PRETREATMENT OF ALGAL AND CYANOBACTERIAL BIOMASS FOR HIGH PURITY PHYCOCYANIN PRODUCTION

by

Sevda Avcı

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PRETREATMENT OF ALGAL AND CYANOBACTERIAL BIOMASS FOR HIGH PURITY PHYCOCYANIN PRODUCTION

APPROVED BY:

Asst. Prof. Dr. Berat Zeki Haznedaroğlu ……
Thesis Advisor

Prof. Dr. Melek Türker Saçan ……

Asst. Prof. Dr. Nadide Seyhun ……

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Hevesle yanan mum sönmez güçsüz bir nefeste…

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ABSTRACT

PRETREATMENT OF ALGAL AND CYANOBACTERIAL BIOMASS FOR HIGH PURITY PHYCOCYANIN PRODUCTION

Phycocyanin is a water-soluble, blue-colored phycobiliprotein, mainly found in cyanobacteria and red algae. Major commercial interest on phycocyanin exists as an important ingredient for food, pharmaceutical and cosmetics sectors. However, dedicated and efficient methods of extraction optimized for different organisms are lacking. In this study, wet and freeze-dried biomass samples from several cyanobacteria (including a local isolate) and a red microalga species were pretreated with sonication, mortar and pestle, freeze-thaw cycling and bead-beating. High concentration yielding phycocyanin extracted from *Phormidium* sp., *Synechocystis* sp., *Desertifilum tharense*, *Nostoc* sp. and *Galdieria sulphuraria* were purified by ammonium sulfate fractionation combined with acetate buffer elution. Bead-beating has been found to be the most successful pretreatment for the extraction of phycocyanin. Phycocyanin from *Synechocystis* sp. and *Phormidium* sp. were further purified with anion exchange chromatography. Overall, food grade phycocyanin extraction has been achieved for all biomass samples except *Scytonema* sp. The highest phycocyanin purity with a higher observed value compared to literature was obtained from *Synechocystis* sp. Furthermore, the effect of various stress conditions (light, salinity, and hydrogen peroxide) on biomass growth and phycocyanin production for *Synechocystis* sp. was investigated after PC extraction with bead-beating. As an alternate method to salting-out, chitosan/activated charcoal was applied as purification step. Antioxidant activities of purified phycocyanin were tested via 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity assay and compared to L-ascorbic acid (vitamin C). In average, ammonium sulfate precipitation gave higher antioxidant activity than activated charcoal and chitosan purification and obtained PC showed lower antioxidant activity than L-ascorbic acid for both purification methods.

ÖZET

ÖN İŞLEM UYGULANAN ALG VE SİYANOBAKTERİ TÜRLERİNDEN YÜKSEK SAFLIKTA FİKOSİYANİN ELDESİ

Fikosiyanin fikobiliprotein grubuna ait, genellikle siyanobakteri ve tek hücreli kırmızı alglerde görülen, suda çözünebilen mavi renkli bir pigmenttir. Gıda, ilaç ve kozmetik sektörlerindeki geniş kullanım alanından dolayı ekonomik olarak değeri yüksektir. Buna rağmen literatürde farklı türler için belirlenmiş verimli bir ekstraksiyon yöntemi bulunmamaktadır. Bu çalışmada, iki farklı biyokütle tipi (ıslak ve liyofilize-kuru) kullanılarak, içlerinde lokal bir izolatın da bulunduğu beş farklı siyanobakteri ve bir kırmızı alg türüne havanda ezme, su banyosu içinde sonikasyon, boncukla parçalama ve dondurup-çözme ön işlem yöntemleri uygulanarak fikosiyanin çıkarılmıştır. Fikosiyanin miktarları ilk ekstraksiyon sonucunda yüksek çıkan *Phormidium* sp., *Synechocystis* sp., *Desertifilum tharense*, *Nostoc* sp. ve *Galdieria sulphuraria* örnekleri amonyum sülfat tuzu ve asetat tamponu ile saflaştırma işlemine alınmıştır. Fikosiyanin ekstraksiyonu için en başarılı ön işlem yöntem, boncukla parçalama olarak belirlenmiştir. Takiben, *Phormidium* sp. ve *Synechocystis* sp. türlerinin fikosiyaninleri iyon değişim kromatografisi ile ileri seviye saflaştırılmıştır. Özetle, *Scytonema* sp. hariç, tüm biyokütle örneklerinden gıda saflığında fikosiyanin elde edilmiştir. En yüksek saflık oranı, literatür değerlerinden yüksek olarak *Synechocystis* sp. türünde elde edilmiştir. Çalışmanın devamında, *Synechocystis* sp. türüne farklı stres koşulları (ışık, tuzluluk ve hidrojen peroksit etkisi) uygulanarak fikosiyanin üretim miktarına ve büyüme eğrilerine etkileri karşılaştırılmıştır. Bu aşamada amonyum sülfat tuzuna alternatif olarak kitosan/aktif karbon saflaştırma yöntemi kullanılmıştır. Saflaştırılan fikosiyaninlerin antioksidan aktivite tayini 1,1-diphenyl-2-picrylhydrazyl (DPPH) yöntemi kullanılarak belirlenmiş ve L-askorbik asit (C vitamini) ile karşılaştırılmıştır. Amonyum sülfat saflaştırması sonucunda elde edilen fikosiyaninlerin, aktif karbon/kitosan yöntemi ile saflaştırılanlara göre ortalama daha yüksek aktivite verdiği ve iki yöntem ile de elde edilen fikosiyaninin L-askorbik asite kıyasla daha düşük antioksidan aktivite gösterdiği gözlenmiştir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

ÖZET vi

TABLE OF CONTENTS vii

LIST OF FIGURES ix

LIST OF TABLES xii

LIST OF SYMBOLS/ABBREVIATIONS xiv

1. INTRODUCTION 1

2. LITERATURE REVIEW 3

2.1. Phycobiliproteins 3

2.1.1. Biosynthesis, Structure and Function of Phycobiliproteins 3

2.1.2. Applications of Phycobiliproteins 6

2.1.2.1. Phycobiliproteins as Dye and Food Additives 6

2.1.2.2. Phycobiliproteins as Fluorescent Probes 7

2.1.2.3. Phycobiliproteins as a Pharmaceutical Agent 7

2.1.3. Activities of Phycobiliproteins 8

2.2. Factors Affecting Growth and Phycobiliprotein Production in Algae and Cyanobacteria 9

2.2.1. Light 10

2.2.2. Temperature 12

2.2.3. pH 13

2.2.4. Salinity 13

2.2.5. Nutrients 14

2.3. Extraction and Purification of Phycobiliproteins 16

2.3.1. Extraction Methods for the Isolation of Phycobiliproteins 16

2.3.2. Purification of Phycobiliproteins 19

2.4. Methods to Measure Antioxidant Activity 22
3. MATERIALS AND METHODS 26
3.1. Optimization of The Extraction Method 26
3.1.1. Cultivation of Cyanobacteria and Algae 26
3.1.2. Extraction and Purification of Phycocyanin 28
3.2. Optimization of Phycocyanin Production 30
3.3. Purification of PC of Stress Reactors 31
3.4. Calculations 32
3.5. Antioxidant Activity Assay 32
3.6. Statistical Analysis 33
4. RESULTS AND DISCUSSION 34
4.1. Optimization of The Extraction Procedure 34
4.1.1. Growth Parameters 34
4.1.2. Crude PC Extraction 35
4.1.3. Further Purification by Salt Precipitation and AEC 44
4.2. Extraction from Stress Applied *Synechocystis* sp. Biomass Samples 48
4.2.1. Growth Parameters for Reactors 48
4.2.2. Extraction of Crude PC from Rectors 50
4.2.3. Purification of the Crude Extracts of Biomass Samples Exposed to Stress Conditions 51
4.3. Antioxidant Activity Measurement by DPPH Assay 53
5. CONCLUSIONS 64
REFERENCES 66
APPENDIX A: PHYLOGENY INFORMATION 84
APPENDIX B: BUFFER PREPARATIONS AND MEDIA RECIPIES 85
APPENDIX C: ANOVA RESULTS 89
APPENDIX D: GROWTH DATA OF BATCHES 105
APPENDIX E: THE PICTURES OF CRUDE EXTRACTS 108
APPENDIX F: L-ASCORBIC ACID STANDARD CURVES 111

LIST OF FIGURES

Figure 1.1. Applications of products that are extracted from algal biomass.....1

Figure 2.1. Different types of PBPs and their colors based on absorption spectra.. 3

Figure 2.2. The representation of the structure of the phycobilisome structure in general.....4

Figure 2.3. Structures of phycobilins found in cyanobacteria.....5

Figure 2.4. Overall representation of natural antioxidants ……...23

Figure 3.1. *D. tharense* (left) and *G. sulphuraria* (right) growing in flasks. 26

Figure 3.2. Overall representation of extraction and purification processes…….....30

Figure 4.1. Absorbance versus time graphs for algal species.. 34

Figure 4.2. Results of the crude extract of *D. tharense* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass)…….....37

Figure 4.3. Results of the crude extract of *G. sulphuraria* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass)….....38

Figure 4.4. Results of the crude extract of *Nostoc sp.* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass).. 40

Figure 4.5. Results of the crude extract of *Phormidium sp*. (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass)……...41

Figure 4.6. Results of the crude extract of *Scytonema sp.* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass)... 43

Figure 4.7. Results of the crude extract of *Synechocystis* sp.....44

Figure 4.8. Absorbance over time graph for stress reactors.....48

Figure 4.9. The change in cell number over time for stress applied reactors.....49

Figure 4.10. The change in pH over time for stress applied reactors.....50

Figure 4.11. Growing of the stress reactors with the control group(left: hydrogen peroxide stress; middle: salt stress; right: control.) 50

Figure 4.12. The 96-well plate design for DPPH assay 53

Figure 4.13. The scavenging activity of the PC obtained from the light stress and purified with the AC/CS method (L-ascorbic acid is used as control).....54

Figure 4.14. The individual graphs for the scavenging activity result of the AC/CS treated PC samples of light stress (left) and L-ascorbic acid control (right) for IC_{50} calculations. 54

Figure 4.15. The scavenging activity of the PC obtained from the light stress and purified with the ASP method (L-ascorbic acid is used as control).....55

Figure 4.16. The individual graphs for the scavenging activity result of the ASP treated PC samples of light stress (left) and L-ascorbic acid control (right) for IC_{50} calculations. 55

Figure 4.17. The scavenging activity of the PC obtained from the control group and purified with the AC/CS method (L-ascorbic acid is used as control)…….. ……. 56

Figure 4.18. The individual graphs for the scavenging activity result of AC/CS treated PC samples of control group (left) and L-ascorbic acid control (right) for IC_{50} calculations.....56

Figure 4.19. The scavenging activity of the PC obtained from the control group and purified with the ASP method (L-ascorbic acid is used as control).… 57

Figure 4.20. The individual graphs for the scavenging activity result ASP treated PC samples of control group (left) and L-ascorbic acid control (right) for IC_{50} calculations 57

Figure 4.21. The scavenging activity of the PC obtained from the hydrogen peroxide stress and purified with the AC/CS method (L-ascorbic acid is used as control). 58

Figure 4.22. The individual graphs for the scavenging activity result of the AC/CS purified PC samples (left) of hydrogen peroxide stress and L-ascorbic acid control (right) for IC_{50} calculations 58

Figure 4.23. The scavenging activity of the PC obtained from the hydrogen peroxide stress and purified with the ASP method (L-ascorbic acid is used as control)... 59

Figure 4.24. The individual graphs for the scavenging activity result of the ASP purified PC samples (left) of hydrogen peroxide stress and L-ascorbic acid control (right) for IC_{50} calculations.....59

Figure 4.25. The scavenging activity of the PC obtained from the salt stress and purified with the AC/CS method (L-ascorbic acid is used as control).….....60

Figure 4.26. The individual graphs for the scavenging activity result of the AC/CS purified PC samples (left) of salt stress and L-ascorbic acid control (right) for IC_{50} calculations 60

Figure 4.27. The scavenging activity of the PC obtained from the salt stress and purified with the ASP method (L-ascorbic acid is used as control).....61

Figure 4.28. The individual graphs for the scavenging activity result of the ASP purified PC samples (left) of salt stress and L-ascorbic acid control (right) for IC_{50} calculations 61

LIST OF TABLES

Table 2.1. Commercial phycocyanin producing companies and their final products 6

Table 2.2. Companies selling phycocyanin as colorant 7

Table 2.3. Companies producing phycocyanin for fluorescence applications 7

Table 2.4. Selected anti-cancer activities of phycocyanin from cyanobacteria. 9

Table 2.5. Selected anti-inflammatory activities of phycocyanin from cyanobacteria……. 9

Table 2.6. Other activities of phycocyanin 9

Table 2.7. Comparison among different cultivation conditions.. 15

Table 2.8. Phycocyanin extraction methods applied for different species. 17

Table 2.9. Several phycocyanin purification methods applied to different species. 20

Table 2.10. Common ROS and the neutralizing antioxidants 24

Table 2.11. Methods of total antioxidant capacity assessment 25

Table 3.1. Species specific growth conditions applied in this study.. 27

Table 3.2. Design details of the optimization of the extraction part 29

Table 3.3. Experimental matrix for tested stress factors. 31

Table 4.1. Salt precipitation and Anion Exchange Chromatography (AEC) results for species. 47

Table 4.2. The phycocyanin amount and intitial purity obtained from the control and the stress reactors. 50

Table 4.3. The purity, yield and recovery results for ASP and AC/CS treatments ……..52

Table 4.4. The % scavenging activity and IC_{50} results for each group after AC/CS purification….62

Table 4.5. The % scavenging activity and IC_{50} results for each group after ASP purification……..62

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
°C	Celsius	
$(NH_4)_2SO_4$	Ammonium Sulfate	
%	Percentage	
C	Carbon	
CO_2	Carbon Dioxide	
$CuSO_4$	Copper Sulfate	mM
Fe	Iron	mM
g	Gram	
H_2O_2	Hydrogen Peroxide	
L	Liter	
M	Molar	
m^2	Meter Square	
mg	Milligram	
mL	Milliliter	
mM	Millimolar	
N	Nitrogen	mM
Na	Sodium	
NaCl	Sodium Chloride	M
nm	Nanometer	
O_2•	Superoxide Radical	
P	Phosphorous	mM
PO_4^{-3}	Orthophosphate	
S	Sulphur	
s	Second	
λ	Lambda	nm
μM	Micromolar	
μL	Microliter	

Abbreviations	Explanation
A_{280}	Absorbance at 280 nm
A_{620}	Absorbance at 620 nm
$ABTS^{\bullet+}$	2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)
AC/CS	Activated Charcoal and Chitosan
AEC	Anion Exchange Chromatography
ALA	Aminolevulinic Acid
ANOVA	One Way Analysis of Variance
AP-1	Activator Protein 1
APC	Allophycocyanin
ASP	Ammonium Sulfate Precipitation
ATPS	Aqueous Two-Phase System
BB	Bead-beating
BG-11	Blue-green-11 Medium
CAT	Catalase
CE	Crude Extract
Chl a	Chlorophyll a
CoA	Coenzyme A
CUPRAC	Cupric Reducing Antioxidant Capacity
DCW	Dry Cell Weight
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylene Diamine Tetra Acetic Acid
EtOH	Ethanol
FDA	Food and Drug Administration
FISH	Fluorescence In-Situ Hybridization
FRAP	Ferric Reducing Antioxidant Power
FT	Freeze-thaw Cycling
GE	Gel Electrophoresis
HSD	Honestly Significant Difference
IEC	Ion Exchange Chromatography
IC_{50}	The Half Maximal Inhibitory Concentration
JNK	c-Jun N-terminal Kinase
LC	Liquid Chromatography

