การวิเคราะห์ไฟโคบิลินในคริปโทโมแนด *Rhodomonas salina*
และไซยาโนแบคทีเรีย *Synechococcus* spp. ในทะเล

นางสาวชนกนาฏ กาญจนภักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EVALUATION OF PHYCOBILINS IN MARINE CRYPTOMONAD *Rhodomonas salina* AND MARINE CYANOBACTERIA *Synechococcus* spp.

Miss Chanoknard Karnjanapak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Marine Science
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ชนกนาฏ กาญจนภักดิ์ : การวิเคราะห์ไฟโคบิลินในคริปโทโมแนด *Rhodomonas salina* และไซยาโนแบคทีเรีย *Synechococcus* spp. ในทะเล (EVALUATION OF PHYCOBILINS IN MARINE CRYPTOMONAD *Rhodomonas salina* AND MARINE CYANOBACTERIA *Synechococcus* spp.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.สุธาพร บุญญเจตน์พงษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ไทยถาวร เลิศวิทยาประสิทธิ, 139 หน้า.

ไฟโคบิลิโปรตีนเป็นรงควัตถุที่สามารถพบได้ในไซยาโนแบคทีเรีย (Cyanophyta) ส่าหร่ายสีแดง (Rhodophyta) และสาหร่ายคริปโทโมแนด (Cryptophyta) สามารถดูดกลืนคลื่นแสงในช่วงแสงสีเขียว สีส้ม และสีแดง ซึ่งเป็นช่วงคลื่นที่คลอโรฟิลล์และคาโรทีนอยด์ไม่สามารถดูดกลืนได้ ในการสกัดคลอโรฟิลล์และคาโรทีนอยด์นั้น ตัวทำละลายที่ใช้กันทั่วไป คือ อะซิโตนและเมทานอล แต่เนื่องจากไฟโคบิลิโปรตีนเป็นสารที่มีขั้วมาก จึงไม่สามารถสกัดด้วยตัวทำละลายดังกล่าว ดังนั้นวัตถุประสงค์ของการศึกษานี้ จึงต้องการพัฒนาวิธีการที่สะดวกต่อการสกัดและวิเคราะห์ไฟโคบิลิโปรตีนในแพลงค์ตอนพืช ซึ่งเพาะเลี้ยงในห้องปฏิบัติการและในธรรมชาติ ทั้งในเชิงปริมาณและคุณภาพ โดยการศึกษานี้ได้เลือกสาหร่ายคริปโทโมแนด *Rhodomonas salina* และไซยาโนแบคทีเรีย *Synechococus spp.* (RS9917) ซึ่งทราบชนิดของไฟโคบิลิโปรตีนที่ผลิต เมื่อทำการวิเคราะห์สัญญาณฟลูออเรสเซนต์ของเซลล์ *Rhodomonas salina* โดยใช้กลีเซอรอลเป็นตัวเหนี่ยวนำไฟโคบิลินให้ปลดออกจากสายโปรตีน (uncoupling protein) พบว่า สัญญาณฟลูออเรสเซนต์เพิ่มสูงขึ้นอย่างมาก ซึ่งให้ผลเหมือนกับที่เคยมีรายงานก่อนหน้าในไซยาโนแบคทีเรีย *Synechococus* ดังนั้นวิธีการดังกล่าวจึงอาจเป็นวิธีการใหม่ที่ใช้ศึกษาไฟโคบิลิโปรตีนในสาหร่ายคริปโทโมแนด ในการศึกษาลำดับถัดมา ได้ทำเก็บเกี่ยวเซลล์โดยการปั่นเหวี่ยง เพื่อศึกษาขั้นตอนการสกัดในบัฟเฟอร์ 4 ชนิด (บัฟเฟอร์ทริสไฮโดรคลอไรด์ บัฟเฟอร์อะซิเตท บัฟเฟอร์โซเดียมฟอสเฟต และบัฟเฟอร์โพแทสเซียมฟอตเฟส) ที่ความเข้มข้น 0.1 โมลาร์ pH 1- 9 โดยวิธีการแช่แข็ง ละลาย และสั่นด้วยคลื่นเสียง จำนวน 3 รอบ เพื่อให้เซลล์แตก จากการศึกษาพบว่า เมื่อบัฟเฟอร์เริ่มมีความเป็นด่าง (pH > 6) สารสกัดจะมีปริมาณคลอโรฟิลล์และคาร์โรทีนอยด์ปนออกมาเพิ่มขึ้น ดังนั้นก่อนที่จะทำการสกัดด้วยบัฟเฟอร์ จึงควรทำการสกัดแยกคลอโรฟิลล์และคาร์โรทีนอยด์ออกก่อน โดยใช้อะซิโตน 90 เปอร์เซ็นต์ การศึกษานี้ยังได้มีการทดลองเติม SDS ที่ความเข้มข้นและ pH ต่างๆ พบว่า SDS สามารถช่วยเพิ่มประสิทธิภาพในการสกัดให้มากขึ้น แม้จะทำให้ค่าการดูดกลืนแสงของสารสกัดเปลี่ยนแปลงไปบ้างในบางสภาวะ แต่เมื่อทดลองเติมยูเรียที่ความเข้มข้น 8 โมลาร์ pH 2 และกรดอะซิติก 20 เปอร์เซ็นต์ ลงในสารสกัดซึ่งมี SDS พบว่า ค่าการดูดกลืนแสงของบิลินสอดคล้องกับที่มีการรายงานก่อนหน้า จึงสรุปได้ว่า การเติม SDS เพื่อช่วยในการสกัดสารไม่ได้ทำลายโครงสร้างของบิลิน นอกจากนี้ยังได้มีการวิเคราะห์บิลินเปปไทด์ โดยการใช้เอนไซม์โปรตีเอส (protease) ในการย่อยไฟโคบิลิโปรตีนและทำการวิเคราะห์ด้วยเครื่อง HPLC พบว่า ควรปรับปรุงวิธีการ โดยเพิ่มความเข้มข้นของเอนไซม์และใช้เวลานานขึ้นในการย่อยไฟโคบิลิโปรตีน

ภาควิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต .....
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CHANOKNARD KARNJANAPAK: EVALUATION OF PHYCOBILINS IN MARINE CRYPTOMONAD *Rhodomonas salina* AND MARINE CYANOBACTERIA *Synechococcus* spp.. ADVISOR: SUTAPORN BUNYAJETPONG, Ph.D., CO-ADVISOR: ASSOC. PROF. THAITHAWORN LIRDWITAYAPRASIT, Ph.D., 139 pp.

Phycobiliproteins, present in Cyanophyta, Rhodophyta and Cryptophyta, harvest green, orange, and red regions of the spectrum where chlorophylls and carotenoids absorb poorly. Phycobiliprotins are not like chlorophylls and carotenoids that are extracted with acetone or methanol; therefore, they are difficult to analyze with methods routinely used for chlorophylls and carotenoids. The long-term goal of this work was to develop procedures for the rapid extraction and analyses, both quantitative and qualitative, of phycobilins that could be used in laboratory-grown cultures and more importantly from field-collected samples. Two model algae with well characterized phycobiliproteins were studied: *Rhodomonas salina* (cryptophyte) and *Synechococus* spp. RS9917 (cyanophyta). Glycerol-induced uncoupling of phycobiliproteins was studied using *in vivo* fluorescence assays of *R. salina* under two growth conditions; results with *R. salina* were surprisingly similar with published results thought to be unique to *Synechococcus*, suggesting this may be a new approach to study phycobiliproteins in cryptophytes. For phycobiliprotein isolation studies, cells were harvested by centrifugation and the cell pellets extracted with four 0.1 M buffers (Tris-HCl buffer; acetate buffer; sodium phosphate buffer; and potassium phosphate buffer) at different pH values (1-9). Pellets were disrupted by three cycles of freeze-thaw-sonicate, followed by centrifugation; supernatants were analyzed by absorption spectrophotometry. Co-extraction of phycobiliproteins and chlorophylls/carotenoids was problematic, especially at pH > 6; hence, cells were pre-extracted in 90% acetone to remove chlorophylls/carotenoids. Phycobiliprotein extraction was tested with different buffers/pH combinations, with and without added SDS and to a lesser extent, different concentrations of urea. SDS enhanced extraction of phycobiliproteins from cells pre-extracted in acetone, frequently yielding complete extraction. SDS resulted in altered spectral properties of phycobiliproteins in some, but not all cases. Treatment of SDS extracts with 8M acidic urea (pH 2) or 20% acetic acid resulted in bilin-protein spectra that were largely in line with published spectra; this observation indicates that inclusion of SDS as part of the extraction buffer does not irreversibly affect the bilins during isolation from cells and that the ‘native’ bilin spectra are recovered following dilution in acidic urea, perhaps amenable to quantitative analyses using published extinction coefficients. Phycobiliproteins were also analyzed both quantitatively and qualitatively by HPLC, using protease digested whole cell extracts; results demonstrated resolution of discrete bilin-peptide conjugates. Given the very high protein concentrations present in crude cell extracts, it is likely that protease treatments need to include very high enzyme concentrations and/or much longer digestion periods.

Department:	Marine Science	Student's Signature	.....
Field of Study:	Marine Science	Advisor's Signature	.....
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ABBREVIATIONS

AB	acetate buffer
AcOH	acetic acid
APC	allophycocyanin
A_{max} (λ_{max})	absorption maximum (wavelength, nm)
BV	biliverdin
Chl	chlorophyll
Chl *a/c*	chlorophyll *a/c* light-harvesting complex
Cys	Cysteine
Em	emission
Ex	excitation
DAD	diode array detector
DBV	dihydrobiliverdin
DG	Diago growth medium
Gly	glycerol
f/2	f/2 growth medium
F-T-S-C	freeze–thaw–sonicate cycles, centrifuge
g	relative centrifugal force
H_2O	water
HPLC	high performance liquid chromatography
K_e	specific growth rate
KDS	potassium dodecyl sulfate
KP	potassium phosphate buffer

L_{CM}	core–membrane linker
MBV	mesobiliverdin
MeOH	methanol
PBS	phycobilisome
PΦB	phytochromobilin
PC	phycocyanin
PE	phycoerythrin
PEB	phycoerythrobilin
PSI	photosynthesis system I
PSII	photosynthesis system II
PUB	phycourobilin
PVB	phycoviolobilin
PXB	phycobiliviolin
NaCl	sodium chloride
$(NH_4)_2SO_4$	ammonium sulfate
NMR	nuclear magnetic resonance
N_0	cell density (cell/mL)
NaP	sodium phosphate buffer
N_t	cell density in days (t) (cell/mL)
OD_{750}	optical density (absorption maximum at 750 nm)
R. salina	*Rhodomonas salina*
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S. griseus	*Streptomyces griseus*

TB	Tris-HCl buffer
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 Overview of Phycobilins/Phycobiliproteins Prevalence and Function

Phycobilins are the major accessory photosynthetic pigments found in Cyanophyta, Cryptophyta and Rhodophyta (Jeffrey & Vesk, 1997; Zhao, Porra, & Scheer, 2011). Phycobilins are important for primary production in freshwater and marine ecosystems.

Functionally, the phycobiliproteins absorb light energy, acting as so-called antenna, before transfer to one of the reaction center complexes, PSI and PSII, required for photosynthetic processes. In photosynthesis, the light reactions are initiated with the absorption of light energy by a pigment-protein complex, usually within an antenna complex. The excitation energy is transferred from one light-harvesting pigment to another before arrival at a reaction center, where charge separation occurs (Glazer, 1989b).

Qualitative and quantitative analysis of phycobiliproteins are important because these pigments provide insights into important ecological questions (e.g., oceanic rate processes, including those associated with remote sensing data) and are useful biomarkers for environmental issues (e.g., identifying harmful algal blooms and/or problems in aquaculture and eutrophication) (Jeffrey & Vesk, 1997; Lawrenz, Fedewa, & Richardson, 2011; Paerl & Otten, 2013; Zimba, 2012).

Remote sensing can be used to monitor ocean color on a global scale from space, providing important information on algal distribution, abundance, and productivity. The distinct absorption signal of phycobiliproteins make these pigments

relatively easy to detect from space; however, it has been problematic to relate these data to biomass and/or productivity and reviewed in (Neveux, Lantoine, Vaulot, Marie, & Blanchot, 1999). Another issue is the relative scarcity of sensors for wavelengths associated with phycobiliproteins; planned sensors such as the European Space Agency's Ocean Land Color Instrument (OLCI) and NASA's Hyperspectral Infrared Imager (HyspIRI) will provide improved spectral and spatial resolution in the near future (Kudela et al., 2015). In this regard, it is important to use quantitative analyses of phycobiliproteins to calibrate pure phycobiliproteins because it is essential to predict the distribution of phytoplankton abundance (e.g. cyanobacteria) and production in the ocean (Jeffrey & Vesk, 1997). Development of methods for qualitative and quantitative analyses of phycobiliproteins is the major focus of this thesis.

1.2 Phycobilins, Apoproteins, and Phycobiliproteins

The phycobilin chromophores are the pigment moieties of bilin-protein complexes known as phycobiliproteins, with the bilin covalently bound to cysteine of apoproteins by thioether bonds (Zhao et al., 2011). Phycobilins consist of an open chain tetrapyrrole. The major pigment-binding apoproteins of phycobiliprotein are two dissimilar polypeptide chains referred to as α and β subunits. Each polypeptide chain carries one or more covalently attached bilins (Glazer, 1989b; Sekar & Chandramohan, 2008).

The phycobiliproteins are organized into macromolecular structures in red algae and cyanobacteria called phycobilisomes (Sekar & Chandramohan, 2008; Zilinskas & Greenwald, 1986). Phycobiliprotein of cyanobacteria and red algae have

the monomer (αβ), with the aggregation states being the trimer $(\alpha\beta)_3$ and hexamer $(\alpha\beta)_6$ (Zhao et al., 2011; Zilinskas & Greenwald, 1986). Other low-abundance proteins in phycobilisomes include linker polypeptides and/or subunit γ (Glazer, 1982, 1989b), which generally, but not always, bind pigments.

Cryptophytes lack true phycobilisomes (Zilinskas & Greenwald, 1986). The phycobiliprotein complex of *Rhodomonas salina*, referred to as PE545, is arguably the most well characterized phycobiliprotein of cryptophytes (Doust, Wilk, Curmi, & Scholes, 2006; MacColl et al., 1998; Wilk et al., 1999). PE545 contains four apoproteins, α_1, α_2, plus two β subunits. These apoproteins are arranged into a complex known as a dimer of αβ monomers (van der Weij-De Wit et al., 2006).

The individual phycobilins have broad (and diverse) absorption spectra *in vivo*, as compared to *in vitro*, in different algae; these differences are related to differences in 'local environments', including pigment-protein interactions, 'package effects', and a number of other considerations (Bricaud & Stramski, 1990; Morel & Bricaud, 1981). The optical absorption of phycobiliproteins is in the range of 500 - 650 nm, wavelengths that are poorly absorbed by chlorophylls and carotenoids (Zhao et al., 2011). For example, the absorption wavelength of blue phycocyanins is approximately 600 nm, the blue-green allophycocyanin is approximately 650 nm, and the red phycoerythrin is approximately 550 nm (Zhao et al., 2011).

Historically, the biochemical analyses of phycobilins, phycobiliproteins, and phycobilisomes benefited from the judicial use of proteases (references - with Glazer front and center). Briefly, protein fractions were isolated (as described above) and then subjected to digestion with protease (e.g., trypsin, pepsin and pronase) (G. Wedemayer, Kidd, Wemmer, & Glazer, 1992). These enzymes digest peptide bonds

in different positions and provide different results, resulting in smaller chromophore-peptides which could be more easily analyzed than the larger structures (e.g., structural determinations by NMR spectroscopy and mass spectrometry) (G. Wedemayer et al., 1992).

1.3 Quantification and Isolation of Phycobiliproteins

Absorption and/or fluorescence spectroscopy historically have been used to characterize phycobiliproteins, both qualitatively and quantitatively. Such measurements could be, and continue to be, made using whole cell suspensions, derived either from natural samples or laboratory cultures (Allali, Bricaud, Babin, Morel, & Chang, 1995; Wyman, 1992; Wyman, Gregory, & Carr, 1985). More recent methods have relied upon advanced technology for whole cell work (e.g., pump-probe fluorescence) (Boulay, Abasova, Six, Vass, & Kirilovsky, 2008).

Early attempts at phycobiliprotein isolation, there are different investigators who have employed a variety of methods for extraction, including chemical and/or enzymatic digestion, grinding with and without glass splinters, and/or addition of detergents (Abalde, Betancourt, Torres, Cid, & Barwell, 1998; Hemlata & Fareha, 2011; Lawrenz et al., 2011; Stewart & Farmer, 1984; Sudhakar, Jagatheesan, Perumal, & Arunkumar, 2015; Viskari & Colyer, 2003; G. Wedemayer et al., 1992). A commonly used protocol, both historically and in current use, for isolation of phycobiliproteins is a freeze-thaw method, sometimes incorporating a mechanical step (e.g., sonication) (Lawrenz et al., 2011).

1.4 The Problem: Qualitative and Quantitative Analysis of Phycobiliproteins from Field Samples

Protocols for isolation and/or quantification of phycobiliproteins have been well developed (see above). However, these protocols invariably involve starting with large algal cultures and/or large amounts of field-collected biomass (e.g., red algae). The protocols involve different extractions methods (frequently species dependent) followed by long and involved purification steps (e.g., ammonium sulfate precipitation, sucrose density gradient centrifugation, column chromatography, PAGE, etc.) (Glazer, 1982, 1989b; Rowan, 1989; G. Wedemayer et al., 1992; Zhao et al., 2011).

These well-developed protocols for phycobiliprotein isolation/quantification are not suitable for many purposes. Two notable examples include: 1) laboratory work with small volumes and/or a requirement for numerous sample points, frequently over short time periods and; 2) field studies involving numerous samples, frequently with low biomass, of which only a fraction is related to phycobilin-containing algae (i.e., samples contain other algae, bacteria, zooplankton and/or detritus).

The most serious limitation for quantification of phycobilins relates to pigment extraction; these covalently linked pigment-protein complexes, unlike the pigment-associated (but not covalently attached) chlorophylls and carotenoids, are not extracted with nonpolar solvent (e.g., acetone or methanol) (Mantoura, Jeffrey, & Llewellyn, 1997). Extraction of phycobiliproteins from some algae, notably cryptophytes, may be relatively straightforward, requiring aqueous buffers with neutral (to slightly acidic) pH values combined with freeze-thaw (and/or sonication) for cell disruption (Lawrenz et al., 2011). Phycobiliprotein extraction from most algae

is more difficult. Excellent summaries of phycobiliprotein extraction are available (Lawrenz et al., 2011; Zimba, 2012).

Given the limitations associated with phycobiliproteins (Lawrenz et al., 2011; Zimba, 2012), it is not surprising that little effort has been placed on quantitative analyses of extracted phycobilins and/or phycobiliproteins (e.g., Zimba used the Bryant-modified equations of (Bennett & Bogorad, 1973) while Lawrence et al. failed to calculate pigment concentrations, reporting results primarily as 'percent'). HPLC methods are available for phycobiliproteins; however, the protocols have been perfected with samples that have undergone extensive purification steps (as outlined above), which may, or may not, be suitable for crude cell extracts and/or samples with low protein concentrations (such as those from field work).

1.5 Research Objectives

1. To determine a suitable technique for extraction of phycobiliproteins
2. To develop a rapid and convenient method for isolation and analyze (The chosen analytical protocol involves a combination of absorption and fluorescence spectroscopy coupled with HPLC.)

1.6 Benefits

This research will provide an important fundamental tool of phycobilin analyses to help Thai researchers understand and monitor many problems occurring in our sea, including eutrophication, oxygen depletion and pollution.

CHAPTER II

LITERATURE REVIEW

2.1 Structure and Function of Phycobiliproteins

Plants and algae have a large number of pigments and proteins in their photosynthetic light-harvesting pigment-protein complexes. These complexes absorb light energy and transfer photons from excited-state energy to photosynthetic reaction centers, where charge separation across the photosynthesis membrane takes place (Becker, Stubbs, & Huber, 1998; Glazer, 1989b). In most marine phytoplankton, chlorophylls (Chl) and carotenoids are the main pigments that preform light-harvesting. However cyanobacteria, red algae and cryptomonad algae utilize carotenoids and, more importantly, accessory photosynthetic pigments called phycobiliproteins; these are water soluble pigment-protein complexes characterized by linear tetrapyrrolic chromophores known as bilins that are covalently bound to cryteines of apoproteins by thioether bonds (Jeffrey & Vesk, 1997; Zhao et al., 2011).

Phycobiliproteins efficiently harvest light in the green, orange and red regions of the spectrum where Chloropylls and carotenoidsabsorb poorly. The phycobiliproteins of marine organisms allow them able to conduct photosynthesis in water depths that have only green light (Zilinskas & Greenwald, 1986).

Phycobiliproteins from cyanobacteria and red algae are found as the major component of macromolecules called phycobilisomes, which are attached to the stroma side of thylakoid membranes (Zhao et al., 2011). Phycobilisomes act like an energy funnel and transfer light energy to reaction centers in membranes (Zilinskas & Greenwald, 1986). Phycobilisomes consist of dissimilar polypeptides, α and β

subunits of apoproteins and each polypeptide chain carries one or more bilin chromophores (Glazer, 1989b; Zhao et al., 2011). The building block of phycobilisomes is the monomer (αβ) with the aggregation states being the trimer $(\alpha\beta)_3$ and hexamer $(\alpha\beta)_6$ (Glazer, 1989b) (Figure 2.1A).

There are three major phycobiliprotein components in phycobilisomes: allophycocyanin (APC) in cores and phycocyanin (PC) and phycoerythrin (PE) in rods. Allophycocyanins absorb wavelength around 650 nm, phycocyanins absorb wavelength around 600 nm and phycoerythrins absorb wavelength around 550 nm (Zhao et al., 2011; Zilinskas & Greenwald, 1986). Moreover all of them are attached by linker polypeptide which are located in central backbone and in the inner hole of the phycobiliprotein complex (Figure 2.1B) (Zhao et al., 2011). At least two of polypeptides contain covalently bound bilin chromophores, which enhance light-harvesting; one of the most important linker is the core–membrane linker (L_{CM}). It attaches phycobilisomes to the photosynthetic membrane and is the terminal donor of excitation. Another well-known linker is the γ-subunit of class II and some class I phycoerythrins (Zhao et al., 2011).

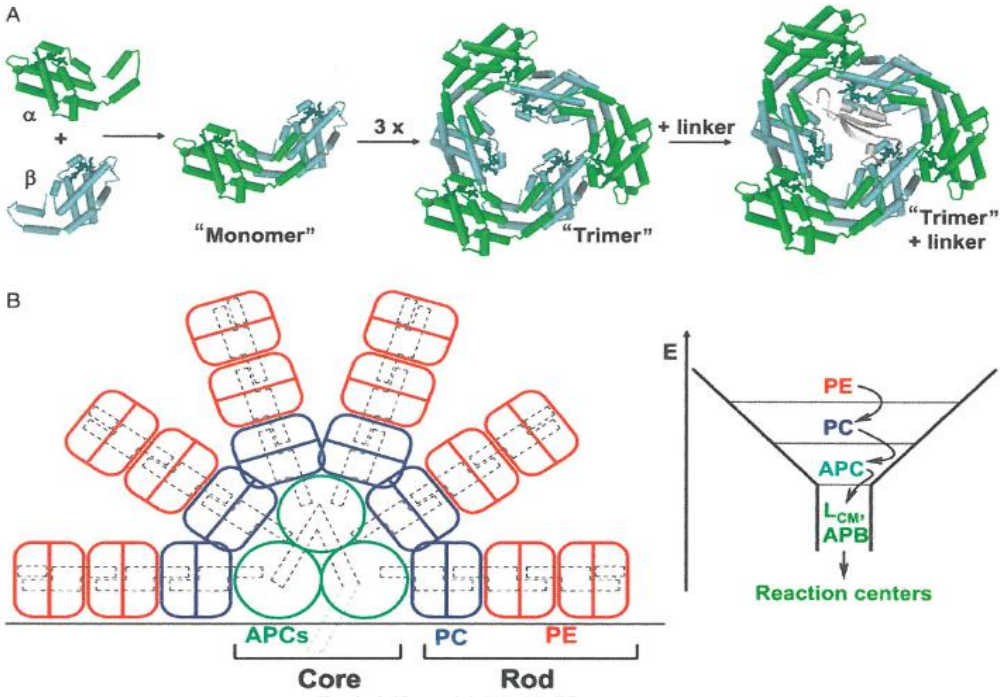


Figure 2.1 Phycobilisome structure.

(A) Assembly of phycobiliprotein trimmers: proteins are α subunit (light green), β subunits (light blue), linker (gray) and chromophores are darker colours.; (B) Phycobilisome structure (left) and energy funnel model (right) (Zhao et al., 2011)

Phycobilisome structure contains three core APCs and six rods of a PC hexamer (blue) and two PE hexamers (red). The linker polypeptides are in the center of rods and the core (dashed line) and the membrane surface is represented as a horizontal black line. The energy model shows energy transfer from higher state to lower state from PE, PC, and APC to reaction centers in photosynthetic membrane.

Phycobiliproteins are found in cryptophyte algae, but cryptophytes lack phycobilisome structures as well as allophycocyanins. The phycobiliproteins are located at the inner surface of the photosynthetic membrane within the lumen (Figure

2.2) (Zhao et al., 2011). Phycoerythrin (PE) and phycocyanin (PC) are the main light-harvesting pigments that absorb yellow and green light and range from red to green and purple to blue spectrum. Either PE or PC will be found in the thylakoid lumen (Mckay, 2015). The β subunits of cryptophytes are evolutionarily similar to the β subunits of cyanobacteria and red algae, but the α subunits have a unique origin; there are two, slightly dissimilar, α subunits, referred to as α_1, α_2 (Wilk et al., 1999). The β subunits are longer than α subunit. These apoproteins are arranged into a complex known as a dimer of αβ monomers (Sekar & Chandramohan, 2008).

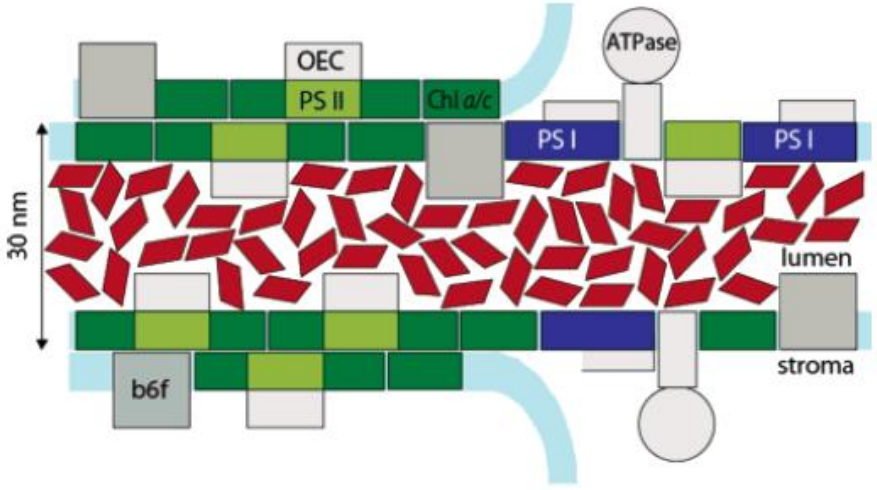


Figure 2.2 Model of the cryptophyte thylakoid membrane.
Phycobiliproteins (red) in the thylakoid lumen; note - the Chl *a/c* complexes (dark green) in addition to the reaction centers, PSII (light green) and PSI (blue) (van der Weij-De Wit et al., 2006)

Each polypeptide subunit contains 1 - 4 chromophores covalently bound to cryteines. Cys-84 is the binding site found in the β subunits of all cyanobacteria, red algae and cryptophyte biliproteins. A number of chromophores are attached by a

single thioether bond at C-3^1, but some of them are attached by a second linkage (e.g. PEB and PUB) (Figure 2.3) (Zhao et al., 2011).

Structurally, phycobilins are 3^1 - Cys - biliverdin (BV, vinyl at ring D), 3^1 - Cys - 15,16-dihydrobiliverdin (DBV), 3^1 - Cys-phycocyanobilin (PCB, ethyl at ring D), 3^1 - Cys-phytochromabilin (PΦB, vinyl at ring D), 3^1(18- Di) – Cys - phycoerythrobilin (PEB), 3^1(18 - Di) - Cys - $\Delta 12^1$ - phycoerythrobilin ($\Delta 12^1$ - PEB), 3^1 - Cys - phycoviolobilin (PVB), 3^1(18^1 - Di) - Cys - phycourobilin (PUB), 3^1 - Cys - mesobiliverdin (MBV, ethyl at ring D) and 3^1 - Cys - $\Delta 12^1$ - 15,16 - dihydrobiliverdin ($\Delta 12^1$ - DBV) (Figure 2.3) (Glazer, 1989b; Zhao et al., 2011)

Four different phycobilins generally have been found in phycobiliproteins of cyanobacteria and red algae. The two most common and abundant are phycocyanobilin (PCB) and phycoerythrobilin (PEB). Phycobiliviolin (PXB) and phycourobilin (PUB) are also present (Six et al., 2007; Zhao et al., 2011). These four phycobilins are isomers, each containing a total of eight double bonds (Glazer, 1989b). The wavelength absorption maximum of phycocyanobilin is around 640 nm, phycobiliviolin absorbs around 590 nm, phycoerythrobilin absorbs around 550 nm and phycourobilin absorbs around 450 nm (Zhao et al., 2011). Cryptophytes contain other bilins, e.g., 15, 16 - dihydrobiliverdin (DBV, λ_{max} ~562 nm), mesobiliverdin (MBV, λ_{max} ~684 nm), 3^1(18 - Di) - Cys - $\Delta 12^1$ - phycoerythrobilin ($\Delta 12^1$ - PEB, bilin 584) and 3^1 - Cys - $\Delta 12^1$ - 15, 16 - dihydrobiliverdin ($\Delta 12^1$ - DBV, bilin 618) (G. Wedemayer et al., 1992; Zhao et al., 2011).

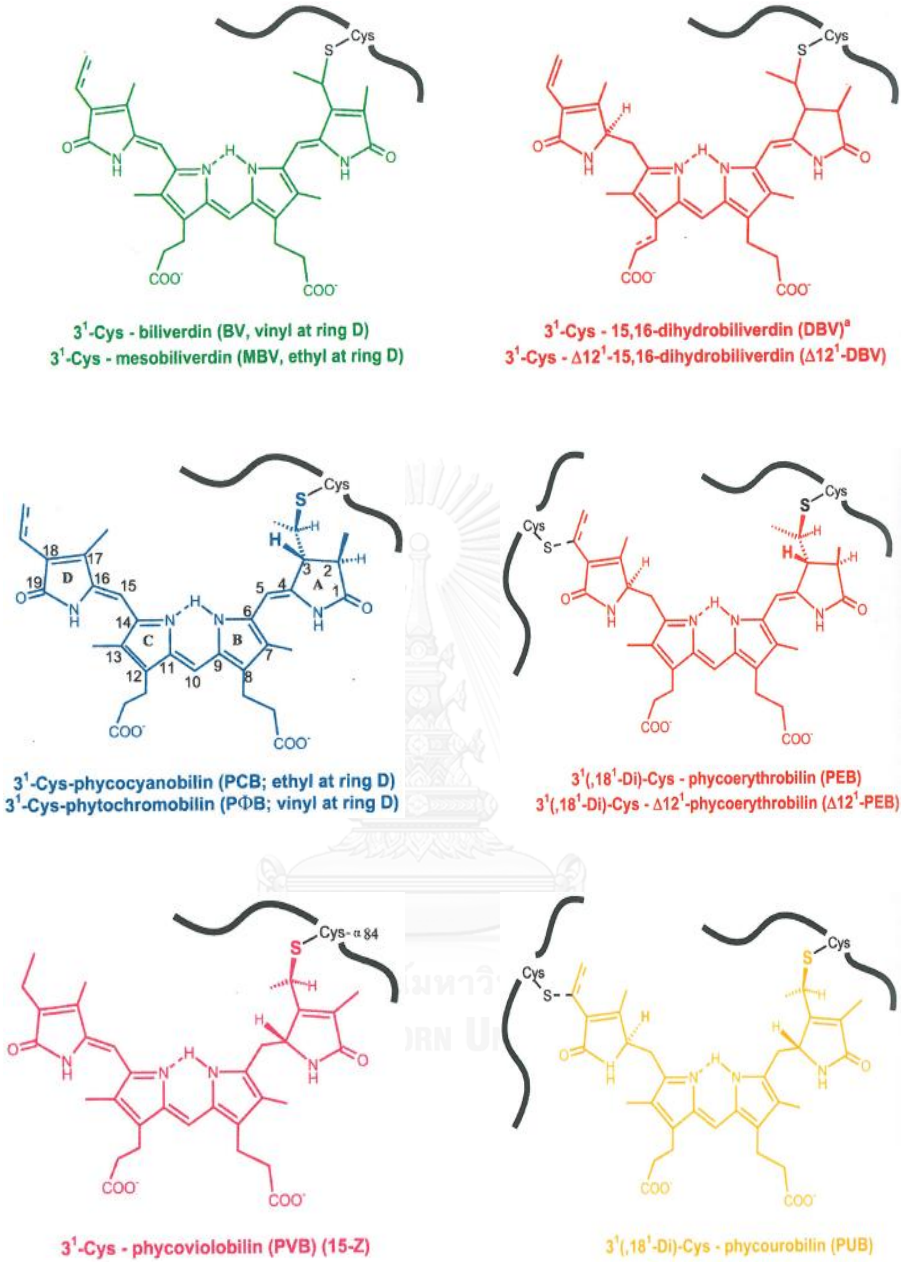


Figure 2.3 The conformation of native phycobiliproteins.

