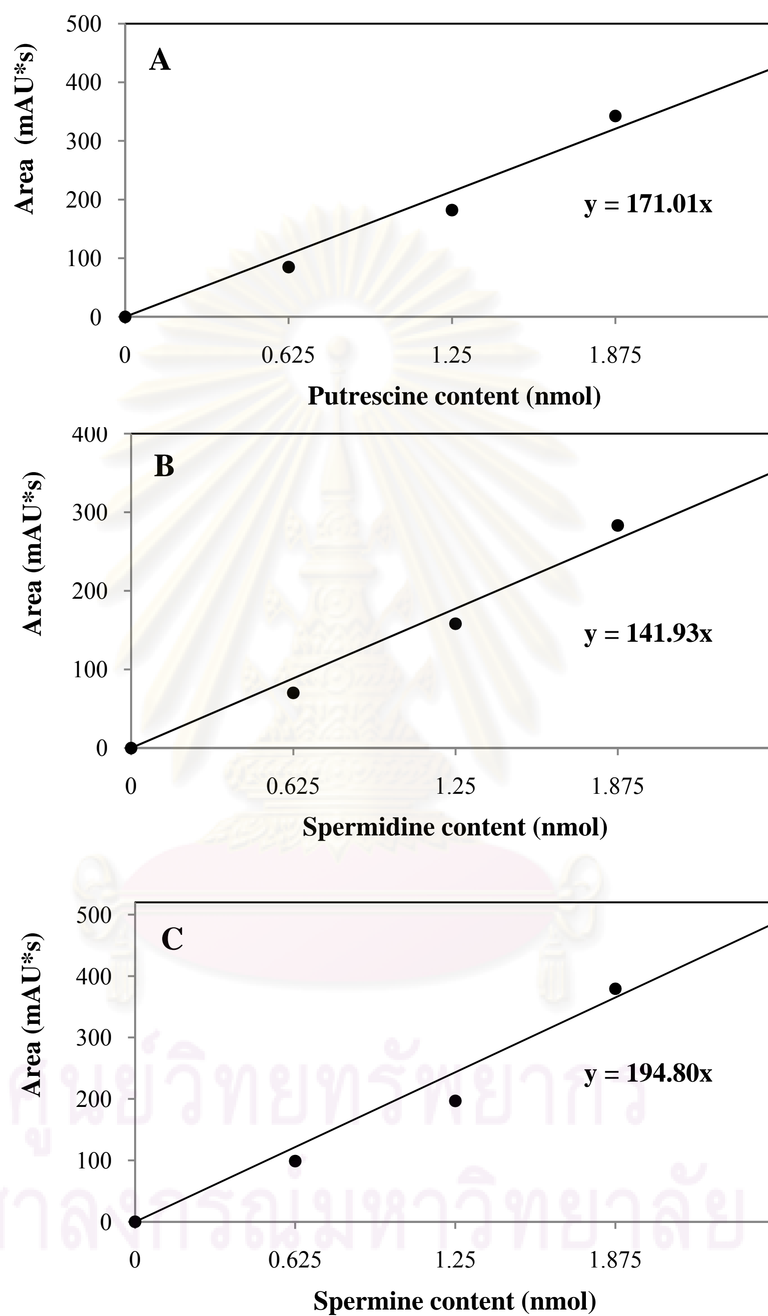

Figure B.2 Standard curve of polyamines (A = Putrescine, B = Spermidine and C = Spermine).

APPENDIX C

RNA extraction buffer

1. Resuspension buffer

   0.3 M sucrose

   10 mM sodium acetate, pH 4.5

2. Lysis buffer

   2% SDS

   10 mM sodium acetate, pH 4.5

3. RNA storage buffer

   20 mM Na-phosphate buffer, pH 6.5

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APPENDIX D

Protein extraction buffer containing:

50 mM Tris-HCl, pH 8.0

1 mM EDTA

50 μM Pyridoxal phosphate

5 mM DTT

0.5 mM PMSF

10 μM Leupeptin

10% (v/v) Glycerol

0.2% Triton X-100

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APPENDIX E

TAE buffer

1. **Working solution**

1X :	0.04 M Tris-acetate
	0.01 M EDTA

2. **Concentrated stock solution (1 L)**

50X :	Tris-base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA, pH 8.0	100.0 ml

Added distilled water to make 1 liter.

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APPENDIX F

Preparation for polyacylamide gel electrophoresis

Stock solution

Acrylamide solution, 100 ml (30% Acrylamide, 0.8% bis-acrylamide)

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

Added distilled water to make 100 ml and stirred until completely dissolved.