Abbreviations	Explanation
MeOH	Methanol
min	Minute
MP	Mortar and Pestle
MPO	Myeloperoxidase
MS	Mass Spectrometry
NF-κB	Nuclear Factor κB
OD	Optical Density
$^{\bullet}$OH	Hydroxide Radical
ORAC	Oxygen Radical Absorbance Capacity
p	Probability
pI	Isoelectric Point
PAR	Photo Active Radiation
PBPs	Phycobiliproteins
PBS	Phycobilisome
PC	Phycocyanin
PCR	Polymerase Chain Reaction
PcyA	Phycocyanobilin Synthase
PE	Phycoerythrin
PGB	Porphobilinogen
PUB	Phycourobilin
PUFA	Polyunsaturated Fatty Acid
PVB	Phycoviolobilin
ROS	Reactive Oxygen Species
rpm	Repetition per Minute
RNA	Ribosomal Ribonucleic Acid
RuBisCO	Ribulose-1,5-bisphosphate Carboxylase/Oxygenase
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SON	Sonication
TPTZ	2,4,6-tri(2-pyridyl)-s-triazine
UV	Ultraviolet

1. INTRODUCTION

Algae including cyanobacteria attract great attention due to their diverse benefits and applications in food, pharmaceutical, cosmetics, agricultural, energy and environmental sectors. They are advantageous to many higher plants due to their faster growth rates, capability to grow in non-arable land, capturing high levels of CO_2, and their fast adaptability to changing conditions in their habitats. They represent a wider diversity compared to plants, and it is also estimated that the diversity of algal compounds is approximately 10 times greater than those produced by plants (Fu et al., 2017). Algae are photosynthetic microorganisms responsible from 25% of the global and 50% of all aquatic productivity (Shalaby, 2011). They can be found in temperate to complex and harsh environments where they need to be well-adopted to survive. For example, marine diatoms have to develop mechanisms to avoid or tolerate pathogens (Smetacek, 2001). One mechanism of survival includes producing a great variety of secondary metabolites and other bioactive compounds (Rodríguez-Meizoso et al., 2010). These value-added bioproducts, i.e. carotenoids, terpenoids, phycobiliproteins (PBPs), xanthophylls, vitamins and polyunsaturated fatty acids (PUFAs), are either released into the growth medium or extracted from the biomass, which carry great potential in their activities such as anti-cancer, anti-microbial, and anti-inflammatory effects (Bhagavathy et al., 2011; De Almeida et al., 2011). There are many different sectors that algal and cyanobacterial compounds can be used such as cosmetics, nutraceuticals, animal feed and pharmaceuticals (Figure 1.1).

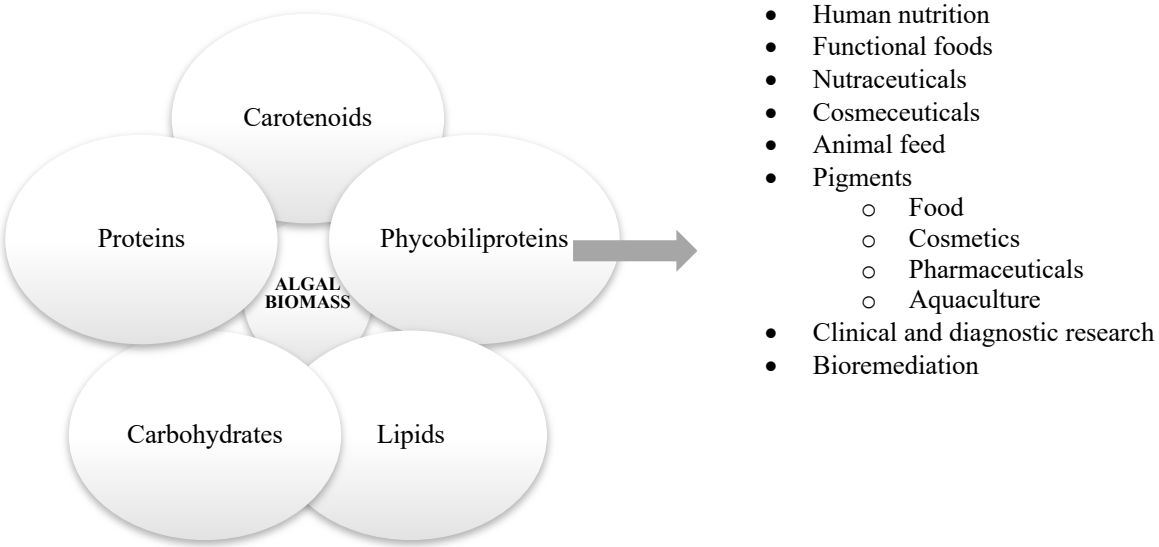


Figure 1.1. Applications of products that are extracted from algal biomass.

Among secondary metabolites, the ones with antioxidant properties are very important as oxidative stress is the main driver of inflammation and inflammation-related diseases such as cancer and diabetes in humans. Under normal conditions, reactive oxygen species (ROS) can be formed via mitochondrial processes, as response to foreign antigens, and/or exposure to various chemicals (Birben et al., 2012). In return, ROS scavenging enzymes such as superoxide dismutase (SOD), catalase, and peroxidase metabolize ROS formed into less harmful compounds (Birben et al., 2012). When this balance is disrupted, oxidative stress increases and results in various abnormalities including cancer and inflammation (Reuter et al., 2011). Inflammatory cells may secrete mutagenic agents that lead to tumor development indicating that all three mechanisms are interconnected. However, it was also proven that the oxidative stress can be relieved via the introduction of antioxidants (Simioni et al., 2018). In addition, increasing incidence rates of cancer and the adverse side effects of chemotherapy such as loss of appetite and fatigue leads to the need for new compound discovery in these fields (Pearce et al., 2017).

Three light harvesting pigments which are chlorophyll, carotenoids and PBPs exist in cyanobacteria and microalgae (Singh et al., 2009). Phycocyanin (PC) with applications in food, cosmetic and pharmaceutical sectors is an advantageous candidate as a natural source of antioxidant. However, its extraction process differs among algal species and not optimized for the best yield and purity. In this study, a comprehensive evaluation of several pretreatment methods to optimize PC extraction from five different cyanobacteria species including one local isolate from Denizli Region in Turkey and one red microalga was performed. Further purification was applied to obtained higher purity PC and antioxidant activities were determined with 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay and compared to L-ascorbic acid (a.k.a. vitamin C).

2. LITERATURE REVIEW

2.1. Phycobiliproteins

2.1.1. Biosynthesis, Structure and Function of Phycobiliproteins

Phycobilisomes (PBSs) are the light harvesting complexes found in cyanobacteria (a.k.a. blue-green algae despite their prokaryotic nature), rhodophytes (red algae, eukaryotic) and certain cryptomonads (algae, eukaryotic) (Román, 2002). The composition of PBSs is changed depending on species and growth conditions. There are four types of phycobiliproteins (PBPs) that constitute the PBS: phycoerythrin (PE, λ_{max} 560 nm), phycoerythrocyanin (PEC, λ_{max} 600 nm), phycocyanin (PC, λ_{max} 615 nm, blue pigment) and allophycocyanin (APC, λ_{max} 652 nm, bluish green pigment) (Figure 2.1) (Kannaujiya et al., 2017). Their characterization is done according to absorption peaks and energy transfer occurs from PE to APC through PC, and finally to chlorophyll a (Chla) (Dumay and Morançais, 2016). In algae, they are located in chloroplasts as bound to the thylakoid membrane (Figure 2.2) (Pagels et al., 2019). The PBPs contain α and β chains, and can be found in either trimeric $(\alpha\beta)_3$ or hexameric form $(\alpha\beta)_6$, and the most common PBP in cyanobacteria is PC and it is found in $(\alpha\beta)_6$ from at low pH and $(\alpha\beta)_3$ form at neutral pH, respectively (Manirafasha et al., 2016). For the red algae and blue-green algae, all three are present even though the most abundant PBP for red microalgae is PE, whereas cryptomonads lack APC (Manirafasha et al., 2016). In phycobilisomes, the energy transfer occurs from PE to PC to APC and finally to chlorophyll a (Viskari et al., 2001).

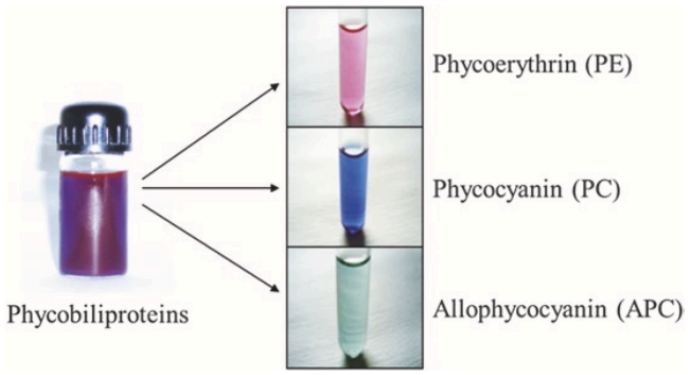


Figure 2.1. Different types of PBPs and their colors based on their absorption spectra.

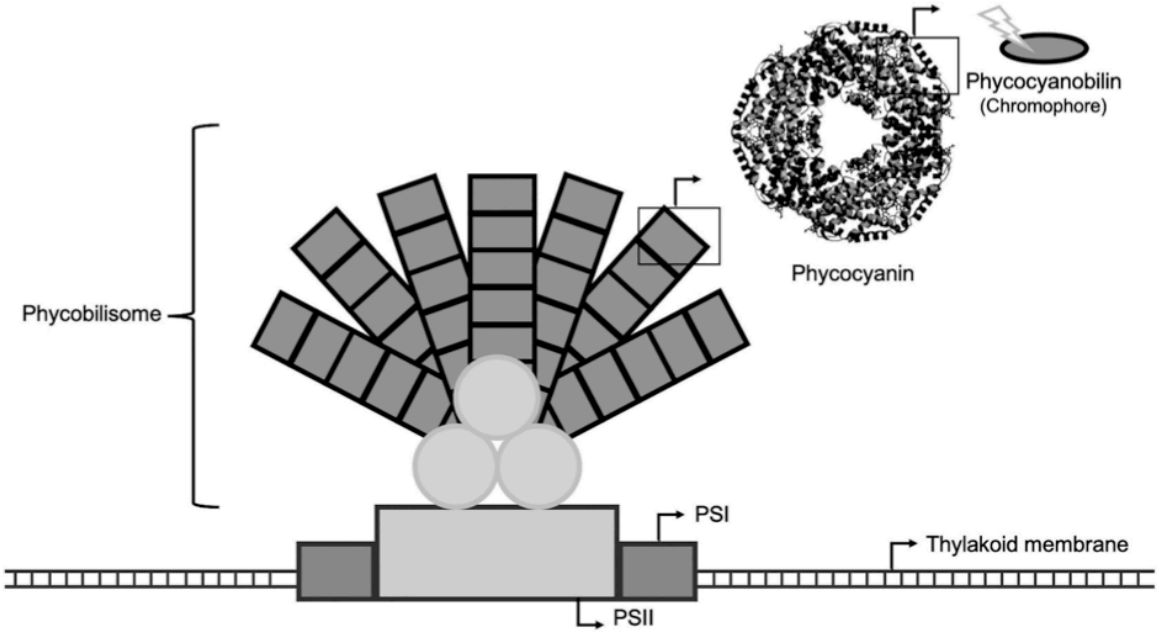
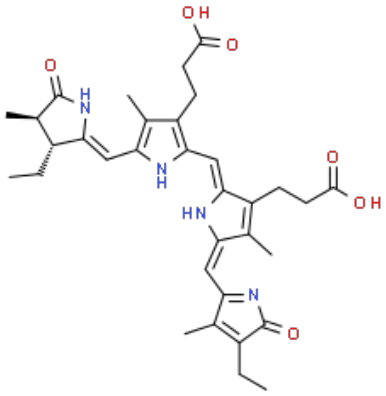
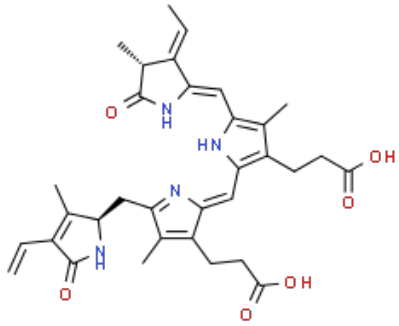


Figure 2.2. The representation of the structure of the phycobilisome structure in general (adopted from Pagels et al., 2019).

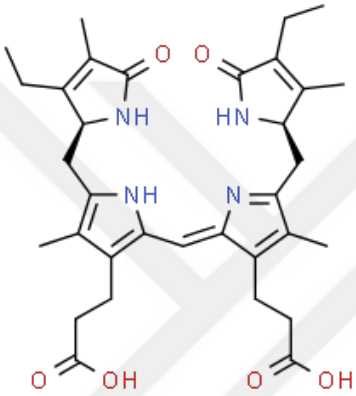
Phycobiliproteins, with an absorption range at 500-660 nm, improve the efficacy of the photosynthesis by widening the absorbance range of incoming light beyond the area that chlorophylls and carotenoids can capture. Cyanobacteria and red algae lack chlorophyll b; therefore, they require to compensate this large gap in the spectrum (Hsieh-Lo et al., 2019). For this purpose, they have a covalently bound phycobilin to a protein backbone. Phycobilins are synthesized from heme by hemeoxygenase which produces biliverdin intermediate from heme (Cornejo and Beale, 1997). After they are synthesized, bilins are linked to cysteine residues by bilin lyases through thioether bonds (Cornejo and Beale, 1997). The light capturing ability of PBPs come from the phycobilins which are light absorbing chromophores. Based on the structure as shown in Figure 2.3, there are four types of phycobilins: phycocyanobilin (blue-$C_{33}H_{38}N_4O_6$), phycoerythrobilin ($C_{33}H_{38}N_4O_6$), phycourobilin (orange-$C_{33}H_{42}N_4O_6$) and phycoviolobilin ($C_{33}H_{42}N_4O_6S$) (Ikeuchi and Ishizuka, 2008). The last two bilins are crucial for the survival of organisms in deeper layers by absorbing at shorter wavelengths (Bishop et al., 1987). PC has phycocyanobilin as its chromophore whereas the bilin of phycoerythrocyanin is phycoviolobilin (Glazer, 1985). Phycocyanobilin and phycoerythrobilin are produced from heme via biliverdin whereas phycoviolobilin and phycourobilin, containing a vinyl group at C3, are synthesized via isomerization because they cannot be cleaved directly from the PBP (Biswas, 2011).