Protein-bound phycobilin with thioether bond between cysteinyl residues on the apoprotein and ethyl or vinyl substituents at the C-3 position on ring A or at C-3 and C-18 positions on ring A and ring D of tetrapyrrole (Zhao et al., 2011)

2.2 Distribution in the Algal Classes

2.2.1 Crytomonads

Crytomonads **(**Cryptophyta) are mostly photosynthetic nanoplanktonic flagellates and go by the common name cryptophytes. Cells range in size from approximately 6 - 20 μm; they are found in marine, estuarine and freshwater habitats (Jeffrey & Vesk, 1997). Usually, cryptophytes cannot be found in field collections when fixation methods are used because these algae are fragile (Hallegraeff, 1981). So, pigment makers are important to detect their presence in oceanic phytoplankton populations (Gieskes & Kraay, 1983). Phycbiliproteins are assessory pigment found in cryptophytes, as outlined above. These pigments are important to study chemotaxonomy of cryptophytes for understanding their role in primary productivity and other activities in the ocean and other aquatic environments.

Rhodomonas salina is one species of cryptophytes (Hill & Wetherbee, 1989)**.** Like all cryptophytes, these algae lack true phycobilisomes (Zilinskas & Greenwald, 1986). The phycobiliprotein complex of *Rhodomonas salina*, referred to as PE545, is arguably the most well characterized phycobiliprotein of cryptophytes, with most studies carried out on a sister species, *Rhodomonas* CS24 (Doust et al., 2006; MacColl et al., 1998; Wilk et al., 1999). PE545 contains four apoproteins, α_1, α_2 plus two β subunits (Figure 2.5). These apoproteins are arranged into a complex known as a dimer of αβ monomers, i.e. $\alpha_1\alpha_2\beta\beta$. Three PEBs (phycoerythrobilins) are covalently attached to each β subunit; a single DBV (15, 16 - dihyrobiliverdin) is covalently attached to each α subunit. In total, PE545 dimers contain four apoproteins and eight phycobilins.

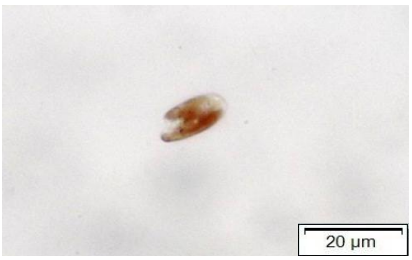


Figure 2.4 *Rhodomonas salina*

Phycobiliprotein: PE545

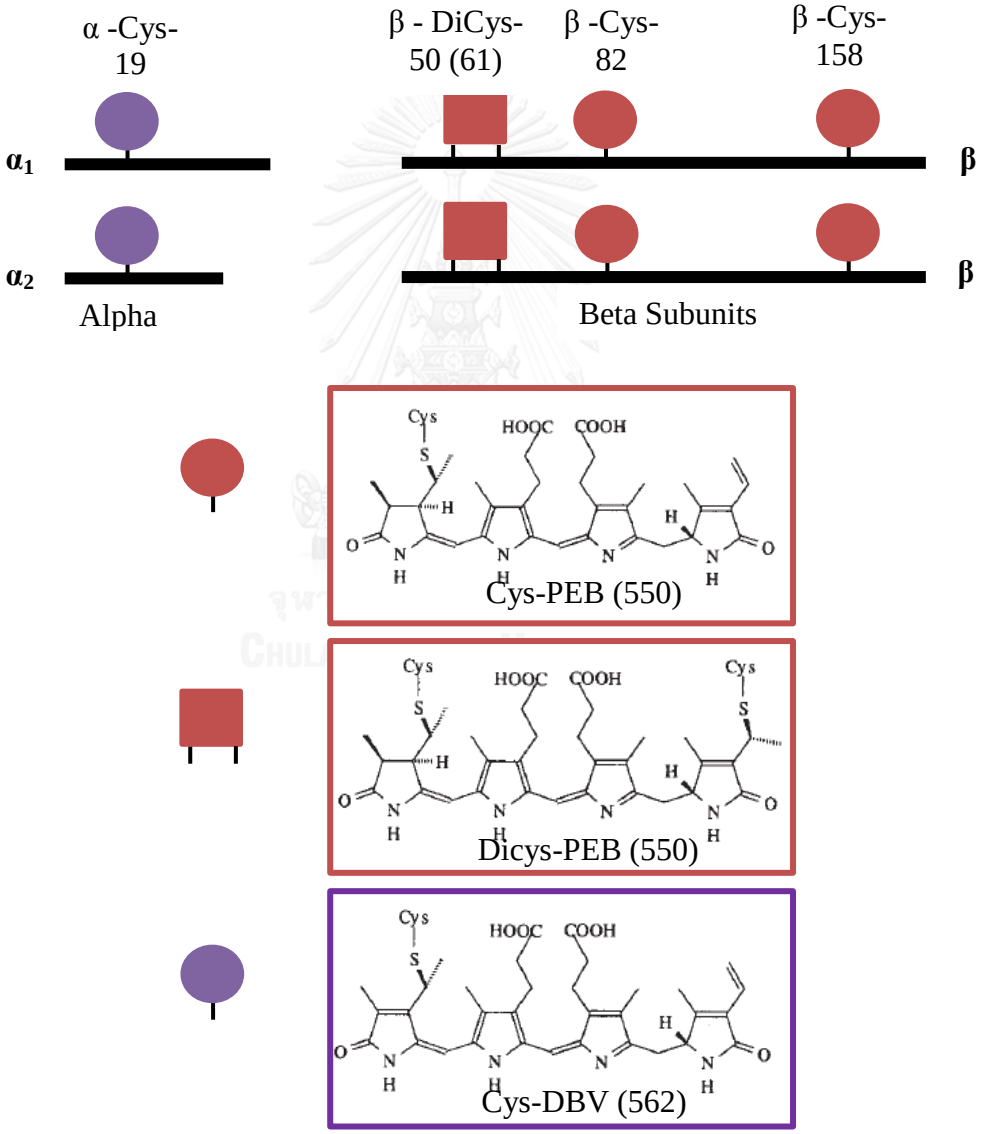


Figure 2.5 PE-545 contains four apoproteins, α_1, α_2 plus two β subunits in *Rhodomonas salina* (Modified from (G. J. Wedemayer, Kidd, & Glazer, 1996)).

Each β-subunit is covalently linked to three phycoerythrobilin (PEB) chromophores, labeled PEB82 Cys, PEB158 Cys, PEB50/61Cys where the numbers indicate the cysteine residues to which the chromophores are linked. The PEB50/61 chromophores are linked to two cysteine residues via their A and D pyrroles. Each α-subunit contains a covalently linked 15,16-dihydrobiliverdin (DBV) chromophore, DBVA and DBVB, respectively, which are spectrally red-shifted compared to the PEBs due to their extended conjugation (Wilk et al., 1999).

Cryptophytes have a unique evolutionary origin (McFadden, 2001) and have a corresponding Gly unique complement of light-harvesting components (Glazer & Wedemayer, 1995). In addition to the already unique features of the phycobiliproteins (as discussed above), cryptophytes also have light-harvesting proteins that are evolutionarily similar to superfamily of LHC of green algae, higher plants and a host of algae with Chl c (Green & Parson, 2003). The Chl *a/c* complexes of cryptophytes play an important role in light harvesting, but their functional inter-relationships with phycobiliproteins remains controversial (Doust et al., 2006; Kereïche et al., 2008).

2.2.2 Cyanobacteria

Cyanobacteria are a diverse group of prokaryotes previously known as blue-green algae; the original name derives from the blue and green pigments now known to be present in the phycobilisomes. Cyanobacteria are important contributors to primary production and food chain dynamics in marine, estuarine and freshwater habitats. Like ‘all’ bacteria, the cyanobacterial cells are small in size, but several species form macroscopic filaments or tufts that are visible to the human eye. The literature on cyanobacterial photosynthesis and/or phycobilisome/phycobiliprotein is

voluminous, such that for the purpose of this M.Sc. thesis, the focus will be narrowed to primarily two important marine species, *Trischodesmium* spp. and *Synechococcus* spp.

Two marine cyanobacteria have tremendous ecological significance. *Trichodesmium* spp. is present globally in tropical and subtropical waters (Capone, Zehr, Paerl, Bergman, & Carpenter, 1997) including the Gulf of Thailand (Lirdwitayaprasit, 2003). This filamentous cyanobacterium was easily identified because it can be very abundant, was visible to the naked eye, and because it was easily obtained in net samples; *Trichodesmium* was shown to be a major contributor of N fixation in many diverse marine environments (Capone et al., 1997). Second, the pico/nano-planktonic *Synechococcus* spp. (0.5 - 1.5 µm) was likely observed more than 100 years ago in microscopic analysis of plankton that passed through nets, but was not conclusively identified and characterized until the 1970's when two seminal manuscripts described its global distribution and major contribution to ocean planktonic biomass (Johnson & Sieburth, 1979). These studies relied upon the orange autofluorescence of cells visible by epifluorescence for enumeration and electron microscopy for characterization (note: most algae have red autofluorscence from Chl a.; the orange signal from *Synechococcus* is due to PE.).

Marine species of *Synechococcus* are environmentally and ecologically significant in the world's oceans and coastal ecosystems (Flombaum et al., 2013). As a genus, marine *Synechococcus* is ecologically and genetically diverse; the genus contains multiple 'clades' or species (Ahlgren & Rocap, 2012; Ferris & Palenik, 1998). The phycobilisomes of marine *Synechococcus* are also incredibly diverse, imparting a visually obvious range of colors to cultures, ranging from red to green to

blue-green to orange to yellowish (Six et al. 2007); the whole-cell coloration is due differential accumulation of three phycobilins, the blue-colored PCB (A_{max} = 620 nm), the red-colored PEB (A_{max} = 550 nm), and the orange-colored PUB (A_{max} = 495 nm).

Furthermore, marine *synechococcus* spp. has only three types based on the phycobiliproteins composition of the rod which are type 1 to type 3. Type 1 have only PC, type 2 have PC and PE I and type 3 have PC, PEI, PEII, but type 3 have subtype (3a-3d) based on ratio of PEB and PUB (Figure 2.6) (Six et al., 2007). *Synechococus* spp. strain RS 9917 is pigment type 1, strain WH8018 is pigment type 2 and strain WH7803 is pigment type 3a.

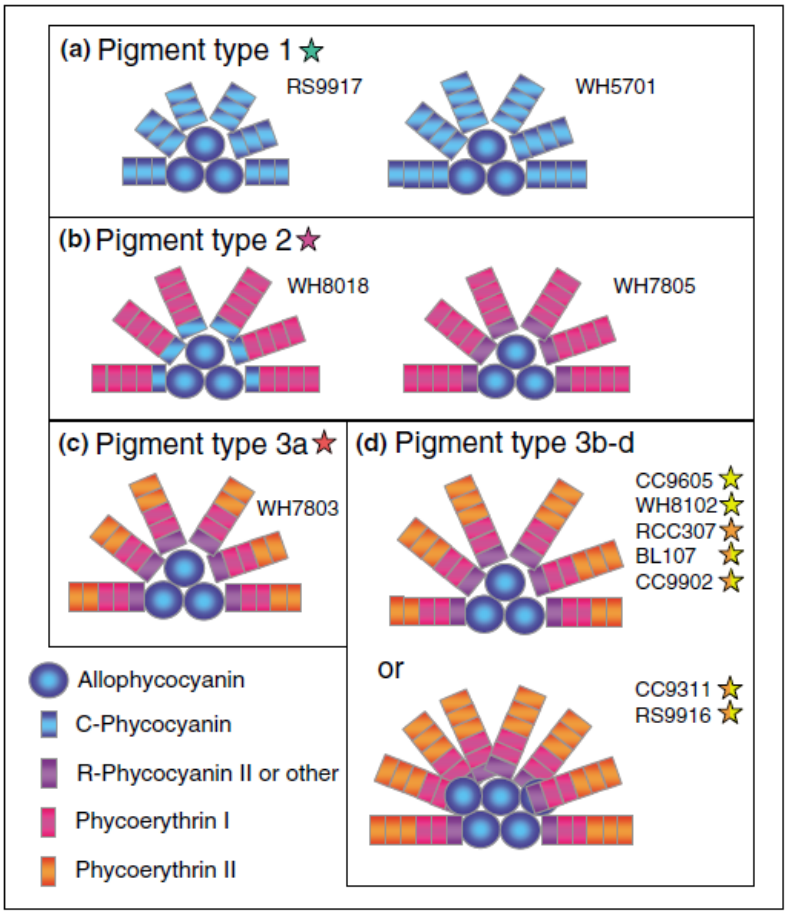


Figure 2.6 Indicated model of PBS structure in different marine *Synechococcus* pigment types and subtypes (Six et al., 2007).

2.3 Extraction and Purification Methods of Phycobiliproteins

Much of the work with phycobiliprotein isolation/characterization has historically relied upon first extracting the macromolecular pigment-protein structures (i.e., the phycobilisome) and relying on the high molecular weight of this structure, combined with high water solubility, as an initial separation/purification step. Several excellent reviews are available (Apt, Collier, & Grossman, 1995; Gantt, 1981; Glazer, 1982, 1985, 1989a; Grossman, Schaefer, Chiang, & Collier, 1993). Early studies focused on first purifying macro-molecular structures and then, over time,

‘deconstructing’ these functionally intact structures to reveal more granular features, such as number and type of apoproteins, number and types of bilins attached, mode of bilin attachment to apoproteins, etc.

Most work with phycobilisome (and/or phycobiliproteins) has started with highly purified material. A brief summary of a ‘typical’ purification scheme is one conducted by (Glazer, 1988) who studied phycobilisome isolation in cyanobacteria suspended in high-ionic strength buffer, potassium phosphate buffer pH 7.5 (≥ 0.75 M). Cells were disrupted by French press and cell debris was removed by centrifugation (10,000 x g); supernatants were further separated by sucrose-density centrifugation in an ultracentrifuge. Furthermore, the macromolecular phycobilisomes could be dissociated to trimer and hexamer in phosphate buffer when the ionic strength was reduced to 50 mM, allowing separation by ion-exchange chromatography. The fractions were stored in $(NH_4)_2SO_4$ (70% w/v) at 6℃ and centrifugation to removed debris and desalted by Sephadex G25.

It is very clear that the long tedious-purification techniques, such as the one outlined in the previous paragraph, have played a crucial role in our understanding of the cellular, molecular, biochemical, and even ecological knowledge of phycobilisomes. On the other hand, these techniques are not always possible. Two important, mostly new, avenues of research with phycobiliproteins are relevant to the research conducted in the M.Sc. thesis, namely, use of phycobiliproteins in commercial enterprises (e.g., cosmetics, biomedical, food color) and field ecology. Some salient points of each will be discussed below.

2.4 Extraction and Purification Methods of Phycobiliproteins – Commercial Applications

Phycobiliproteins are commercially important in a variety of settings, ranging from biomedical to cosmetics to enhanced food products (Kannaujiya & Sinha, 2016). Although it is generally possible to grow (or collect from nature) large amounts of algae for production of phycobiliproteins, there is still an economic rationale for maximizing extraction efficiencies and/or stability of the purified products. Because these commercial endeavors frequently (if not always) require the highest possible degree of purification, most of the detailed steps of phycobiliprotein isolation that were outlined above have been followed, but with an eye on improved methods (e.g., cell breakage is required but by what means is best? which buffer and what pH provides maximum extraction? are stabilizing preservatives useful, and if yes, which ones are best?). One example is given (in the following paragraph) that relates to the current M.Sc. thesis.

Hemlata and Fareha (2011) studied the effect of different buffers (0.1 M) on the extraction of total phycobiliproteins in cyanobacteria. The buffers used were acetate buffer (pH 6.0), carbonate buffer (pH 9.6), citrate buffer (pH 5.0), double distilled water, potassium phosphate buffer (pH 7.0), sodium phosphate buffer (pH 7.0) and Tris chloride (pH 7.2). They found that sodium phosphate buffer was the best buffer to extract phycobiliproteins as it provided the highest total amount of phycobiliproteins (mg/g). Addition of NaCl (up to 0.15 M) also proved helpful. They also studied the effect of varying pH of sodium phosphate buffers for phycobiliprotein extraction and found that pH 7.5 was the best. Freeze-thaw was the best studied

method of pigment extraction, superior to lytic-enzyme-lysozyme extraction, heat-shock treatment, or homogenization.

2.5 Extraction and Purification Methods of Phycobiliproteins – Analysis of Two Marine Planktonic Cyanobacteria

Two of the major difficulties of working with phycobiliproteins from field samples relates to: 1) phycobiliprotein-containing algae are generally present in low numbers; and 2) the phycobiliproteins are not soluble in acetone, which is the 'gold standard' for analyses of photosynthetic pigments in field studies (Capone et al., 1997). Some useful 'work around' techniques for dealing with these water (but not acetone) soluble pigments have been developed and will be discussed.

One of the first methods developed for extraction of phycobiliproteins from marine cyanobacteria as encountered in field work was developed for *Trichodesmium*, an abundant, globally-distributed, ecologically important diazatrophic genus. PE can be extracted from these cells via a combination of freeze - grinding in 0.1 M phosphate buffer of neutral pH (Moreth & Yentsch, 1970). The method, sometimes with slight modifications, has been widely used (Neveux, Tenírio, Dupouy, & Villareal, 2006). The method is not universally applicable, for instance with cells such as *Synechoccocus*, which are resistant to mechanical breakage (Glover, Keller, & Guillard, 1986).

A rather long, labor-intensive protocol was developed by (Stewart & Farmer, 1984) for estuarine cryptomonads and cyanobacteria. Following filtration, the filter is incubated in a lysozyme-containing 0.25 trizma Base and 10 mM disodium EDTA, sonicated, the pH adjusted to 5 with HCl, followed by incubation first at high

temperature (37℃) for 2 hr then at low temperature (1.5 ℃) for 20 hr, before the pH is adjusted to 7.0 with 0.1 N NaOH and then pigment analysis. This protocol has been used by others (Mitchell & Holin-Hansen, 1990), but in general is too labor intensive for routine work and/or when large numbers of samples are involved.

Wyman et al. (1985) addressed the PE issue with *Synechococcus* at least in the laboratory; using Gly to decouple PE from the PS system, Wyman et. al. observed a linear relation between the *in vivo* steady state fluorescence emission intensity of PE measured in the presence of Gly and the PE content of cells of *Synechococcus* sp. strain WH7803. This relationship between PE content and steady state fluorescence was subsequently extended to field populations of *Synechoccocus* treated with Gly, (Wyman et al., 1985). This method is not, strictly speaking, used for isolation of PE, but rather is a something of a hybrid method, resulting in dissociation of PE from the reaction centers such that PE spectra are similar to what is observed for purified PE. This method has also proven to be of widespread usage (Neveux et al., 2006).

Finally, there are a few important manuscripts focused on methods for isolation of phycobiliproteins from a wide range of algae. The two most recent, and comprehensive, are those by Lawrenz et al. (2011) and Zimba (2012). These articles cover most of the earlier research, so only details from these studies will be covered in detail. Suffice it to say, despite earlier work to extract phycobiliproteins from algae, especially from natural/field populations of microalgae, the techniques perfected to date are effective with a limited number of species and/or are too laborious for routine research.

Importantly, both of these recent studies (Lawrenz et al., 2011; Zimba, 2012) attempted to purify phycobiliproteins in their ‘native state’, which has been the

approach universally employed. This approach is not too surprising, given the long and complicated history of ‘artifacts’ in the very early days of phycobiliprotein research (Carra & Heocha, 1966; De Marsac, 2003; O'Carra, Murphy, & Killilea, 1980).

Lawrenz et al. (2011) were able to reach 100% extraction efficiencies with a cryptophyte (*Rhodomonas salina*) while efficiencies were lower with cyanobacteria, even with more prolonged extraction periods (i.e., up to 96 hr); it is not clear from the data presented if extraction was complete or if bilin stability (or lack of bilin stability) resulted in pigment-free cell pellets, giving a false reading of extraction. In 2012, Zimba (2012)Found that Asolectin-CHAPS extractions yielded higher concentrations of phycobilins extracted with lower variation compared to phosphate buffer in a number of different cyanobacterial strains; pH 6.7 was more effective than pH 3.75. Very high extraction efficiencies, with low coefficients of variation between samples, were observed in some cyanobacteria with Asolectin-CHAPS.

2.6 Extraction and Purification Methods of Phycobiliproteins – Use of Detergents

Detergents have played an important role in biochemical studies, including phycobiliproteins. One of the important early publications involving SDS was conducted by (Berns, Scott, & O'Reilly, 1964) using SDS to determine the minimum molecular weight of phycocyanin. A number of subsequent studies with SDS (Fujiwara-Arasaki, Yamamoto, & Kakiuchi, 1984) lead to confusing and/or contradictory results; in general, most studies have used SDS as part of SDS-PAGE, which in some cases can result in bilin-protein complexes that retain their native coloration though generally at very low levels (Raps, 1990).

Triton X-100 and other ‘gentle’ detergents have also played a role in phycobiliprotein studies. The initial characterization of phycobilisomes that has since widely been regarded as a definitive step utilized Triton X-100 to release phycobilisomes from membranes of a red alga (Gantt & Conti, 1965). In general, these detergents have proven effective in terms of releasing phycobilisomes from thylakoid membranes and/or preventing unnatural aggregation of phycobiliproteins in various buffers (usually of low ionic strength). Other gentle detergents that have been used include zwitterionics, e.g., CHAPS used by (Zimba, 2012), Miranol and Deriphat used by (Glazer, Williams, Yamanaka, & Schachman, 1979). These detergents help solubilize proteins, but do not lead to denaturation at the concentrations and conditions used.

2.7 Extraction and Purification Methods of Phycobiliproteins – Use of 8M Urea pH 2

The absorption properties of the phycobiliproteins are highly dependent on their aggregation state, pH, ionic strength, and protein concentration. In other words, the spectral data shown thus far are not quantitatively accurate (Schluchter, 2002). For quantitative analyses, Glazer and co-workers demonstrated that the most reliable method to determine the concentration of phycobiliprotein solutions is to measure the absorption spectrum of the peptide-bound bilins by dissolving an aliquot of the protein in 8 M urea, pH 1.9 or in 10 mM TFA (trifluoroacetic acid) or 20% acetic acid. With these treatments, the protein-bound bilins ‘act’ as if the protein is not present.

In other words, the bilin-protein (with acidic urea) has absorption properties similar/identical to the non-bound bilin. To date, the use of acidic urea, TFA and AcOH have only been shown to be effective with highly purified biliproteins.

2.8 Extraction and Purification Methods of Phycobiliproteins – Use of Proteases

Proteases have also played an important role in phycobiliprotein research. An early study compared phycobilisome isolated with trypsin instead of Triton X-100 (Hiller, Post, & Stewart, 1983). Phycobilisomes prepared by the two protocols had identical absorbance and fluorescence properties while the polypeptide composition, as revealed by SDS-PAGE suggested that a lamellar peptide was cleaved by trypsin to release the intact phycobilisome.

More recent work with proteases have been a corner-stone of research performed in the Glazer laboratory. For instance, in 1992 Wedemayer and others studied the structures of phycobilins in cryptophycean. Before determining the structures with NMR spectroscopy and mass spectrometry, they isolated phycobilins from their protein complexes by digesting with three different enzymes, trypsin, pepsin and pronase. These enzymes digested peptide bonds in different positions and provided different results, which were used to identify bilin-binding sites.

Briefly, Trypsin is produced in the pancreas and cleaves peptides on the C-terminal side of lysine and arginine amino acid residues at pH 7 - 9. Pepsin is from pocrine gastric musisa; it hydrolyzes only peptide bonds and cleaves at the carboxyl side of phenylalamine and leucine at pH 2 - 4. Pronase is from culture broth of *S. griseus*; it contains a number of different endo- and exo-proteases that hydrolyzes

peptide bond on carboxyl side of glutamic or aspartic acid at pH 8.8 and/or from the terminal residues.

2.9 Extraction and Purification Methods of Phycobiliproteins – HPLC Methods

HPLC has also proved to be very useful for analyses of phycobiliproteins. Earlier work, which tended to focus on isolation of the larger, macromolecular complexes (e.g., phycobilisomes, hexamers, trimers) relied on sucrose density gradients and/or column chromatography using gravity flow, both of which are more useful for the 'preparative' (or large) amount of material involved and for larger (higher molecular weight) analytes. SDS-PAGE was generally used to characterize the proteins following chromatography.

HPLC played an important part as studies progressed, for instance, in demonstrating the presence of different subunits in various phycobilisome preparations. For instance, Swanson and Glazer (1990) introduced C4 reverse HPLC that was rapid, required less starting material and, if used with diode array detectors, provide immediate identification of bilin content and subunit stoichiometry. Over time, HPLC played an increasing Gly important role in identifying bilin-peptide fragments, produced by protease digestion (as covered in section 7, above). The methods-based manuscript of (Schluchter & Bryant, 2002) provides interesting background on various HPLC methods.

2.10 Extraction and Purification Methods of Phycobiliproteins – Final Thoughts

Many basic methods of phycobiliprotein isolation and characterization have been developed, including those for cellular, molecular, biochemical and/or

biophysical research. Methods developed for commercial purposes tend to follow the same protocols, including several purification steps after the initial extraction. Research directed toward field sampling programs also follow the same basic procedures during extraction, but then forego the more elaborate, time-consuming steps of purification.

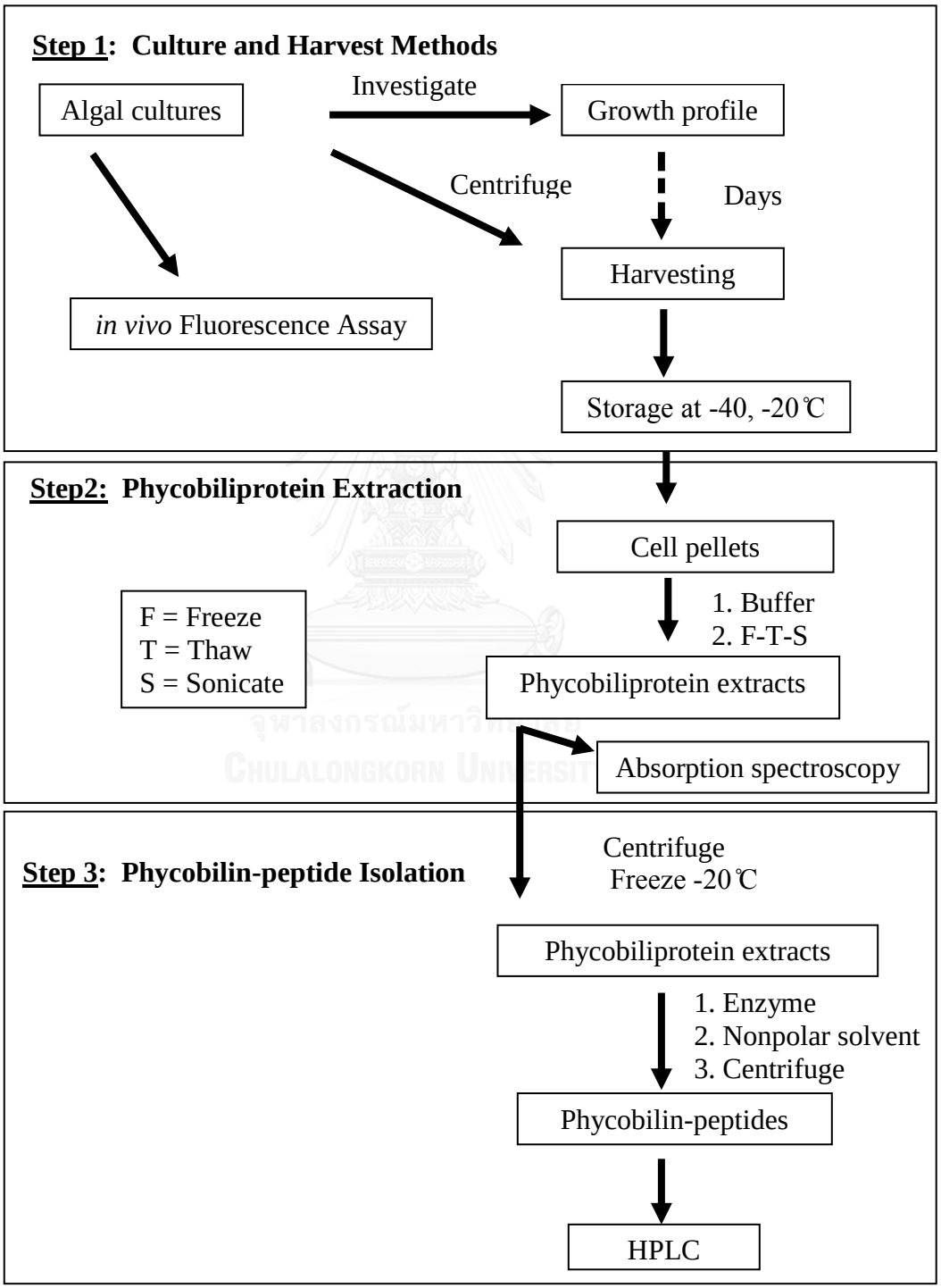
A brief summary of the results to date include:

1. The extraction of large multi-subunit complexes is difficult and, as such, extraction efficiencies are remarkably low, especially when working with low amounts of starting material.
2. The protocols are generally conducted with very large volume, high-biomass cultures (i.e., even with low extraction efficiency, sufficient material for further work is available).
3. Methods for releasing large macromolecules frequently rely upon instrumentation that is tedious and is suitable only for larger volumes (e.g., French Pressure Cells).
4. The cell wall of many algae can be incredibly resistant to breakage and/or lysis. In fact, the cell wall of cyanobacteria is generally considered to be more like that of gram positive bacteria than gram negative bacteria (Mehta, Evitt, & Swartz, 2015), though evolutionarily speaking cyanobacteria are clearly in the gram negative group.
5. The macro-molecular complexes are generally unstable in the presence of detergents (except ‘gentle’ detergents at very low concentrations) and high molarity buffers are required to prevent dissociation.

The work presented in this M.Sc. thesis attempts to address these five issues such that protocols are available for extraction and analyses of phycobiliproteins that utilize crude-cell extracts and are amenable to study of natural/field populations. Further, attempts were made to develop methods for phycobiliprotein analyses that could be employed following extraction of Chlorophylls and carotenoids with acetone, thus making it possible to determine ‘all’ photosynthetic pigments from a single sample.