2.0 M Tris-HCl, pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

1.5 M Tris-HCl, pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

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1.0 M Tris-HCl, pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane l 2.10 g

Adjusted pH to 6.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

0.5 M Tris-HCl, pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjusted pH to 6.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

20% Sodium dodecyl sulfate (SDS), 100 ml

Sodium dodecyl sulfate 20.0 g

Added distilled water to make 100 ml and stored at room temperature.

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10% Ammonium persulfate (APS), 1 ml

Ammonium persulfate 0.1 g

Added distilled water to a total volume of 1 ml.

22.2% Glycerol, 100 ml

100% Glycerol	22.2 ml

Added distilled water to a total volume of 100 ml.

Working solution

Solution B (for SDS-PAGE), 100 ml

2 M Tris-HCl, pH 8.8	75 ml
10% SDS	4 ml

Added distilled water to a total volume of 100 ml.

Solution C (for SDS-PAGE), 100 ml

1 M Tris-HCl, pH 6.8	50 ml
10% SDS	4 ml

Added distilled water to a total volume of 100 ml

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SDS-PAGE

12% Separating gel (for 2 gel)

30% Acrylamide solution	4.17 ml
Solution B	2.50 ml
Distilled water	3.33 ml
10% APS	50.00 µl
TEMED	5.00 µl

5% Stacking gel (for 2 gel)

30% Acrylamide solution	1.67 ml
Solution C	2.50 ml
Distilled water	5.80 ml
10% APS	50.00 µl
TEMED	5.00 µl

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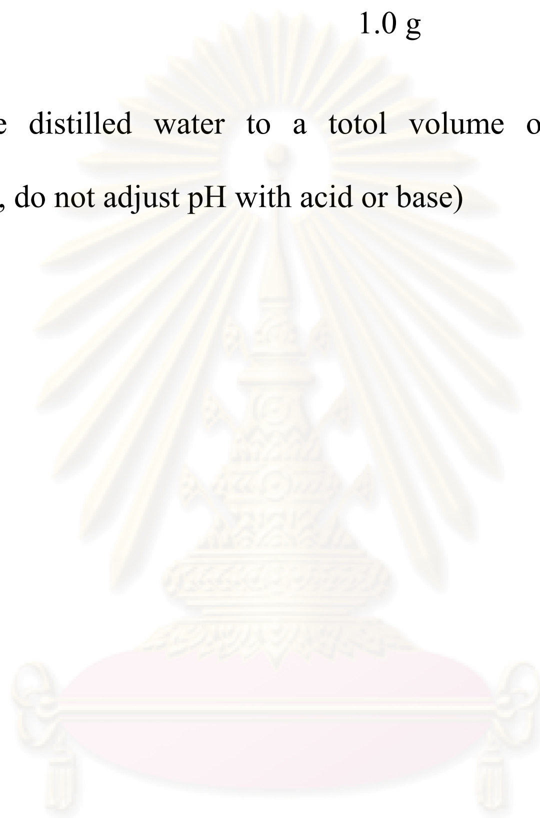
Electrophoresis buffer, 1 L (25 mM Tris, 192 mM Glycine, 0.1% SDS)

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

Added the distilled water to a totol volume of 1 liter. (Final pH is approximately 8.3, do not adjust pH with acid or base)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APPENDIX G

Western blotting buffer

10X Blotting solution (Used time = 1X)

100 mM Tris-HCl, pH 9.5

100 mM NaCl

10 mM $MgCl_2$

10X TBS (Tris-buffer-saline) (Used time = 1X)

200 mM Tris-HCl, pH 7.5

5 M NaCl

T-TBS

1X TBS

0.05% Tween-20

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Blocking solution

5% Skim milk in TBS

Antibody buffer

1% Skim milk in T-TBS

Coomassie Gel Stain, 1 L

Coomassie Blue R-250	1.0 g
Methanol	450 ml
Glacial acetic acid	100 ml

Added distilled water to a total volume of 1 liter.

Coomassie Gel Destain, 1 L

Methanol	100 ml
Glacial acetic acid	100 ml

Added distilled water to a total volume of 1 liter.

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5X Sample Buffer, 10 ml

1 M Tris-HCl, pH 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1.0 ml

Added distilled water to a total volume of 10 ml. Stored at 4 ^{o}C for weeks or -20 ^{o}C for months

BIOGRAPHY

Miss Apiradee Pothipongsa was born on February 17, 1985 in Songkhla provience, Thailand. She has graduated with a Bachelor of Science degree in Chemistry, Faculty of Science, Thaksin University in 2006. She has further studied for the Master of Science degree in Biochemistry, Faculty of Science, Chulalongkorn University since 2007.