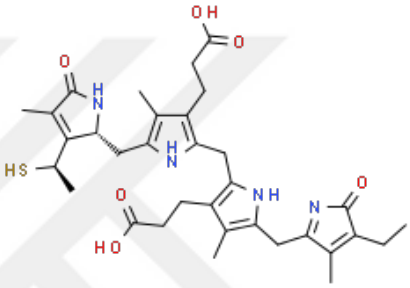
Phycocyanobilin



Phycoerythrobilin



Phycourobilin



Phycoviolobilin

Figure 2.3. Structures of phycobilins found in cyanobacteria.

Among PBPs, phycocyanin has greater importance due to its wide application areas including biotechnological and pharmaceutical (Manirafasha et al., 2016). PC is a water-soluble pigment with equal numbers of α and β chains. The molecular mass of each chain varies from 18 to 22 kDa (Santiago-Santos et al., 2004). The total number of α and β chains is dependent on the species and the overall mass of PC is in between 140 and 210 kDa (Santiago-Santos et al., 2004). Phycocyanin is found in cyanobacteria, red algae and cryptophytes (Patel et al., 2005; Patil and Raghavarao, 2007). Its dark blue color originates from covalently attached linear tetrapyrrole prosthetic groups (Benedetti et al., 2006). At moderate pH and ionic strength, PC is found in trimeric state whereas its oligomerization state differs with pH, ionic strength and protein concentration (Manirafasha et al., 2016). Phycocyanin can comprise up to 20% of algal dry cell weight (DCW) (Troxler and Bogorad, 1966).

2.1.2. Applications of Phycobiliproteins

As synthetic products developed for pharmaceutical industry might cause side effects, prospecting efforts for natural products with similar or superior functionalities are on the rise. Phycocyanin is one of the most demanded bioproducts with a wide range of application areas. It is the first natural food colorant from algae and cyanobacteria approved by Food and Drug Administration (FDA) of the United States by being non-toxic (de Morais et al., 2018). Yet, the applicability of PC extends beyond the food sector as a natural colorant. It can be used as florescent probes with numerous pharmaceutical applications and health-promoting properties. There are 55 patents on PBP production, 30 patents on applications in medicine, functional foods and similar areas, and 236 patents on applications utilizing the fluorescence properties of PBPs (Sekar and Chandramohan, 2008). Currently, the prices of PBP products range from 3 to 25 US $/mg for native pigments and can reach up to 1500 US $/mg for certain cross linked PBPs (with antibodies or other fluorescent molecules). In Table 2.1, a few companies producing commercial PC are listed.

Table 2.1. Commercial phycocyanin producing companies and their final products (adopted from Hsieh-Lo et al., 2019).

Compound	Product	Biomass Source	Manufacturer
Phycocyanin	PC powder	*Spirulina*	Xi'an Pincredit Bio-tech Co.
	Lyophilized C-PC	*Spirulina*	Sigma-Aldrich
	Native PC protein	*Arthrospira platensis*	Abcam
	Linablue®	*Spirulina*	Earth Rise Nutritional

2.1.2.1. Phycobiliproteins as dye and food additives. Due to possible toxic effects of synthetic dyes, there is an increasing demand for natural colorants and inks in food, pharmaceutical, cosmetics, textile and printing industries. PC is used as natural dyes for food (candy, chewing gum, ice creams, dairy products, soft drinks, etc.), and cosmetics replacing synthetic colorings (Deshmukh and Puranik, 2012; Patel et al., 2005). For food applications, the stability of the PC at low pH is crucial since many food items such as drinks and confections are acidic. In a study by Sonani et al. (2014), it was found that the blue pigment produced from *Phorphyridium aerugineum* does not change with pH and was stable under light. Even though, it was found to be sensitive to heat, the produced blue color is stable at 60°C for 40 minutes within a pH range of 4 to 5. Pepsi® and Bacardi Breezer® add the blue color to beverages without heat application and they preserve their color for at least 1 month at room temperature.

Table 2.2. Companies selling phycocyanin as colorant (adopted from de Morais et al., 2018).

Company	Product	Application
DIC Corporation	Linablue®	Food colorant
Japan Algae Co., Ltd	*Spirulina* pigment	Food colorant
Ozone Naturals	PC color	Food colorant
Northland Biotech	Natural PC	Cosmetics colorant

2.1.2.2 Phycobiliproteins as fluorescent probes. Having a large Stokes shift and lower interference with other fluorescing particles, being resistant to quenching, being able to be stored at low temperatures and being highly soluble in aqueous environments make PBPs ideal for fluorescent tags (Sekar and Chandramohan, 2008). In addition, having an isoelectric point close to 4.65 make them easily bound to antibodies (Jiang et al., 2017). These properties make them powerful and highly sensitive fluorescent reagents to serve as labels for antibodies, receptors and other biological molecules in fluorescence-activated cell sorter (FACS) assays, immune-labelling experiments, fluorescence microscopy, diagnostics, fluorescence in-situ hybridization (FISH), and gel electrophoresis (GE) applications (Table 2.3). They can also be used to detect cyanobacterial presence in water bodies as an indication of water quality and safety (Eriksen, 2008).

Table 2.3. Companies producing phycocyanin for fluorescence applications (adopted from de Morais et al., 2018; Sekar and Chandramohan, 2008).

Company	Product	Application
ProZyme, Inc.	PhycoPro	Immunological assays
Hash Biotech Labs	C-PC	Fluorescence microscopy
Martek Bioscience Corporation	SensiLight dyes	Immunodiagnostics
Cyanotech Corporation	C-PC	Flow cytometry

2.1.2.3 Phycobiliproteins as pharmaceutical agents. The microbial resistance to already existing products is increasing. Also, there are various side effects of synthetic products. With anti-inflammatory, anticancer and antioxidant activities, the PC is a good candidate as a pharmaceutical agent (Sekar and Chandramohan, 2008).

2.1.3. Activities of Phycobiliproteins

Reactive oxygen species are the leading causes of many health problems including cancer, inflammation and neurodegenerative disorders. During the day, humans experience many natural ROS generating activities including routine metabolic processes and external factors such as cigarette smoke, stress, chemical exposure, etc. Therefore, ROS reduction is highly crucial to sustain a healthy life. Due to its antioxidant characteristics, PC has potential to alleviate the damaging effects of ROS. When evaluated as an antioxidant in vitro, PC was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and inhibit microsomal lipids peroxidation (Eriksen, 2008). Inhibitory effects on allergic inflammation was shown through the ability of PC to protect red blood cells against lysis induced by peroxyl radicals (Kovárová-Kovar et al., 2000).

Phycocyanin is inhibits cancer cells whereas it does not have any known negative effects on normal cells (Ravi et al., 2015). Anti-cancer activity occurs through either inhibition of the cell proliferation or enhanced apoptosis of tumor cells. Anti-inflammatory activity of PC occurs via NF-κB and MAPK signaling pathways which are responsible for the synthesis of the pro-inflammatory mediators (IL-1β, TNF-α) (Leung et al., 2013). Another mechanism is the inhibition of COX-2 which is the key in the production of prostanoids, a pain reliever and mediator (Leung et al., 2013). Phycocyanin also reduces the levels of tumor necrosis factors, such as TNF-α in the blood serum of mice-treated with endotoxin and shows neuroprotective effects in the rat cerebellar granule cell cultures (Ljubuncic et al., 2008).

The main biological sources of PC are cyanobacteria and red algae. Therefore, there are various studies investigating potential effects of PC isolated from these species. For example, PC isolated from Spirulina (*Arthrospira platensis*, *A. fusiformis*, and *A. maxima*) enhanced the survival rate of mice injected with liver tumor cells (Belay et al., 1993) and suppressed the IgE specific antigen which in turn reduces the inflammation (Nemoto-Kawamura et al., 2004). In addition, PC also reduces mouse ear oedema via PGE2 pathway (Romay et al., 1998). Several other proven activities of PC extracted from various algae species are listed in Table 2.2, 2.3 and 2.4. As can be seen from the literature, the activities of PC span a wide range of anti-cancer, anti-viral, anti-bacterial, anti-obesity and anti-inflammation.

Table 2.4. Selected anti-cancer activities of phycocyanin from cyanobacteria (adopted from Pagels et al., 2019).

Cyanobacterial Source	Cell line	Cancer type	Main mechanism of action	Reference
Limnothrix sp. 37-2-1	LNCap	Prostate	Caspase 9 and Caspase 3 activation	(Gantar et al., 2012)
Microcystis aeruginosa	HepG2	Hepatoma	Caspase 3 activation	(Wang et al., 2012)
Oscillatoria tenuis	HT-29	Lung	Cell cycle arrest	(Thangam et al., 2013)
Arthrospira platensis	PANC-1	Pancreatic	Caspase 3 activation	(Liao et al., 2016)

Table 2.5. Selected anti-inflammatory activities of phycocyanin from cyanobacteria (adopted from Pagels et al., 2019).

Cyanobacterial source	Mechanism of action	Reference
Arthrospira platensis	Inhibition of COX-2 activity	(Leung et al., 2013)
Arthrospira platensis	Reduction of autoimmune response	(Cervantes-Llanos et al., 2018)
Arthrospira maxima	Reduction of Myeloperoxidase (MPO) activity	(Shih et al., 2009)

Table 2.6. Other activities of phycocyanin (adopted from Pagels et al., 2019).

Cyanobacterial source	Bioactivity	Mechanism of action	Reference
Anabaena oryzae	Anti-bacterial	Bacterial growth inhibition	(Han et al., 2006)
Arthrospira platensis	Anti-viral	Inhibition of cytopathic effects	(Sitohy et al., 2015)
Arthrospira platensis	Anti-obesity	Pancreatic lipase activity inhibition	(Chakdar and Pabbi, 2017)

2.2. Factors Affecting Growth and Phycobiliprotein Production in Algae and Cyanobacteria

The optimization of the cultivation conditions is as important as the selection of the most efficient strain for PBP synthesis. Cultivation of algae and cyanobacteria in photobioreactor (PBR) systems gives the advantage of controlling and modifying fundamental bioprocessing parameters that have key effects on cellular metabolism. By manipulating the growth conditions, metabolites synthesized by algae and cyanobacteria can be changed in both quantity and composition (Kong et al., 2013). The most commonly tested growth conditions include but not limited to adjustment of

temperature, pH, CO_2, macronutrient (such as nitrogen, phosphorus) availability and light intensity. Temperature, being the key regulator of metabolic processes, has different effects on the chemical composition of the biomass (Huseby et al., 2013). Adjustment of pH facilitates the absorption of nutrients in a culture medium and in/directly affects cellular membrane functions. Light availability strictly governs the photosynthetic mechanism and phototrophic growth. In addition, the developmental stage of the culture and harvest time, can also change the metabolite composition of the cells (Barofsky et al., 2010; Lakeman et al., 2009; Vidoudez and Pohnert, 2012). Overall, all of these factors have high potential to enhance diversity and synthesis of bioactive compounds including PBPs that can be obtained from algae and cyanobacteria.

2.2.1. Light

Light is the primary source of energy for photosynthesis. Higher light intensities promote the growth of photosynthetic organisms until a critical point where photoinhibition occurs. Photoinhibition decreases cellular growth in both algae and cyanobacteria as it leads to the formation of ROS such as hydroxide radical, singlet oxygen, superoxide radical and hydrogen peroxide. In order to compensate for fluctuations in light regime and photoperiod, the chromatic adaptation which is the adaptation of the photosynthetic apparatus through the changing of the total number of photosystems, the PSI to PSII ratio or the PBS structure occurs (Fujita et al., 2006). There are four groups defined by Tendeau de Marsac; no PC to PE conversion, only the change in PE levels in response to green or blue light, the change in both PE and PC depending on the light color and the change in chromophore association with bilin proteins (Tandeau De Marsac, 1977). *Fremyella diplosiphon* changes its PCB to PEB in red light whereas PEB is converted into PCB when it was cultivated under green light (Bennett and Bogorad, 1973; Kehoe and Gutu, 2006).

The intensity of light affects species differently. A portion prefers low or moderate light intensities for the optimum production of PBPs. Under low light intensities, some algae prefer to enhance density of its PBPs to capture more light. In addition, higher light intensities might be detrimental by causing photo oxidative damage. In a study by Gris et al. (2017), the light intensities from 15 to 650 µmol photons/m^2s was examined for the amount of PC production for *Aponinum* species and it was found that PC production was increased until 100 µmol photons/m^2s and it was decreased after that point. In a similar study by Ma et al. (2015), the light intensities of 10, 30, 60, 90 and 120 µmol photons/m^2s were examined for their effects on the PC production for *Nostoc sphaeroides*. Until 90 µmol photons/m^2s, the PC production increased whereas the dramatic decrease was observed at 120 µmol photons/m^2s. For *Nostoc* sp. strain UAM206, the higher PC production

was observed under 10 μmol photons/m^2s for both pH 7 and 9 than 100 µmol photons/m^2s light intensity (Poza-Carrión et al., 2001). For *Geitlerinema sulphureum*, the highest PC concentration was achieved under approximately 10 μmol photons/m^2s (700 lux) [8.94% of dry cell weight (DCW)] (Kenekar and Deodhar, 2013). The purity index (1.15) was also the highest among other conditions. However, the cultivation under approximately 20 μmol photons/m^2s yielded higher productivity than at 10 μmol photons/m^2s.

In contrast, some species prefer higher light intensities for their optimum production of PBPs. In a work by Chaneva et al. (2007), the light intensities of 50-100-150 and 300 µmol photons/m^2s were studied for their effect on the production of PC. Even though the highest PC productivity was achieved at 150 µmol photons/m^2s (23% of DCW), 300 µmol photons/m^2s did not result in tremendous decrease in PC production (18.4% of DCW). In a similar study by Chen et al. (2010), 750, 1500 and 3000 µmol photons/m^2s light intensities were applied to see how the production of PC is changed. When the light color was red, 3000 µmol photons/m^2s gave the highest PC productivity (0.152 ±0.042 g/g DCW).

Additionally, the photoperiod is also as affective as the light intensity for the production of PBPs. In a study, optimum light intensity and the length of photoperiod were studied on *Gloeocapsa* sp., *Lyngbya* sp. and *Synechocystis* sp. (Maurya et al., 2014). For the light intensity, increase from approximately 6,75 μmol photons/m^2s (500 lux) to 40 μmol photons/m^2s (3000 lux) was examined while culturing at 35 °C in BG-11 medium. For the light period from 8 to 24 h light conditions were tried. The optima for light intensity and photoperiod were found to be 27 μmol photons/m^2s (2000 lux) and 16:8 h L/D cycle, respectively. For *Synechocystis* sp., approximately 74 μmol photons/m^2s (5500 lux) light intensity with 16:8 h L/D cycle was found as optimum in another study where cyanobacteria was cultivated in BG-11 medium at 24℃ and pH 10.3 (Deshmukh and Puranik, 2012). For *Anabaena* sp. strain NCCU-9, 16:8 L/D cycle was also reported as the condition where highest PBP synthesis was observed whereas the cultivation under continuous light reduces the PBP productivity 69% (Hemlata and Fatma, 2009).