CHAPTER III

METHODOLOGY



3.1 Culture and Harvest Methods

3.1.1 Algal cultures

In order to accomplish the first objective, algal selection is the first challenge to consider. Cyanobacteria and cryptomonad were chosen. A marine cryptophyte, *Rhodomonas salina* was kindly provided by Saskia Ohse and Dr. Alexandra Kraberg (Alfred Wegener Institute, Helgoland, Germany). Other three marine *Synechococcus* spp. strains RS9917, WH8018 and WH7803, which were obtained from the Roscoff Culture Collection (Roscoff, France).

For the culture of *Rhodomonas salina,* cells were cultured with either f/2 or Daigo IMK medium (Appendix IV) at 24 ºC under a photon flux of 41 µmol m^{-2} sec^{-1} with light: dark cycles of 12:12 hr.

The marine *Synechococcus* spp. strains WH7803, WH8018 and RS9917 were cultured in 250 mL Erlenmeyer flasks with 125 mL in PCR-S11 medium for stock culture (APPENDIX IV) (Rippka et al., 2000). Cells were cultured at 24 ºC under an irradiance of 10 µmol m^{-2} sec^{-1} and dark: light cycle of 12:12 h. at 730 laboratory culture rooms. The strain of WH7803 was isolated from Sargasso Sea in July 1978 at 33º45′E, 67º30′ N at a depth of 25 m by L. Brand, the WH8018 was isolated from East coast USA in June 1980 at 41º31′E, 70º40′ N by J. Waterbury and the RS9917 was isolated from Red sea in November 1999 at 29º28′E, 34º55′ N at a depth of 10 m. by N. Fuller. All these strains were from the Roscoff Culture Collection (RCC), Roscoff, France.

3.1.2 Growth profile

Rhodomonas salina

Cell density was determined with a Neubauer Improved Brightlinehemo cytometer (0.0025 mm^2). Triplicate 150 mL cultures were inoculated and followed over time; triplicate 1.0 mL aliquots were taken at each time point from each culture and triplicate 10 µl subsamples were counted from each aliquot.

Calculate growth specific by (Guillard, 1975) formula:

$N_t = N_0 e^{Ket}$

when N_0 = Cell density (cell/mL)

N_t = Cell density in days (t) (cell/mL)

K_e = specific growth rate

t = time (day)

$N_t = N_0$, at t = 0

$\log N = \log N_0 + K_e t \log(e)$

$= \log N_0 + (0.4343) K_e t$

***Synechococcus* spp.**

Chlorophyll concentration was measured by spectrofluorometric analysis. Cell stock cultures (150 - 250 mL) were grown in 1500 mL medium. The cultures (1 mL) were filtered through 47 mm GF/F filters, added $MgCO_3$ solution, folded in half, wrapped in aluminum foil and stored in freezer (-20℃) until analysis. For analysis, chlorophyll pigment was extracted by 90% acetone, cut filters to small pieces, kept in 10 mL falcon, added 10 mL 90% acetone (to avoid formation of degradation chlorophylls; therefore, should be wrapped 10 mL falcon by aluminum foil.),

sonicated at 8 mins, kept at -20℃ almost 6 - 8 hr, centrifuged to removed cellular debris and after that sample were analyzed by spectofluorometric (Model Perkin Elmer instrument, LS55 Luminaescence Spectrometer). Then, the growth specific was calculated using the formula, $\mu = (\ln C_2 - \ln C_1) / (t_2 - t_1)$ when C = chlorophylls concentration and t = day (Guillard, 1975; Tanoi, Kawachi, & Watanabe, 2011).

3.1.3 Algal havesting

Rhodomonas salina

For biochemical analyses, 150-200 mL of cells was transferred to 1,200 mL in 2 L flasks. Replicate 35-50 mL samples of culture (8 - 10 days old; ~1.1 - 1.8 X 10^7 cells) were harvested by centrifugation (3,500 x g for 10 min); the resuspended pellets were transferred to microcentrifuge tubes and collected at 10,000 rpm for 3 min. Cell pellets were stored at -40, -20 ºC.

***Synechococcus* spp.**

For biochemical analyses, 150-250 mL of cells was transferred to 1,200 mL in 2 L flasks. Replicate 35-50 mL samples of culture (10 days old; ~0.1800 - 0.1900 g wet weigh) were harvested by centrifugation (3,500 x g for 15 min); the resuspended pellets were transferred to microcentrifuge tubes and collected at 10,000 rpm for 5 min. Cell pellets were stored at -40 or -20 ºC.

3.1.4 *In vivo* fluorescence assay of PE545 in *Rhodomonas salina*

Whole cell fluorescence assays of *R. salina* were conducted as described by Wyman (1992). Briefly, cells from f/2 and DG were diluted 50:50 in either the culture medium or in Gly. Fluorescence spectra were recorded on a Perkin Elmer model LS

55 Luminescence Spectrometer, with emission and excitation slits of 2.5 and 2.5 nm, respectively. Samples were excited at 520 nm and fluorescence emission detected at 530 - 750 nm.

3.2 Phycobiliprotein Extraction

Phycobiliprotein extraction was expedited by three freeze - thaw - sonicate cycles (3X-FTS). Potassium phosphate (KP), sodium phosphate (NaP), Tris-HCl (TB), or acetate (AB) buffers, all at 0.1M with pH values from 1 - 9, were tested. Cell debris was removed by centrifugation (10,000 rpm for 5 min). Supernatant absorption spectra were recorded on an Analytikjena SPECORD 200 PLUS; spectra were normalized at OD_{750} before plotting. The pellets, frequently enriched in pigments, were processed as described below. In some cases, 90% acetone was used to extract non-polar pigment before extraction of phycobiliproteins in KP, NaP, TB, or AB.

To quantitate phycobiliprotein extraction efficiencies, cell pellets from the 3X-FTS procedure were resuspended in fresh buffer and the 3X-FTS process repeated; SDS was added during the final extraction cycle.

Phycobiliprotein stability was evaluated using the supernatants from 3X-FTS; supernatants were transferred to fresh tubes following centrifugation to eliminate pigments in the cell pellet from interfering with the analyses. Samples were stored for various intervals at either -20 °C or 4 °C in darkness before spectroscopic analysis.

Furthermore, SDS, urea and acetic acid were used to test with phycobiliprotin extraction. 0.1%, 0.5%, 1% and 2% SDS were use in the experiment. 8M urea pH 2 and 20 % AcOH were added in supernatant (from sample in buffer with or without SDS) after 3X-FTS and centrifuge. The samples were analyzed by spectroscopy.

3.3 Phycobilin-peptide Isolation

3.3.1 Phycobilin isolation

The outlined above yields crude whole cell extracts, containing phycobiliproteins as well as a number of other soluble proteins, nucleic acids, sugars and etc. To clarify this complexity, a High Performance Liquid Chromatography (HPLC) method was developed for phycobilin analysis and quantification. In this step, before injecting phycobiliprotein samples to HPLC, we used 'Pronase' enzyme for digestion of bilin-peptide conjugates from phycobiliproteins. Then, acetone was applied to isolate phycobilins from others.

3.3.2 HPLC method

For the quantitative HPLC method, the solid-phase C18 column (VYDAC Model, CAT. # 201TP5) was used to analyze samples. The HPLC brand used is Varian (Prostar model). The DAD detector scanned from 220 nm to 800 nm. The UV-visible was set up at 528 nm. The fluorescence excitation (520 nm) was recorded at an emission wavelength of 570 nm. The solvent system was shown below:

Time	Flow(mL/min)	H_2O	MeOH
0	1	95	5
2	1	95	5
20	1	0	100
25	1	0	100
27	1	95	5
32	1	95	5

CHAPTER IV

RESULTS

4.1 Culture and Harvest Methods

4.1.1 Growth profile

Rhodomonas salina

Rhodomonas salina cell density profiles when grown in f/2 and Daigo IMK were similar (Figure 4.1; Appendix I). Cells in f/2 had a slightly longer lag phase; the small cell density differences during station phase were not significantly different and were similar in both media when cells were harvested (day 8 - 10). The number of cells decreased, and cells were phase of decline in day 20 - 25 (Appendix I). Furthermore, specific growth rate was 0.7598 and 0.866 in f/2 and Daigo IMK, respectively. Phycobiliprotein extraction with very high efficiencies could be accomplished from cells grown in f/2 with simple freeze - thaw - sonicate treatments; this was not the case for cells grown in Daigo IMK.

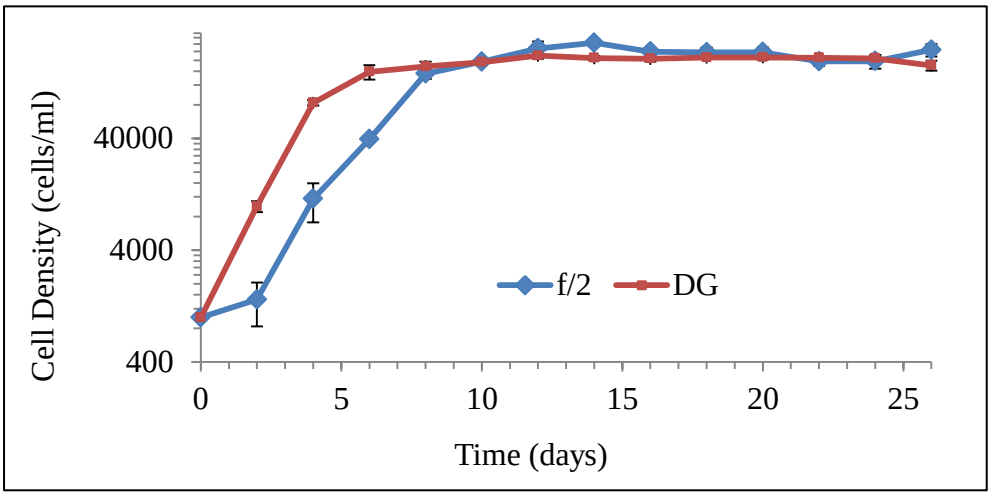


Figure 4.1 Cell density profiles of *Rhodomonas salina* in f/2 and Daigo IMK media.

***Synechococcus* spp. strains RS9917, WH8018, and WH7803**

Synechococcus spp. strains RS9917, WH7803, and WH8018 are picoplankton. Therfore, cell counting is not appropriate to study growth profiles. In this study, they were investigated by measurement of chlorophyll concentration. Growth profile was approximately 28 days in all strains (Figure 4.2 - 4.4; Appendix I) and was harvested at day 10 to for the next extraction step.

The RS9917 chlorophyll concentration fluctuated during four days (lag phase). Chlorophyll concentration was increasing at day 6 to 20 (Logarithmic phase) and then decreasing slightly (stationary phase). Finally, growth profile was reaching the decline phase at day 28. For the WH8018 culture, it had longer logarithmic phase and the concentration of chlorophyll was declined after day 22. The chlorophyll concentration of the WH7803 culture was decreasing at the first couple day, then increasing from day 4 to 20 (logarithmic phase) and declined at the last day.

Their specific growth rates of RS9917, WH8018 and WH780 were 0.287, 0133 and 0.318, respectively.

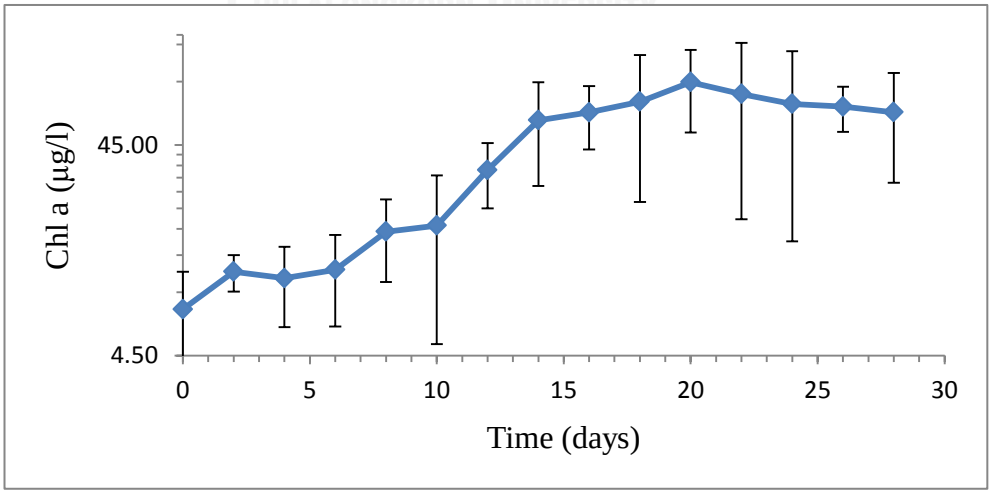


Figure 4.2 Chlorophyll concentration profile of *Synechococus* sp. (strain RS9917) in PCR-S11 media.

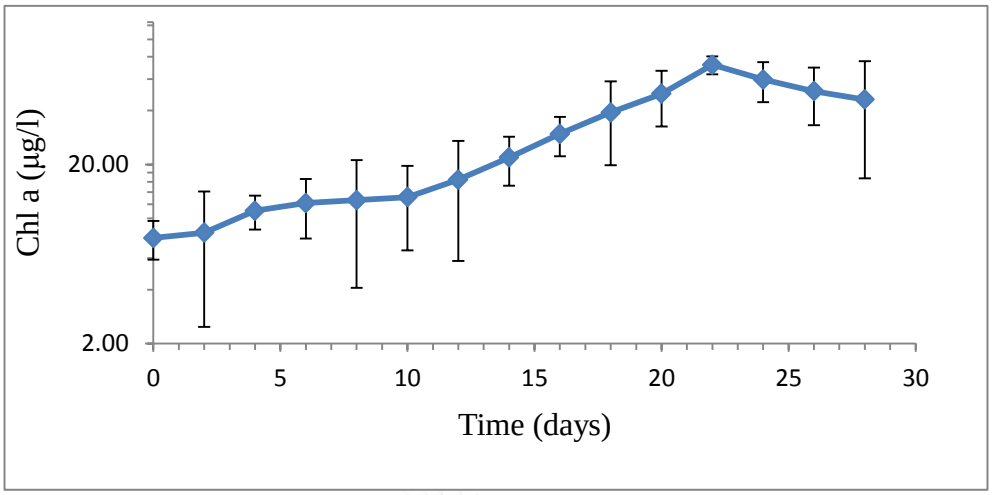


Figure 4.3 Chlorophyll concentration profile of *Synechococus* sp. (strain WH8018) in PCR-S11 media.

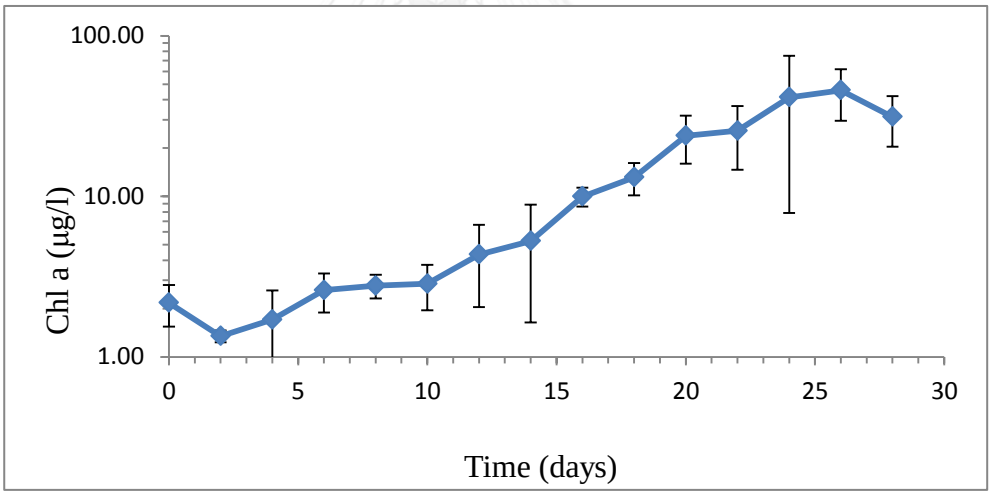


Figure 4.4 Chlorophyll concentration profile of *Synechococus* sp. (strain WH7803) in PCR-S11 media**.**

4.1.2 Fluorescence of whole cell, *Rhodomonas salina*

The effect of Gly on the fluorescence emission from *R. salina* in f/2 and DG medium. The culture in DG medium was approximately 2X more dense than the culture in f/2 medium; both were in logarithmic phase (8 days). Cell culture samples were resuspended in 50% (v/v) aqueous Gly (f/2 + Gly, DG + Gly) or their medium (f/2 + f/2, DG + DG). Samples were excited at 520 nm and fluorescence emission detected at 530 - 750 nm.

The ratio of fluorescence intensity between Gly and no Gly in DG medium is 265/11.7 (22.6 units), Figure 4.5A; furthermore**,** the ratio in f/2 medium is 75/6.4 (11. 7 units), Figure 4.5B. The ratio (+Gly/-Gly) should be relatively independent of cell density (except at much higher cell densities, where fluorescence intensity would decrease as cell densities increase due to reabsorption). In other words, it appears that the amount of PE fluorescence is about 11X greater in DG than in f/2 (Figure 4.5C) when Gly is added. The baseline fluorescence for PE (i.e., no Gly) differs by approximately 2X (i.e., 11.7 vs 6.4) Figure 4.5D which is roughly equal to the difference in cell density i.e., DG cells were about 2X the concentration of f/2 cells. The baseline fluorescence for Chl (~685 nm) is about 2.6 vs 3.1.

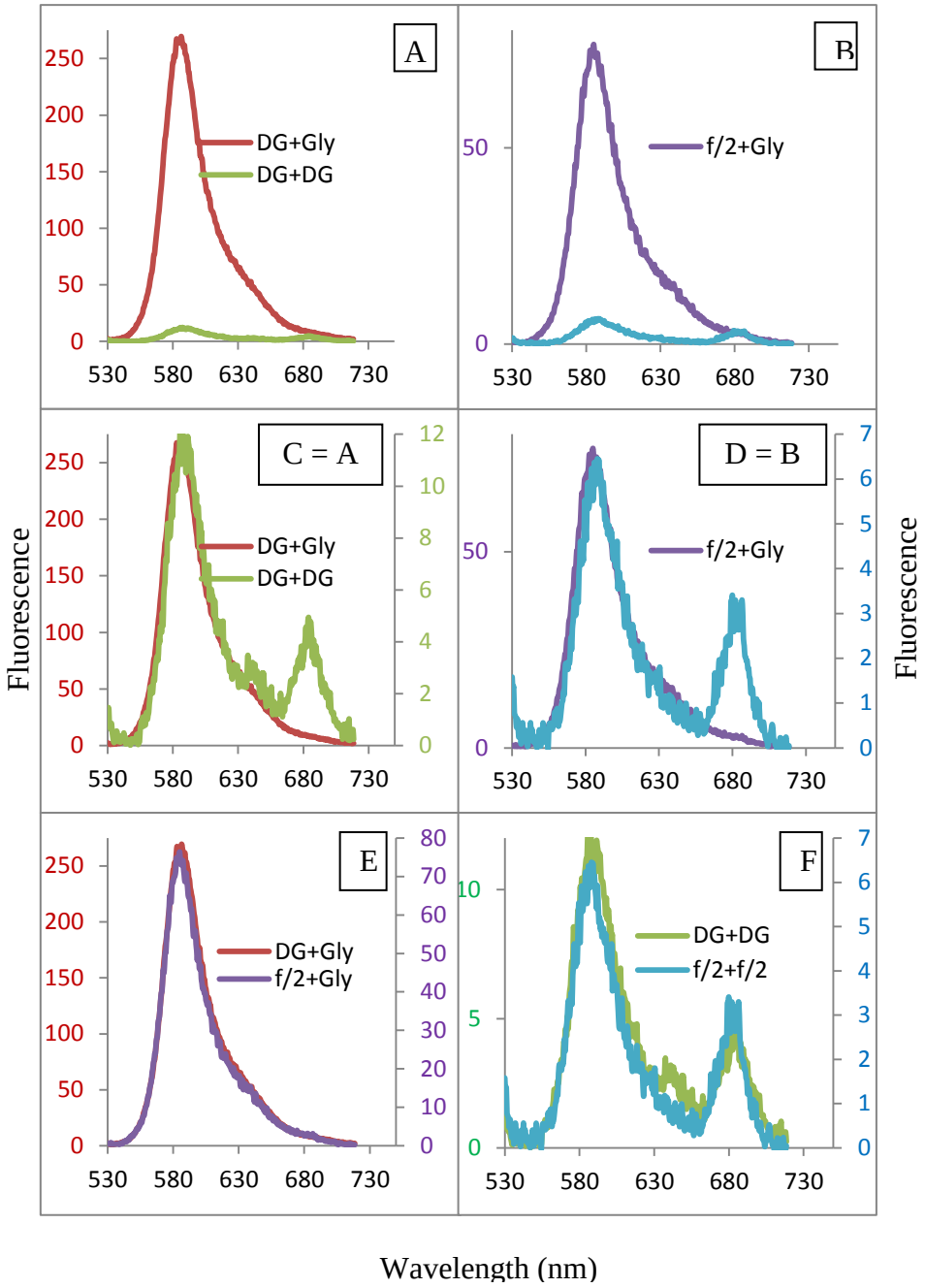


Figure 4.5 Fluorescence of whole cells of *Rhodomonas salina* in 50 % Gly and medium (DG and f/2).

A), C) PE fluorescence intensity between Gly (red) and no Gly in DG medium (green); B), D) PE fluorescence intensity between Gly (purple) and no Gly in f/2

medium (green); E) PE fluorescence intensity between Gly in DG and in f/2 medium; F) PE fluorescence intensity between no Gly in DG and in f/2 medium

4.2. Phycobiliprotein Extraction

Rhodomonas salina

4.2.1 Extraction of PE545 in different buffers/pH

The solubility and properties of biliproteins depend upon the particular biliprotein, its concentration, and the pH and ionic strength of the buffers used for purification (Glazer, 1982). KP and/or NaP buffers are frequently used for isolation of phycobiliproteins. Here, three buffers (KP, TB, AB) at nine pH values were tested for extraction of phycobiliproteins following 3X-FTS (Figure 4.6).

AB buffer at low pH was generally ineffective at solubilization of PE545 while considerable pigment was recovered at pH 5 - 9 (Figure 4.6A). For TB, higher extraction was noted at pH 3 - 8. In KP, pH 4 - 8 was effective. Some buffer/pH combinations yielded extracts enriched in phycobiliproteins with absorption spectra similar to that observed in whole cells; the supernatants were 'pink' (A_{max} at 545-550 nm) and were generally associated with buffers of pH 6 and 7 (e.g., AB, TB and KP at pH 6; Figure 4.6A and B).

Several buffer/pH combinations yielded extracts enriched in PE545, but also enriched in chlorophylls and carotenoids (note: the peaks at ~440 ~500 and ~670 nm; Figure 4.6A). These supernatants were 'red' in color and tended to prevail at pH values $\geq$ 6 (Figure 4.6B). At the two lowest pH values the peaks were red shifted by 5-10 nm and were 'violet' in color (e.g., TB pH 1 - 2; Figure 4.6A and B).Very little PE545 was detected at pH 9 in TB and KP and the seeming Gly high absorption of

PE545 in AB at pH 9 is likely enhanced by Chloropylls and carotenoids (Figure 4.6A).

None of the buffer/pH combinations were completely efficient at solubilization of PE545 from these cells. More drastic extraction protocols (e.g., elevated temperatures, longer sonication times, addition of β-mercaptoethanol and/or SDS) were attempted. Success was limited, frequently either PE545 was degraded and/or enhanced recovery of chlorophylls and carotenoids was observed in the aqueous phase; these preliminary results also suggested that Na salts were preferable to K salts, hence NP was subsequently tested along with KP. A different approach was required.

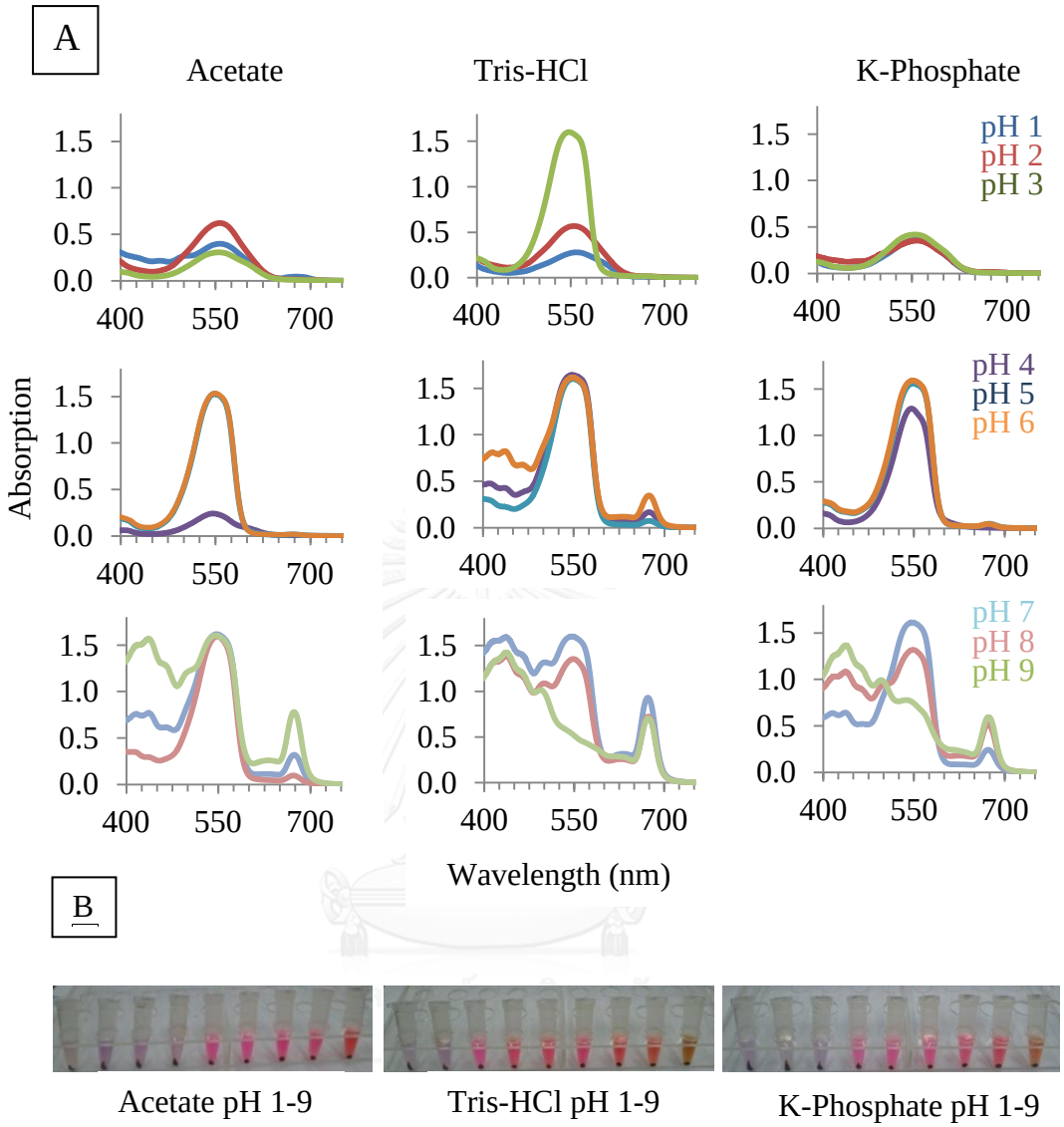


Figure 4.6 Absorption spectra of PE545-containing cell extracts prepared in one of three buffers at nine pH values.

(A) along with color photographs of the extracts and the cell pellets/debris: PE545-containng extracts in AB (left column), TB (center column) and KP (right column) buffers at pH 1-3 (top row), pH 5-6 (middle row) or pH 7-9 (bottom row); (B) Color photographs of AB, TB and KP (left to right, respectively) at pH 1 – 9 (left to right, respectively), for each buffer

4.2.2 Extraction of PE545 from cells pre-extracted with acetone

Co-extraction of phycobiliproteins and chlorophyll/carotenoids was problematic, especially at pH 6 and above (Figure 4.6A); hence the later pigments were extracted in 90% acetone. The absorption profile of whole cells (in Daigo IMK media), cell pellets resuspended in KP buffer at pH 6, solubilized PE545, and the acetone extract are shown in Figure 4.7A. The absorption spectra are difficult to normalize, especially for the whole cells in media and the resuspended cell pellets due to the well-known pigment 'package effect' as well as scattering from cell walls and other cellular debris (Bricaud & Stramski, 1990; Morel & Bricaud, 1981). Despite these limitations, the spectra (Figure 4.7A) are generally consistent with published spectra of *Rhodomonas* spp. Importantly, the isolated PE545 retains the expected absorption spectra with peaks near 545 nm and a prominent shoulder at 567 nm, associated with phycoerythrin and DBV, respectively. Deconvolution of the PE545 absorption spectra, following the guidelines by Billo (2004) and using the expected position and line shapes of the eight pigments in PE545 (Doust et al., 2004), yielded a model with good fit to the observed data for PE545 isolated in TB buffer at pH 6 (Figure 4.7B); similar good model fits were observed in other buffers/pH combinations that yielded the expected 'pink' color associated with PE545 (not shown); modeled peaks were generally at, or near, 506, 521, 535, 553, 565, and 572 nm. These results confirm that extraction of cells with 90% acetone prior to extraction in AB, TB and KP do not meaningfully alter the spectral properties of PE545.

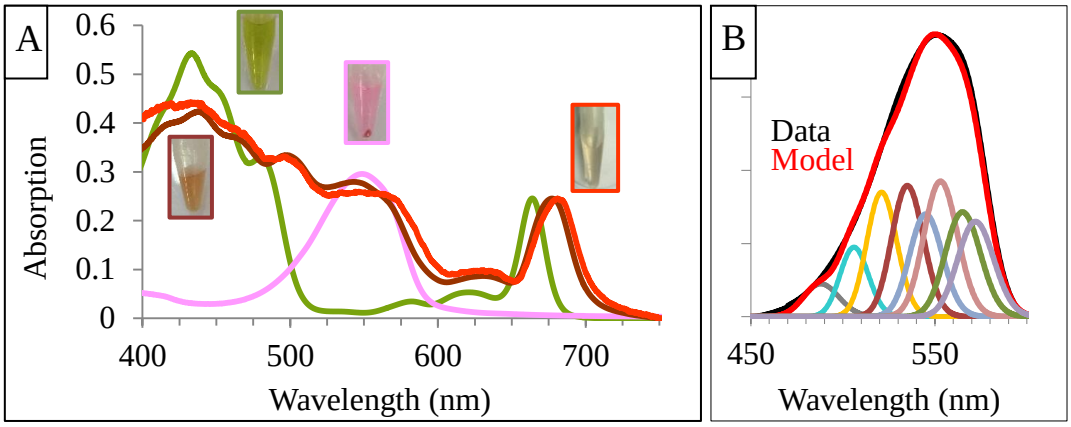


Figure 4.7 Absorption spectra of whole cells, cell pellets, acetone extracts, and solubilized PE545.

(A) Whole cell spectra recorded in Daigo IMK media; cell pellets (normalized against talcum powder), acetone extracts, and PE454 were prepared as described in Methods. Whole cell and cell pellet data are normalized at their Chl a absorption in the red and adjusted to the acetone sample; the acetone extract and PE545 are drawn to scale relative to the number of cells. Inserts are pictures of cell pellets solubilized in TB (pH 8), acetone extract, solubilized PE545, and whole cells in Daigo IMK, (from left to right, respectively). The colors of the absorption curves are the same as the lines surrounding each sample type.