With respect to light conditions, the wavelength of the light also has a significant effect on PC content as well as biomass concentration. In response, cyanobacteria adopt the number and size of PBPs, the PE levels or the ratio of PE to PC (Kehoe, 2010). The relative levels of RNAs encoding PC and APC vary with light intensity (Hsieh-Lo et al., 2019). When the light color was changed from white to red, the PBP content of *Cyanobium* sp. strain LEGE 06.113 was increased from 72.0 mg/g to 116.7 mg/g DCW. In another study by Luimstra et al. (2018), the highest biomass concentration

(5.11 g/L) and the highest specific growth rate (1.918/day) were obtained from *Synechococcus* sp. strain PCC 6715 by the cultivation under red light in 16:8 h L/D photoperiod. However, the highest PC content was obtained when the cultivation was performed under blue light, even though the growth rate is lower in blue light because of the less efficient usage during photosynthesis by cyanobacteria (Luimstra et al., 2018). In a similar study by Klepacz-Smółka, the highest PC yield was obtained when the photoperiod (16:8 L/D) with the blue light was applied for *Synechococcus* sp. strain PCC 6715 (Klepacz-Smółka et al., 2020). The growth under green light (36.1±3.8 µg/mg DCW) resulted in lower phycocyanin production than red light (94.4±5.2 µg/mg DCW) for *Nostoc* sp. strain UAM206 (Poza-Carrión et al., 2001). During the cultivation of *Arthrospira platensis*, the highest biomass concentration was achieved with the combination of red (70%) and blue (30%) light (Lima et al., 2018). Interestingly, there was no observable growth when the cultivation was performed with only blue light. Hence, an initial cultivation with red light to increase biomass and then a second cultivation under blue light to accumulate PC can be a good strategy for the production of PC at higher amounts. This strategy was studied on *Arthrospira platensis* where at the first period of the cultivation, the combination of red and blue light was applied until the OD was reached to 1.4-1.6, then the light was changed to only blue (Lee et al., 2016). As a result, 1.28 mg/mL PC with 2.7 purity was achieved. On the contrary, the highest PBP production for *Anabaena* sp. strain NCCU-9 was achieved under white light (Hemlata and Fatma, 2009).

2.2.2. Temperature

Temperature is one of the most fundamental factors affecting PC accumulation among the cultivation conditions. It affects nutrient availability and uptake, cell membrane fluidity and PS II (Hsieh-Lo et al., 2019). The survival and growth are highly affected depending on the temperature. When it is excessive, proteins and enzymes are denaturated, and growth is inhibited. In addition, the dissolved-oxygen level in aquatic environments are highly dependent on temperature, and it is the primary cause of the stress in photosynthesis. In a study by Hemlata and Fatma, the optimum temperature for the PBP production from *Anabaena* sp. strain NCCU-9 was determined as 30 °C (Hemlata and Fatma, 2009). When the growth temperature was reduced to 20 °C, the production was inhibited by 23.6%. Also, the production was diminished by 40% at 40 °C. For *Arthronema africanum*, the highest PC production was observed at 35 °C under 150 µmol photons/m^2s whereas PC content was decreased at 40 °C (Chaneva et al., 2007). The PC productivity was inversely affected in *Geitlerinema sulphureum* when it was cultivated at 20 °C, the PC concentration was 5.73% DCW which is higher than the cultivations under 25 °C (5.27%) and 30 °C (2.88%) (Kenekar and Deodhar, 2013).

2.2.3. pH

The pH affects the metabolic process and biochemical composition of the cell by affecting the solubility and the bioavailability of macro- and micronutrients, the membrane transport, the activities of the enzymes, the electron transfer during photosynthesis and the osmotic pressure of the cytoplasm (Gerloff-Elias et al., 2005; Tipton and Dixon, 1979). Growth ability of microalgae and cyanobacteria at high pH generally facilitates axenic growth, whereas the content of pigments and proteins are reduced after a certain threshold (Poza-Carrión et al., 2001). The optimum pH for the growth and the pigment production varies among species. In a study by Deshmukh and Puranik (2012), the cultivation of *Synechocystis* sp., isolated from Lake Lonar (Maharashtra, India), from pH 5 to pH 12 was performed. The highest PC content was achieved from the cultivation under pH 10 at 25℃ with the 16:8 L/D cycle of 75 µmol photons/m^2s light intensity for 12 days. In another study by Hong and Lee (2008), the optimum pH for the highest PBP production was observed around 7.3 for *Synechocystis* sp. PCC 6701. For *Phormidium* sp. strain BTA-1048, the effect of pH on the production of PC was studied and the lowest PC content (148.13±9.20 µg/mg) was observed at pH 9.0 whereas the highest concentration (168.15±9.58 µg/mg) was achieved at pH 6.0 (Keithellakpam et al., 2015). Hemlata and Fatma have reported that the optimum PBP production from *Anabaena* sp. strain NCCU-9 was at pH 8.0 which was reduced to 19% when the cultivation was performed at pH=10.0 (Hemlata and Fatma, 2009).

2.2.4. Salinity

Salt concentration affects proper cell functioning including ion regulation, osmotic balance and metabolic activity (Kannaujiya et al., 2017). *Arthrospira maxima* increased its PBPs from 11.08% to 15.63% of DCW when it was grown in 0.1 M NaCl instead of commonly preferred cultivation salinity of 0.02 M NaCl (Abd El-Baky and El-Baroty, 2012). For PC amount, it was also enhanced from 2.60% of DCW to 3.92%. In another study by Hemlata and Fatma, *Anabaena* sp. strain NCCU-9 increased its PBP content under the lowest concentration of salt (10 mM) (Hemlata and Fatma, 2009).

2.2.5. Nutrients

Nitrogen is the second most abundant element found in living organisms and the main component of nucleic acids (DNA and RNA), amino acids and various pigments (Latasa and Berdalet, 1994). Therefore, nitrogen limitation affects overall PC accumulation, as it is broken down to generate nitrogen reserves within the cells (da Silva et al., 2009). In a study by Khazi et al. (2018), *Pseudoscillatoria* sp. was cultivated under continuous light with 1.5 g/L concentration of nitrogen (in the form of $NaNO_3$) and total PBP content was found to be 19.9% DCW. Setyoningrum and Nur (2015) have shown that 0.1 g/L nitrogen supplied in the form of urea for *Spirulina* sp. resulted in the best PC accumulation (114.74 mg/L) when cultured under continuous light. In a similar study, the accumulation of the PBPs reached to 16.09% DCW when *Arthrospira* sp. was cultivated with 0.5 g/L nitrogen supplied in the form of $NaNO_3$ (Chentir et al., 2018). Differences between these two studies were the light intensity, nitrogen source and growth medium indicating how cultivation conditions affect algal metabolism and PC accumulation accordingly. In another work by Kenekar and Deodhar (2013), varying nitrogen concentrations of $NaNO_3$ (from 1.5 to 4.5 g/L) were supplied to *Geitlerinema sulphureum* where highest PC productivity (9.9% DCW) was achieved at 4.5 g/L of $NaNO_3$. Under standard Zarrouk's culture medium recipe, corresponding to 2.5 g/L $NaNO_3$, the PC concentration was found as 5.27% DCW proving that increase in the nitrogen concentration enhances PC production (Kenekar and Deodhar, 2013).

As carbon source, rather than inorganic CO_2, organic carbon is also used. There are certain species among algae and cyanobacteria that can utilize organic carbon. When organic carbon and light were applied simultaneously, algae grow mixotrophically whereas the supplementation of only the organic carbon source leads to heterotrophic growth (Table 2.7). There are many studies for the PC extraction from the heterotrophically grown *G. sulphuraria* (Carfagna et al., 2018; Sørensen et al., 2013; Wan et al., 2016). Heterotrophic growth inhibits phycocyanin accumulation whereas mixotrophy results in higher PC accumulation then both autotrophy and heterotrophy. In addition, mixotrophy is also advantageous to obtain higher growth (Das et al., 2011; Setyoningrum and Nur, 2015; Sloth et al., 2006).

Table 2.7. Comparison among different cultivation conditions.

Growth mode	Energy source	Carbon source	Light requirement
Autotrophy	Light	Inorganic	Required
Mixotrophy	Light and organic	Organic and inorganic	Not required
Heterotrophy	Organic	Organic	Not required

With respect to cultivation conditions under varying nutrient conditions, another strategy involves two-stage cultivation. In the first stage, the aim is to obtain highest biomass amount, whereas second stage growth conditions get optimized to increase the overall accumulation of targeted compound. As a result, product yield increases while the drawbacks of nutrient stress conditions on the growth are minimized. One such example was shown for *G. sulphuraria*, where heterotrophic cultivation followed by autotrophy, referred as "Sequential Heterotrophy-Dilution-Photoinduction" increased overall PC accumulation to the 13.88% of its DCW which is 147-fold higher than the standard photoautotrophic cultivation (Wan et al., 2016).

There are additional studies where nutrient conditions are coupled with a stress factor to improve PC accumulation. For example, Kumar et al. (2015), applied photo acclimation combined with salt addition for *Nostoc muscorum* and *Phormidium foveolarum*. Three different light intensities (25, 75, 225 μmol photons/m^2s PAR) were exposed for 25 days followed by 30 mM and 90 mM NaCl exposure for three days. In the control group (no NaCl exposure), light intensity was inversely correlated with PC accumulation for both species and overall PC production was decreased in the salt applied batches. In another study by Kenekar and Deodhar (2013), two different growth conditions are compared for the end-concentration of PC. One is the standard condition (in Zarrouk's medium, for 15 days, under 13.5 μmol photons/m^2s light intensity and at 27 °C). The compared condition was with Zarrouk's medium containing 3.5 g/L $NaNO_3$ and 6.35 g Na_2CO_3, for 15 days, under 19 μmol photons/m^2s light intensity and at 30 °C. The latter one resulted in higher PC yield (0.071g/L) than the standard (0.021g/L).

2.3. Extraction and Purification of Phycobiliproteins

The isolation of the PBPs follows various steps including cell disruption or breakage, crude PC extraction and isolation, purification, drying, and analytical characterization. As a first step, a variety of physical and chemical methods are used to disrupt cells because the cell wall structure differs among species and a method that works well for one organism may not be a suitable method for the other. Extraction of PBPs from certain cyanobacteria can be extremely difficult due to their small size (0.2- 2 μm) (Kannaujiya and Sinha, 2016a), and they possess exceptionally resistant multi-layered cell walls (Viskari et al., 2001). As photosynthetic proteins are regularly arranged in parallel rows on the thylakoid membrane, the efficiency of extraction depends on the rupture of the cell walls. Physical methods for the extraction of the crude proteins involve several steps and may require a combination of bead-beating, sonication, cavitation, mortar-pestle, osmotic shock, and repeated freeze-thaw cycles (İlter et al., 2018; Sonani et al., 2016; Sørensen et al., 2013). Per chemical methods, usage of acids, alkali, detergents, enzymes, and their combination thereof are also reported (Moraes et al., 2011). In general, one or more of the physical and chemical methods are combined to obtain optimal cell breakage. These methods are explained more in detail in the following section.

2.3.1. Extraction Methods for the Isolation of Phycobiliproteins

Extraction yield and purity of PBPs varies significantly based on applied physical and/or chemical extraction methods, which also affect their bioactivity. As summarized in Table 2.8 applicable physical pretreatment methods include bead-beating (or -milling), homogenization with mortar and pestle, sonication and freeze-thaw cycling. Ultrasonic treatment in a water bath provides cavitation for the disruption of the cell wall and highly beneficial by being operated easily and in short time, causing minimum loss and providing high PC yield (Kannaujiya et al., 2017). Intensity and the process time are the most important parameters of the sonication (Benedetti et al., 2004). Freeze-thaw cycling, on the other hand, provides crystallization which bursts the cells and is advantageous by being robust, highly reproducible and operating in short time.

In a comparative study, bead-beating, vortexing and high pressure homogenizer techniques were compared for *C. merolae* (Rahman et al., 2017), and the highest PC extraction was achieved with vortexing (0.55 mg/mL). However, *C. merolae* lack cell wall and it is easier to extract its PC content. The lower mechanical force increases the purity of crude extract because the chlorophyll and other cellular constituents are not extracted with PC. Therefore, it is important to optimize the best extraction method to reduce the cost of purification.

The combination of the previously mentioned physical extraction methods are also advantageous to increase the extracted PC yield. In a work by Moraes et al. (2011), the addition of beads to sonication at 50 kHz with 1:1.1 (biomass to bead) ratio increased the extraction yield (43.75 mg/g wet weight) which is higher than freeze-thaw by 56% whereas the yield by only sonication was 0.57 mg/g wet weight. In another study, freeze-thaw cycles was combined with mortar-pestle for the PC extraction from *Nostoc* sp. strain HKAR-11, and 0.124 mg/mL PC yield was obtained (Kannaujiya and Sinha, 2016a). In a similar study, the sonication was applied prior to freeze-thaw cycles to enhance the extraction yield of the PC, and 0.08 mg/mL PC yield was measured from *Nostoc* sp. strain HKAR-2 (Kannaujiya and Sinha, 2016b).

Meanwhile, chemical pretreatment methods include acid-, detergent-, and enzyme-treatment. Except enzymatic treatment, the others are not preferred because of their possibility to cause to the protein denaturation.

Table 2.8. Phycocyanin extraction methods applied for different species.

Species	**Extraction Method**	**CE Purity**	**Reference**
G. sulphuraria	**Frozen** (-80°C) biomass was disrupted for 2×4 min at 3500 rpm in a **bead milling device**. Then slurry was washed out with 50 mM **K-phosphate buffe**r and centrifuged for 90 min at 25000g.	< 0.1	(Sørensen et al., 2013)
Arthrospira platensis	**Aqueous two-phase extraction** with 12.28% (w/w) polyethylene glycol (PEG4000), potassium phosphate as 11.63% at pH of 7.2. The mixture was stirred at room temperature for 1 h. Two phases were separated by centrifuge for 3 min at 4000g.	1.18	(Patil et al., 2006)
Arthrospira platensis	20 g **wet** biomass in 200 ml of 0.05 M **Na-phosphate buffer** (pH=7.0) was **frozen (-20°C) and thawed** repeatedly, and then **sonicated** for 3 min. Finally, it was centrifuged at 10000g for 30 min.	1.14	(Zhang and Chen, 1999)
Arthrospira platensis	**Dried biomass powder** was suspended with 20 mmol/L **Tris-HCl** buffer containing 10 mmol/L EDTA (pH=6.5) at 0.06 g/mL ratio. Then **freeze-thaw cycling** (-20°C, RT) was applied. Finally, slurry was centrifuged at 5000 rpm for 10 min.	N.A.	(Zhang et al., 2014)

Table 2.8. (continued)

Arthrospira platensis	10 g **wet** biomass was extracted in 200 mL of 0.1M **Na-phosphate buffer** (pH=7.0) with 100 μg/mL **lysozyme** and 10 mM EDTA. After incubation for 24 h in shaking bath at 30°C, it was centrifuged at 40000g for 1 h.	0.90	(Boussiba and Richmond, 1979)
Arthrospira platensis	100 g **dry** biomass was extracted in a 1L **high pressure extraction** vessel at 5000 bars, 20°C for 15 min. After the high-pressure treatment, 12 h incubation in water at 4°C was performed twice to obtain crude extract.	0.91	(Seo et al., 2013)
Limnothrix sp. *strain 37-2-1*	100 mg **freeze dried** biomass in 10 ml of 0.1 M **phosphate buffer** (pH=7.0) was extracted at 4 °C overnight.	N.A.	(Gantar et al., 2012)
Arthrospira maxima	10 g **wet** biomass was extracted in 100 mL of 0.05 M **phosphate buffer** (pH=6.7) at 4°C in dark overnight. Then it was centrifuged at 10000g for 15 min at 4°C.	N.A.	(Abd El-Baky and El-Baroty, 2012)
Synechococcus sp. *(Anacystis nidulans* BD1*)*	10 mg **dry** biomass was extracted within 1 mL **HEPES** (100 mM HEPES, 1 mM EDTA, pH=8.0) containing 1 mg/mL **lysozyme**. After 16 h incubation at 37°C, it was centrifuged at 6000g for 15 min at 4°C	2.18	(Gupta and Sainis, 2010)
Cyanidioschyzon merolae	200 mg **wet** biomass was extracted in 2 mL **ultrapure water** at room temperature for 300 min. With centrifugation at 15000g, crude extract was obtained.	9.92	(Rahman et al., 2017)
Phormidium autumnale	1 g **freeze-dried** biomass was extracted with 50 mM **Na-phosphate buffer** (pH=6.8) in a **mortar and pestl**e. Then it was centrifuged at 20000 rpm for 10 min at 4°C.	N.A.	(Rodrigues et al., 2015)

One of the common methods used for extraction of PBPs is to homogenize the cell suspension in dilute phosphate buffer or distilled water, which results in osmotic shock that results in the breakage of cell walls (Furuki et al., 2003). Acetate buffer, HEPES buffer, Tris-HCl are also commonly preferred buffers for this procedure (Amarante et al., 2020; Kumar et al., 2014). A study by Bermejo et. al. (2016), the methods leading to osmotic shock for the release of PC were compared. Higher efficiency was achieved, compared to distilled water and 1M Na-phosphate buffer (pH=7.0), when osmotic shock was performed with 1M acetic acid at pH=5.0. In addition, the buffer type with its pH, and length and the temperature of the extraction are also important parameters on the yield and the purity of the crude extracts (Moraes et al., 2010). In a study by Rahman et al. (2017), the PC amount increased from 0.28 mg/mL at 100 min to 0.81 mg/mL at 150 min, and it was not increased after 150 min. In another study by Pan-utai et al. (2018), the effect of the biomass type (oven-dried and freeze-dried.) on the extraction efficiency was studied. Freeze-dried biomass extracted with 0.01M Na-phosphate buffer at 0.06 g/mL ratio gave the highest PC yield (183.11±11.4 mg/g DCW). Along with the biomass type, the process parameters of the pretreatment techniques affect the extraction

efficiency significantly. A study by Lawrenz et al. (2011), freezing at -20°C and thawing at 5°C for 24 h gave higher yield than freezing at -50°C and thawing at 5°C 24 h. In the same study, harvesting type also effected the extraction yield in which harvesting by centrifugation resulted in higher PC extraction than harvesting on filter paper. In a similar study by Akoğlu (2012), freezing at -65°C and thawing at 30°C resulted in the highest (123.80±3.6 mg/g DCW) PC amount compared to freezing at -21°C or thawing at 4°C. However, when freeze-thaw was combined with sonication, the PC amount was increased slightly (126.62±1.9 mg/g DCW).

2.3.2. Purification of Phycobiliproteins

The purity of PC is generally determined by the ratio of the absorbances taken at 615 to 625 nm (maximum for PC) over 280 nm (representative of all proteins) wavelengths of the final product. Purity ratio of ≥0.7 is accepted as food grade, up to 3.9 as reactive grade, and purity ratios ≥4 are considered as analytical grade PC, respectively (Patel et al., 2005).

Once crude PC extract is obtained as described in previous section, various purification steps can be applied to achieve a specific purity level. One of the most common purification methods is salting out, where ammonium sulfate salt is used due to its non-toxic, highly water soluble, bacteriostatic characteristics, and it does not damage the integrity of the proteins (Benedetti et al., 2006; Boussiba and Richmond, 1979; Sørensen et al., 2013). During salting out process, proteins precipitate due to the aggregation as a result of the competition between protein and saline ions for water molecules (da Silva et al., 2009). In a study by Patel et al. (2005), it was reported that the crude extracts of PC from *Arthrospira* sp., *Phormidium* sp. and *Lyngbya* sp. were fractionated by precipitation with solid ammonium sulphate ($(NH_4)_2SO_4$) first at 25% w/v and then at 50% w/v saturation levels. Followed by acetate buffer precipitation and anion exchange chromatography (AEC) purification, PC purities were enhanced above analytical grades (Table 2.9).

Besides salt precipitation, chromatographic methods are also commonly used for PC purification (Patel et al., 2005; Patil et al., 2006; Reis et al., 1998). These methods include ion exchange chromatography (IEC) and gel filtration, hydrophobic interaction chromatography (HIC), chromatography on hydroxyapatite and expanded bed adsorption (EBA) chromatography (Bermejo et al., 2003; Galland-Irmouli et al., 2000; Soni et al., 2008). During chromatography, the separation based on molecule size, charge, pI and affinity can be achieved. In addition, gel filtration chromatography (GFC) facilitates the removal of salt from the extracted PC. Chromatographic methods can increase PC purity ratios above analytical grades (Table 2.9).

There are relatively new techniques in use for the purification of PC such as density gradient centrifugation (Gray et al., 1973), usage of rivanol (Minkova et al., 2007) and chitosan-activated carbon (Patil and Raghavarao, 2007). Chitosan is a polysaccharide which facilitates the removal of organics and proteins at the proper pH. Active carbon, on the other hand, removes smaller impurities by creating larger surface area for absorption. In a work by Gantar et al. (2012), the optimum active carbon and chitosan concentrations to obtain the highest PC yield from *Limnothrix* sp. was examined. The 0.2% chitosan at pH 6.9 with 12% active carbon was found the suitable method to obtain a highly pure PC. In a study by Gray et al. (1973), *Nostoc* spp. grown under 73 μmol photons/m^2s light intensity for 6 to 8 days were harvested, and 1 g of wet biomass was crushed in 10 mL phosphate buffer (pH=7.0) with French-press. Following the extraction, discontinuous sucrose gradient (0.25M-0.50M-1M-2M) was prepared and the supernatant was loaded. After centrifugation at 40000 g, orange top layer indicating carotenoids followed by turquoise for APC and red for PE layers were obtained. In the 1 M region, the purple band corresponding to PC, APC and PE in combination was observed indicating partial PC purification.

Table 2.9. Several phycocyanin purification methods applied to different species (adopted from Eriksen, 2008).

Purification techniques and involved steps	**Species**	**Purity**	**Yield (%)**	**Reference**
1. $(NH_4)_2SO_4$ precipitation, 2. Hydroxyapatite chromatography, 3. AEC	*A. platensis*	4.15	N.A.	(Boussiba and Richmond, 1979)
1. $(NH_4)_2SO_4$ fractionation, 2. AEC, 3. Gel filtration	*A. platensis*	5.06	N.A.	(Zhang and Chen, 1999)
1. Expanded bed adsorption chromatography, 2. AEC	*A. platensis*	3.64	8.7	(Niu et al., 2007)
1. HIC, 2. AEC	*Synechococcus* sp.	4.85	N.A.	(Abalde et al., 1998)
1. $(NH_4)_2SO_4$ precipitation, 2. Size exclusion chromatography, 3. AEC	*Oscillatoria quadripunctulata*	3.31	44.2	(Soni et al., 2006)
1. $(NH_4)_2SO_4$ precipitation, 2. Hydroxyapatite (electrostatic interaction) chromatography	*A. flos-aquae*	4.78	N.A.	(Benedetti et al., 2006)
1. $(NH_4)_2SO_4$ fractionation, 2. HIC	*Phormidium fragile*	4.52	62.0	(Soni et al., 2008)

Table 2.9. (continued)

1. Two-phase aqueous extraction, 2. ultrafiltration, 3. $(NH_4)_2SO_4$ precipitation	*Arthrospira. maxima*	3.8	29.5	(Rito-Palomares et al., 2001)
1. Chitosan adsorption, 2. Two-phase aqueous extraction, 3. AEC	*Arthrospira platensis*	6.69	N.A.	(Patil et al., 2006)
1. Osmotic shock, 2. Stream-line DEAE expanded bed columns, 3. Gel filtration, 4. AEC	*Arthrospira platensis*	N.A.	12	(Bermejo et al., 2006)
1. $(NH_4)_2SO_4$ fractionation, 2. Two-phase aqueous extraction, 3. AEC, 4. Ultrafiltration	*G. sulphuraria*	4.5	39	(Sørensen et al., 2013)
Activated charcoal and chitosan	*Synechococcus* sp.	4.72	90	(Gupta and Sainis, 2010)
1. $(NH_4)_2SO_4$ fractionation, 2. Sephadex G-150, 3. AEC	*Lyngbya* sp.	5.53	60.2	(Sonani et al., 2014)
1. $(NH_4)_2SO_4$ fractionation, 2. Acetate buffer	*G. sulphuraria*	4.1	81.93	(Moon et al., 2014)
Density gradient centrifugation	*Nostoc* spp.	N.A.	N.A.	(Gray et al., 1973)
1. $(NH_4)_2SO_4$ fractionation, 2. Acetate buffer, 3.AEC	*Arthrospira,* *Phormidium,* *Lyngbya* spp.	4.42 4.43 4.59	45.6 35.2 36.8	(Patel et al., 2005)

Moreover, there is also an approach to extract impurities beforehand to increase the PC purity followed by using supercritical CO_2 in the extraction. In a study by Marzorati et al. (2020), the carotenoids, the chlorophyll a and b were extracted by supercritical CO_2, and the PC was extracted from the remaining *A. platensis* residue via water. The working parameters of the initial extraction were 300 bar, 15 mL/min CO_2 flow and 10% ethanol (1.5 mL/min flow rate), 45°C with 31.4 g DCW. After water extraction, the extract was saturated to 39% $(NH_4)_2SO_4$ and the final yield and purity were 250 mg/g DCW and 2.22 respectively.

2.4. Methods to Measure Antioxidant Activity

Commonly referred as ROS, free radicals include superoxide, hydrogen peroxide and hydroxyl radical. They are highly reactive and electrically charged molecules by having an unpaired electron and derive from oxygen, sulfur and nitrogen elements (Carocho and Ferreira, 2013). Hence, they react with other molecules easily via electron acceptance/donation and reducing radicals. The targets of free radicals are usually proteins, DNA-RNA, lipids and sugars (Carocho and Ferreira, 2013). Free radicals modify amino acids, mediate peptide cleavages, form cross-linkages, cause base modifications, deletions, frame-shift mutations and strand breaks in DNA and RNA (Lobo et al., 2010).

Reactive compounds can be generated either from metabolic or external environmental conditions (Birben et al., 2012). Mitochondria through xanthine oxidase, peroxisomes, inflammation processes, phagocytosis and physical exercise are the main free radical generating metabolic activities (Ugya et al., 2020). The organisms performing aerobic metabolism are highly susceptible to ROS as more than 5% of inhaled oxygen is converted to ROS (Phaniendra et al., 2015). Oxidative burst from the bacteria and virus destruction by phagocytes (white blood cells) during an infection is another ROS generating mechanism (Paiva and Bozza, 2014). Xenobiotic metabolism, which is the detoxification of harmful substances, also generates ROS (Banerjee et al., 2016).

As external sources, smoking, stress, drug usage, and exposure to environmental pollutants and radiation can be given (Birben et al., 2012). When the humans are exposed to harmful substances such as cigarette smoke, hazardous chemicals and vigorous physical exercise, the body's oxidant load increases. When the balance between ROS formation and the defense system is disrupted, oxidative stress occurs (Birben et al., 2012). Oxidative stress is associated with more than 60 health issues including cancer, inflammation, aging and cardiovascular disorders (atherosclerosis) (Carocho and Ferreira, 2013). The high production of ROS leads to modifications on proteins and lipids due to the change in DNA structure, and aging, carcinogenesis, neurodegenerative and autoimmune diseases occur (Birben et al., 2012). As more fundamentally, the lipid bilayer of the cell is affected from the modification of the lipids, and the permeability of the membrane increases. Reactive oxygen species also have an impact on the signal transduction including NF-κB and AP-1 in inflammatory response, and JNK, p38 in cell proliferation, differentiation and apoptosis (Birben et al., 2012).

To facilitate defense against free radicals, there are antioxidants which are substances that either scavenge ROS or inhibit their synthesis (Lobo et al., 2010). The natural antioxidants can be divided

into two groups as enzymatic and non-enzymatic antioxidants (Figure 2.4). Enzymatic antioxidants constitute superoxide dismutase (SOD), catalase (CAT), glutathione peroxide, glutathione reductase and glucose-6-phosphate dehydrogenase (Carocho and Ferreira, 2013). Glutathione peroxide donates two electrons to peroxides and make them unavailable for Fenton reactions (Valko et al., 2007). Superoxide dismutase turns superoxide radical into hydrogen peroxide, which is then turned into water and molecular oxygen by CAT (Wang et al., 2018). On the other hand, indirect mechanisms via the involvement of glutathione reductase and glucose-6-phosphate dehydrogenase, the regeneration of primary enzymatic antioxidants are facilitated, more free radicals can be neutralized (Carocho and Ferreira, 2013).

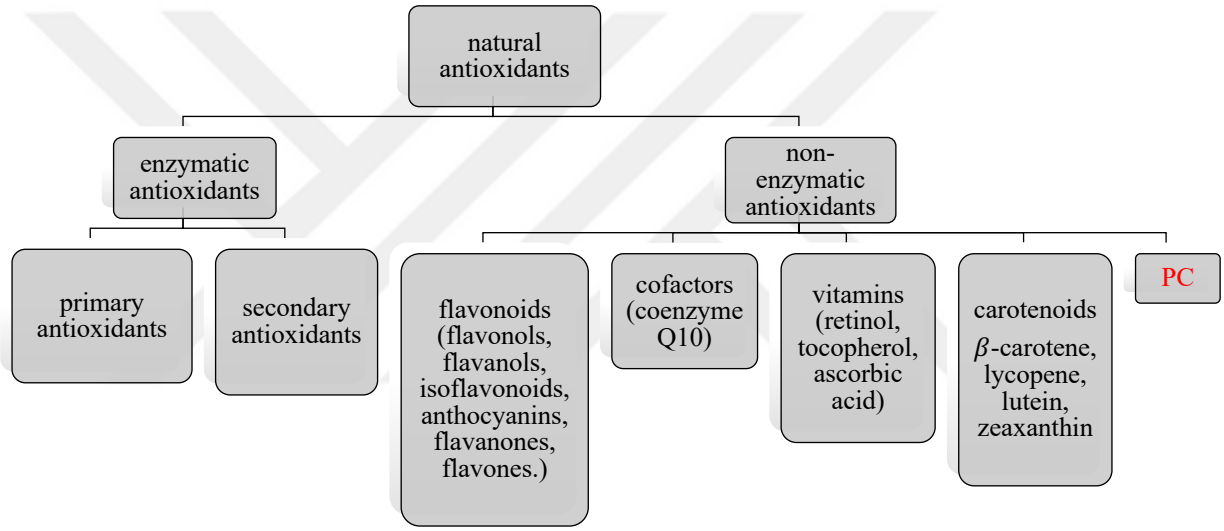


Figure 2.4. Overall representation of natural antioxidants (adopted from Carocho and Ferreira, 2013).