(B) The deconvoluted absorption spectra of PE545 extracted in Tris-HCl, pH 6.0. The curves in black (original data) and red (modeled data) are shown with eight smaller bands associated with the eight pigment moieties in PE545. The X and Y axes scales in (B) are enhanced relative to (A) for illustrative purposes

Four buffers were tested at nine pH values to identify conditions that facilitated phycobiliprotein extraction from the acetone pellets (Figure 4.8). As

observed previously (with non-acetone-extracted cells; Figure 4.6), AB at low pH was not effective, while AB at pH 5 - 8 (and somewhat at pH 9) was more effective at solubilization of PE545. TB at pH 4 -8 solubilized considerable PE545. Both KP and NaP were ineffective at pH 1 - 4, with NaP showing greater extraction than KP at pH 5, 6 and 7, while KP was more effective at pH 8 than NaP (Figure 4.8A). The results indicated that at pH values of 6 and 7, regardless of the buffer, the PE545-enriched supernatants showed pronounced peaks in the 445 - 450 nm range. In contrast, the two lowest pH buffers yielded red shifted peaks (556 - 564 nm); at pH 8 the peaks tended to shift toward the blue by a few nm, while at pH 9 the peaks were very blue shifted (518 - 536 nm; Figure 4.8A). Buffers at pH values of 6 or 7 tended to produce peaks with the greatest absorption, due to a combination of high extraction efficiencies and/or stability of extracted pigments; peak heights were lower at both pH extremes.

The photographs in Figure 4.8B show pigment-enriched cell pellets in all buffer/pH combinations. In other words, pigment extraction was incomplete under all tested conditions.

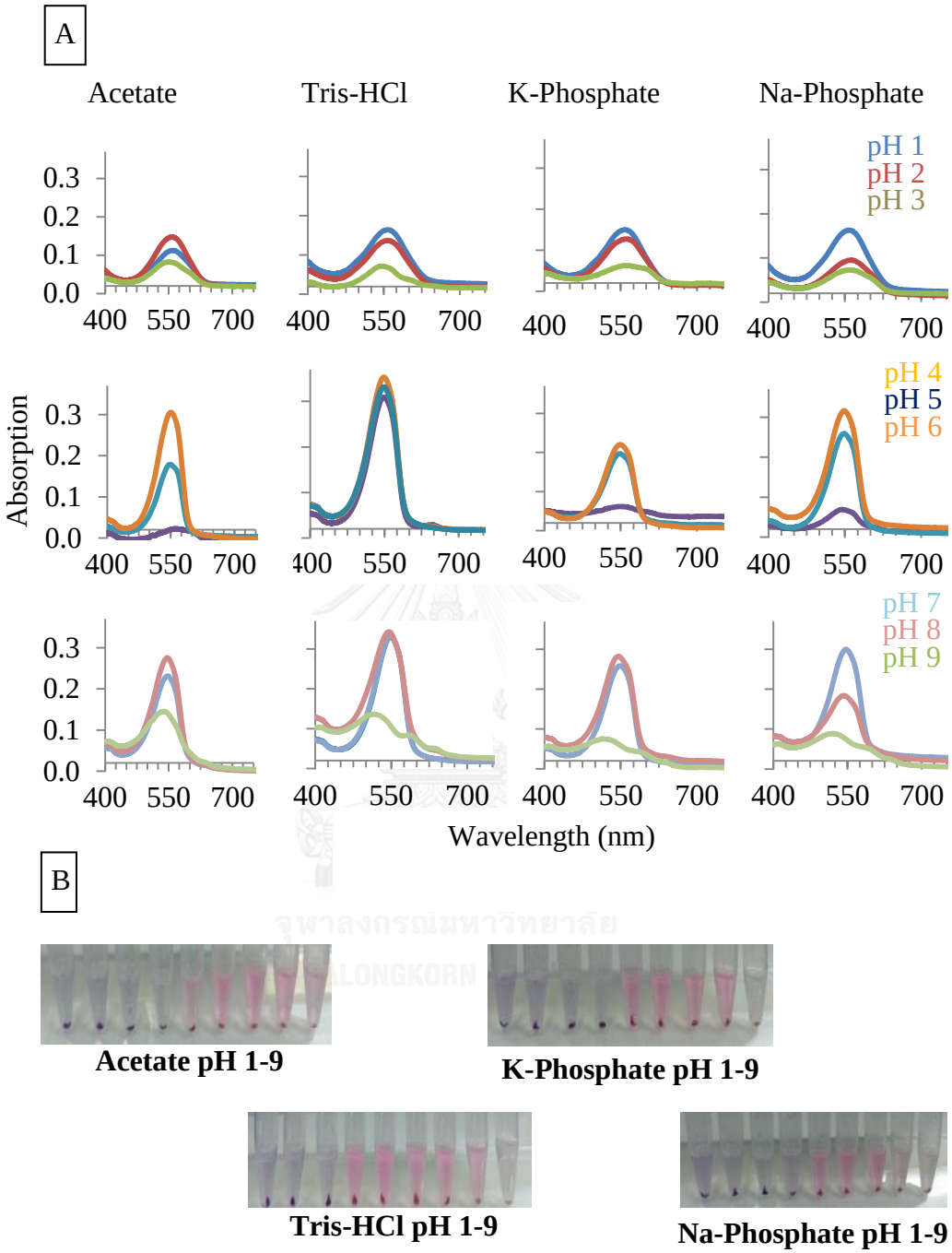


Figure 4.8 Absorption spectra of PE545-containing cell extracts prepared in one of four buffers at nine pH values, along with color photographs of the extracts and the cell pellets/debris following extraction.

Cells were extracted in acetone prior to PE545 extraction with aqueous buffers. As described in Figure 4.8, with addition of Na-phosphate (NaP) in the far right column, the PE545-containing supernatant fractions are referred to in the text as $PE545_{3X\text{-}FTS}$ and are used for analyses shown in Figures 4.9 and 4.10.

4.2.3 Efficiency of PE545

To determine how much PE545 remained in the pellets, extraction efficiencies were computed based on the amount of pigment present in the supernatant following 3X-FTS ($PE545_{3X\text{-}FTS}$; Figure 4.9A) compared to the total amount of pigment ($PE545_T$; Figure 4.9B) in the sample; $PE545_T$ was computed based on the total pigment extracted following two additional rounds of 3X-FTS plus an additional 3X-FTS extraction that included 1% SDS. $PE545_T$ was remarkable similar (no significant differences) for AB, TB and NaP at pH 6 (Figure 4.9B). $PE545_T$ was consistently high for NaP and TB over broad, but slightly different, pH ranges centered around pH 6. The high $PE545_T$ values at pH 6 -7 are not surprising; similar results have been shown repeatedly (Glazer, 1982, 1989b; G. Wedemayer et al., 1992; Zhao et al., 2011). Somewhat unexpectedly, $PE545_T$ in AB was high only at pH6, declining markedly at both pH 5 and pH 7. $PE545_T$ in KP was a noted exception in most cases, due to what appeared to be insoluble KDS particles that co-precipitated with cell debris, including phycobiliproteins. These presumptive KDS particles reduced $PE545_T$ at all pH values tested. Two final comments relate to $PE545_T$ at the lowest and highest pH extremes: 1) at the lowest pH values (pH 1 - 3), pigment was frequently present in the cell pellets for AB, TB, and NaP), and 2) at the higher pH values, substantial alterations in spectroscopic profiles were observed (e.g., pH 9 in all buffers where the PE545

absorption peaks are very blue shifted, indicative of pigment degradation).

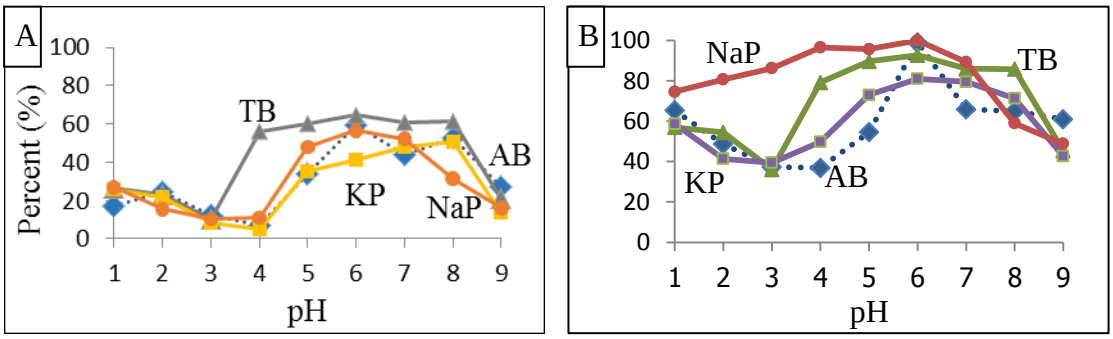


Figure 4.9 'First-roundextraction' efficiency of PE545 following 3X-FTS treatment ($PE545_{3X\text{-}FTS}$; A) and 'total extraction'efficiency' ($PE545_T$; B).

Turning now to $PE545_{3X\text{-}FTS}$ (the extraction efficiency during the first round of 3X-FTS; Figure 4.9A), extraction efficiency was low in all four buffers at both the three lowest (i.e., pH 1 - 3) and highest (i.e., pH 9) values tested (Figure 4.9A). $PE545_{3X\text{-}FTS}$ was more variable between pH 4 - 8 in the different buffers, though pH 5 - 7 tended to provide relatively high extraction in all four buffers. Importantly, $PE545_{3X\text{-}FTS}$ never exceeded 65%.

4.2.4 Stability of PE545

The long-term stability of the $PE545_{3X\text{-}FTS}$ extracts was examined for all buffer/pH combinations. The $PE545_{3X\text{-}FTS}$ samples were transferred to clean microfuge tubes (to eliminate interference from pigments trapped in the cell pellets), frozen at -20 °C for 2 days, thawed, and then incubated at 4 °C for 1 and 12 hr before recording absorption spectra (Figure 4.10).The initial absorption spectra were considered time T_0 for each buffer/pH combination and the value was set to 100% for

each buffer/pH combination (Figure 4.10); because T_0 was set at 100%, the values in Figure 4.10 are difficult to compare to those in Figure 4.10. For instance, for NaP pH 1, pH 3, and pH 6, the $PE545_{3X\text{-}FTS}$ values (taken from Figure 4.9) were 27%, 10%, and 56%, respectively; these values were converted to 100% for the T_0 values in Figure 4.10. One of the striking features of the stability curves revealed in Figure 4.10 is the high stability of pigments at the lower pH values (pH 1 and, in some cases, pH 2), with a rapid decrease in stability at pH 3 or 4 (depending on the buffer). In short, this corresponds to 'low extraction efficiency (Figure 4.9), but high stability once extracted (Figure 4.10). There was a prominent 'V' shape to the stability profiles, as stability increased rather dramatically beyond pH 4 (pH 3 for TB) as the pH increased. Stability decreased above pH 7 (pH 6 for AB and pH 8 for TB). These complex stability profiles are difficult to explain, but are likely related to different aggregation states of PE545 (MacColl et al., 1998), which is likely to impact both extraction efficiencies (Figure 4.9) and long-term stability (Figure 4.10).

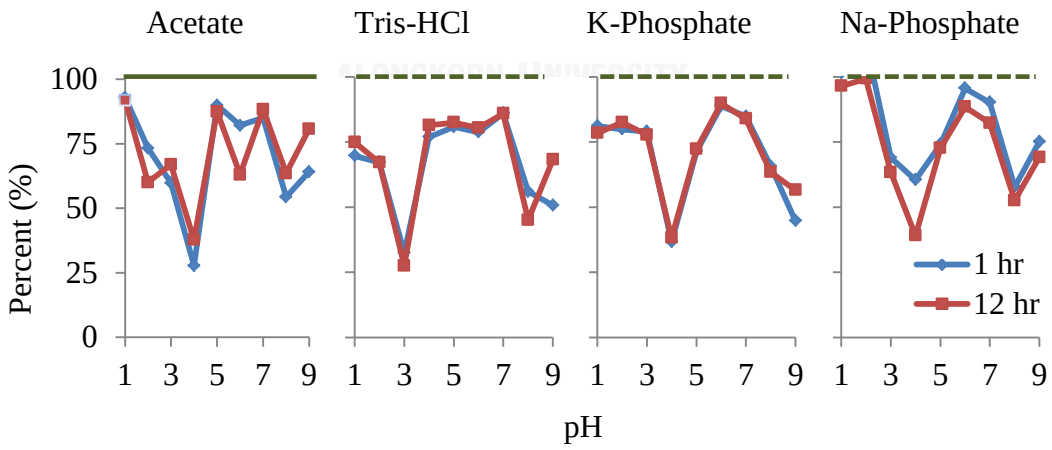


Figure 4.10 Stability of $PE545_{3X\text{-}FTS}$ (from Figure 4.8) during long-term storage. The initial pigment content for each buffer/pH combination was set to 100% and is referred to as T_0 in the text – and is drawn as a dashed green line in the plots above.

None of the buffer/pH combinations allowed complete phycobiliprotein extraction, even if the 3X-FTS was repeated several times (Figure 4.8A). Treatment of samples with SDS, but not urea, tended to result in complete extraction with several buffer/pH combinations; KP was a noted exception, due to what appeared to be insoluble KDS particles that co-precipitated with cell debris, including phycobiliproteins.

4.2.5 Study PE545 with different concentration of SDS

Based on results shown in Figure 4.11, it is clear that pigments are not easily extracted from *R. salina* unless SDS is included in the extraction buffer. The following experiment, rather preliminary in nature, was conducted to gain insights into the use of SDS during the initial 3X-FTS-C. Cells were first extracted in 90% aceteone and cell pellets recovered by centrifugataion. For resuspesion and extraction, four different SDS concentrations (0.0, 0.1, 0.5 and 1.0%) were tested in four buffers at seven different pH values (pH 3 - 9).

Results were unexpectedly complicated, both from a qualitative and quantitative aspect. First, SDS generally improved pigment extraction, as judged by increased color in the supernatant (Figure 4.11A) and decreased pigmentation in the pellet (Figure 4.11 B). Higher SDS concentrations were generally more effective than lower concentrations in terms of pigment extraction. An interesting difference was observed when KP was used as extraction buffer: there was very little phycobiliprotein observed in the supernatant, while the pellet was highly pigmented (Figure 4.11B). The sequestration of phycobiliprotein in the pellet is likely due to the formation of KDS aggregates, which co-precipitated with pigment-protein complexes.

The solubility of KDS would be increased at higher temperature, potentially liberating the phycobiliprotein from the KDS aggregates; however, this would likely to lead to pigment destruction.

A second way the results with SDS were unexpected relates to the color of the supernatants. As shown in earlier work (Figure 4.9) and as repeated here (Figure 4.11), extracts prepared without SDS in buffers with pH values around 5 - 7 (is this correct) are pink in color and have SDS at 0.1, 0.5 and 1.0% with many buffer/pH combinations yielded purple (533 nm) supernatants; unexpectedly, purple supernatants were evident in some buffers at pH values which provided pink supernatants in the absence of SDS. Interesting Gly, the spectra of the purple supernatants in TB (pH 3) and AB (pH 4) with 1% SDS were only slightly blue shifted (541 nm) compared with the pink supernatants (543 - 550 nm), suggesting SDS had only minimal impacts on the native absorption spectra under these buffer/pH conditions.

Overall, this preliminary experiment demonstrated that inclusion of SDS at various concentrations (from 0.1 - 1.0%) improved extraction of phycobiliproteins, resulting in some cases in cell pellets that were white/gray and largely, if not completely, devote of pigments. On the other hand, inclusion of SDS in the extraction buffer led to shifts in the absorption spectrum of biliproteins under some buffer/pH combinations, indicating that further work was needed (i.e., are the shifts due to permanent pigment damage/destruction or are the shifts reversible?).

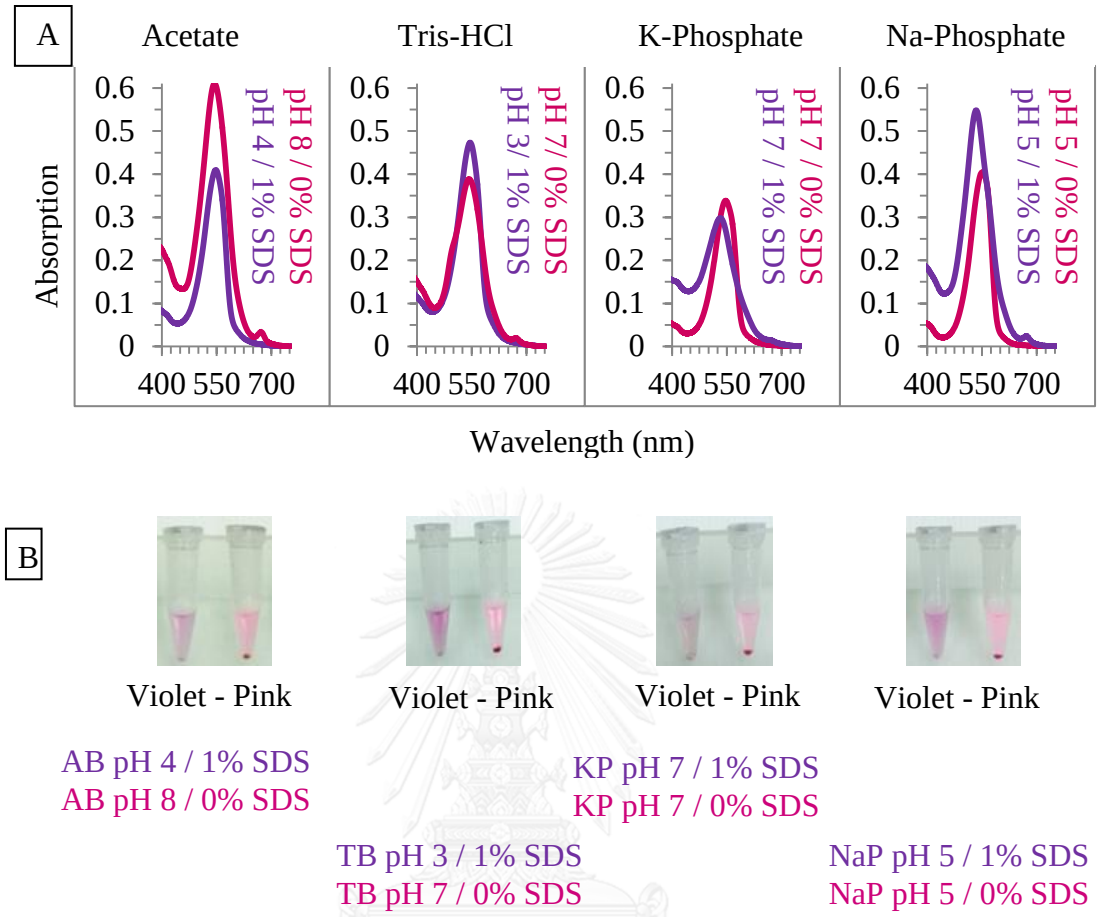


Figure 4.11 Absorption spectra of PE545-containing cell extracts prepared in one of four buffers at nine pH values with or without SDS, along with color photographs of the extracts and the cell pellets/debris following extraction.

Cells were extracted in acetone prior to PE545 extraction with aqueous buffers. Only a few selected spectra and/or photographs are shown (A - absorption spectra of pink and violet supernatants from each buffer; B - photographs of pink and violet supernatants and pellets).

4.2.6 Study the effects of SDS on pigment stability

The previous experiment (Figure 4.9AB) confirmed that SDS, with specific buffer/pH combinations, could enhance extraction of phycobiliproteins from *R. salina* cells previously extracted with 90% acetone. As mentioned above, the results with SDS (Figure 4.11) were confusing, in part because it was not always clear if SDS action was due to enhanced extraction and/or enhanced destruction of pigment either during or after extraction (i.e., were the color shifts from pink (545 - 550 nm) to purple (520 - 530 nm) due to negative effects of SDS on pigment stability?). To address this question more directly, a slightly different approach was utilized. Cells were extracted in acetone, then resuspended in buffer (TB at pH 3, 6 and 9) without SDS, and subjected to 3X-FTS-C. At this point, the supernatants were recovered and the pellets discarded. SDS was then added at either 0.0 or 1% to the supernatants. This revision of extraction protocol completely removes SDS from the extraction process and focuses solely on the action of SDS following extraction. Absorption profiles were recorded over time to determine the effects of SDS on pigment stability.

As expected, TB buffer at pH 3, 6, and 9 provided different extraction efficiencies (compare the signal amplitude in the left most columns, Figure 4.12A). The TB (pH 6) extract had the largest signal followed by TB (pH 3). The absorption of the extract from TB (pH 9) was reduced in size and substantially blue shifted (~510 - 515 nm). The supernatant in TB (pH 9) also shows a pronounced shoulder (~590 nm), which is tentatively ascribed to DBV, not PE; if correct, this implies a red shift of DBV (from 562 nm to 590 nm).

The addition of 1% SDS resulted in a reduced absorption signal at all three pH values tested in TB (Figure 4.12, compare top panels with vs without SDS). A

reduction in absorption in the presence of SDS is most likely due to conformational changes in the phycobiliprotein, an effect that has been noted previously (Glazer & Hixson, 1975). The inclusion of SDS in the TB (pH 6) supernatant resulted in a blue shift of the signal (to ~535 nm); SDS did not appreciably alter the λ_{max} in the other buffers (TB, pH 3 and TB, pH 9).

A major objective of this experiment was to test the effects of SDS on pigment stability. All samples were kept in the refrigerator (3 - 5 ºC) and then scanned at 0 hr, 12 hr and 24 hr; except, the TB (pH 6) samples were scanned at 0 hr, 12 hr, 24 hr, and Day 9. The TB (pH 3 and pH 9) samples were almost colorless after 9 days.

First, it is important to examine the pigment spectra without SDS during this stability experiment (Figure 4.12A, middle panels; Table 4.1; Appendix II). Pigments were more stable in TB (pH 6) than in TB (pH 3); the TB (pH 9) data were not informative.

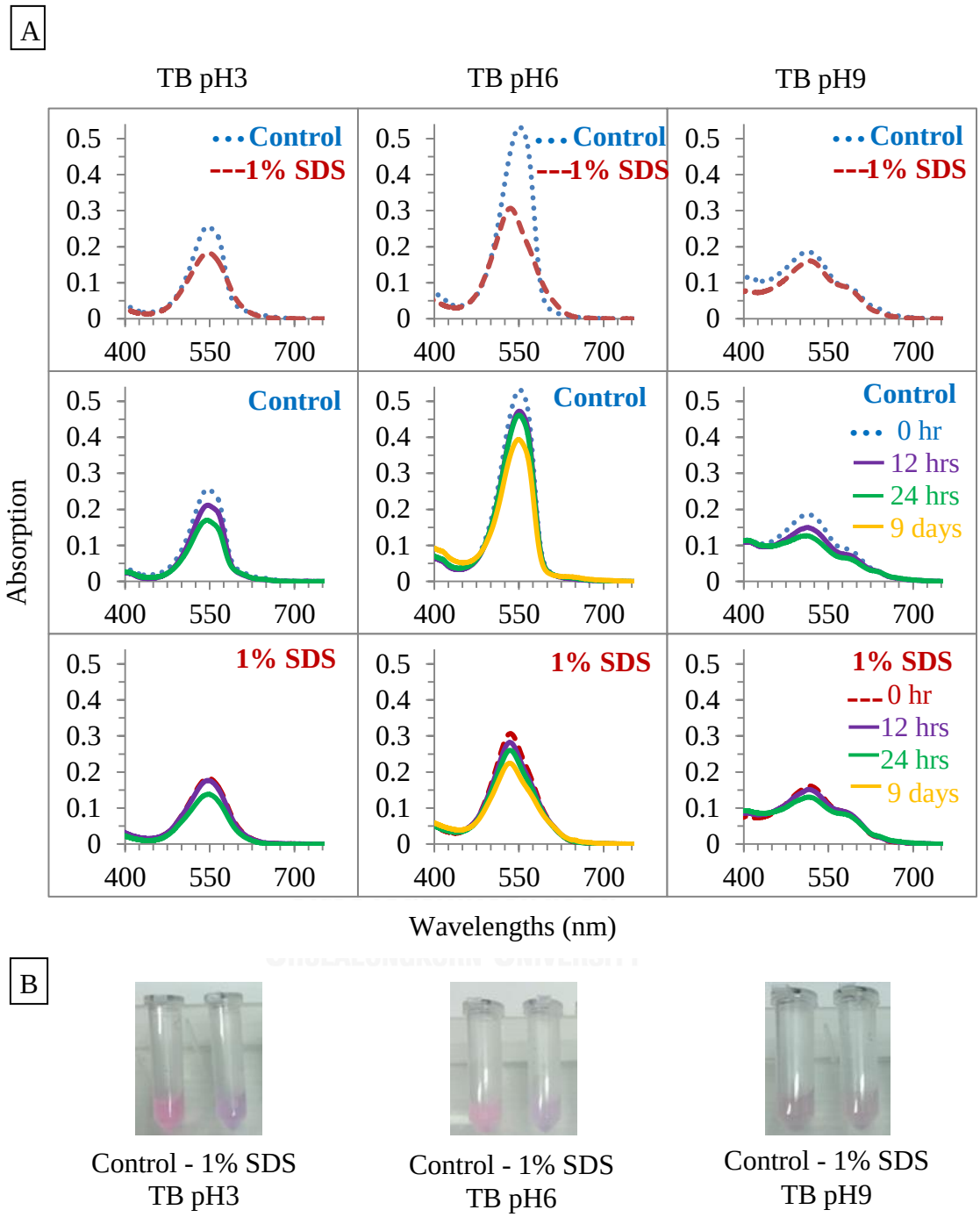


Figure 4.12 The effects of SDS on pigment extraction/stability of PE545 in TB at three pH values.

A) Pigment spectra with / without SDS during this stability experiment: PE545 containing extracts in TB pH 3 (left column), pH 6 (center column) and pH 9 (right column). PE545 containing extracts without 1% SDS at 0, 12, 24 hr and 9 days (middle row) and with 1% SDS at 0, 12, 24 hr and 9 day (bottom row).

B) photographs of TB(pH 3, 6, 9) with / without 1% SDS: color photographs of TB pH 3, 6, 9 (left to right, respectively) without and with 1%SDS (left to right, respectively).

Table 4.1 Summary of results based on data from Figure 4.12

Data presented as % of pigment remaining									
Buffer /pH	λ_{max} (nm)	Time(0 hr)		Time(12 hr)		Time (24 hr)		Time (9 days)	
		Control	1% SDS	Control	1% SDS	Control	1% SDS	Control	1% SDS
TB 3	547	100	100	83	97	66	76	ND	ND
TB 6	551	100	100	89	91	87	84	74	72
TB 9	512	100	100	80	94	68	81	ND	ND

As it is clear that the pigments are undergoing rapid destruction at elevated pH. Pigment stability was not appreciably impacted by addition of SDS during 24 hr (TB, pH 3 and TB, pH 6) or after 9 days of extended storage (TB, pH 6).

Overall, this experiment provided two important insights. First, the absorption spectra of PE may, or may not, be altered qualitatively by the addition of SDS following extraction (i.e., TB, pH 6 + 1% SDS is blue shifted to ~535 nm; in contrast, TB, pH 3 + 1% SDS remains largely unchanged relative to its control without SDS). Second, the addition of SDS does not lead to a dramatic loss of absorption signal. Once pigments are extracted at least during the first 24 hr.

4.2.7 Study effects of SDS and urea on phycobiliprotein extraction and stability

As described above for cells not extracted in acetone (i.e., Section 3.3, stability of PE545), there is a complex interaction between phycobiliprotein extraction efficiencies and phycobiliprotein stability as influenced by pH. Briefly, phycobiliprotein extraction was decreased at low pH, while phycobiliprotein stability was enhanced (Figure 4.9 and 4.10). Further, SDS was required for complete pigment extraction (Figure 4.9). Subsequent work (Figure 4.12) indicated that SDS did not substantially affected PE stability, following PE extraction from acetone-extracted cells, at pH 3 and pH 6.

The current experiment was conducted to ascertain the combined, and possibly synergistic, effects of SDS and urea on phycobiliprotein extraction and stability. Briefly, cells extracted in acetone were resuspended in TB buffer at either pH 3 or pH 6, with various concentrations of SDS and/or urea. As shown repeatedly, PE extraction at pH 3 is very low, presumably related to aggregation of the beta subunits (MacColl et al., 1998). Extraction is enhanced with urea, increasing in parallel with increasing urea concentrations from 1 to 4 to 8M (Figure 4.13). The absorption profile of PE extracted with 1% SDS in TB (pH 3) is substantially enhanced, relative to those with and/or without urea (Figure 4.13). The combination of urea and SDS in TB (pH 3) resulted in absorption profiles that urea interfered with PE extraction (i.e., peaks were small when urea + SDS were used, relative to SDS alone); however, the peak heights may be affected by unfolding of the phycobiliproteins in the presence of urea to a greater/lesser/different extent than when only SDS is used as a denaturant.

The PE absorption peak was, as expected, of greater magnitude in TB (pH 6) relative to that in the TB (pH 3) extract (Figure 4.13 and 4.14). The inclusion of urea

in the extraction buffer with TB (pH 6) resulted in diminished absorption relative to the control (Figure 4.14); however, the peak heights in both TB (pH 6) and TB (pH 3) were of approximately equal intensity, suggesting that the reduced signal amplitude with urea is a direct reflection on phycobiliprotein conformation, not on phycobiliprotein extraction. The addition of SDS to the TB (pH 6) buffers resulted in a slightly reduced PE signals relative to the control (Figure 4.14), regardless of SDS concentration. When both SDS and urea were included in the TB (pH 6) extraction buffers, the absorption was relative unchanged with 1 and 4M urea, regardless of the SDS concentration; with 8M urea, the absorption was substantially decreased at all three SDS concentrations tested.

The above comments are all related to pigment strength (i.e., peak heights). Extractions performed with SDS, urea, and combinations of SDS + urea also resulted in qualitative changes in absorption, primarily blue shifts, resulting in extracts that were visually distinguished by a change from pink to violet (Figure 4.13 - 4.14). The hypochromic (i.e., blue shifted, with lower absorption) shifts were evident with both TB (pH 3) and TB (pH 6), but were more pronounced at pH 6; extraction buffers with SDS, urea, and SDS+ urea all resulted in formation of violet extracts.

Another objective of this experiment was to continue evaluating various buffer/pH combinations with and without SDS and/or urea in terms of long-term PE stability; in this experiment, samples were stored for 16 hr at 3 - 5℃ (refrigerator). PE was surprising Gly stable in this experiment in buffers of pH 3 and pH 6, with and/or without inclusions of urea, SDS or combinations of urea + SDS (Table 4.2 - 4.3; Appendix II), generally greater than 90% over a 16 hr period. The reasons for the enhanced stability of PE in this study (Table 4.2 - 4.3) relative to earlier work (Table

4.1; Appendix II) are unclear, but perhaps related to the use of only the supernatants in the earlier work (Table 4.1) vs use of whole cell extracts (Table 4.2).

Overall, this experiment provided additional insights into PE extraction and stability at two pH values with and/without inclusion of SDS, urea (or combinations of urea + SDS) in the extraction buffer. After completion of this experiment, it was realized that inclusion of urea in the extraction buffer resulted in pH changes; with 8M urea, the TB (pH 6) buffer became (pH 6.9), while the TB (pH 3) buffer was pH 5.59 (Table 4.4; Appendix II). Because of these urea-induced pH changes, it is difficult to unambiguously equate urea effects to changes in pH vs changes in phycobiliprotein conformational changes due to urea-induced unfolding; bilin stability also remains unknown under the different pH, SDS and urea combinations.

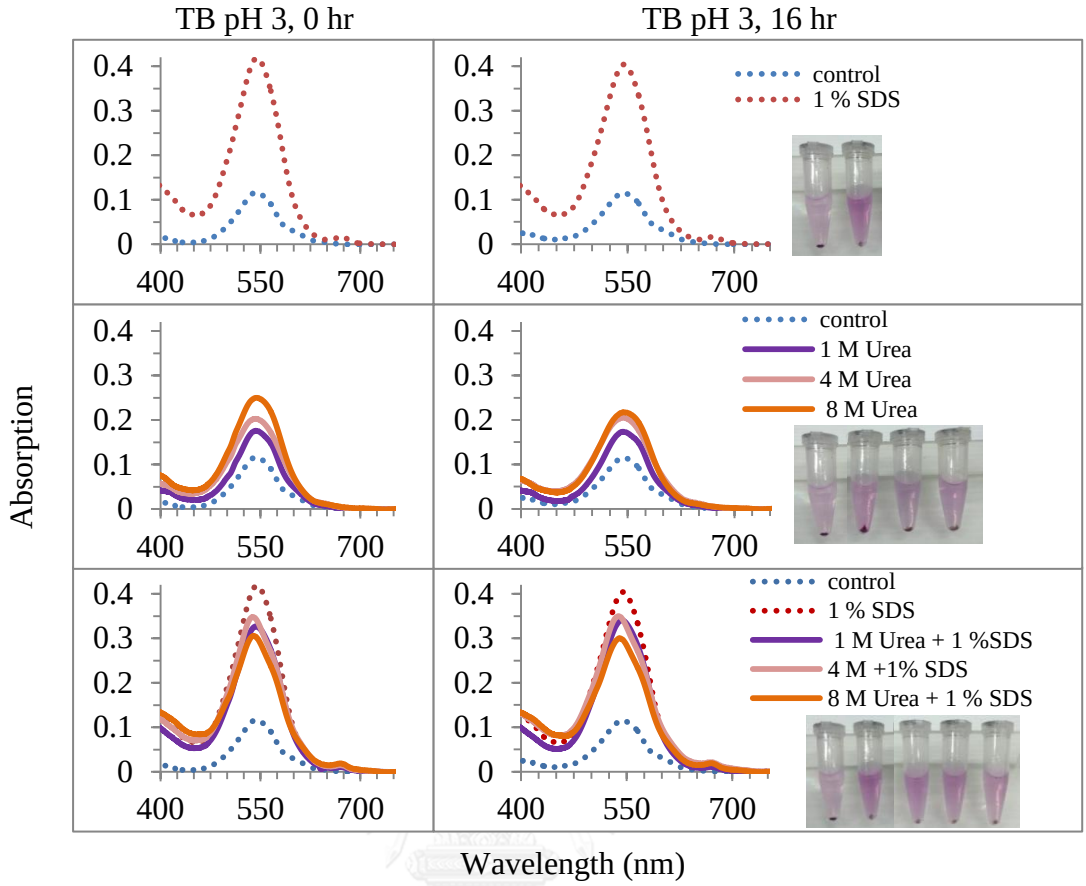


Figure 4.13 The absorption profile of PE545 extracted with 1% SDS in TB pH 3 relative to those with and/or without urea.

PE545-containing extracts in TB pH 3 at 0 hr (left column) and 16 hr (right column). With and without 1% SDS (top row), with and without 1 M, 4M, 8M Urea (middle row) and with and without 1% SDS and 1 M, 4M, 8M Urea +1 %SDS (bottom row).

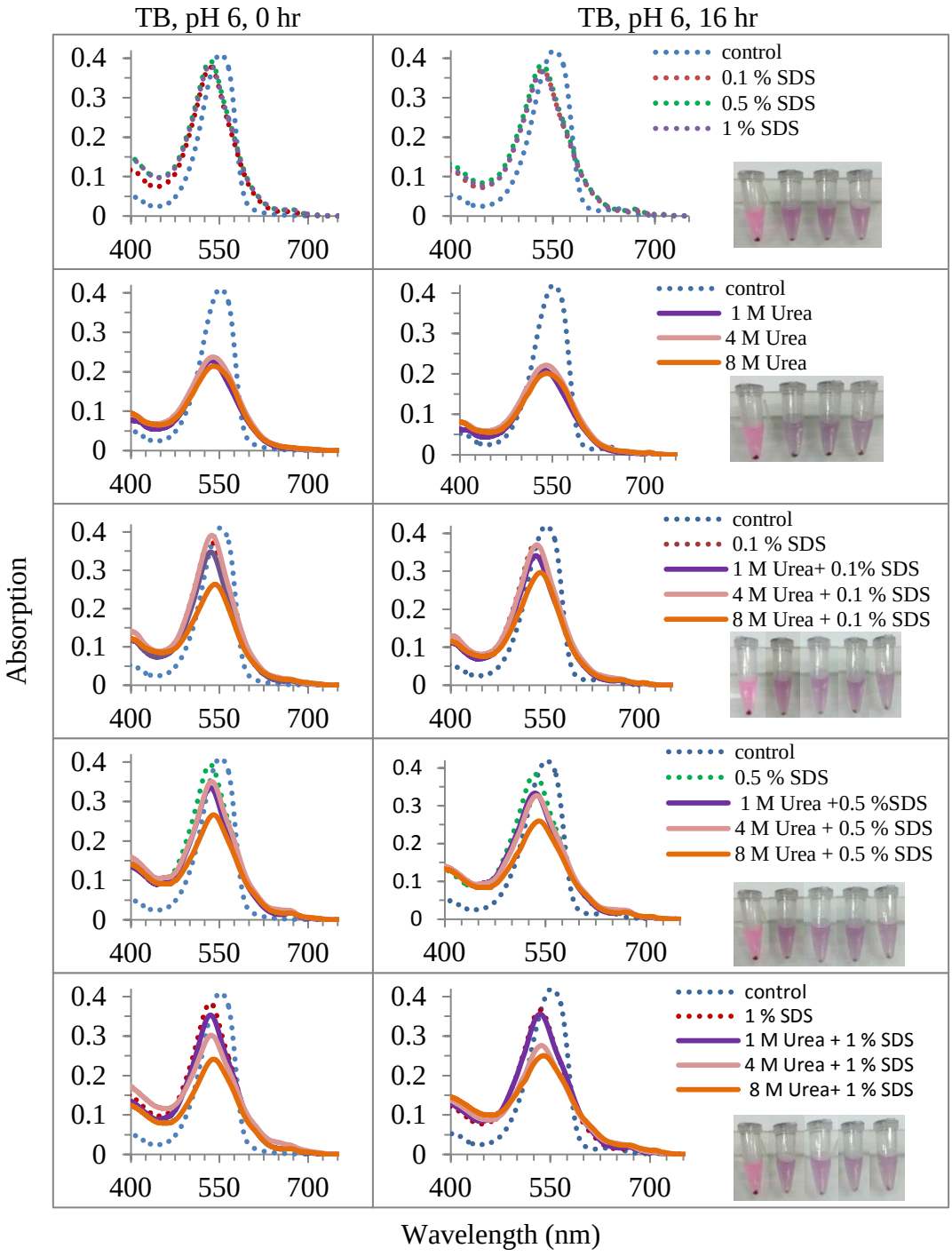


Figure 4.14 The absorption profile of PE545 extracted with 1% SDS in TB (pH 6) relative to those with and/or without urea.

PE545-containing extracts in TB (pH 6) at 0 hr (left column) and 16 hr (right column), with and without SDS (top row), with and without 1 M, 4M, 8M Urea

(secondly row), with and without 0.1% SDS and 1 M, 4M, 8M Urea + 0.1 %SDS (third row), with and without 0.5% SDS and 1M, 4M, 8M Urea + 0.5 %SDS (fourth row) and with and without 1% SDS and 1 M, 4M, 8M Urea + 1 %SDS (bottom row)

Table 4.2 Signal strength at λ_{max} (as percent) remaining after 16 hr of PE545 extracted with 1% SDS and/or urea in TB (pH 3) relative to those with and/or without urea.

Condition (Buffer pH 3)	**λ_{max}**	**Percent remaining after 16 hr**
Control	544	99
1% SDS	545	97
1 M Urea	543	99
4 M Urea	543	101
8 M Urea	544	87
1M Urea + 1% SDS	544	104
4 M Urea + 1 % SDS	538	100
8 M Urea + 1 % SDS	540	98

Table 4.3 Signal strength at λ_{max} (as percent) remaining after 16 hr of PE545 extracted with 1% SDS and/or urea in TB (pH 6) relative to those with and/or without urea.

Condition (TB, pH 6)	**λ_{max}**	**Percent remaining after 16 hr**
Control	551	102
0.1 % SDS	534	98
0.5 % SDS	534	97
1% SDS	534	95
1 M Urea	536	92
4 M Urea	540	88
8 M Urea	540	94
1 M Urea + 0.1% SDS	535	98
1 M Urea + 0,5% SDS	535	99
1M Urea + 1.0% SDS	535	101
4 M Urea + 0.1 % SDS	538	94
4 M Urea + 0.5 % SDS	537	93
4 M Urea + 1 % SDS	536	91
8 M Urea + 0.1 % SDS	543	113
8 M Urea + 0.5 % SDS	540	98
8 M Urea + 1.0 % SDS	540	104

Table 4.4 The pH change when urea (8M) was added to TB buffer at original pH 3 or pH 6**.**

Condition	pH
TB pH 3 + 8 M urea	5.59
TB pH 6 + 8 M Urea	6.9

4.2.8 Study PE545 with and without 8M Urea pH 2 or 20% acetic acid

The primary objective of this experiment was to determine bilin levels in control and SDS-treated extracts. This was accomplished by subjecting extracts to either 20% acetic acid (AcOH) or 8M urea, pH 2 (acidic urea); both treatments have been shown to provide bilin spectral properties that are (largely) independent of the biliprotein (i.e., these treatments disrupt bilin-bilin and bilin-protein interactions). Cells were extracted 2X in 90% acetone then resuspended in TB (pH 3 or pH 6) without or with SDS (0.1, 0.5 or 1%) and subjected to 3X-FTS-C. Beta-mercaptoethanol (BME) was added at 5 mM to half the samples.

A few caveats are required before describing the results. First, extracts prepared in TB (pH 3) with SDS were blue shifted in this experiment (i.e., from 550 to 530 nm; Figure 4.15 and 4.16, normally SDS extracts prepared in TB (pH 3) have only a small (or no) blue shift (e.g., Figure 4.15 and 4.16). Second, both controls (TB, pH 3 and pH 6, without SDS) had elevated absorption in the region of 450 nm (Figure 4.15 and 4.16). The absorption in the 450 nm region was enhanced with addition of SDS; although SDS has been shown to result in slight increases in absorption at 450 nm in earlier experiments (e.g., Figure 4.15 and 4.16), the increase was larger in this experiment. Further to the second point, treatment of extracts with AcOH or acidic urea led to even larger increases in 450 nm absorption. Elevated absorption at 450 nm is generally asssociated with bilin conformational changes (Schluchter & Bryant, 2002). The reason (or reasons) for the elevated 450 nm absorption are unclear, but may be related to either extracting cells 2X in 90% acetone (instead of only once), the longer over-night extraction in acetone (vs a few hours in previous experiments), or the use of fresh TB buffers (made a few days/weeks before this experiment was

conducted). It seems most likely the effect was caused by acetone (probably protein dehydration); this represents future work that should be addressed. SDS at all three concentrations (0.1, 0.5 and 1%) increased the absorption profiles in both TB (pH 3) and TB (pH 6) to approximately the same extent (i.e., all concentrations appeared to be equally effective at both pH values; e.g., Figure 4.15 and 4.16). As mentioned above, the peaks were blue shifted when extraction buffers included SDS. BME generally did not impact pigment profiles, except the control in TB (pH 3), where BME appears to have led to a substantial (almost 2X) increase in pigment extraction; BME had little effect in other samples and will not be described further.

Addition of AcOH to 20% resulted in a red shift in the PE signal (e.g., Figure 4.15and 4.16) in both control samples as well as in the samples treated with the highest SDS (1%). The signal in the controls was approximately 560 nm, which likely reflects the 555 nm signal from PE, plus the 562 nm signal of DBV. The extracts prepared in 1% SDS also had substantial signal strength centered at 560 nm, but also retained substantial signal in the 545-550 nm region, likely reflecting bilins not yet impacted by AcOH. Glazer and Fang (1973) emphasized that spectrophotometric measurements should be made within 3 min after adding AcOH, but the earlier work involved purified phycobiliproteins, not crude extracts like those used in this study. A second line of future work should be to test the AcOH incubation time with crude cell extracts to determine when (or if) the 'native' bilin profiles become evident in SDS-treated extracts, as judged by formation of a 560 nm signal similar to that in the controls. An unexpected result was that the AcOH treated extracts prepared with the lowest SDS concentration (i.e., 0.1%) provided spectrophotometric signals that were impossible to comprehend. The cell suspension became somewhat cloudy, almost like

a gel, resulting in low amplitude signals with very little sign of bilins. Extracts prepared in the intermediate SDS concentration (0.5%) gave signals that were intermediate to those of the low and high concentration of SDS (i.e., understandable, but still rather confusing).

Treatment of extracts with acidic urea resulted in a blue shift in all samples, both controls as well as SDS-containing extraction buffers (Figure 4.15 and 4.16). Signals were rather broad, as expected, and centered around 560 nm, again likely due to a predominance of PE (555 nm) and lesser amounts of DBV (562 nm). Other than the elevated 'baseline' at 450 nm, these spectra are as anticipated and, importantly, suggest that SDS does not appreciably impact the bilins.

Despite the overall positive results shown with AcOH (at high SDS) and acidic urea in this experiment, there are still issues that remain to be resolved. In addition to the issues described above (e.g., elevated 450 nm baseline; time-dependent AcOH incubation), one important observation is the large increase in absorption observed in extracts once diluted in acidic urea (i.e., compare the Y axis scale in Figure 4.15 and 4.16 for urea treated extracts). In general, addition of AcOH and/or acidic urea are expected to result in a diminished absorption signal (i.e., bilins have increased absorption in the native state, relative to the denatured state). This reduction was generally observed in the AcOH controls, but not with SDS-treated extracts, probably because SDS had already resulted in protein denaturation. However, the extracts prepared in acidic urea had substantially increased absorption relative to the samples before urea treatment. Bryant 'quantitatively compensated' for this by diluting the sample 5X in buffer and also 5X in acidic urea, then comparing the absorption profile of both dilutions. This is meant to eliminate (or at least minimize) the effects

of bilin-protein concentrations on absorption spectra. Clearly, this is another avenue of future research that should be evaluated in greater detail.

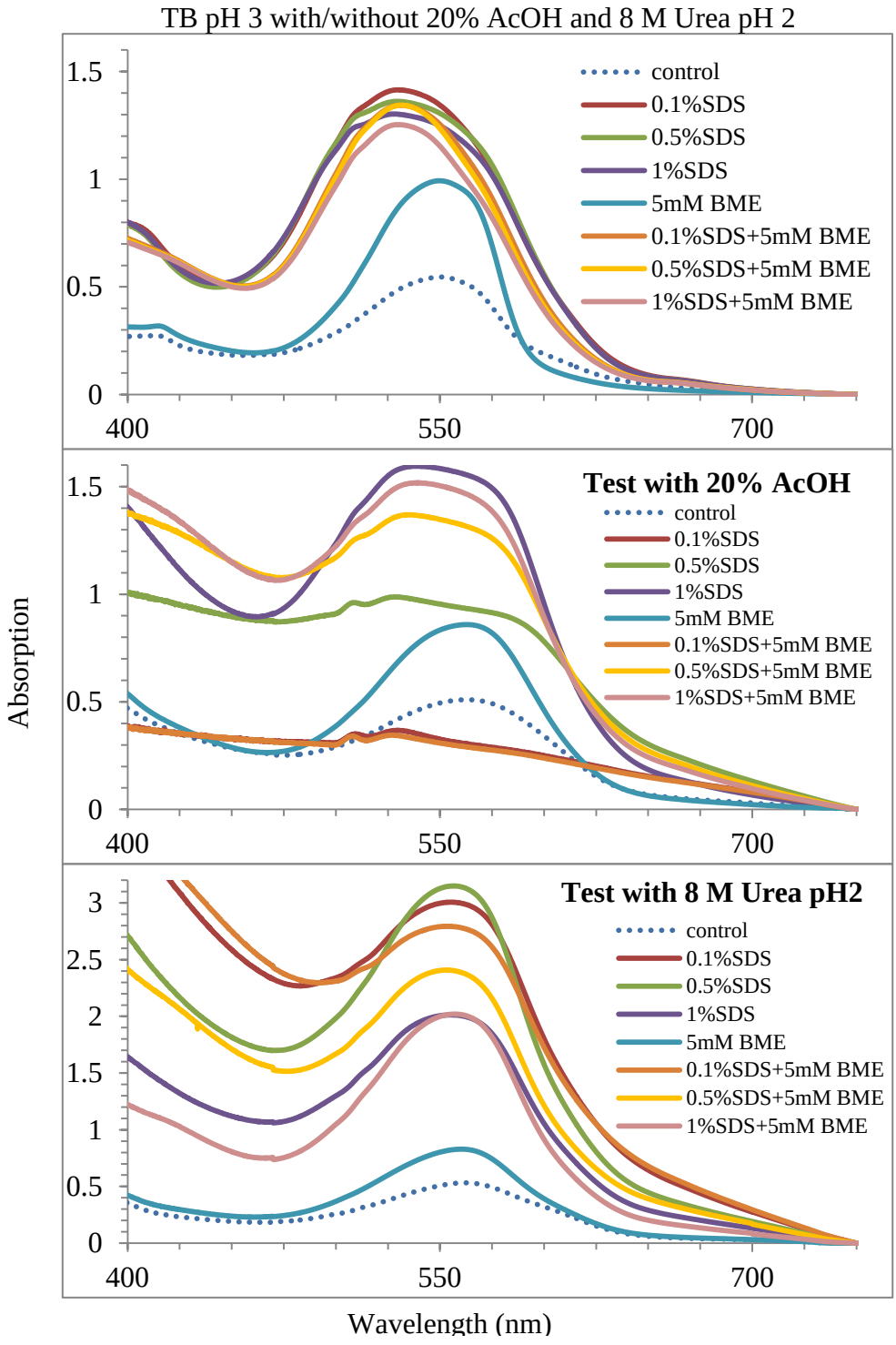


Figure 4.15 TB, pH 3, with/without 20% AcOH and 8 M Urea pH 2.

PE545 containing extracts in TB, pH 3 (top row), with/without 20% AcOH (middle row) and 8 M Urea pH 2 (bottom row).

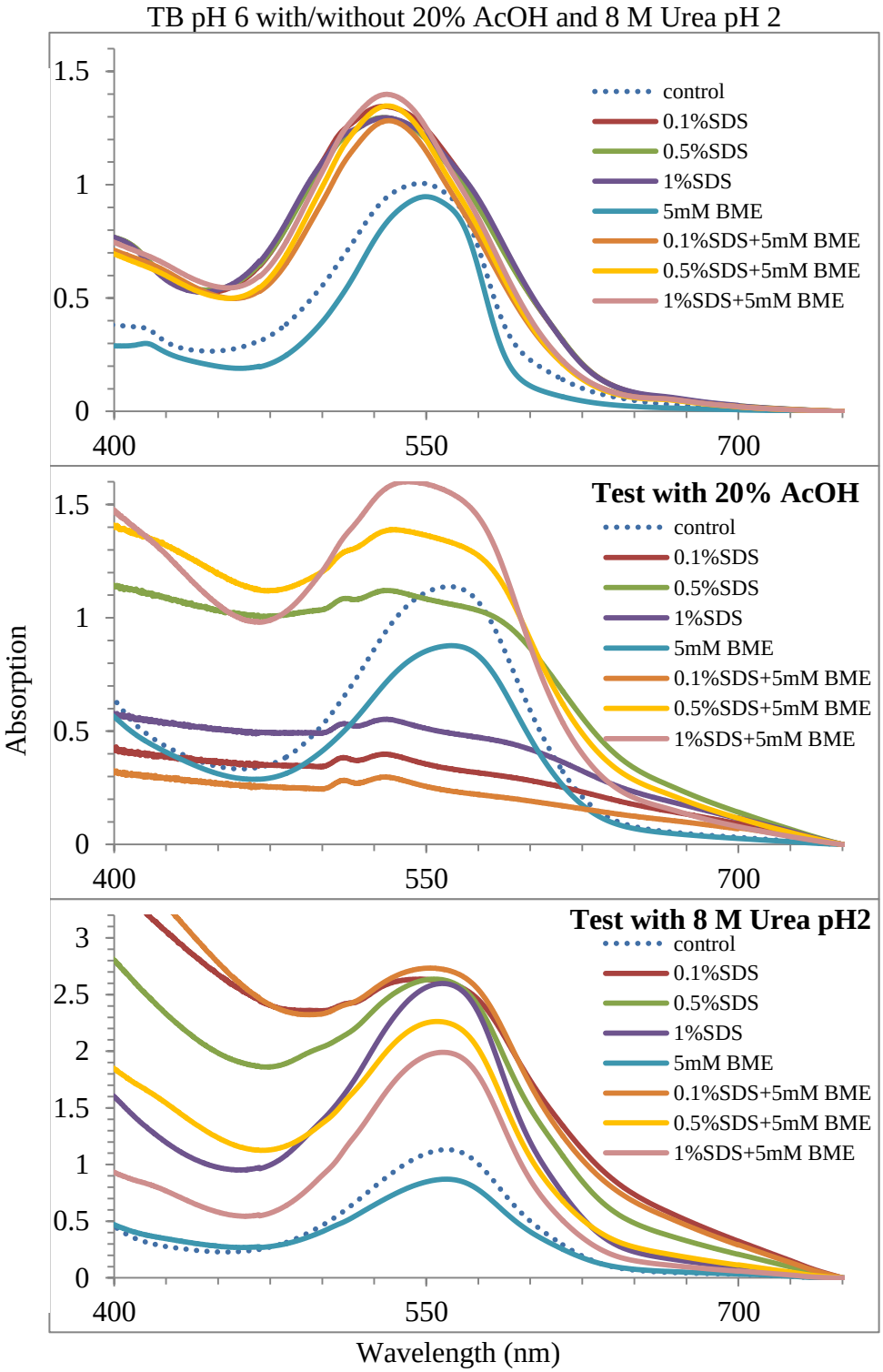


Figure 4.16 TB, pH 6, with/without 20% AcOH and 8 M Urea pH 2.

PE545 containing extracts in TB, pH 3 (top row), with/without 20% AcOH (middle row) and 8 M Urea pH 2 (bottom row)

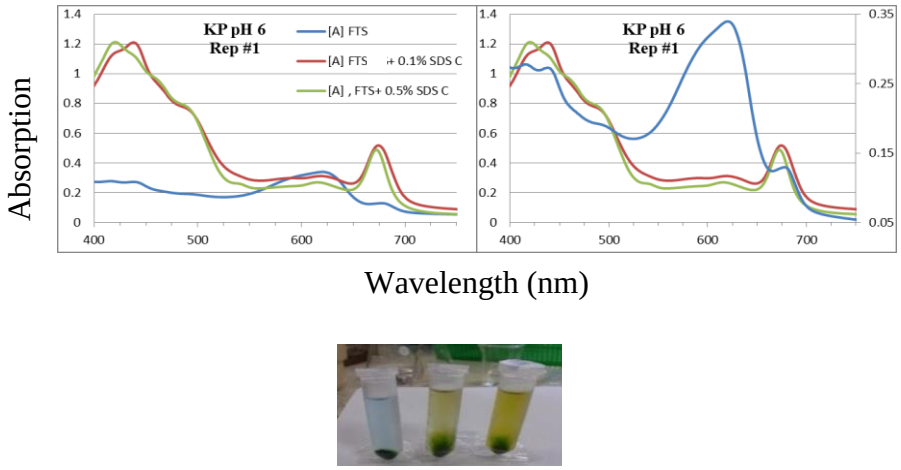
***Synechococcus* spp.**

4.2.9 Extraction of PC with and without 0.1, 0.5 % SDS

The first, very preliminary, work with RS9917 was a test of extraction with and without added SDS (0.1 and 0.5%). KP (pH 6) buffer was chosen, as this buffer/pH combination has been routinely used for extraction of phycobiliproteins from a large number of cyanobacteria. Cell pellets were resuspended in KP buffer and subjected to single round of FTS-C (i.e., not 3X-FTS-C as later became routine).

A blue pigmented supernatant was obtained in the control samples (i.e., no SDS), which had an absorption peak at ~ 623 nm, with a blue-shifted shoulder at ~595 nm (this can be seen most clearly in the righ-most panel, which has the control peak at full scale). The addition of SDS caused a considerable increase in the extraction of Chl and carotenoids, as evidenced by the appearance of bands absorption bands at ~670 and ~440 nm (Chl) and 487 nm (carotenoid). The supernatants were also clearly green, or brownish green, in extracts with SDS, in contrast to the blue extracts prepared without SDS (Figure 4.17).

The effects of SDS extraction/stability are not clear from this experiment. The inclusion of SDS in the extraction buffer appears to have caused a reduction in the blue signal (623 nm and/or 595 nm), but the strong signal from Chl tends to complicate this region of the spectrum. As the pellets in the SDS-treated samples are somewhat smaller than those in the control, the SDS effect on phycocyanin may be related to bilin stability rather than phycobiliprotein extraction.



FTS, FTS + 0.1%SDS C, FTS + 0.5%SDS

Figure 4.17 The effects of 0.1 and 0.5 % SDS on extraction of phycobiliproteins of *Synechococcus* spp. (RS9917).

PC containing extracts in KP with and without SDS (left and right).

4.2.10 Extraction of PC with and without 0.1, 0.5, 1, 2 % SDS

The next, still preliminary, experiment was conducted using two buffers, 0.1 M KP, pH 6.38 and 0.1 M Tris-HCl, pH 8. SDS was used at 0, 0.1, 0.5, 1.0 and 2.0%. Resuspended cell pellets were subjected to 3X-FTS-C cycles to release PC. The PC signal was substantially increased in TB (pH 8) than in KP (pH 6.38) (Figure 4.18, left-most panels); the PC peaks, drawn to full scale (Figure 4.18, right-most panel) both had the expected signal strength at long wavelength, ~612 nm in KP and ~617 nm in TB. Both PC peaks had pronounced shoulders at shorter wavelengths (~590 - 595 nm in both cases). The PC 'baseline' expected around 500 nm was elevated in both samples, but more so in KP than TB. The pounced shoulder (~590 – 595 nm) and

the elevated baseline (at 500 nm) are both signs of altered biliprotein environments, with corresponding changes in bilin absorption profiles.

Inclusion of SDS in the extraction buffers increased the amount of chlorophylls and carotenoids in the supernatants as visually evident in photographs of the samples (Figure 4.18B, lower) and elevated signals in the red (~670 nm) and blue (~440 - 487 nm). Signals attributable to PC were not evident in the photographs or in the spectral profiles. However, it is noteworthy that supernatants in both buffers have elevated absorption in the ~520 - 610 nm region as well as a rapidly increasing signal starting at about 520 nm that overlaps with the carotenoid signal as it 'bleeds' into the blue region of the spectrum. The importance of these signals is described and discussed below, but in general, are likely due to SDS-induced changes in biliproteins and the corresponding changes in bilin absorption.

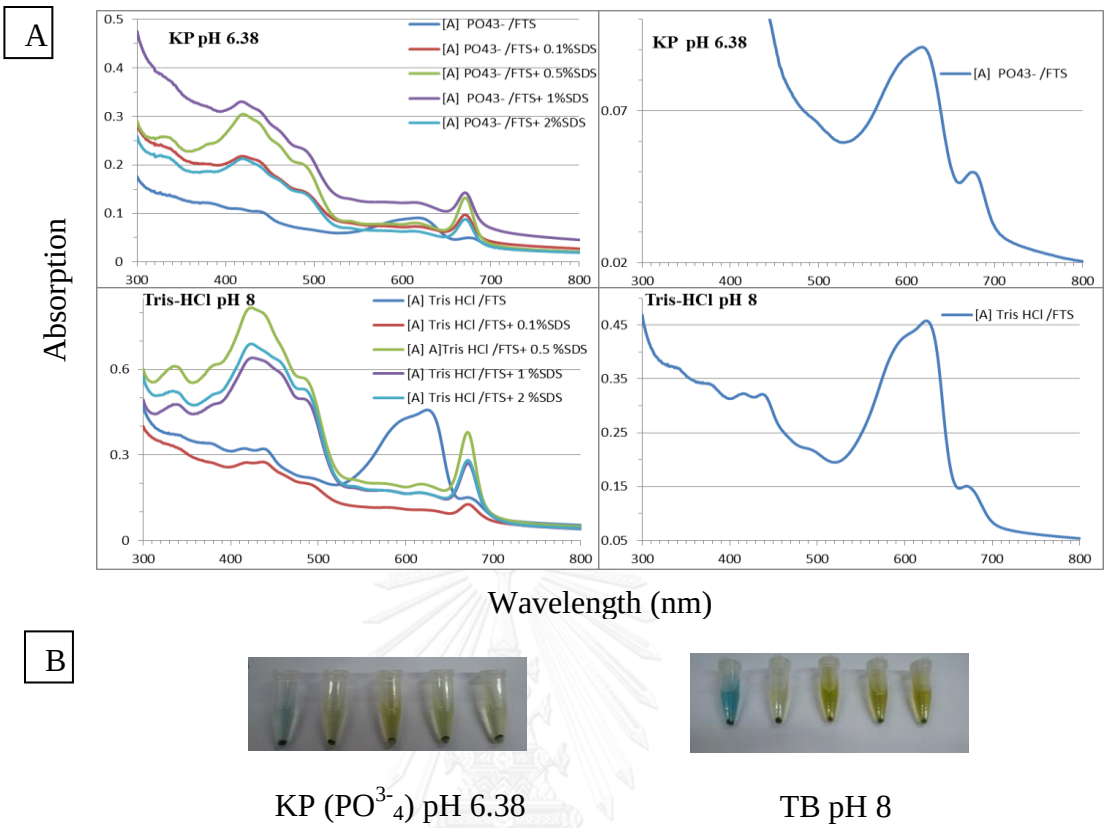


Figure 4.18 The effects of 0.1, 0.5, 1, 2 % SDS extraction with phycobiliprotein in *Synechococcus* spp. (RS9917).

A) The effects of 0.1, 0.5, 1, 2 % SDS extraction with phycobiliprotein in KP (pH 6.38) and TB (pH 8): The PC signal was substantially increased in TB (pH 8) than in KP (pH 6.38) (left-most panels).

B) Photographs of the samples, the blue supernatant in KP (pH 6.38) and TB (pH 8): Tubes are control FTS (without SDS) and other samples are FTS with 0.1, 0.5, 1, 2 % SDS.

4.2.11 PC extraction with and without prior extraction of chlorophylls and carotenoids in 90% acetone

A third and final, preliminary experiment with RS9917 tested PC extraction with and without prior extraction of in 90% acetone. Following acetone extraction, the supernatants were green in color and the pellets were blue; both results were as expected (Figure 4.19, left). The cell pellets were substantially reduced in size following acetone extraction (Figure 4.19, center photo). The acetone-extracted cell pellets, as well as the controls (no acetone treatment) were resuspended in 0.1 M Tris-HCl, pH 8 and subjected to 3X-FTS-C to release PC. Extracts from the control (no acetone) cells had clear signs of PC, it was blue in color (Figure 4.19, right most photo) and with prominent absorption peaks at ~615 and a shoulder around ~590 - 595 nm (data not shown). Extracts from acetone-treated cells were very lightly pigmented (Figure 4.19, right most photo) and the pellet remained heavily pigmented, suggesting the acetone treatment interfered with PC extraction. The supernatants from the acetone-treated cells had an absorption spectrum that was small in amplitude, but generally consistent with the PC signal in the control; however, the PC signal in acetone-extracted cells was perhaps more similar to that of SDS-treated cells (e.g., Figure 4.19).

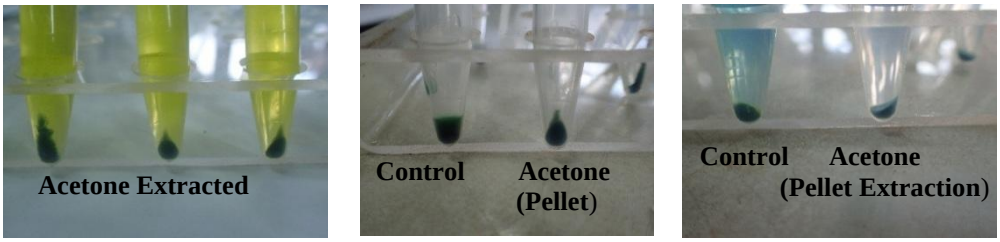


Figure 4.19 PC extractions from *Synechococcus* RS9917 with and without prior extraction of Chlorophylls and carotenoids in 90% acetone.