As non-enzymatic antioxidants, vitamins, carotenoids, flavonoids, phenolic acids and cofactors can be listed (Pisoschi and Negulescu, 2012). Vitamin A (retinol) prevents lipid peroxidation by neutralizing peroxyl radicals (Moussa et al., 2019). Coenzyme Q10, which is present in all cells and membranes, also stabilize peroxyl radicals and serve as the precursor of vitamin E (tocopherol), whereas glutathione contributes by donating a hydrogen atom or an electron (Bullón et al., 2015). However, there also are exogenous antioxidants such as tocopherol (vitamin E) which scavenges peroxyl radicals involved in lipid peroxidation and ascorbic acid (vitamin C) which has a wide range scavenging capacity including superoxide and hydroxyl radicals, singlet oxygen and hydrogen peroxide (Bouayed and Bohn, 2010). Flavonoids act as hydrogen donors, reducing agents, and superoxide radical scavengers (Kasote et al., 2015). The antioxidant activity of carotenoids comes from singlet oxygen quenching ability in which they become excited and dissipate the energy via

vibrational and rotational movements and turn into their unenergetic state to quench more radicals (Stahl and Sies, 2003; Truscott, 1990).

Table 2.10. Common ROS and the neutralizing antioxidants.

ROS	Neutralizing Antioxidants
Hydroxyl radical	Vitamin C, Glutathione, Flavonoids
Superoxide radical	Vitamin C, Glutathione, Flavonoids, SOD
Hydrogen peroxide	Vitamin C, Glutathione, Flavonoids, Vitamin E, β-carotene
Lipid peroxides	Glutathione peroxidase, Flavonoids, Vitamin E, β-carotene

Methods which are used to determine antioxidant capacity are listed on Table 2.11. 1,1-diphenyl-2-picrylhydrazyl is a stable free radical and produces violent purple color due to its spare electron (Alam et al., 2013). When it is reduced by taking a hydrogen atom of the antioxidant molecule, the intense color is diminished (Pisoschi and Negulescu, 2012). The reduction in absorbance is directly correlated with the concentration of the antioxidant molecule. 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid (ABTS) is a cation radical which absorbs at 743 nm and its bluish-green color comes from the loss of an electron by the nitrogen atom of it (Pisoschi and Negulescu, 2012). When the antioxidant molecule is present, nitrogen takes the hydrogen atom and decolorization correlated with the concentration occurs (Pisoschi and Negulescu, 2012). Ferric Reducing Antioxidant Power (FRAP) assay relies on the reduction of ferric ion-2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) complex, which gives intense navy blue color, via the antioxidant molecule (Malta and Liu, 2014). Oxygen Radical Absorbance Capacity (ORAC) assay measures the antioxidant capacity against peroxyl radical (Zhong and Shahidi, 2015). Fluorescein is used as a fluorescent probe and it loses its fluorescence when it reacts with peroxyl radical. So, the fluorescence loss is lower in the presence of the antioxidant molecule because antioxidant molecule reacts with peroxyl radical instead. The temperature control is important in this assay since the reaction is temperature-sensitive (Badarinath et al., 2010). In another method referred as Cupric Reducing Antioxidant Capacity (CUPRAC), $CuSO_4$ and neocuproine are mixed with antioxidants (Pisoschi and Negulescu, 2012). The reaction depends on the reduction of Cu to Cu(I) (Pumas et al., 2011). In hydrogen peroxide scavenging activity assay, reaction of antioxidant with H_2O_2 is determined at 230 nm after a short period of incubation (10 min) (Ruch et al., 1989). With the same principle, β-carotene/linoleic acid method measures antioxidant activity (Terpinc and Abramovič, 2010). In this method, linoleic acid is used to create color. When water oxygenated, ROS are formed. The inhibited reaction between ROS and linoleic acid with the antioxidant test molecule can be detected via spectrometry.

Table 2.11. Methods of total antioxidant capacity assessment (adopted from Pisoschi and Negulescu, 2012).

Antioxidant capacity assay	**Reaction molecule**	**End-product determination**
Spectrometric techniques		
DPPH	An organic radical	Colorimetry
ABTS	An organic cation radical	Colorimetry
FRAP	A Fe(III) complex	Colorimetry
CUPRAC	Cu reduction to Cu(I)	Colorimetry
ORAC	Peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein
H_2O_2 Scavenging Activity	Hydrogen peroxide	Colorimetry
β-carotene/linoleic acid	Oxygenated water	Colorimetry
Chromatographic techniques		
Gas Chromatography	Separation of the molecule depending on the partition between mobile gas phase and stationary liquid phase.	Flame ionization, thermal conductivity detection
High Performance Liquid Chromatography	Repartition between solid stationary phase and liquid mobile phase	UV-VIS detection, fluorescence, mass spectrometry

3. MATERIALS AND METHODS

3.1. Optimization of The Extraction Method

3.1.1. Cultivation of Cyanobacteria and Algae

Maintained preservation cultures of algae and cyanobacteria were kept in a plant growth chamber (GC 401, Nüve, Ankara, Turkey) operating under 25°C±1, at 65% relative humidity, illuminated with cool white fluorescent bulbs providing approximately 16 μmol photons/m^2s (1200 lux) light intensity under 12:12 h L:D cycle. All of selected species for the study, except *Desertifilum tharense*, which was a local isolate, obtained from Sammlung von Algenkulturen Göttingen Culture Collection of Algae (SAG), University of Texas Culture Collection of Algae (UTEX), and American Type Culture Collection (ATCC) (details are provided in Appendix A).

To initiate the seed cultures, algae and cyanobacteria maintained in solid agar plates or liquid cultures in tissue culture flasks with 20 mL medium were transferred into 1L or 3L Erlenmeyer flasks. Once cultures reached mid-exponential growth phase, they were seeded into Erlenmeyer flasks operated as batch reactors in triplicate (Figure 3.1) with around $5x10^6$ cells /mL density whereas, for the species that cell count is not possible, optical density at 680 nm was set around 0.1 as starting OD.



Figure 3.1. *D. tharense* (left) and *G. sulphuraria* (right) growing in Erlenmeyer flasks.

As shown in Figure 3.1, cultures were placed on top of a magnetic stirrer continuously mixed at 400 rpm. Reactors were kept in front of white LED lights at 14:10 h of L:D cycles. Filtered dry air (0.22 µm pore size, EMD Millipore, USA) was purged into the reactors through air stones at 0.33 mL/min flow rates. Syringe filters (0.45 µm pore size) at the exhaust were used to keep the reactors

working under axenic conditions. Prior to usage, all reactor components were sterilized at 121°C for 15 min in the autoclave (Nüve-OT430, Ankara, Turkey).

All species were grown at RT (25±2°C), except *G. sulphuraria*, which was cultivated at 40±2 °C due to its thermo acidophilic characteristics. Accordingly, growth medium pH was also adjusted to around 2.5 with 1N HCl. Specific growth media and modifications (if any), reactor volumes, light intensities, harvest day at early stationary growth phase for each species are reported in Table 3.1. Individual components of each culture media are given in Appendix B. Growth curves were constructed by taking daily OD values at 680 nm via a spectrophotometer (Hach DR3900, US), and by cell counting every other day with a hemocytometer (Neubauer improved, ISOLAB, Eschau, Germany).

Table 3.1. Species-specific growth conditions applied in this study.

Species	**Strain**	**Growth media**	**Modification to Growth Media**	**Flask and medium volumes**	**Light Intensity ((μmol photons/m^{2}s) or (lux))**	**Temp. (°C)**	**Day of culture harvest**
Phormidium sp.	SAG 14.92	SWES	None	In 2L flask with 1.75L	67.56 (5000)	RT	4th
G. sulphuraria[1]	SAG 21.92	Cyanidium	3X $(NH_4)_2SO_4$ and no glucose	In 2L flask with 1.8L	108 (8000)	40	7th
Synechocystis sp.	ATCC 27184	BG-11	2X K_2HPO_4	In 2L flask with 1.75L	203 (15000)	RT	7th
D. tharense[2]	Local isolate	BG-11	None	In 3L flask with 2L	108 (8000)	RT	11th
Scytonema sp.	UTEX LB 1163	MB3N	None	In 3L flask with 2L	142 (10500)	RT	11th
Nostoc sp.	UTEX B EE5	BG-11	None	In 3L flask with 2L	76 (5600)	RT	8th

[1] Red algae; [2] Isolated from Denizli Region, Turkey.; RT: 25 ±2 °C

Additional to OD-based growth monitoring, dry cell weight measurements were performed for *Scytonema* sp. and *Nostoc* sp. by filtering 10 ml of cultures through 0.45 µm pore size, 47 mm diameter glass fiber filter papers (Merck-Millipore, Darmstadt, Germany) ignited at 450°C for 2 h and pre-weighted. Filter papers were dried in an oven (Daihan ON-105, Korea) at 105 °C overnight.

The difference between the filter paper with biomass and pre-weighted filter paper was recorded as dry cell weight (g/L). This procedure was also performed for each reactor at corresponding harvest day to determine final concentration in each reactor.

3.1.2. Extraction and Purification of Phycocyanin

Biomass samples were harvested by centrifugation (M240, Boeco, Germany) at 4500 rpm and 25°C for 5 min except *Scytonema* sp. which was harvested by vacuum filtering using Whatman® Grade 4 filter paper (Sigma). All biomass samples were aliquoted and stored as wet and freeze-dried biomass samples. Freeze-dried biomass samples were prepared using a lyophilizer (Hypercool H4055, Hanil Scientific, Republic of Korea) overnight. Freeze-dried biomass samples were stored at -80 °C and wet biomass samples were stored at -20 °C freezers until extraction.

Prior to phycocyanin extraction, biomass samples were subjected to several pretreatment methods including sonication, freeze-thaw cycling, mortar and pestle, and bead-beating. Sonication was performed in a water-bath type sonicator (Sonorex Super RK 102 H, Bandelin, Germany) at 35 kHz for nine cycles of two min sonication followed by two min rest on ice (Khazi et al., 2018). For bead-beating (Minilys, Bertin, France), the cycle of 90 seconds beading at 4000 rpm with 90 sec rest on ice was repeated six times in screw-capped bead-beating tubes filled with 0.1 g of 0.5 mm and 0.3 g of 0.1 mm glass beads. Glass beads were washed with 1N HNO_3 prior to usage and rinsed with water. After drying in oven at 105°C, they were weighted in screw-capped vials and autoclaved. Freeze-thaw cycling was performed in two consecutive days by freezing at -80 °C and thawing at 25 °C. When pretreatments were completed, slurry was centrifuged at 10000 g for 20 min at 4°C by a microcentrifuge (M240R1, Boeco, Germany). The supernatants were collected and referred as crude extracts.

Pretreatment procedures were performed in 0.01 M Na-phosphate buffer (pH=7.0) with 1:10 (v/w) ratio applied for freeze-dried biomass samples. For wet biomass samples, 2 mL of buffer was added for sonication and freeze-thaw experiments whereas 1.5 mL of the buffer was used for bead-beating (Table 3.2) whereas mortar-pestle was not applied to wet biomass samples. To compare freeze-dried and wet biomass samples, a portion of wet biomass samples of each species were dried in oven at 60°C for two days. The weight before drying and the weight after drying were subtracted from each other to determine the humidity of wet biomass samples and it was used to compare yield values of two different types of biomass samples.

Table 3.2. Design details of the optimization of the extraction part.

Species	Biomass type	Buffer amount used (mL)				Biomass amount (mg)
		BB	SON	MP	FT	
Nostoc sp.	lyophilized	3	3	3	3	30
	wet	1.5	2	-	2	283
Scytonema sp.	lyophilized	3	3	3	3	30
	wet	1.5	2	-	3	350
G. sulphuraria	lyophilized	3	3	3	3	30
	wet	1.5	2	-	2	242
Phormidium sp.	lyophilized	3	3	3	3	30
	wet	1.5	2	-	2	229
D. tharense	lyophilized	3	3	3	3	30
	wet	1.5	2	-	2	261
Synechocystis sp.	lyophilized	3	3	3	3	30

BB: Bead-beating; SON: Sonication; MP: Mortar and pestle; FT: Freeze-thaw cycling.

As purification processes, a modified version of Patel et al. (2005) was used, which include two-step ammonium sulfate precipitation (20% to 50%), acetate buffer precipitation, dialysis and column chromatography as shown in Figure 3.2. Crude ammonium sulfate to 20% saturation was added into the crude extracts and tubes were incubated overnight. After the centrifugation at 10000 g for 20 minutes at 4°C, the supernatants were collected and further brought to 50% saturation. After overnight incubation and a following centrifugation at 10000 g for 20 minutes at 4°C, the pellets were resuspended in acetate buffer (pH=4.5). Next day, they were centrifuged at 10000 g for 20 minutes at 4 °C and supernatants were collected. At this point, the purity and concentration of PC in the extracts were measured. To change the acetate buffer to phosphate buffer, the extracts were once more precipitated with 50% ammonium sulfate addition. After centrifugation, the pellets were dissolved in 0.01 M Na-phosphate buffer (pH=7.0) and they were loaded into dialysis membranes (Sigma-D9277). Prior to dialysis, membranes were cutted and washed out from the glycerol by leaving them under the running tap water for 3-4 h, were acidified with 0.2% (v/v) sulfuric acid and washed with hot water to remove the acid (Marzorati et al., 2020). Until the usage, they were preserved in the 20% ethanol. Dialysis was performed as two consecutive days against 0.01 M sodium phosphate buffer (pH=7.0) (1st 1:25; 2nd 1:120). 500 µl of dialysis samples was loaded onto the glass column filled with DEAE-Sepharose CL-6B resin (Sigma-DCL6B100) and gravity was used as elution force. Column was stabilized with extraction buffer before each run and 1 M NaCl was applied after each run to wash out remaining contaminants. During step-wise elution from 0.05 M NaCl to

0.3 M NaCl, eluents were collected at 2.5 mLs. In this stage, purities and concentrations were measured with nanophotometer (Implen-P360, Germany).



Figure 3.2. Overall representation of extraction and purification processes.

3.2. Optimization of Phycocyanin Production

Among the algae and cyanobacteria species studied during the course of this work, the highest PC concentration and purity were achieved with *Synechocystis* sp. Therefore, this cyanobacteria species was selected for further optimization experiments to improve PC production.

In order to improve PC production in *Synechocystis* sp., varying growth conditions such as light and salt stress, exposure to hydrogen peroxide were applied, and compared to a control group as shown in Table 3.3. Except the applied stress factor, all remaining cultivation parameters were kept constant among the reactors as previously described in subsection 3.1.1. The salt was added in the form of NaCl because it is the most applicable salt form for growth medium recipes. In addition, the

aim was to create osmotic imbalance and oxidative stress on cyanobacteria cells by adding NaCl. By doing so, the PC production was tried to be triggered. The rationale behind the hydrogen peroxide addition was to create oxidative stress which in turn to enhance the PC production. The concentrations were determined after the examination of transcriptomics paper prepared by Sinetova and Los (Sinetova and Los, 2016). However, the parameters were reduced in amount because the applications in the study were in minutes whereas the seven days growth was performed in this study. The cultures were grown for seven days till early stationary phase. In the beginning of the cultivation procedure, one drop of polyethylene glycol with 2 mL syringe via filtration was added into the reactors as anti-foaming agent. After centrifugation at 4500 rpm for 5 min, harvested biomass samples were washed with 1X PBS for twice and freeze-dried. 40 mg of this lyophilized biomass was mixed with 1.5 mL of 0.01 M Na-phosphate buffer (pH=7.0) and homogenized by bead-beating in tissue homogenizer (Precellys, Bertin, France) for 90 seconds with 5 seconds intervals for 6 cycles at 4500 rpm, 10°C. After this pretreatment step, the slurry was centrifuged at 10000 g for 20 min at 4°C and supernatants were collected, PC concentration and purity were determined by spectrophotometry. Extracted samples were aliquoted to improve purity values by salting out and activated carbon/chitosan treatment (AC/CS). For salting out, exact procedure described in subsection 3.1.2 was followed.