4.2.12 The effects of SDS on PC extraction/stability

Further experiments were conducted with RS9917 to determine the effects of SDS on PC extraction/stability. Cell pellets were resuspended in either NaP (pH 6) or NaP (pH 7) buffers with various concentrations of SDS (0.0, 0.5, 1.0 and 2.0%); both control cells as well as acetone extracted cells were used. Extraction included 3X-FTS-C, as routinely developed for *R. salina*.

PC extraction was greater at pH 6 (Figure 4.20A and 4.20C) than at pH 7 (Figure 4.20B and 4.20D) in cells that had not been pre-extracted in acetone. The PC signal at pH 6 was also more like the native complex reported by others, e.g., a prominent signal at ~620 nm, but with a notable shoulder at ~ 595 nm (Figure 4.20C); the 595 nm signal in PC at pH 7 was slightly larger than the 620 nm signal (Figure 4.20D), a potential sign of pigment instability. There is a moderately strong Chl signal in both samples (Figure 4.20C and 4.20D) as most easily evidenced by the peak at ~675 nm; the co-extraction of Chl with PC is one of the main reasons it is important to remove Chl by acetone pre-treatment, as described below.

The inclusion of SDS in the extraction buffer reduced the signals easily attributable to PC at both pH 6 and pH 7 (Figure 4.20A and 4.20B). The putative PC signal in SDS-containing extracts, marked as ~611 nm (Figure 4.20A and 4.20B), is difficult to decipher, in large part because it is swamped by the much large signal from Chl.

RS9917 cells were extracted in acetone to remove Chlorophylls and carotenoids, then resuspended in NaP buffer at pH 6 and pH 7, with and without SDS before 3X-FTS-C. PC extraction and/or stability were greatly diminished in acetone extracted cells (compare A to E and B to F in Figure 4.20); virtually no PC was

detectable without SDS at either pH. When PC spectra are drawn full scale, it becomes more obvious that PC at pH 6 with and without SDS has similar spectral absorption profiles with and without SDS (Figure 4.20G and 4.20H). In contrast, the absorption profiles at pH 7 are blue shifted to ~ 555 - 560 nm.

In 1975, Glazer and Hixson (1975) studied the effect of SDS on R-PC from a red alga (*Porphyridium cruentum*), which, in an unusual case, has two bilins, both PCB and PEB, as components of its PC. At pH 7, the addition of 1% SDS to extracts of purified R-PC resulted in a reduction of absorption by approximately 3 - 4 X in 0.02M acetate buffer at 24℃. Further, the PCB chromophore was blue shifted to approximately 560 nm with added SDS. It appears that the results presented here largely mirror those of Glazer and Hixson (1975); at pH 7 (with 0.5 and 2% SDS, but not 1.0% SDS), the PC signal at ~550 - 560 nm has an amplitude of ~0.7 A units (Figure 4.20H) whereas the signal without SDS in the control sample (Figure 4.20A and 4.20D) is about 1.60 A units. In other words, when care is taken to keep the samples cold (in this work) and not at elevated temperature (24℃ by Glazer and Hixon), the effects of SDS on PC strength is lessened (i.e., 3 - 4 X at 24 C vs ~ 2.2X when samples are maintained on ice, as was done in this work). Interesting Gly, at pH 6, the effects of SDS are in one way more dramatic (i.e., the signal is reduced ~ 16 X, from 0.28 to 0.017 A units), however, at the same time, the signal at pH 6 does not show the dramatic blue shift observed at pH 7.

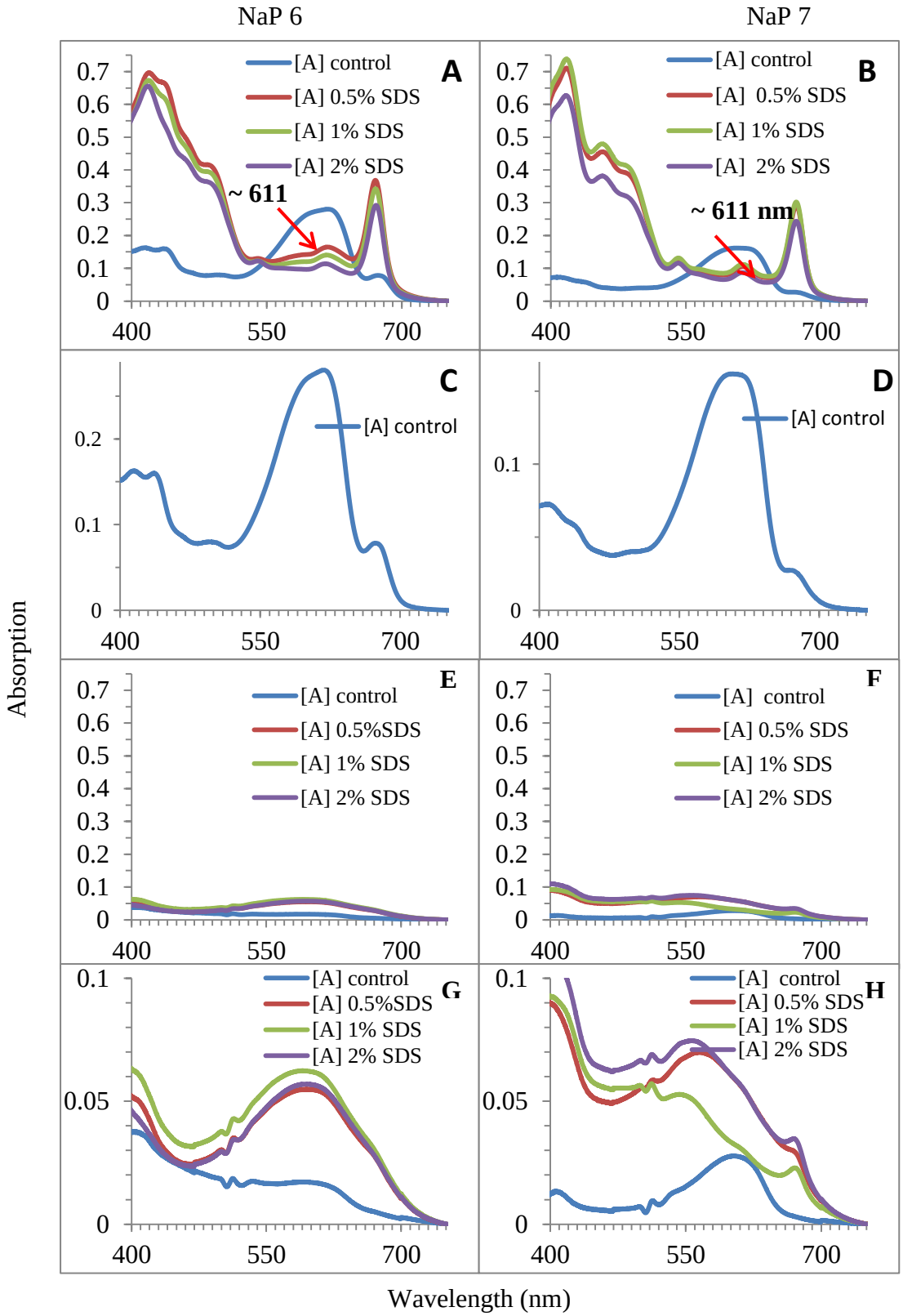


Figure 4.20 The effects of SDS on PC extraction/stability from *Synechococcus* spp. RS9917.

(A-D cells that had not been pre-extracted in acetone; (E-H)cells that had been pre-extracted in acetone; PC containing extracts in NaP (pH 6) with and without SDS (left column) and PC containing extracts in NaP (pH 7) with and without SDS (right column).

4.2.13 Extraction of PC in different buffers/pH

The purpose of this experiment was to confirm the earlier, largely preliminary, studies regarding the effects of different buffer/pH combinations on extraction and stability of PC from the cyanobacterium RS9917. Overall, PC extraction/stability appeared to be highest when the pH of the buffer was between pH 5 and 8 (Figure 4.21); there was a considerable buffer-to-buffer variation at these pH values. The buffer/pH variations included the amount of Chl extracted (e.g., always greater with higher pH, but especially in Tris-HCl) as well as the PC spectral profile (e.g., lambda max tended to be closer to 617 nm with a pronounced shoulder ~590 nm at pH 5 - 6, Buffers at pH 3 failed to extract PC in all three buffers, while pH 4 buffers provided low (AB buffer), moderate (NaP buffer) or high (Tris-HCl) extraction/stability (Figure 4.21).

A second objective of this experiment was to test the effect of SDS on PC stability. PC-enriched supernatants were removed from the pellet and SDS (1%), along with Gly (30%), were added; Gly was included because it is generally included as part of the SDS-PAGE buffer and it was guessed that it may provide some stability to PC. The inclusion of SDS (and Gly) resulted in sharp and dramatic decreases in PC absorption (spectra with and without SDS/Gly were corrected for dilution so a direct comparison is possible; Figure 4.22, compare upper row to lower row). As noted

before (e.g., Figure 4.21), the presence of Chl at higher pH values interferes with interpretation of the PC signal, but a few salient points are possible. Qualitatively, the PC lambda max remains well above 600 nm for several buffer/pH combinations, notably when the pH is 6, but especially at pH 4 and 5 (Figure 4.21); blue shifts are very evident at pH 7 and especially pH 8. Quantitatively, the PC signals were reduced with SDS/Gly by 3-4 X (e.g., in A, NaP and Tris-HCl buffers at pH 6, the control (no SDS) absorption vs the control were: 0.605 units vs 0.189 units = 3.2X; 0.71 vs 0.22 = 3.2X and; 0.58 vs. 0.17 = 3.4X, respectively) in Tris-HCl, pH 6. Admittedly, the presence of Chl make these calculations very approximate, but still informative. Interesting Gly, the 3-4X reductions in absorption observed here (Figure 4.22) are in line with those reported by Glazer and Hixson (1975) for highly purified R-PC with added SDS at neutral pH.

One final observation about the effects of SDS on PC, as shown in Figure 4.22, are warranted. There was considerable buffer-to-buffer variation and considerable pH-dependent variation in the PC absorption profiles when SDS was added (Figure 4.22), however, the absorption was generally about 0.2 A units, which are a combination of both PC and Chl signals. This value will be used below to explain the usefulness of SDS.

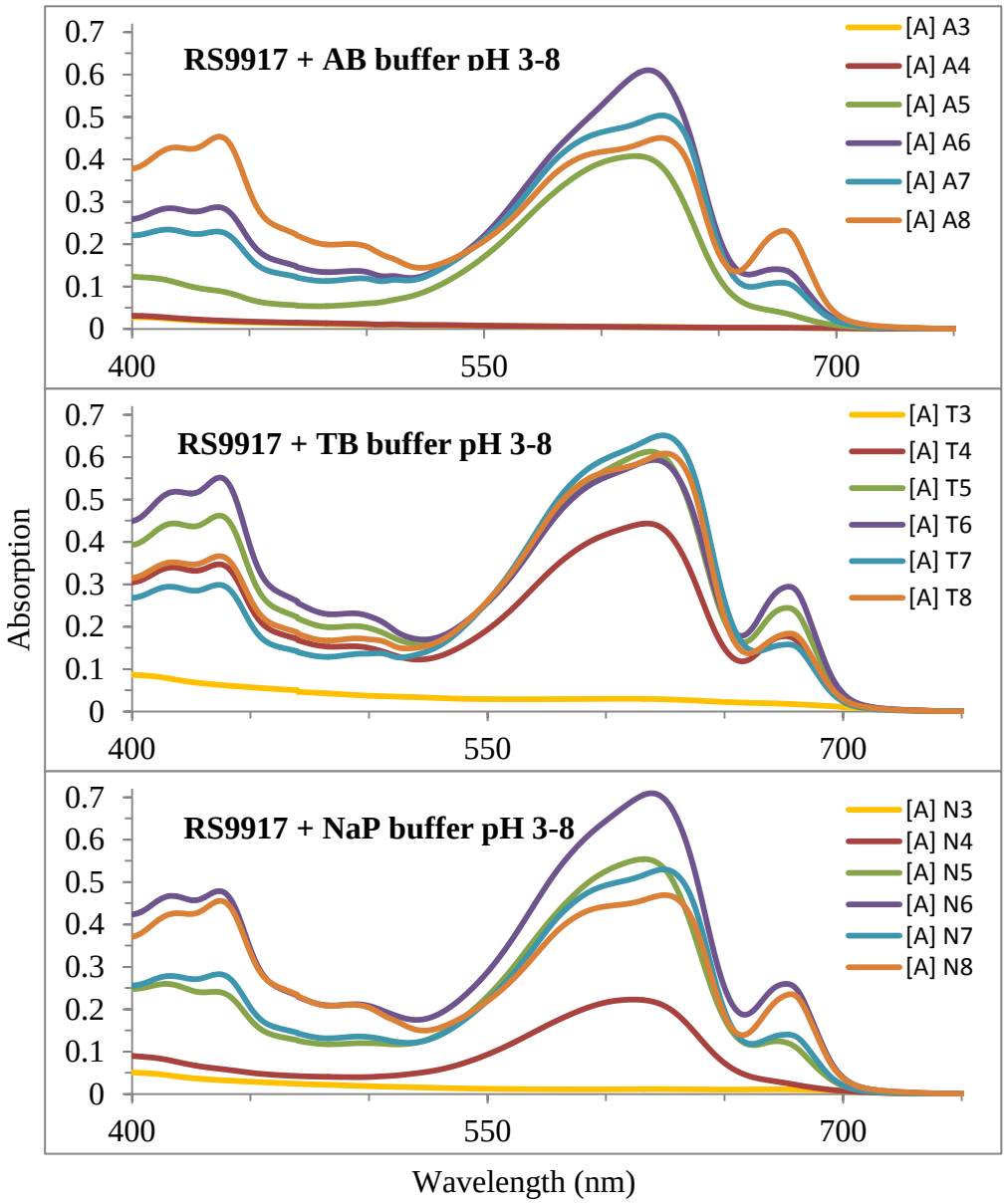


Figure 4.21 The effects of different buffer/pH (AB, TB, NaP pH 3-8) combinations on extraction and stability of PC.

PC containing extracts in AB, A3-A8; AB (pH3-8) (Top row), in TB, T3-T8; TB (pH3-8) (Middle row) and in NaP, N3-N8; NaP (pH3-8) (Bottom row)

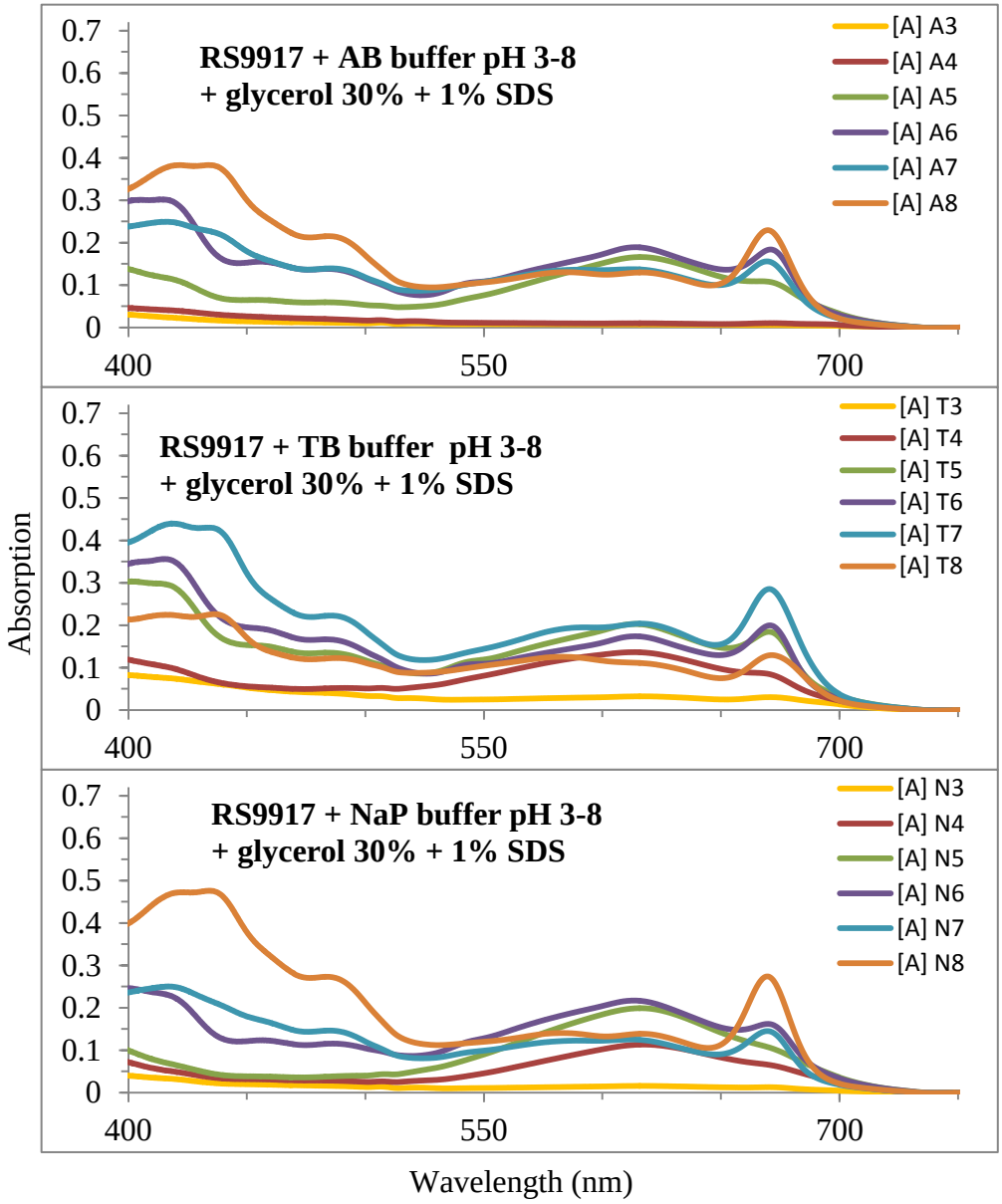


Figure 4.22 The effect of SDS and Gly on PC stability from the cyanobacterium RS9917.

PC containing extracts in AB + 30% Gly+1% SDS, A3-A8; AB (pH3-8) (Top row), in TB+ 30% Gly+1% SDS, T3-T8; TB (pH3-8) (Middle row) and in NaP+ 30% Gly+1% SDS, N3-N8; NaP (pH3-8) (Bottom row)

4.2.14 Extraction of PC in different buffers/pH (cells pre-extracted in acetone)

Based on the continued interference of Chlorophylls/carotenoids with analysis of PC (e.g., Figure 4.21), the following experiments were conducted with cells extracted in 90% acetone. Three buffers (AB, TB, and NaP) were tested at pH 3, 6, and 8 with SDS at either 0, 0.5, or 1% (Figure 4.23).

As shown repeatedly, SDS was essential for phycobiliprotein extraction following acetone extraction, regardless of the buffer pH. Further, as shown before, buffers with a pH above ~7 are generally either not effective at extraction and/or stabilization of phycobiliproteins, including PC (Figure 4.23). SDS, at both concentrations tested, were roughly equal in terms of PC extraction/stability. Tris-HCl buffer at pH 3 provided the best results, both qualitatively (i.e., the PC lambda max was ~617 nm with only a small blue shoulder at ~590 nm) and qualitatively (i.e., absorption was slightly > 0.3 units, compared with ~0.2 A units with other buffer/pH combinations). Again, when the pH is > 7, the spectra are enriched in blue signals (i.e., all three buffers at pH 8; Figure 4.23).

Finally, it is interesting to compare the absorption profiles, representative of pigment extraction and pigment stability, following acetone extraction and the addition of SDS. As highlighted for Figure 4.23 (preceding Figure), the inclusion of SDS to supernatants of cells not extracted in acetone was generally about 0.2 A units (i.e., see comments above). These 0.2 A units were a combination of absorption from both PC and Chl. In contrast, in cells extracted in acetone then in SDS-containing buffers (Figure 4.21, above), the PC absorption is generally > 0.2 A units, approaching 0.3 A units (or more) with Tris-HCl, pH 3. Importantly, there is virtually

no Chl signal associated with the PC signal in these experiments (Figure 4.23), emphasizing the utility of SDS for pigment extraction/stability.

Despite the overall positive results with PC extraction/stability from acetone-extracted cells, several questions remain. The most obvious questions relate to PC stability during extraction and/or following treatment with SDS. Treatment of PC-enriched supernatants with acidic urea (as was done with *Rhodomonas salina*, Figure 4.15 - 4.16) would be very useful in terms of answering these questions.

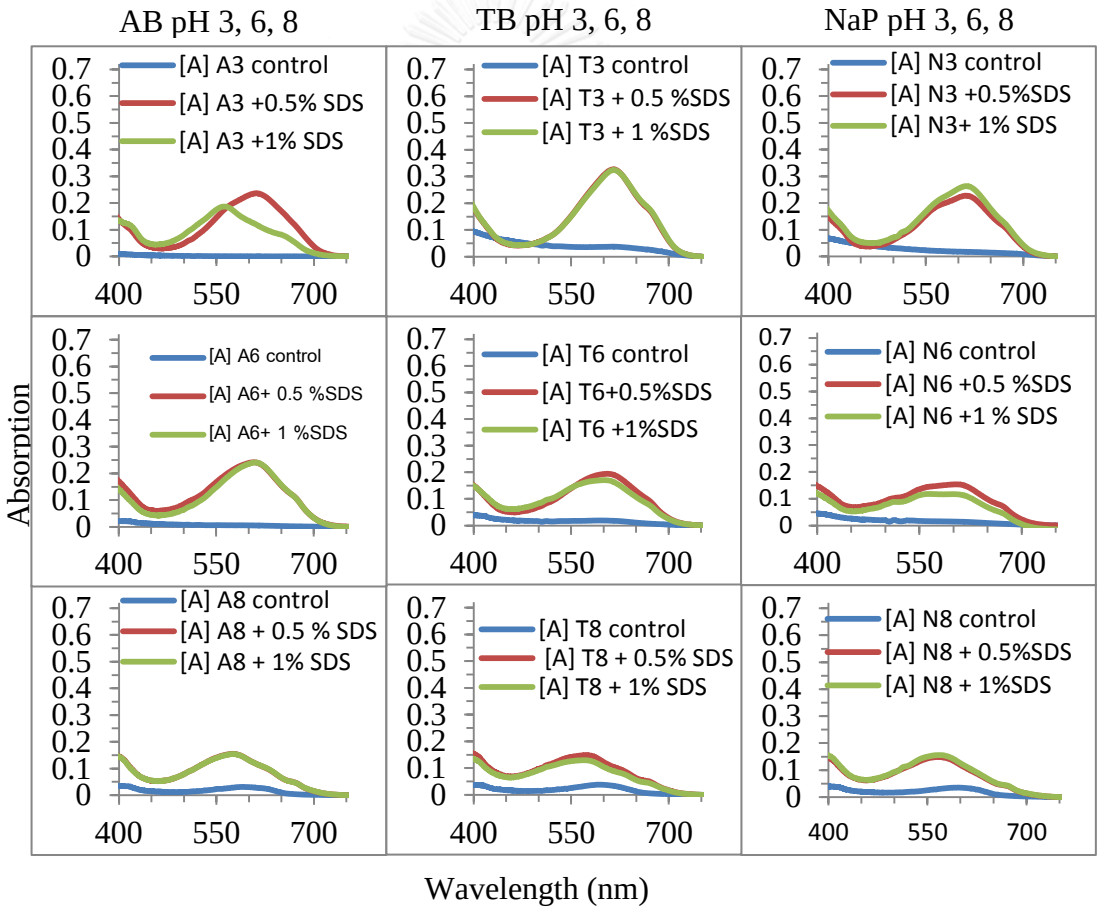


Figure 4.23 Three buffers (AB, TB and NaP) were tested at pH 3, 6 and 8 with SDS at either 0, 0.5 or 1%. Cells were extracted in 90% acetone.

PC containing extracts in AB (left column), in TB (center column) and in NaP (bottom column), pH 3 with and without SDS (top row), pH 6 with and without SDS (middle row), pH (8) with and without SDS (bottom row)

4.2.15 RS9917 treated with 20% acetic acid

As mentioned repeatedly, it is difficult to accurately predict bilin amounts based on absorption spectra of crude cell extracts and/or purified biliprotein complexes; difficulties arise owing to the influence of different buffers, pH, and protein concentrations, all of which influence bilin-protein and/or bilin-bilin interactions, which in turn influence absorption. The most reliable method for determining bilin concentration relies upon treatment of purified phycobiliproteins in 8 M urea at pH 2 - 3. As shown for *Rhodomanas salina* (Figure 4.15 - 4.16), a similar effect of acidic urea can be obtained by diluting samples to 20% acetic acid. When supernatants of RS9917 were treated brought to 20% acetic acid, the absorption spectra were difficult to interpret, largely because the PC signal almost completely overlapped that of Chl (i.e., PCB in 20% acetic acid is expected to have absorption of ~662 nm, while the acid-treated Chl would be ~670 nm). In fact, this is what was seen with RS9919; the acid-treated PC and Chl signals completely overlapped.

A few of the RS9917 supernatants had very low levels of Chl and moderate-to-high levels of PC; a few of these samples are redrawn in Figure 4.24 (above) before and after treatment with 20% AcOH. The PC signals in the non-acid-treated supernatants have lambda max of ~605 - 620 nm. In contrast, the PC signals for the AcOH-treated samples are shifted to ~662 nm, as expected for PCB that are not

encoumbered by protein and/or bilin interactions. This simple treatment confirms that the PCB chromophores are not substantially destroyed during the extraction process.

AcOH treatment also allows a quantitative assessment of bilin levels in each supernatant. The RS9917 supernatant in AB buffer at pH 6 had ~0.67 A units without AcOH and ~0.21 A units following acid treatment. The molar extinction coefficients for PEB are known (Zhao et al., 2011), suggesting it is possible to relate the AcOH-treated absorption spectrum to bilin and biliprotein amounts; however, since these coefficients were generated with purified phycobiliproteins, it is important to perform additional tests on these crude-cell extracts before attempting to be so quantitatively precise. While precise bilin levels are not yet available for these samples (Figure 4.24, above), it is interesting that the bilin absorption spectra (i.e., the AcOH-treated samples) in A buffer and NaP buffer were very similar, with OD values of ~0.2 A units. This suggests that each of these two extraction buffers provided the same level of bilin extraction and/or stability. The amount of bilin in Tris-HCl appears to be somewhat higher than in A and NaP buffers (i.e., ~0.26 vs ~0.2), which is consistent with earlier observations on the benefits of using Tris-HCl for bilin extraction/stabilization.

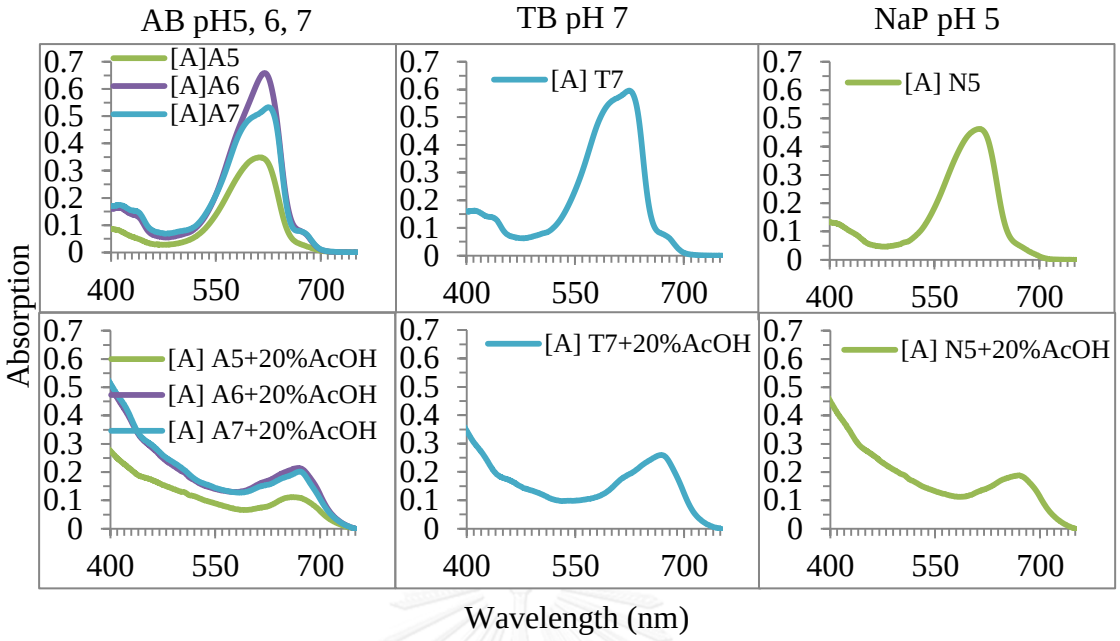


Figure 4.24 RS9917 extracts treated with 20% acetic acid.

PC containing extracts in AB, pH 5, 6, 9 (left column), in TB, pH 7 (middle column) and in NaP, pH 5 (right column), without 20% AcOH (Top row) and with 20 % AcOH (Bottom row)

4.3 Phycobilin-peptide Isolation

4.3.1 Protease digestion and HPLC

The pronase digests were subjected to HPLC for analysis. Extracts from four whole-cell pellets (pre-extracted with acetone) were resuspended in buffer with 0.5% SDS (160 ul); four preparations were combined (640 ul) and treated with 50 mM $CaCl_2$ (80 ul) and 10 mg/mL pronase enzyme (80 ul). Aliquots were incubated at 37 °C for various periods of time before enzyme activity was stopped by addition of acetone (80% final). Samples were centrifuged to remove insoluble material and the supernatants were injected onto the HPLC.

The amount of pigment remaining in solution, following addition of acetone, was very low (i.e., Figure 4.25, lower panel). The low pigment amounts resulted in very low HPLC chromatogram signals when a UV/VIS detector was used (data not shown). When a fluorescence detector was used, several bilin-peptide signals were observed (Figure 4.25; Appendix III)., which were clearly distinguishable from the small signals observed with blanks (e.g., acetone or buffer + reagents; both of which gave small signals at ~3 X min and again at 17 - 20 min). Signals from the early and late time points, corresponding to 'blanks', were not evaluated further, though it is likely that bilins may have been present in the peaks of some samples during the 9 hr incubation.

There was a clear distinction between elution times of bilin-peptides at the 10 min time point (which was the 'processing time for samples to be mixed, transferred to a new tube, and killed with acetone; all samples were done at room temperature) and all later time points (compare panel '10 min' vs other digestion times in Figure 4.25).

If earlier reports are to be believed, that pronase cleaves bilins from the peptide (O'Carra et al., 1980), then it would be expected that the HPLC chromatograms of *R. salina* would have two bilin peaks, assuming complete pronase digestion (i.e., one peak for PEB and one for DBV, with the PEB peak larger because it is three-times more abundant in PE; see Figure 2.5, Chapter 2). However, if more recent work is correct (G. Wedemayer et al., 1992; G. J. Wedemayer et al., 1996), that pronase, even extensive pronase, treatment leaves several amino acid residues covalently attached to the bilin, then it is reasonable to expect several bilin-protein signals following pronase digestion (i.e., one for PEB attached at two Cys residues,

one for PEB attached at position 82, one PEB attached at position 158 and either one, or two, DBV residues attached to the two slightly different α subunits; see Figure 2.5, Chapter 2). Although more work is required, it appears that the there are four bilin-peptide conjugates at the 9 hr time point. The appearance of more peaks (e.g., six peaks at the 3 hr time point; data not shown) likely represents incomplete digestion.