Table 3.3. Experimental matrix for tested stress factors.

Parameter	**Control**	**Light**	**Salt**	**H_2O_2**
Light intensity	15000 lux (approximately 203 μmol photons/m^2s)	22000 lux (approximately 297 μmol photons/m^2s)	15000 lux (approximately 203 μmol photons/m^2s)	15000 lux (approximately 203 μmol photons/m^2s)
Aeration rate	0.33 L/min	0.33 L/min	0.33 L/min	0.33 L/min
Temperature	25±2ºC	25±2ºC	25±2ºC	25±2ºC
H_2O_2 conc.	-	-	-	0.25 mM
NaCl conc.	-	-	0.25M	-

3.3. Purification of PC of Stress Reactors

Equal volume was used for salting out and activated carbon/chitosan purification treatments after biomasses were harvested and lyophilized. For salting out, the procedure in 3.1.2 was repeated with the addition of three s mixing at 60 rpm at 4 °C before overnight incubation for both of the saturation values. For chitosan part, 20 mg/mL of prewashed activated charcoal and 0.02% (w/v) chitosan dissolved in 1% acetic acid was mixed with crude extracts with vortex at each for 4 h at 4°C. After

centrifugation at 11000 g, 4°C for 20 min, the supernatants were collected, and absorbance was measured.

3.4. Calculations

At each step, PC purity was checked with A_{620}/A_{280} ratio via UV-vis nano-photometer (Implen, Pearl, Germany) and PC concentrations were determined based on the modified version of calculations given in Bennett and Bogorad (Equation 3.1) (Bennett and Bogorad, 1973). Calculations for *G. sulphuraria* was applied based on the equation given in Kursar and Alberte (Equation 3.2) (Kursar and Alberte, 1983). Extraction yields and recovery percentages between purification steps were calculated.

$$PC\ (mg/mL) = (A_{620} - 0.474 \times A_{650}) / 5.34 \quad (3.1)$$

$$PC\ (\mu g/mL) = (166 \times A_{620} - 108 \times A_{650}) \quad (3.2)$$

$$\underset{(mg/g\ DCW)}{Yield} = \underset{(mg/mL)}{Concentration} \times \underset{(mL)}{volume\ of\ extracts} / \underset{(g)}{dry\ weight} \quad (3.3)$$

$$Recovery\ (\%) = ((final\ concentration\ (mg/mL) \times volume\ of\ extract\ collected\ (mL)) \times 100) / (initial\ concentration\ (mg/mL) \times volume\ of\ extract\ started\ (mL)) \quad (3.4)$$

3.5. Antioxidant Activity Assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a purple stable radical and it turns into yellowish after it reacts with antioxidant compounds. The color results from the odd electron of the DPPH radical and it is reduced by taking a hydrogen atom from the antioxidant compound (Kedare and Singh, 2011). Correlation among absorbance of the color change indicates the antioxidant activity reported as % scavenging activity (Sharma and Bhat, 2009). For activity detection (Tarozzi et al., 2004), 50 µl of 60-120-180-240-300-600-900 µg/mL of AC/CS and ASP treated PC was mixed with 250 µL of 100 µM DPPH solution in methanol in 96-well microplates. After 30 minutes incubation at dark, absorbance at 525 nm was measured by spectrophotometer (SpectraMax i3, USA). L-ascorbic acid

was used for standard curve generation and as the positive control. For each sample, the sample blank was applied. Scavenging activity was calculated as follow:

$$\text{DPPH Scavenging Effect (\%)} = [1- (Abs_{sample}-Abs_{blank})/Abs_{control}] \times 100 \quad (3.5)$$

3.6. Statistical Analysis

All of the experiments of this study were conducted in biological triplicates (unless specifically indicated as duplicates) and the calculated data values were reported as mean ± standard deviation (SD). For statistical analysis, one-way analysis of variance (ANOVA) with post-hoc Tukey's honestly significant difference (HSD) tests were performed by IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). The results were considered significant if $p \leq 0.05$. Full results of statistical analyses are presented in Appendix C.

4. RESULTS AND DISCUSSION

4.1. Optimization of The Extraction Procedure

4.1.1. Growth Parameters

Optical density changes with time for each species were represented in Figure 4.1. As can be seen from the graph, the day of reactor closing (biomass harvest) is around the early stationary phase for each species. The OD values of each flask are provided in Appendix D. During the cultivation, flasks were wrapped with parafilm on the tap to prevent leakage and contamination. For the cultivation of filamentous cyanobacteria, diffusor was removed from the system because a significant percent of the biomass was gathered around the diffusor and the growth is restricted. For *Synechocystis* sp., growth medium was modified by doubling the concentration of K_2HPO_4 to compensate pH increase above 10.5. For *G. sulphuraria*, the nitrogen concentration was tripled, and glucose was removed from the cyanidium medium recipe. This change was performed to increase the PC production as referenced in "Sequential Heterotrophy-Dilution-Photoinduction" method (Wan et al., 2016). There is no growth data for *D. tharense* because its growth was not measurable with either OD or dry weight measurements. For further studies, there are designed apparatus that can measure growth from the PC content with external measurements.

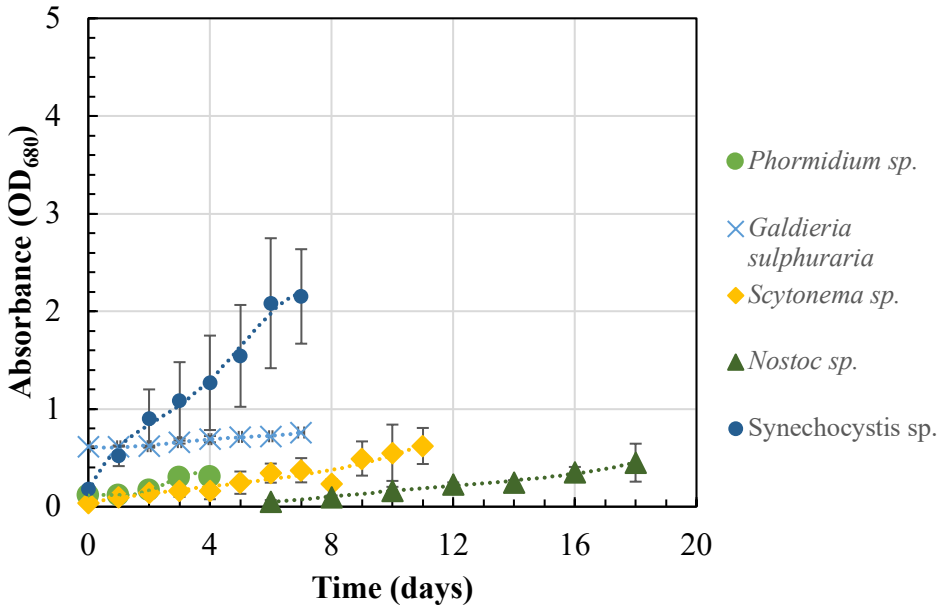


Figure 4.1. Absorbance versus time graphs for algal species.

4.1.2. Crude PC Extraction

There is limited information available on different pretreatment methods for the extraction of high-quality PC from cyanobacteria and red algae (Diez and Ininbergs, 2013; Gutiérrez et al., 2016; Yu et al., 2017). *Arthrospira platensis* and *Arthrospira maxima* (commonly referred as *Spirulina*) are among the most studied species where several methods are compared (İlter et al., 2018; Vernès et al., 2015). Meanwhile, there are additional potential cyanobacteria and algae species that can be evaluated for large scale production of PC for commercial purposes. Hence, this study aimed to explore the effects of different pretreatment techniques for the extraction and quality of PC from several potential cyanobacteria and algae biomass samples. Physical techniques such as sonication, freeze-thaw cycling, mortar and pestle, and bead-beating were primarily selected over chemical techniques such as lysozyme due to their non-reactive nature and cost-effectiveness.

As the extraction solvent, Na-phosphate buffer with low molarity was used to disrupt algal and cyanobacterial cell integrity by creating pressure inside the cell to release the PC free from the thylakoid membrane into the extraction solvent. Since PBPs are most stable around neutral pH levels, the pH of the Na-phosphate buffer used was adjusted to 7.0 to maintain the integrity of extracted PC. As being denaturants, the extracts were kept away from direct sun light by wrapping the flasks with aluminum foil and preserved at low temperature. In the previous trials with *A. maxima* and *A. variabilis*, it was observed that the extraction with 0.01 M Na-phosphate buffer gave better results than the extraction with 0.05 M Na-phosphate buffer; therefore, 0.01 M Na-phosphate buffer was used as extraction buffer. Also freeze-thaw cycling was performed by freezing at -80°C rather than -20°C. Even though -20°C was the preferred method in lots of the studies, the trial of PC extraction from *Synechocystis* sp. did not yield detectable PC concentration when the freezing was performed at -20°C.

As crude extract results, PC in liquid with different colors were obtained. The reason of difference in color for the crude extract solutions was the difference in the composition of the extract in terms of the PE, PC and APC. When it is greener, it is the sign of the more APC presence in the solution (Figure 2.1). The colors of the crude extract solutions were given in Appendix E. For *D. tharense,* the obtained supernatants labeled as crude extracts were greenish in color which indicates the presence of APC in the extract. For the ones with lower PC concentrations in the crude extract were white in color whereas, for the highest PC detected species, the color of the solution was intense blue.

As being isolated from Denizli Region in Turkey by Akdeniz University, promising results were obtained from *D. tharense* (Figure 4.2). Before cultivation, it was filtered through grade 4 filter, and then grown in nitrogen free BG-11 medium to eliminate the growth of contaminating species. Also, this is the very first prospective report of PC production and extraction from *D. tharense* in literature. The phycocyanin concentrations after each treatment were compared (one-way ANOVA, df = 5, F = 13.051, p = 0.000). The wet biomass extracted with freeze-thaw cycling did not result in detectable amount of PC. Mortar and pestle treatment resulted in significantly lower concentration of PC than the bead-beating applied freeze-dried biomass (Tukey's post-hoc, p = 0.006); however it was not significantly different than the rest of the methods. The concentration results of the bead-beating applied lyophilized biomass (33.623±6.039 mg/g DCW) was also significantly higher than freeze-thaw cycling applied lyophilized, sonication applied wet and sonication applied lyophilized biomass samples' results (Tukey's post-hoc, p = 0.001, 0.000 and 0.001 respectively). The phycocyanin concentration after the bead-beating treatment of the wet biomass (20.288±11.520 mg/g DCW) was also significantly higher than lyophilized biomass extracted with the freeze-thaw cycling, and wet and lyophilized biomass samples extracted with the sonication methods (Tukey's post-hoc, p = 0.049, 0.022 and 0.048 respectively). Therefore, bead-beating might be an efficient method to extract PC from *D. tharense*. For the purity part, when the statistical analysis was performed, none of the methods were significantly different than the other (one-way ANOVA, df = 5, F = 1.005, p = 0.456). The bead-beating applied freeze-dried biomass (0.393±0.096) and wet biomass (0.326±0.098) resulted in the highest average purity among all methods whereas the freeze-thaw cycling gave the lowest average purity (0.178±0.054). Since there is no optimization of growth conditions, it is validated as a potential PC producer at food-grade. However, its concentration was not enough to further purify it with AEC due to the lower recovery of the CE between purification steps. The reason of obtaining high standard deviations for the wet biomass samples might be resulted from the fact that the separation of *D. tharense* biomass depending onto its growth shape which is a ball like structure.

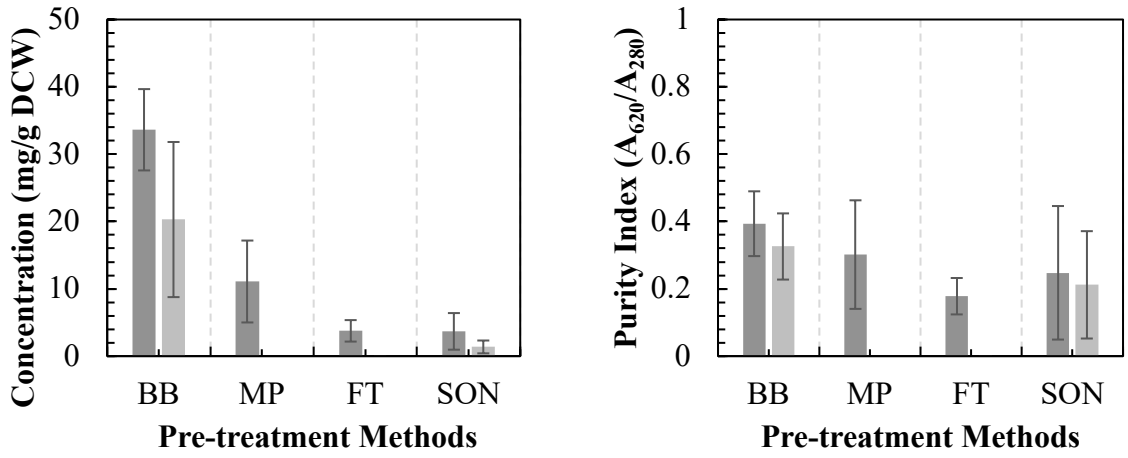


Figure 4.2. Results of the crude extract of *D. tharense* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass).

There is a limited number of studies for the large-scale production of PC. For the large scale, outdoor cultivation becomes more prominent. Even though *Arthrospira* species are the main source of PC, their outdoor cultivation is not as effective as wanted. As an alternative to *Arthrospira* species, *G. sulphuraria,* which is an acidophilic and thermophilic red algae species, is a potential alternative for the large-scale production in open ponds. Besides being able to grow up to 56°C, its ability to grow at low pH reduces the risk of contamination and the cost of stabilizing culturing parameters in the open ponds. In addition, *G. sulphuraria* can also be cultivated mixotrophically and heterotrophically. Even though heterotrophic and mixotrophic cultivation results in higher biomass productivity, the PC production is reduced. In this study, *G. sulphuraria* was cultivated at 40 °C under 8000 lux (approximately 108 μmol photons/m^2s) light intensity autotrophically, and the PC yields and purities after each treatment were compared (Figure 4.3). When concentration results were analyzed (one-way ANOVA, df = 6, F = 8.651, p = 0.000), mortar pestle treatment did not result in significantly different results than the other methods whereas bead-beating applied lyophilized biomass gave significantly higher concentration (5.609 ± 1.793 mg/g DCW) than freeze-thaw cycling applied wet and lyophilized biomass samples, and sonication applied wet biomass (Tukey's post-hoc, p = 0.022, 0.026 and 0.005 respectively). In addition, the concentration obtained after the treatment of wet biomass with the bead-beating was significantly higher than freeze-thaw applied wet and lyophilized biomass samples, and sonication applied wet and freeze-dried biomass samples (Tukey's post-hoc, p = 0.005, 0.006, 0.001 and 0.025 respectively). Even though mortar and pestle, and bead-beating were not significantly different than each other, bead-beating resulted in higher concentration than the rest of the methods for both of the biomass type. Hence the bead-beating treatment could be selected as an efficient technique for the extraction of PC from *G. sulphuraria*. For the purity comparison among methods, when the analysis was performed by comparing all methods, there was

no significant difference (one-way ANOVA, df = 6, $F = 0.638$, $p = 0.698$). When the results of the freeze-thaw cycling and sonication of lyophilized biomass samples which having higher standard deviations were excluded from the analysis (one-way ANOVA, df = 4, $F = 10.563$, $p = 0.001$), bead-beating applied lyophilized and wet biomass, and freeze-thaw cycling applied wet biomass results were significantly higher than sonication applied wet biomass result (0.076±0.046) (Tukey's post-hoc, $p = 0.009$, 0.001 and 0.014 respectively.). The lower concentration and purity from the sonication might be resulted from the generation of lower shear force and heat in the solution even though cooling was applied in between cycles.