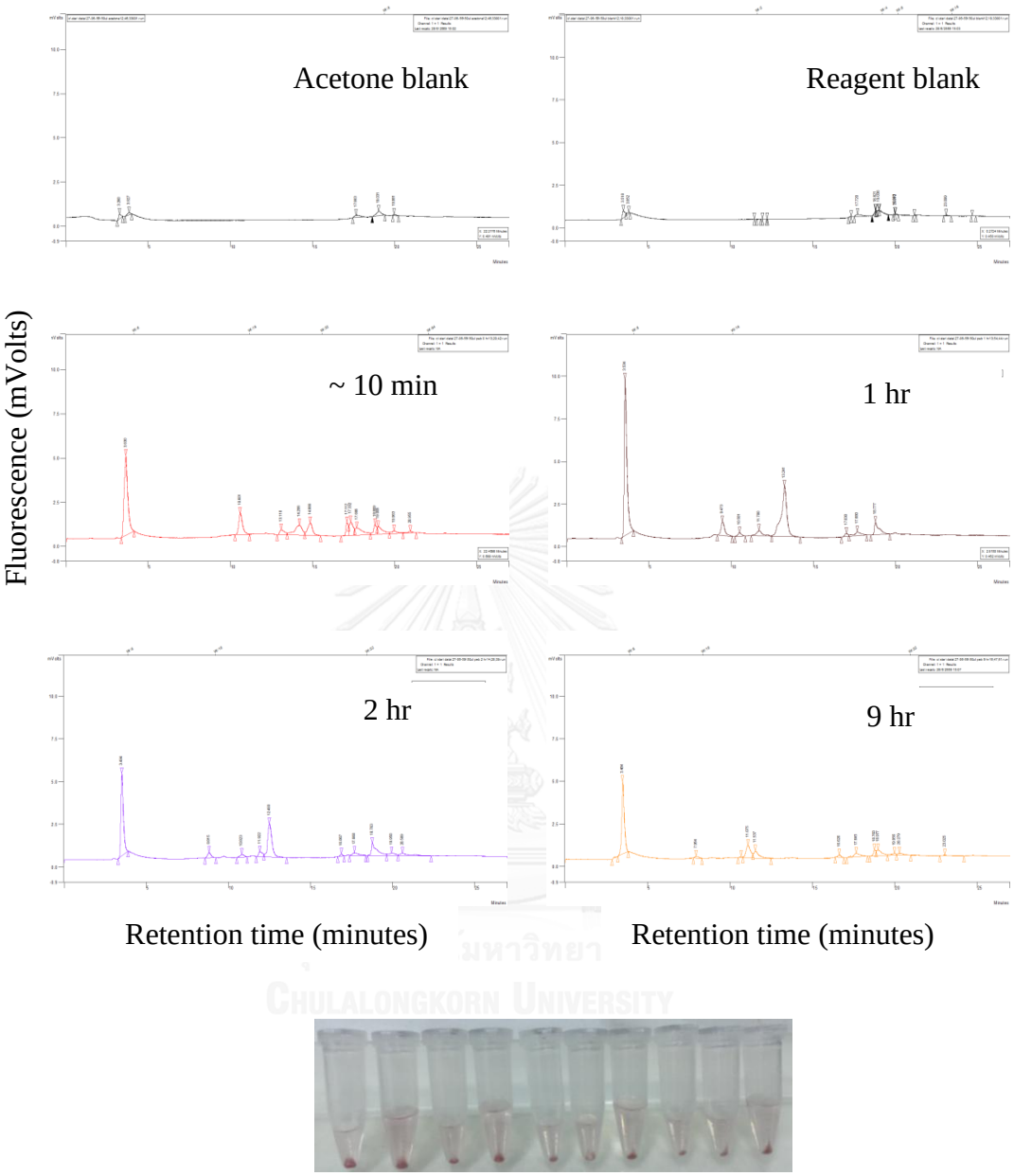
Several notable shortcomings of the current work warrant mention. First, the detectors used for this type of work should be capable of providing spectral scans of the peaks. While it is virtually certain that the peaks identified in the preceding paragraphs are due to bilin-peptides, the inclusion of either excitation and/or emission spectra of the peaks would substantially enhance these conclusions. Although it is possible to collect HPLC peaks as they elute from the HPLC, this is a difficult, if not impossible, feat to accomplish with field samples (which are expected to be very large in number – and with very low pigment amounts). Clearly, more up-to-date equipment is required for this work to progress.

Second, it is clear that the acetone treatment caused precipitation of bilin - proteins and/or bilin-peptides (Figure 4.25, lower panel). While it is important to stop the enzyme reaction, perhaps it would have been better to use acid treatment, such as formic acid (Wemmer, Wedemayer, & Glazer, 1993). Pronase should have very reduced activity at low pH (i.e., ~2-3) and the bilin-peptides and/or bilin-proteins would remain in solution, available for HPLC analyses. Formic acid (added to 5% final) would also result in less pigment dilution, which would improve detection during HPLC, and enhance analyses of field-collected samples.

Third, it is not clear if the pronase was completely effective in protein digestion; however, it seems likely that digestion, even after 9 hr, was incomplete

(based on the size and color of the acetone pellets). Given the enhanced stability of bilins at low pH, perhaps a better enzyme than pronase would be a so-called acid protease, such as pepsin, which is active at very low pH, e.g., 1.2. Unfortunately, pepsin is not resistant to SDS, hence it is necessary to identify an SDS-resistant acid protease for future work.

Fourth, it is not clear if the bilins are stable at 37 °C, especially over the long incubation times (i.e., 9 hr, Figure 4.25) required for digestion. Hence, the search for an SDS-resistant acid protease should be focused on enzymes that are active at lower temperatures, especially around 4 °C. Such enzymes are increasing Gly available for work in molecular biology, because there are other temperature/pH sensitive analyses that must be performed under these conditions and because of the financial incentives to find such enzymes for use in the potentially lucrative fields of molecular biology.



The extracts following pronase digestion 10 min, 1, 2, 3, 4, 5, 6, 7, 8, 9 hr, respectively

Figure 4.25 HPLC chromatograms and photographs of samples of *R.salina*. Phycobiliprotein extracts digested for various times with pronase.; (upper) Fluorescence chromatograms (570 nm Em, with 520 nm Ex) are shown for the acetone blank, the reagent + buffer blank, plus several time points (~10 min and 1, 2,

and 9 hr are shown; other time points provide similar results; not shown).; (lower) Photographs of the extracts following pronase digestion. Enzyme activity was stopped by addition of acetone (80% final). Samples were centrifuged before photographing. From left to right, samples are 10 min, 1, 2, 3, 4, 5, 6, 7, 8, 9 hr pronase.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Phycobiliproteins play an important light-harvesting role in the photosynthetic processes of three groups of algae, all important in the marine and/or freshwater ecosystems. Considerable information about the cellular, biochemical, and molecular biology of the phycobilins has been accumulated over the past 50+ years (Glazer, 1977). However, many very fundamental questions remain, most prominently addressed in this M.Sc. thesis work was the development of procedures for the rapid extraction and analyses, both quantitative and qualitative, of phycobilins, especially those from field-collected samples. While the end goal is to further develop the work presented here, such that it is applicable to field-collected samples, it was important to first demonstrate so-called 'proof-of-concept' using laboratory-grown cultures. The algal cultures chosen have been well characterized in terms in many respects, including detailed information about their phycobiliproteins; the two chosen algae were *Rhodomanas salina* (marine cryptophyte) and *Synechococcus* RS9917 (marine cyanobacterium). The most salient points of the present work include the following observations:

1) crude-cell extracts of algae that are rapid and easy to prepare can be used in many of the same qualitative/quantitative assays previously developed for highly purified phycobiliproteins that require extensive time, labor, and large amounts of starting material; however, the protocols for crude-cell extracts

require more research to reach the levels obtained from the hundreds (if not thousands) of studies devoted to purified phycobiliproteins.

2) *Rhodomonas salina* phycobiliproteins (PE) can be assayed using the same Gly-induced uncoupling procedures previously thought to only apply for analyses of certain cyanobacteria; this finding is potentially important in near-shore ecosystems where *Rhodomonas* spp. and/or other cryptophytes co-exist with cyanobacteria (i.e., previously observed signals attributed to cyanobacteria may be due in part to cryptophytes).

An overview of future research to address these findings, as well as their shortcomings, occupies the majority of Chapter 5. However, before future suggested research is discussed, a brief overview of the results and their implications is provided.

A major focus of this M.Sc. thesis research was directed toward identification of buffers and pH conditions required for extraction of so-called 'native' phycobiliproteins (i.e., biliproteins with absorption spectra unaltered, or only slightly changed, from that observed in whole cells and/or in the published literature). The major difference between the current work and the voluminous literature on similar topics is that our goal was to develop techniques/protocols that were applicable to whole-cell (or 'crude') cell extracts. In short, the literature since the 1950's is filled with research devoted to purified phycobiliproteins (either as phycobilisomes, hexamers, trimers, dimers, monomers, or highly purified bilin-peptide conjugates; reviewed by (Gantt, 1981; Glazer, 1982; Grossman et al., 1993) These highly purified preparations were required for the detailed biochemical and biophysical analyses that have led to the current corresponding Gly highly developed summary on

phycobiliprotein synthesis, assembly, function, ecological adaptations, and, in some cases, degradation.

Phycobiliproteins have been extensively studied at the biochemical and biophysical levels, both *in situ* and in purified pigment-protein complexes. Isolated phycobiliprotein analyses generally include extraction in an aqueous buffer followed by ammonium sulfate precipitation and then one or more rounds of chromatography and/or different purification steps (Glazer, 1982, 1989b); phycobiliproteins can be estimated by the ratio of absorption of the pigment to that of the protein (e.g., A_{545}/A_{280}), where a low ratio is considered 'crude' (i.e., a preparation contaminated with many proteins, e.g., RuBPCase, one of the most abundant proteins in algae) while a high ratio is indicative of high purity. Protein concentration is an important component of phycobiliprotein stability (Glazer (1982); low protein concentrations being deleterious to maintaining the native conformation.

A majority of research in this M.Sc. thesis was focused on the cryptophyte, *Rhodomonas salina*, which accumulates a phycobiliprotein complex known as PE545. In general, PE545 as well as other phycobiliproteins of cryptophytes, are easy to extract, requiring a simple (or repeated freeze-thaw treatments). However, here it was observed that extraction of PE545 from *Rhodomonas salina* grown in Daigo IMK medium was incomplete following procedures normally effective with cryptophytes (i.e., freeze-thaw-sonicate). Different buffers that covered a wide range of pH values (from pH 1 – 9) were tested to determine if PE545 extraction was enhanced. No buffer/pH combination was effective, with several buffer/pH combinations leading to co-extraction of chlorophylls and carotenoids. Whole cell fluorescence in the presence of Gly (Figure 4.5, Chapter 4) demonstrated that PE545 was disconnected by the

treatment in both f/2 and Daigo IMK, but also revealed an enhancement of Chl *a/c* in Daigo IMK, the medium that also gave the difficult-to-extract PE545 phenotype. It is not clear if the 'extra' Chl a/c in Daigo-grown cells is related to the resistant PE545, but it is interesting in that there has been hypothesized to be two pools of PE545 in cryptophytes, one that is more easily dissociated from membranes and a second pool that is more tightly attached to membranes (Mckay, 2015); it seems reasonable to hypothesize that cells grown in Daigo have an enhanced pool of PE545 that is difficult to extract (relative to f/2 cells) and that this pool of PE545 is associated with the Chl *a/c*.

Research reported in this M.Sc. thesis demonstrates that, for the most part, phycobiliproteins in crude cells extracts have spectral properties similar to those of purified preparations. The bilins are generally far less stable at higher pH (especially pH 9), but there were a few surprises (e.g., the unexpected stability of PE at pH 8 in buffer AB; Figure 4.6, Chapter 4). While bilin stability was adversely impacted at higher pH values, there was a corresponding increase in bilin extraction, especially as compared to low pH buffers (e.g., pH 3; see Figure 4.6; Chapter 4). Further, extractions carried out at higher pH values tended to result in extraction of chlorophylls and carotenoids, which interfere with spectroscopic analyses of phycobilins. The co-extraction of phycobilins, Chloropylls and carotenoidsis likely one reason highly purified phycobilins are used for research (i.e., the purification methods remove chlorophylls and carotenoids). Overall, based on the results presented in this thesis, it is easy to understand why most current work utilizes extraction buffers with pH values of ~5 to 6.5 (perhaps pH 7) and why elaborate purification schemes are in broad usage.

While comprehensive isolation and purification protocols are required for many studies, these protocols are not feasible when dealing with natural samples, either because the abundance of algae is very low and/or the number of samples involved is so large that multiple time-consuming and tedious steps cannot be routinely completed. Hence, it is virtually impossible to utilize pigment/protein ratios (e.g., A_{545}/A_{280}) as a measure of purity and it is difficult/impossible to maintain high protein concentrations in samples that are 'thin' in algal abundance. Hence, other approaches are required.

One of the goals of the current M.Sc. research was to begin development of protocols applicable to field-collected samples, especially for unicellular (or filamentous) marine algae in the water column. The pigments in these algae are routinely measured in acetone (or methanol or other non-polar reagent), which is effective at extraction of chlorophylls (Chl) and carotenoids (Mantoura et al., 1997). Results shown here clearly demonstrate two important points: 1) Chl, and to a lesser extent carotenoids, interfere with interpretation of phycobiliprotein spectroscopy (e.g., Figure 4.6; Chapter 4); and 2) acetone extraction prior to phycobiliprotein extraction greatly decreases phycobiliprotein extraction efficiencies (e.g., Figure 4.8; Chapter 4), likely a result of biliprotein dehydration/aggregation, unless detergents such as SDS are utilized.

Given that acetone-extracted Chl, especially when analyzed by HPLC, is the so-called 'gold standard' of algal pigment analyses (Mantoura et al., 1997), it is likely that researchers would want to use the same samples for both chlorophylls, carotenoids and phycobilins. Hence, the research focus of this M.Sc. was drawn to methods that would enhance phycobiliprotein extraction and stability in acetone-

extracted algal samples. In short, the objective was to develop methods that could be used with field-collected samples that were first extracted in acetone (for chlorophylls and carotenoids) and then in aqueous buffers (for phycobiliproteins). A similar method, but in reverse (i.e., phycobiliproteins extracted followed by acetone extraction) has been attempted with *Trichodesmium* spp. (Subramaniam, Carpenter, Karentz, & Falkowski, 1999), but the pigments of this alga are relatively easy to extract (Moreth & Yentsch, 1970).

For research presented here, different buffers/pH combinations were tested, with and without added SDS (e.g., Figures 4.11, 4.12, 4.13; Chapter 4). Urea, another often-used agent for protein extraction and phycobiliprotein purification, was also tested (e.g., Figures 4.13, 4.14 and 4.15, Chapter 4), again with different buffers/pH combinations and with and without SDS. The overall conclusion from these experiments demonstrated: 1) SDS enhanced extraction of phycobiliproteins from cells pre-extracted in acetone, frequently yielding complete extraction (e.g., Figure 4.9 Chapter 4); and 2) that SDS resulted in altered spectral properties of phycobiliproteins in some cases (e.g., Figures 4.11 Chapter 4), but not in all cases (e.g., Figures 4.11 Chapter 4).

Importantly, treatment of SDS extracts with 8M acidic urea (pH 2) or 20% acetic acid resulted in bilin spectra that were largely, but not totally, like those of non-detergent extracts (e.g., Figures 4.15, 4.16, Chapter 4). It is likely that the SDS-induced changes in phycobilin absorption are analogous to those reported for Triton X-100, which alters the absorption of both PEB attached to protein and to PEB in solution(K. H. Zhao et al., 2002). In other words, the use of SDS to extract

phycobiliproteins does not appear to negatively impact bilin stability, under the proper conditions of buffer composition and pH.

The final method used to analyze phycobiliproteins, both quantitatively and qualitatively, as part of this M.Sc. thesis research involved HPLC analyses of protease digested extracts. Protease digestion/HPLC has been widely used in phycobiliprotein research, aimed almost exclusively at very highly purified fractions with the goal of identifying the amino acid attachment sites for each bilin and each protein (insert various references from Glazer's laboratory here). The basic premise is that protease digestion will reduce the number of amino acids attached to the bilin and make subsequent analyses (e.g., amino acid sequencing) far easier, while also providing unambiguous identification of amino acid residues involved in bilin attachment.

The HPLC results shown here (Figure 4.25, Chapter 4) demonstrate an important 'proof of concept', namely, that it is possible to analyze phycobiliproteins as bilin-peptide conjugates, using crude cell extracts. Given the very high protein concentrations present in the crude cell extracts, it is likely that protease treatments will need to be conducted with high enzyme concentrations and/or longer periods of time (also at reduced temperatures) to provide more reliable results. Additional suggestions for improvement of HPLC results are found in Section 3.9, Chapter 4.

In conclusions, work presented here represents initial steps aimed at combining acetone extraction and detergent-aided phycobiliprotein extraction from single samples, such as those collected during on-board research cruises. Simple, but not necessarily broadly applicable, methods based on fluorometric analyses of phycobiliproteins have been presented (Moreth & Yentsch, 1970), but the extraction protocols appear to limit widespread utilization of the method (Wyman, 1992).

The inclusion of an acetone extraction step is advantageous because it potentially allows analyses of all photosynthetic pigments (chlorophylls, carotenoids, and phycobilins) from the same sample, a noted advantage when dealing with samples from field studies and/or at other times when sampling is limited by time, conditions, or circumstances. If both acetone extraction and SDS-induced solubilization are to be combined, it is important to understand the impacts of SDS on phycobiliprotein stability as well as the likely impacts on pigment extinction coefficients. Especially important will be the extinction coefficients of SDS-extracted biliproteins in 8 M acidic urea. Once these laboratory studies with single algal species are complete, it will be important to start research with mixed cultures grown in the laboratory and/or with field-collected samples.

CHAPTER VI

SUGGESTIONS FOR FUTURE STUDIES

Some suggestions for future research are provided at three points in Chapter 4, once at the completion of studies with *Rhodomonas salina* (i.e., Figure 4.25, Chapter 4), a second time after completion of work with *Synechococcus* RS9917 (i.e., Figure 4.25 - 4.25, Chapter 4), and again following the HPLC results (i.e., Figure 4.25, Chapter 4). The suggestions given at these earlier points of this M.Sc. thesis represent those pertinent to the specific experimental protocols involved. In contrast, the following suggestions are much broader in scope and are aimed at providing insights aimed at achieving the overall goals of this research, which are related to analyses of phycobiliproteins from field-collected samples of microalgae.

6.1. Phycobiliprotein Extraction and/or Stabilization

Phycobiliprotein extraction and, in parallel, stabilization, remains problematic. Possible future experimental work is outlined below.

6.1.1. Stabilizing agents during extraction – (edible, food grade) preservatives

As described in Chapters 1 and 2 and as observed in Chapter 3 and 4 of this thesis, methods for phycobiliproteins remain a stubborn issue that requires further attention. This is especially important when working with field-collected samples, where algae (and their pigments) are expected to be present at low levels (relative to laboratory cultures).

Hand-in-hand with phycobiliprotein extraction is the question of phycobiliprotein stability. For instance, numerous studies have focused on

phycobiliprotein stability in the food technology field (Kannaujiya & Sinha, 2016; Mishra, Shrivastav, & Mishra, 2008; Mishra, Shrivastav, Pancha, Jain, & Mishra, 2010; Sudhakar et al., 2015; Wu, Wang, Xiang, Li, & He, 2016); the beautiful colors of phycobiliproteins provide enhanced marketability of several food products that are otherwise not pleasing to the eye. These studies have invariably focused on phycobiliproteins that have already undergone rather extensive purification processes (e.g., cell breakage, ammonium sulfate precipitation, one or more rounds of sucrose-density centrifugation and/or column chromatography, etc. etc.). Such studies have shown that purified phycobiliproteins retain greater stability to heat, pH, and light when treated with reagents such as sucrose, sodium chloride, citrate or benzoic acid. A question left unanswered by these experiments is: would use of preservatives increase phycobiliprotein stability during the isolation process (e.g., during cell breakage, ammonium sulfate precipitation, sucrose density gradient centrifugation, etc.). Experiments conducted with these preservatives (plus others, such as EDTA and/or EGTA) during the extraction and the purification processes could be very informative.

It is likely that these food grade preservatives enhance phycobiliprotein stability due to enhanced stability of higher order structures (e.g., phycobilisomes, hexamers and/or trimers, all of which are generally more stable than the denatured structures). Hence, it is possible that these preservatives could be useful in conjunction with treatments required to rupture/penetrate/permeabilize cell walls, prior to release/extraction of phycobiliproteins. For instance, would citrate and/or benzoic acid, two of the most positive preservatives, enhance phycobiliprotein extraction/stability when cells are disrupted by SDS (see more on SDS below).

6.1.2. Stabilizing agents during extraction – proteinase inhibitors and/or microbial inhibitors

Another method to stabilize (and/or perhaps aid in extraction) phycobiliproteins relates to use of proteinase inhibitors and/or antimicrobial agents in the extraction buffers. Phycobilisome synthesis and degradation can be very dynamic in nature, with algae actively altering pigments amounts and/or ratios in response to changes in nutrition (e.g., nitrogen or sulfur) and light (both quality and quantity). Although phycobiliproteins may be diluted by growth (i.e., as cell numbers increase, a lack of new synthesis would reduce the pigment/cell), it is also clear that active (i.e., proteinase - driven) degradation occurs in many strains; an excellent review of phycobilisome degradation is provided by Bienert, Baier, Volkmer, Lockau, and Heinemann (2006). Commercial protease inhibitor 'cocktails' are commercially available that are active against a broad range of proteases (e.g., Sigma-Aldrich; CalBiochem). PMSF, a fast acting, but short-lived, protease inhibitor may be of particular interest; it acts as a 'suicide' inhibitor, eliminating protease activity when cells are initially broken (i.e., during the first 'thaw' cycle), but would not be problematic if subsequent protease activity was desired (e.g., prior to HPLC analysis; see Figure 4.25, Chapter 4). EGTA and/or EDTA may also inhibit protease activity, as many of these enzymes require metals at their active site.

Antimicrobials also may be beneficial. Although care was taken in work presented here to minimize microbial activity (e.g., sterilization of buffers and supplies/equipment in direct sample contact; low temperatures), it is possible that inclusion of NaN_3 (or similar) could be helpful, especially as this work progresses to field-collected samples, which are expected to be greatly enriched in bacteria.

6.1.3. Use of detergents to aid extraction

The use of detergents during phycobiliprotein extraction also warrants additional consideration. Considerable work has focused on Triton X-10 (Gantt and Lipschultz (1972); Wyman (1992)) or zwitterionic detergents (e.g., Glazer et al. (1979) PNAS USA) and has been reviewed by Gantt (1981) These are generally considered ‘gentle’ detergents and allow for purification of so-called ‘native’ phycobilisomes and/or phycobiliproteins with higher-order structure (e.g., trimers, hexamers, etc.). Gentle detergents were frequently used in earlier studies in conjunction with high ionic strength buffers, which help stabilize phycobilisomes, but at the same time can cause un-natural aggregation (the detergents tend to prevent aggregation in high salt buffers).Gentle detergents such as Triton X-100 can also help in phycobilisome/phycobiliprotein extraction, especially useful from recalcitrant cells such as small unicellular cyanobacteria (Gantt, 1981; Wyman, 1992). Notably missing from such studies is a more fundamental question related to phycobiliprotein extraction: would inclusion of detergents, especially ‘harsher’ detergents, aid in complete recovery of phycobiliproteins, even if these detergents negatively impacted the higher order structures (e.g., phycobilisomes, hexamers, trimers and even the monomers). Clearly, these ‘harsh’ detergents have been avoided, because the result in both quantitative and qualitative changes to the spectral properties of the phycobiliproteins. However, if research is focused on algal species with well-characterized bilins and phycobiliproteins, it is possible to determine which detergents (plus required buffers, pH, etc.) cause destruction and/or chemical changes in bilins (e.g., isomerization) that preclude qualitative analyses vs those detergents (plus buffers, pH, etc.) that do not irreversibly alter bilin spectral properties. Research

presented as part of this M.Sc. thesis clearly indicates that the harsh detergent SDS can facilitate pigment extraction (e.g., Figure 4.11, 4.12) and that the bilins are only qualitatively, but not quantitatively impacted by inclusion of SDS in the extraction buffers (e.g., compare bilins in 20% acetic acid (Figure 4.15) and/or 8 M acidic urea (Figure 4.15)). Importantly, the bilins in 8M acidic urea or 20% acetic acid in the crude cell preparations generally retained absorption profiles similar to those of highly purified phycobiliproteins.

6.1.4. Use of proteases to aid extraction

The suggestions here, the use of proteases to aid phycobiliprotein extraction, appears to be a direct contradiction, where it was argued that extraction buffers should include protease inhibitors. However, there is a potentially valid argument for inclusion of proteases as soon as possible during the extraction process. The rational is to ‘stand dogma on its head’ and to attempt to disrupt the higher order phycobiliprotein structures (e.g., phycobilisomes, hexamers, dimers, and even monomers) as soon as possible, hence facilitating their extraction from cells that may be difficult to render permeable. In other words, the argument is that it may be easier to extract small bilin-peptides from recalcitrant cells than it is to extract bilin-proteins (especially higher order bilin proteins that are likely present in high ionic strength buffers, such as the 0.1M solutions used throughout this study).

6.1.5. Use of low ionic strength buffers and/or denaturants such as urea, phenol and/or guanidine isothiocyanate to aid extraction

To repeat, the emphasis on phycobiliprotein extraction in virtually every recent publication has been on maintaining the native state of the bilins during the extraction process. Is it time to reverse this thinking? Low ionic strength buffers (especially with detergents) are known to be effective in converting higher order phycobiliproteins to lower order structures (e.g., hexamers to dimers). On the other hand, the formation of monomers from dimers requires additional 'work', with denaturants such as urea commonly employed (Glazer & Fang, 1973). Clearly, urea is generally held in high regards in terms of acting as a protein solubilizing agent, especially when used together with SDS(Hummon, Lim, Difilippantonio, & Ried, 2007). The use of high concentrations of urea, especially at low pH values, has been very beneficial for the characterization of purified phycobiliproteins Glazer and Hixson (1975) and (possibly) could be equally useful for earlier steps, notably extraction. Inclusion of urea in this study was somewhat effective in phycobiliprotein extraction from acetone-treated cells (Figure 4.15- 4.16, Chapter 4), but clarification of urea effects on extraction requires additional work. A more robust method of protein extraction may be advantageous; recent work (Hummon et al. (2007); Mehta et al. (2015)) could serve as guides for future exploration.

6.1.6. Gly-induced quantification of phycoerythrin in cryptophytes

Wyman and coworkers (Wyman, 1992; Wyman et al., 1985) demonstrated a precise linear relationship between PE levels in *Synechococcus* spp. and the fluorescence emission of Gly-treated cells. The technique is eloquent: it is simple,

easy, fast, inexpensive and robust.The method has been used widely in marine environments(Wyman, 1992). As part of the work conducted for this M.Sc. thesis, it was found that Gly-treated cells of *Rhodomonas salina* exhibit fluorescence emission spectra very similar (if not identical) to those of *Synechococcus*. Gly enhanced PE fluorescence in cells grown in both f/2 medium and in DG medium (Figure 4.5, Chapter 4).

Wyman et al. (1985) was careful to use very dilute samples for analyses; culture samples were diluted to < 0.05. This dilution is required to prevent re-adsorption of light emissions. This precaution was not taken in this work and represents one issue that needs to be addressed in future studies; however, cultures used here were very dilute and the adjustments required, if any, are likely to be small. Of more importance is the larger question: do other cryptophytes also share the phenomenon or is this unique to *R. salina*. Hence, it would be important to test other cryptophyte species that utilize PE and, in addition, species that utilize PC for light harvesting. Assuming other cryptophytes share the 'Gly effect' on phycobiliprotein coupling, it would be interesting to collect samples from coastal environments known to have both *Synechococcus* and cryptophytes, such as the upper Gulf of Thailand (e.g., *Synechococcus* as reported by Gunbua, Paphavasit, and Piusomboon (2012); cryptophytes as reported, indirectly as *Mesodinium rubrum*, by Lirwitayaprasit). Such studies would necessarily involve both fluorescence measurements and cell identification (e.g., light microscope work and/or molecular probes or techniques for each group).

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APPENDIX

Appendix I

Table 4.5 Cell density of *Rhodomonas salina* in f/2 medium

Days	Cell/mL (rep1)	Cell/mL (rep2)	Cell/mL (rep3)	Cell/mL (average)	SD
0	1000	1000	1000	1000	0
2	1815	1778	741	1444	609.7317
4	14667	13370	6482	11506	4399.523
6	38889	41222	38111	39407	1619.064
8	155296	165000	134852	151716	15389.62
10	209444	182778	191667	194630	13577.96
12	214074	292593	259225	255297	39406.35
14	304444	272778	282963	286728	16165.61
16	255370	236667	225926	239321	14900.63
18	230000	262222	214074	235432	24529.38
20	267037	220370	220370	235926	26942.97
22	178519	196296	212963	195926	17225.24
24	203333	219630	165926	196296	27534.74
26	234444	284815	225185	248148	32089.98
28	171111	222592	214444	202716	27672.07
30	164815	165185	199630	176543	19994.27
32	161111	148889	132963	147654	14114.55

Table 4.6 Cell density of *Rhodomonas salina* in DG-IMK medium.

Days	Cell/mL (rep1)	Cell/mL (rep1)	Cell/mL (rep1)	Cell/mL (average)	SD
0	1000	1000	1000	1000	0
2	10123	10864	8641	9876	1131.896
4	88641	79753	81728	83374	4667.025
6	184938	145185	143950	158024.3	23316.1
8	190123	155308	183086	176172.3	18408.43
10	192592	198024	187283	192633	5370.617
12	227654	228395	204074	220041	13832.79
14	213209	198024	217037	209423.3	10055.94
16	197407	202345	220370	206707.3	12087.07
18	211605	208889	215679	212057.6	3417.611
20	224074	204321	209506	212633.7	10241.19
22	214449	208148	210000	210865.7	3238.469
24	210741	201605	212222	208189.3	5750.08
26	200987.7	171234.6	168395.1	180205.8	18053.56

Table 4.7 Chlorophyll concentration of *Synechococus* sp. (strain RS9917) in PCR-S11 media.

Days	Chl(μg/l) (rep1)	Chl(μg/l) (rep2)	Chl(μg/l) (rep3)	Chl (μg/l) (Average)	SD
0	9.88	9.43	3.10	7.47	3.79
2	11.67	13.26	8.89	11.27	2.21
4	7.09	9.00	15.36	10.48	4.33
6	8.71	8.18	17.64	11.51	5.31
8	10.73	25.35	16.27	17.45	7.38
10	32.21	5.11	18.66	18.66	13.55
12	34.55	45.75	22.35	34.22	11.70
14	24.61	81.28	70.89	58.92	30.17
16	47.47	88.18	56.92	64.19	21.30
18	20.69	80.13	115.70	72.17	48.00
20	51.49	127.09	89.29	89.29	37.80
22	27.05	142.20	66.27	78.51	58.54
24	41.36	36.38	133.69	70.48	54.80
26	55.01	63.45	86.79	68.41	16.46
28	29.18	98.19	65.64	64.34	34.52

Table 4.8 Chlorophyll concentration of *Synechococus* sp. (strain WH8018) in PCR-S11 media.

Days	Chl(μg/l) (rep1)	Chl(μg/l) (rep2)	Chl(μg/l) (rep3)	Chl(μg/l) (Average)	SD
0	6.53	9.94	6.85	7.77	1.89
2	14.88	6.32	3.72	8.31	5.84
4	9.89	13.76	9.46	11.04	2.37
6	8.86	10.44	17.23	12.17	4.45
8	22.52	7.81	7.62	12.65	8.55
10	14.83	5.94	18.69	13.15	6.54
12	9.43	11.19	28.72	16.45	10.67
14	27.05	14.35	24.31	21.90	6.69
16	36.41	30.68	21.81	29.63	7.35
18	61.08	25.98	30.08	39.04	19.19
20	50.59	32.19	66.33	49.70	17.09
22	62.87	74.60	78.96	72.14	8.32
24	75.80	46.09	57.12	59.67	15.02
26	31.51	67.17	55.43	51.37	18.18
28	37.85	21.74	78.80	46.13	29.41

Table 4.9 Chlorophyll concentration of *Synechococus* sp. (strain WH7803) in PCR-S11 media.

Days	Chl(μg/l) (rep1)	Chl(μg/l) (rep2)	Chl(μg/l) (rep3)	Chl (μg/l) (Average)	SD
0	2.90	1.81	1.80	2.17	0.629
2	1.34	1.23	1.47	1.35	0.116
4	1.99	0.71	2.42	1.71	0.890
6	1.83	3.22	2.77	2.60	0.710
8	2.42	2.61	3.31	2.78	0.466
10	3.87	2.16	2.52	2.85	0.902
12	3.58	6.93	2.52	4.34	2.303
14	4.08	9.43	2.52	5.27	3.626
16	8.98	9.43	11.50	9.97	1.343
18	16.49	12.09	10.78	13.12	2.992
20	32.36	22.46	16.76	23.86	7.893
22	29.05	13.33	34.37	25.58	10.944
24	76.81	37.32	10.11	41.41	33.537
26	45.61	62.14	29.70	45.82	16.218
28	35.83	18.81	39.11	31.25	10.893

Appendix II

Table 4.10 The effects of SDS on pigment stability (PE545)

Buffer/ pH	λ_{max} (nm)	Control	Control 12 hr	Control 24 hr	Control 9 days
TB 3	547	0.256	0.212	0.170	ND
TB 6	551	0.532	0.472	0.461	0.393
TB 9	512	0.186	0.150	0.127	ND
'percent remaining'					
TB 3	547	100	83	66	ND
TB 6	551	100	89	87	74
TB 9	512	100	80	68	ND
Buffer/ pH	λ_{max} (nm)	1%SDS	1%SDS 12 hr	1%SDS 24 hr	1%SDS 9 days
TB 3	547	0.183	0.177	0.138	ND
TB 6	551	0.262	0.237	0.221	0.189
TB 9	512	0.160	0.151	0.130	ND
'percent remaining'					
TB 3	547	100	97	76	ND
TB 6	551	100	91	84	72
TB 9	512	100	94	81	ND

Table 4.11 Percent remaining after 16 hr of PE extracted with 1% SDS in TB (pH 6) relative to those with and/or without urea.

Condition	λ_{max}	Absorption (0 hrs)	Absorption (16 hrs)	Percent Remaining
Control	551	0.4103	0.4196	102
0.1% SDS	534	0.3775	0.3712	98
0.5% SDS	534	0.3937	0.3838	97
1% SDS	534	0.3837	0.3657	95
1M Urea	536	0.2277	0.2096	92
4 M	540	0.2372	0.2084	88
8 M Urea	540	0.214	0.2013	94
1 M Urea + 0.1% SDS	535	0.3477	0.3411	98
1 M Urea + 0.5% SDS	535	0.3351	0.3334	99
1M Urea + 1.0% SDS	535	0.353	0.3555	101
4 M Urea + 0.1 % SDS	538	0.3909	0.3691	94
4 M Urea + 0.5 % SDS	537	0.3505	0.3266	93
4 M Urea + 1 % SDS	536	0.3022	0.2758	91
8 M Urea + 0.1 % SDS	543	0.2632	0.2967	113
8 M Urea + 0.5 % SDS	540	0.266	0.2594	98
8 M Urea + 1.0 % SDS	540	0.2413	0.2505	104

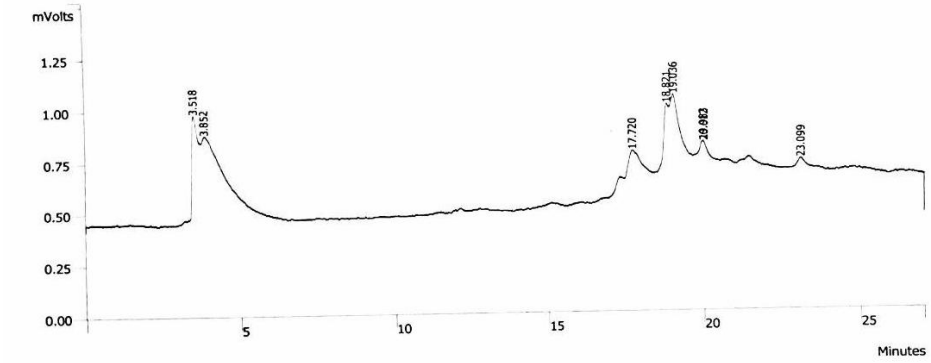
Table 4.12 Percent remaining after 16 hr of PE extracted with 1% SDS in TB (pH 6) relative to those with and/or without urea**.**

Condition	λ_{max}	Absorption (0 hrs)	Absorption (16 hrs)	Percent Remaining
Control	544	0.1169	0.1155	99
1% SDS	545	0.4183	0.4041	97
1 M Urea	543	0.1758	0.1734	99
4 M Urea	543	0.2018	0.2048	101
8 M Urea	544	0.2499	0.217	87
1M Urea + 1% SDS	544	0.327	0.3403	104
4 M Urea + 1 % SDS	538	0.3479	0.3496	100
8 M Urea + 1 % SDS	540	0.3054	0.3002	98

Appendix III

HPLC Chromatogram

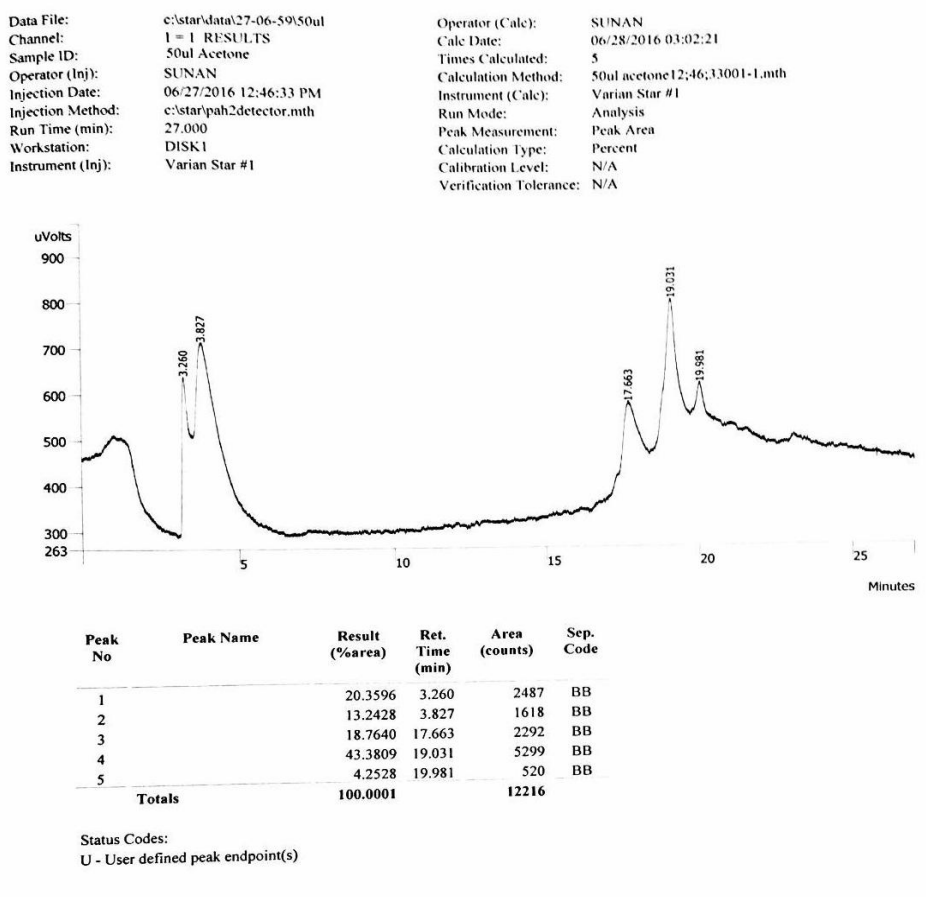
Data File:	c:\star\data\27-06-59\50ul	Operator (Calc):	SUNAN
Channel:	1 = 1 RESULTS	Calc Date:	06/28/2016 03:03:49
Sample ID:	50ul Blank	Times Calculated:	5
Operator (Inj):	SUNAN	Calculation Method:	50ul blank12;18;33001-1.mth
Injection Date:	06/27/2016 12:18:33 PM	Instrument (Calc):	Varian Star #1
Injection Method:	c:\star\pah2detector.mth	Run Mode:	Analysis
Run Time (min):	27.000	Peak Measurement:	Peak Area
Workstation:	DISK1	Calculation Type:	Percent
Instrument (Inj):	Varian Star #1	Calibration Level:	N/A
		Verification Tolerance:	N/A



Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		47.3593	3.518	4699	BV
2		12.9913	3.852	1289	VB
3		24.2808	17.720	2409	BB
4		1.5286	18.821	152	BV
5		3.0141	19.036	299	BV
6		2.5511	19.983	253	BV
7		3.2110	20.012	319	VB
8		5.0637	23.099	502	BB
	Totals	**99.9999**		**9922**	

Status Codes:
U - User defined peak endpoint(s)

Figure 4.26 Chromatogram of blank in samples of *R. salina* PE545 extracts digested with pronase.

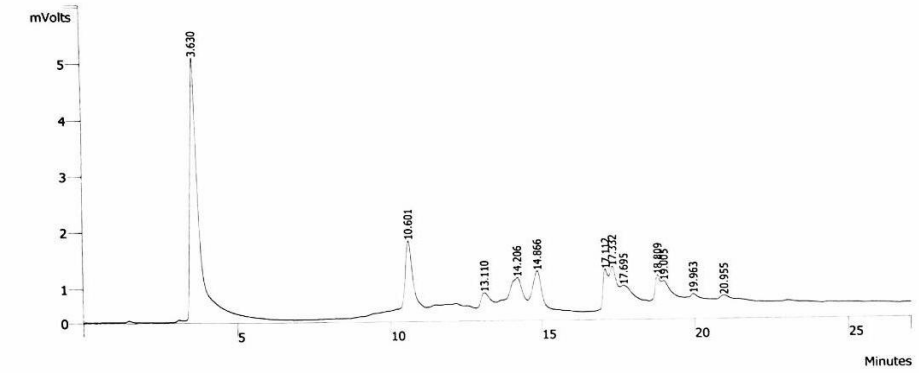


Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		20.3596	3.260	2487	BB
2		13.2428	3.827	1618	BB
3		18.7640	17.663	2292	BB
4		43.3809	19.031	5299	BB
5		4.2528	19.981	520	BB
	Totals	**100.0001**		**12216**	

Status Codes:
U - User defined peak endpoint(s)

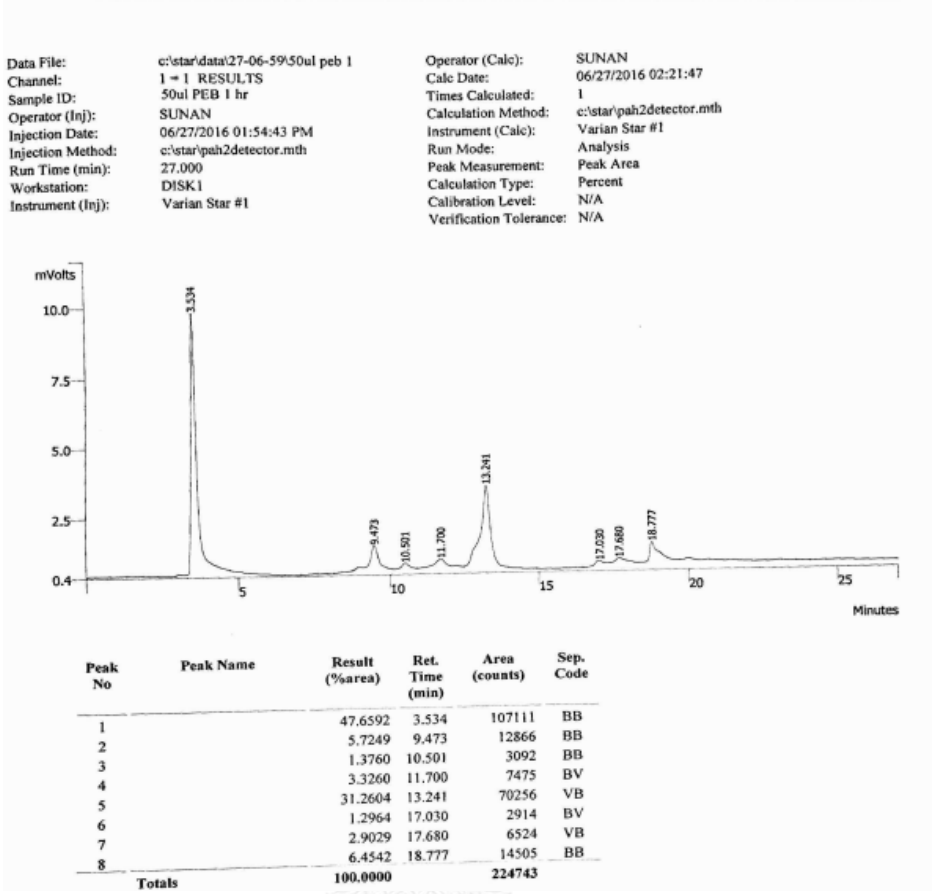
Figure 4.27 Chromatogram of acetone (blank) in samples of *R. salina* PE545extracts digested with pronase.

Data File:	c:\star\data\27-06-59\50ul peb 0	Operator (Calc):	SUNAN
Channel:	1 = 1 RESULTS	Calc Date:	06/27/2016 01:47:45
Sample ID:	50ul PEB 0 hr	Times Calculated:	1
Operator (Inj):	SUNAN	Calculation Method:	c:\star\pah2detector.mth
Injection Date:	06/27/2016 01:20:42 PM	Instrument (Calc):	Varian Star #1
Injection Method:	c:\star\pah2detector.mth	Run Mode:	Analysis
Run Time (min):	27.000	Peak Measurement:	Peak Area
Workstation:	DISK1	Calculation Type:	Percent
Instrument (Inj):	Varian Star #1	Calibration Level:	N/A
		Verification Tolerance:	N/A



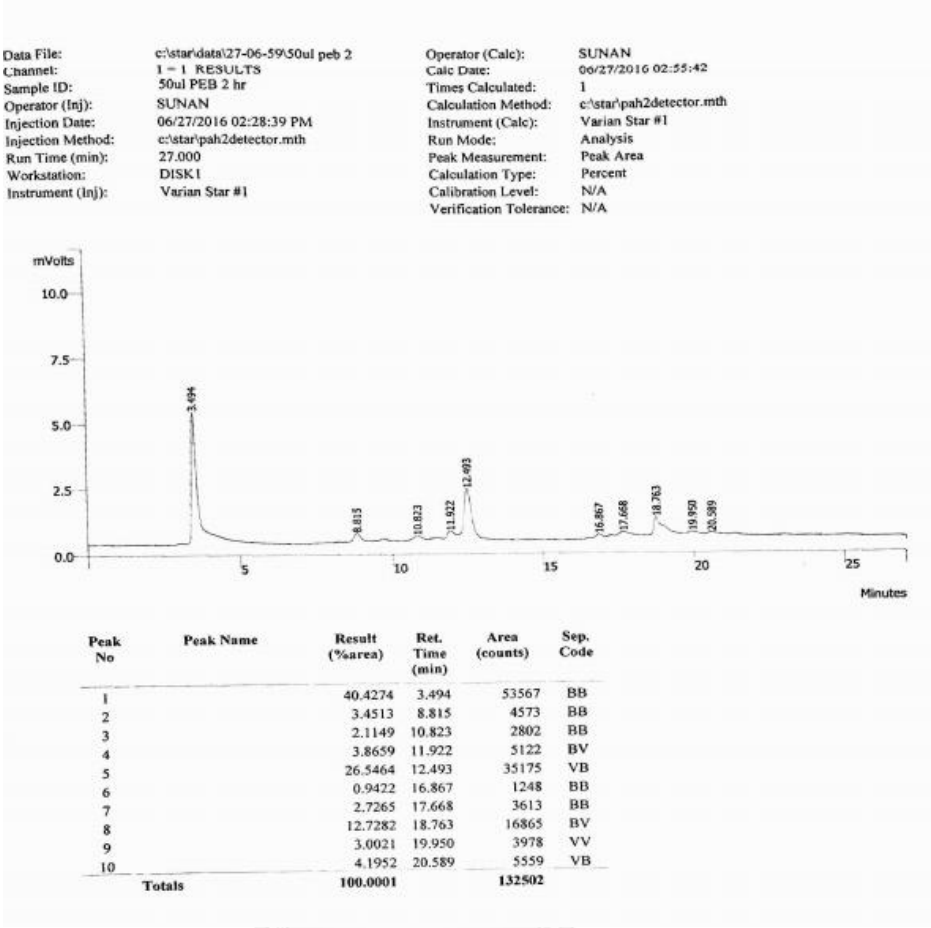
Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		37.2939	3.630	70918	BB
2		10.3789	10.601	19737	BB
3		3.1669	13.110	6022	BV
4		9.7960	14.206	18628	VV
5		7.6155	14.866	14482	VB
6		4.2975	17.112	8172	BV
7		7.0608	17.332	13427	VV
8		7.3412	17.695	13960	VV
9		4.1691	18.809	7928	VV
10		5.4418	19.005	10348	VV
11		2.3973	19.963	4559	VV
12		1.0409	20.955	1979	VB
	Totals	**99.9998**		**190160**	

Figure 4.28 Chromatogram of bilin peptide (0 hr) in samples of *R. salina* PE545 extracts digested with pronase.



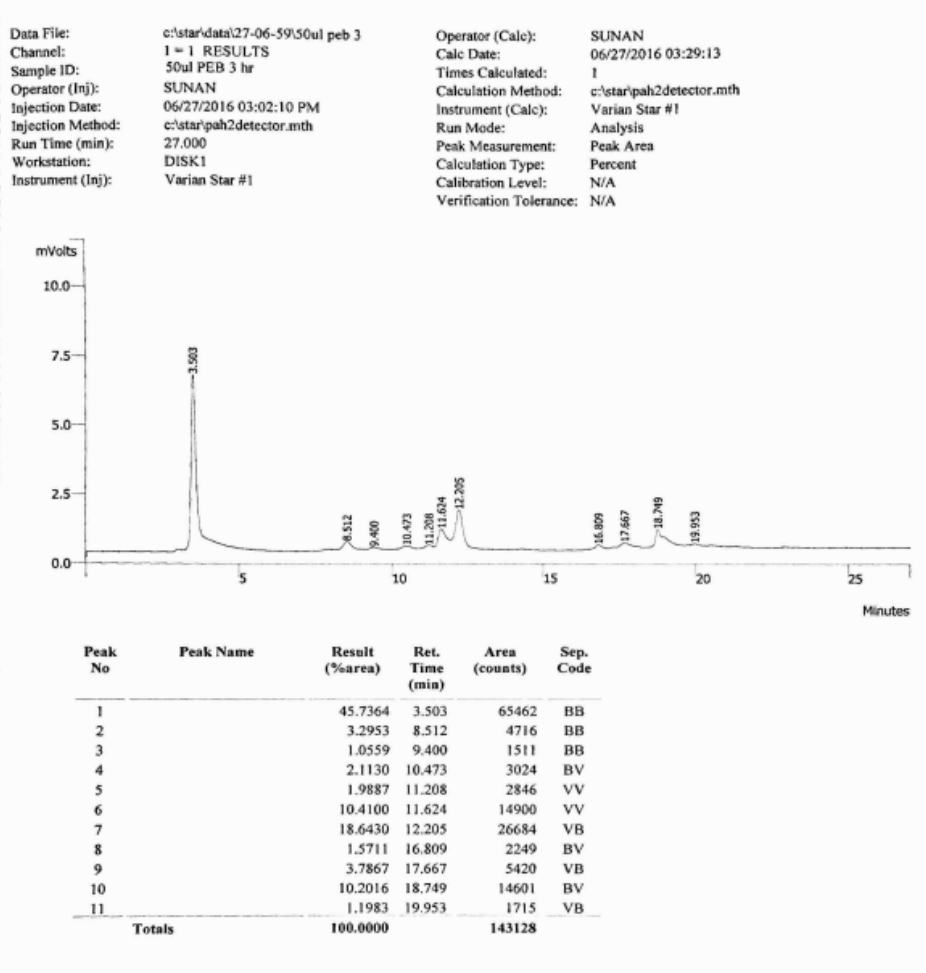
Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		47.6592	3.534	107111	BB
2		5.7249	9.473	12866	BB
3		1.3760	10.501	3092	BB
4		3.3260	11.700	7475	BV
5		31.2604	13.241	70256	VB
6		1.2964	17.030	2914	BV
7		2.9029	17.680	6524	VB
8		6.4542	18.777	14505	BB
	Totals	**100.0000**		**224743**	

Figure 4.29 Chromatogram of bilin peptide (1 hr) in samples of *R. salina* PE545 extracts digested with pronase.



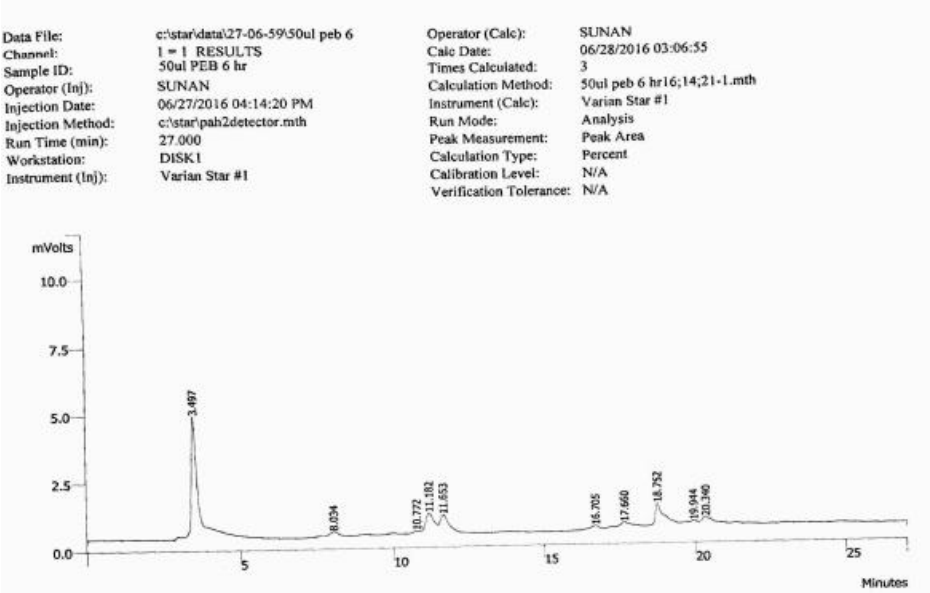
Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		40.4274	3.494	53567	BB
2		3.4513	8.815	4573	BB
3		2.1149	10.823	2802	BB
4		3.8659	11.922	5122	BV
5		26.5464	12.493	35175	VB
6		0.9422	16.867	1248	BB
7		2.7265	17.668	3613	BB
8		12.7282	18.763	16865	BV
9		3.0021	19.950	3978	VV
10		4.1952	20.589	5559	VB
	Totals	**100.0001**		**132502**	

Figure 4.30 Chromatogram of bilin peptide (2 hr) in samples of *R. salina* PE545 extracts digested with pronase.



Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		45.7364	3.503	65462	BB
2		3.2953	8.512	4716	BB
3		1.0559	9.400	1511	BB
4		2.1130	10.473	3024	BV
5		1.9887	11.208	2846	VV
6		10.4100	11.624	14900	VV
7		18.6430	12.205	26684	VB
8		1.5711	16.809	2249	BV
9		3.7867	17.667	5420	VB
10		10.2016	18.749	14601	BV
11		1.1983	19.953	1715	VB
	Totals	**100.0000**		**143128**	

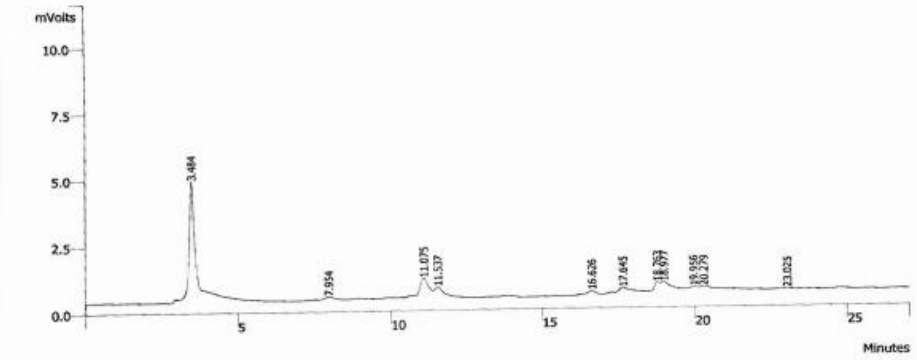
Figure 4.31 Chromatogram of bilin peptide (3 hr) in samples of *R. salina* PE545 extracts digested with pronase.



Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		40.3722	3.497	47649	BB
2		2.0965	8.034	2474	BB
3		1.1278	10.772	1331	BV
4		12.8629	11.182	15181	VV
5		12.7706	11.653	15073	VB
6		2.2844	16.705	2696	BV
7		4.9287	17.660	5817	VB
8		14.7727	18.752	17436	BV
9		3.0434	19.944	3592	VV
10		5.7408	20.340	6776	VB
	Totals	100.0000		118025	

Figure 4.32 Chromatogram of bilin peptide (6 hr) in samples of *R. salina* PE545 extracts digested with pronase.

Data File:	c:\star\data\27-06-59\50ul peb 9	Operator (Calc):	SUNAN
Channel:	1 = 1 RESULTS	Calc Date:	06/28/2016 03:07:43
Sample ID:	50ul PEB 9 hr	Times Calculated:	4
Operator (Inj):	SUNAN	Calculation Method:	50ul peb 9 hr16;47;51-1.mth
Injection Date:	06/27/2016 04:47:51 PM	Instrument (Calc):	Varian Star #1
Injection Method:	c:\star\pah2detector.mth	Run Mode:	Analysis
Run Time (min):	27.000	Peak Measurement:	Peak Area
Workstation:	DISK1	Calculation Type:	Percent
Instrument (Inj):	Varian Star #1	Calibration Level:	N/A
		Verification Tolerance:	N/A



Peak No	Peak Name	Result (%area)	Ret. Time (min)	Area (counts)	Sep. Code
1		48.2162	3.484	47625	BB
2		1.5997	7.954	1580	BB
3		14.6567	11.075	14477	VV
4		8.8194	11.537	8711	VB
5		1.7861	16.626	1764	BB
6		6.0050	17.645	5931	BB
7		3.7067	18.763	3661	BV
8		8.0413	18.977	7943	VV
9		2.9331	19.956	2897	VV
10		3.1760	20.279	3137	VB
11		1.0600	23.025	1047	BB
	Totals	**100.0002**		**98773**	

Figure 4.33 Chromatogram of bilin peptide (9 hr) in samples of *R. salina* PE545 extracts digested with pronase.

Appemdix IV

Preparation medium

PCRS11

1. To 1L of seawater

2. Under hood, to seawater, add these nutriments beforehand autoclaved (except vitamin):

Table 4.13 Preparation of PCRS11

Quantity	Compound	Final concentration
1.0 mL	Hepes-NaOH 1M (pH 7.5)	1 mM
1.0 mL	Na_2-EDTA/$FeCl_3$	8 µM
1.0 mL	Sodium Phosphate ($NaPO_4$) 50mM (pH 7.5)	50 µM
1.0 mL	Ammonium Sulfate 400mM $(NH_4)_2$-SO_4	400 µM
0.1 mL	Trace metals "Gaffron+Se"	-
0.1 mL	Cyanocobalamin 10mg/L (Vit. B12)	1 µg/L

3.Filter the medium

Hepes-NaOH 1M

To 250 mL of H_20, add gradually 119.15g of Hepes. Adjust pH at 7.5 and complete the volume at 500 mL. Store in refrigerator.

Na_2-EDTA/$FeCl_3$

- To 40 mL of HCl 0.1 N, add gradually 1,080 g of $FeCl_3$
- To 40 mL of NaOH 0.1 N, add gradually 1.488 g of Na_2-EDTA
- Mix both solutions
- Complete final volume to 2 L of sterile water
- Store in refrigerator

Sodium Phosphate

- Prepare two solutions:

  - Monosodium dihydrogen phosphate (NaH_2PO_4) at 50mM (6 g in 1 L)
  - Disodium hydrogen phosphate (Na_2HPO_4) at 50 mM (3.55 g in 500 mL)

- Make an equimolar mixture of these two solutions and adjust the pH at 7.5

Trace metals "Gaffron+Se"

- To 500mL of H_20, add gradually these nutriments:

Table 4.14 Preparation of trace metals Gaffron+Se

Quantity (mg/L)	Compound	Final concentration in media (nM)
186	Boric acid (H_3BO_3)	150
101	Manganese (II) Sulfate Monohydrate ($MnSO_4.H_2O$)	30
1.98	Sodium Tungstate dihydrate ($Na_2WO_4.2H_20$)	0.3
5.16	Ammonium molybdatetetrahydrate ($(NH_4)_6MO_7O_{24}.4H_2O$)	1.45
7.14	Potassium bromide (KBr)	3
4.98	Potassium iodide (KI)	1.5
17.25	Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	3
9.25	Cadium Nitrate ($Cd(NO_3)_2.4H_2O$)	1.5
8.76	Cobalt (II) Nitrate ($Co(NO_3)_2.6H_2O$)	1.5
7.5	Copper (II) Sulfate ($CuSO_4.5H_2O$)	1.5
7.1	Nickel Chloride ($NiCl_2.6H_2O$)	1.5
2.4	Chromium (III) Nitrate ($Cr(NO_3)_3.9H_2O$)	0.3
1.5	Vanadyl Sulfate Pentahydrate ($VOSO_4.5H_2O$)	0.3
28.4	Aluminium Potassium Sulfate ($KAl(SO_4)_2.12H_2O$)	3
3.3	Selenium (IV) Oxyde (SeO_2)	1.5

f/2 medium

Metall Mix

1. Prepare metall mix:

   150 mg $ZnSO_4 .H_20$

   100 mg $CuSO_4.5H_20$

   120 Mg $CuSO_4.7H_20$

   2000 mg$MnSO_4.H_20$

- Put all in 100 mL A.dest

2. Put 500 mg $FeCl_3.H_2O$ in 100 mL A.dest
3. Put 65 mg $Na_2MoO.2H_2O$ in 100 mL A.dest
4. Put 5000 mg $Na_2EDTA.2H_2O$ in 100 mL A.dest

- For 1 L Metall mix: 10 mL solution 1, 2, 3 +100 mL solutions 4

Vitamin Mix

-Prepare metall mix:

1 mL vitamin B_{12}	1	g/L
10 mL Biotin	0.1	g/L
100 mL Thiamin B_1	2	g/L

- Put in 100 mL A.dest and store in a Freezer < -1℃

Nutriumnitrat

Add 75 g $NaNO_3$ in 1000 mL

Di – Natriumhydrogen phosphate

Add 5 g Na_2HPO_4 in 1000 mL A.dest

Mix 1 L filter seawater

1. 1 mL Metall Mix

2. 1 mL Vitamin Mix
3. 1 mL Natriumnitrat ($NaNO_3$)
4. 1 mL Di – Natriumhydrogen phosphate (Na_2HPO_4)

Daigo IMK

To 1000 mL of seawater, add gradually these nutriments 0.252 g and adjust to pH 8.

Nutriments

To add gradually these nutriments:

Table 4.15 Preparation of Daigo IMK medium

Quantity(g)	Compound
200	$NaNO_3$
1.4	$NaHPO_4$
5	K_2HPO_4
2.68	NH_4Cl
5.2	Fe.EDTA
0.332	Mn.EDTA
37.2	Na_2EDTA
0.023	$ZnSO_4.7H_2O$
0.014	$CoSO_4.7\ H_2O$
0.0073	$Na_2MoO_4.2\ H_2O$
0.0025	$CuSO_4.5\ H_2O$
0.0023	Na_2SeO_3
0.0015	Thiamin.HCl
0.0015	Biotin
0.0015	Vitamin B_{12}
0.18	$MnCl_2.4H_2O$
1000 mL	Seawater

VITA

Miss Chanoknard Karnjanapak was born on May 11, 1990 in a southern province of Thailand, Nakhonsrithammarat. She obtained her bachelor of science from the Department of Chemistry at Kasetsart University in 2012. Then, she was admitted to the master degree program at the Department of Marine Science at Chulalongkorn University in 2013. During her master study, she received the research fund from Chulalongkorn University's graduate school on the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). She finished her master's degree of science in July 2016.