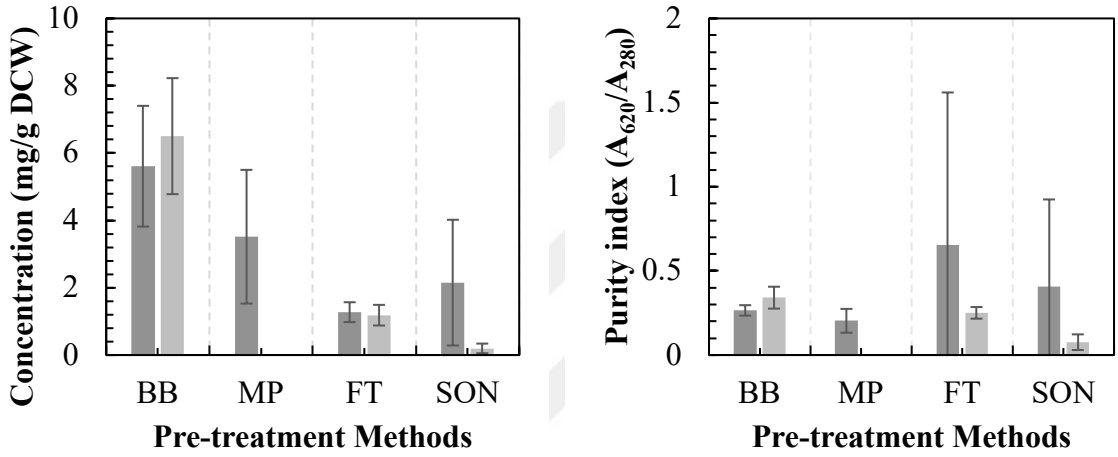


Figure 4.3. Results of the crude extract of *G. sulphuraria* (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass).

In a comparable study by Moon et al. (2014), PC with purity greater than 4 was obtained from freeze-thawed wet biomass of *G. sulphuraria* UTEX 2919 after the purification of crude extract with ammonium sulfate precipitation and acidic acetate buffer (pH=4.5). In a similar study by Eriksen (2008), the 0.1 purity of crude PC from *G. sulphuraria* 074G was increased to 4.5 after it was treated with ammonium sulfate precipitation, followed by ATPE and AEC. In this study, the purity of the crude PC extract from lyophilized biomass samples of *G. sulphuraria* treated with bead-beating was enhanced to 0.71±0.32 after ammonium sulfate salt precipitation (Table 4.1). In another study by Sørensen et al. (2013), the purity of PC was also reported to be 0.7 following ammonium sulfate fractionation applied to heterotrophically grown *G. sulphuraria*. In this study, AEC purification was not applied as the remaining PC left in the extract of particular aliquots were low in concentration. It should also be noted that, *G. sulphuraria* was cultivated under autotrophic conditions using modified Cyanidium medium supplemented with three times more ammonium sulphate to sustain sufficient nitrogen reserves because the PC is used as a nitrogen source when nitrogen is depleted and glucose suppresses PC production (Simeunović et al., 2013; Sloth et al., 2006). In a notable study by Wan et

al. (2016), “Sequential Heterotrophy-Dilution-Photoinduction” model was applied, and the PC content was increased to 13.2% when the growth conditions were changed from heterotrophy to autotrophy with a dilution step. Even though, it was tried for *G. sulphuraria*, the mentioned results could not have been obtained in this study. In another study by Graveholt and Eriksen (2007), *G. sulphuraria* was cultivated heterotrophically and final PC content was 2.7%. In a similar study by Schmidt et al. (2005), PC productivity was 0.3% when *G. sulphuraria* was cultivated heterotrophically. Overall, food grade PC was obtained after salt precipitation under autotrophic and thermophilic growth conditions using bead-beating pretreated lyophilized biomass samples of *G. sulphuraria* comparable to other studies.

Nostoc sp. has wide biotechnological application area including food, pharma and environmental sectors (Abed et al., 2009; Sigamani and Natarajan, 2016; Thajuddin and Subramanian, 2004). Its growth does not require additional nitrogen because it is a diazotrophic species which reduces the PC production-cost. Even though it is highly studied in diverse sectors and a less expensive way to obtain PC, the pretreatment methods were not extensively studied. In this study, four different pretreatment methods were tried, and the methods were compared by the final purity and the concentration of the PC obtained (Figure 4.4). When the analysis was performed for the concentration (one-way ANOVA, df = 6, F = 4.884, p = 0.007), only the freeze-thaw cycling applied lyophilized biomass generated significantly higher concentration (3.294±0.832 mg/g DCW) than the wet biomass treated with the same method (Tukey’s post-hoc, p = 0.019). When the analysis was redone by excluding the mortar-pestle and sonication applied lyophilized biomass results (one-way ANOVA, df = 4, F = 24.460, p = 0.000), the concentration obtained from the bead-beating applied lyophilized and wet biomass samples were significantly lower than the result of the freeze-thaw cycling applied lyophilized biomass (Tukey’s post-hoc, p = 0.000 for both). In addition, the concentration obtained from the freeze-thaw cycling applied lyophilized biomass was significantly higher than the results of wet biomass samples treated with freeze-thaw cycling and sonication (Tukey’s post-hoc, p = 0.000 for both). In the case of wet biomass samples, the sonication gave the highest average concentration and purity (0.751±0.432 mg/g DCW and 0.120±0.063 respectively); however, it was not significantly different than the other methods. When the analysis was performed for the purities (one-way ANOVA, df = 6, F = 4.663, p = 0.008), the bead-beating applied freeze-dried biomass result was significantly lower than mortar and pestle and freeze-thaw cycling treated lyophilized biomass results (Tukey’s post-hoc, p = 0.033 and 0.011 respectively). In addition, freeze-thaw cycling of lyophilized biomass resulted in significantly higher purity than the wet biomass of the same method (Tukey’s post-hoc, p = 0.031). Moreover, sonication treatment for both wet and lyophilized biomass samples did not generate any significance, they were excluded from the analysis

(one-way ANOVA, df = 4, F = 11.695, p = 0.001). After that, bead-beating applied wet biomass result was significantly lower than freeze-thaw cycling applied lyophilized biomass result (Tukey's post-hoc, p = 0.042). The purity obtained after the treatment of freeze-dried biomass with mortar and pestle was significantly higher than the purity of the wet biomass treated with freeze-thaw cycling (Tukey's post-hoc, p = 0.017). As previous analysis, the purity obtained after the treatment of lyophilized biomass with the bead-beating method was significantly higher than mortar and pestle, and freeze-thaw cycling results of the lyophilized biomass (Tukey's post-hoc, p = 0.006 and 0.002 respectively). Lastly, the freeze-thaw cycling applied to wet biomass resulted in significantly lower purity than lyophilized biomass treated with the same method (Tukey's post-hoc, p = 0.005).

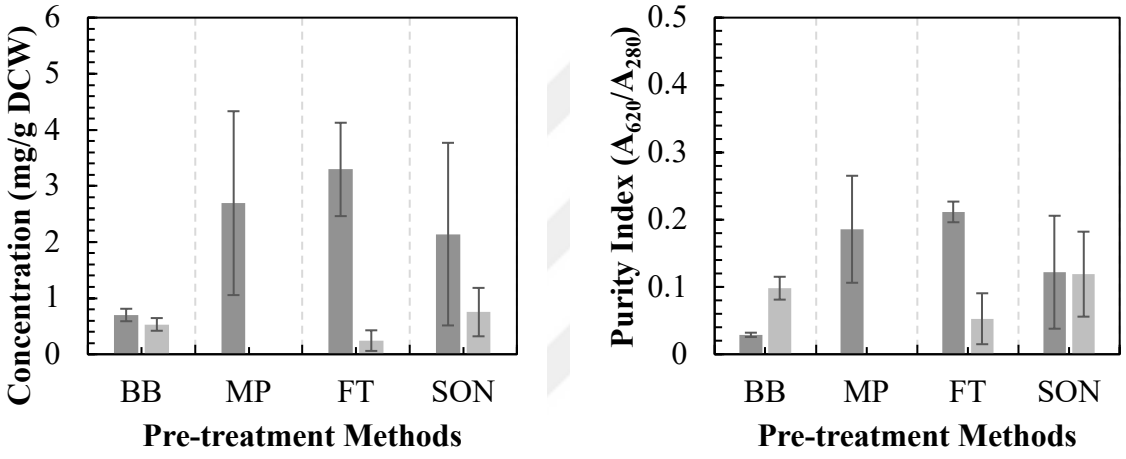


Figure 4.4. Results of the crude extract of *Nostoc* sp. (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass)

In a study by Gray et al. (1973), freeze-thaw cycling followed by salting out with ammonium sulfate resulted in PC extraction from *Nostoc* sp. with the purity ratio of 2.8. In a comparable study by Oliveira (2014), the effect of different light intensities on the PC production was tested. At 20 µmol photons/m^2s, 0.1 mg/mL PC was extracted from the French-pressed *Nostoc* sp. F105 biomass samples extracted with sodium acetate buffer (pH=5.5). In another study, the PC was extracted with distilled water at 5^{o}C for 24 and 72 hours, and the obtained purities were 0.72 and 1.4 respectively (Lee et al., 2017). In addition, mortar and pestle followed by repeated freeze-thaw cycles was used for the PC extraction from *Nostoc* sp. strain HKAR-11 (Kannaujiya and Sinha, 2016a). The 0.14 purity of crude extract was increased to 1.36 after salting out and gel filtration chromatography of Sepharyl S-100 HR. When compared, the crude extract purities in this study were higher for mortar and pestle (0.186±0.080) and freeze-thaw (0.211±0.015) pretreated lyophilized biomass samples, and after salt precipitation, the purity index was increased to 0.842±0.165 for the CE obtained from the freeze-thaw cycling applied lyophilized biomass sample.

The wet and lyophilized biomass samples of *Phormidium* sp. were extracted with either bead-beating, sonication, freeze-thaw cycling and the mortar and pestle. The final PC concentrations were compared and none of the methods were significantly different than the other (one-way ANOVA, df = 6, $F = 0.877$, $p = 0.536$). Therefore, the determination of a more efficient method for the extraction of PC from *Phormidium* sp. could not have been achieved by this study. When the average values were compared, the highest average concentration was obtained from the freeze-thaw cycling of freeze-dried biomass (35.558±21.941 mg/g DCW) followed by mortar-pestle (21.006±15.363 mg/g DCW), bead-beating (19.927±15.585 mg/g DCW), and sonication (17.458±7.737 mg/g DCW) (Figure 4.5). For the purity, there is no significant difference among methods (one-way ANOVA, df = 6, $F = 0.413$, $p = 0.858$). The highest average purity was obtained with the freeze-thaw cycling pretreatment of wet biomass (0.422±0.136) whereas the lowest average purity value was obtained from the bead-beating of the wet biomass (0.230±0.058).

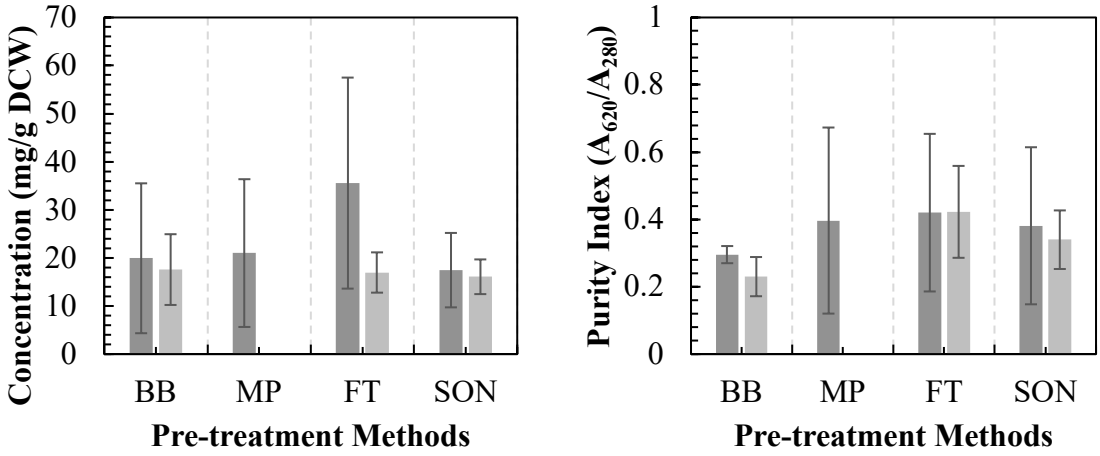


Figure 4.5. Results of the crude extract of *Phormidium* sp. (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass).

In a study by Keithellakpam et al. (2015), the species (*Phormidium* sp. strains BTA-52, BTA-75 and BTA-1048) that are isolated from the same lake were studied for the similarity in their PBPs. *Phormidium* sp. strain BTA-52 produced APC and PC at dominant levels. *Phormidium* sp. strain BTA-75 produced PE as the dominant PBPs whereas PC was the dominant one in *Phormidium* sp. strain BTA-1048 indicating that the difference of strain also affects the PC content. In a comparative study by Madamwar et al. (2015), *Phormidium* sp. strain A27DM was pretreated with freeze-thaw cycling and extracted PC was purified with two step ammonium sulfate precipitation and gel permeation chromatography. The purity obtained was nearly 1.5, whereas in this study, freeze-thaw cycling coupled with AEC column purification resulted in an average purity ratio of 2.17 (Table 4.1),

which is higher than the Madamwar's study could be based on the utilization of a different strain. In another study by Patel et al. (2005), PC purities obtained from biomass samples of *Phormidium* sp. (a local isolate), after the purification with 20% and 50% ammonium sulfate precipitation, and ion exchange chromatography on DEAE-Sepharose CL-6B, were 0.69, 0.73, 1.62 and 4.43 respectively, which suggests that under certain conditions, crude biomass can also yield to high purity PC. Meanwhile, Pumas et al. (2011) obtained 165.47 mg/g dry weight PC from wet paste biomass samples of *Phormidium* sp. PD40-1 after the sonication in Tris-HCl buffer. Sonication pretreatment applied to *Phormidium* sp. was resulted in much lower concentrations in this study. When lyophilized biomass samples of *Phormidium* sp. EGEMACC 72 was extracted with $CaCl_2$ (1.5%), purified by two-step ammonium sulfate precipitation following dialysis and separation on Sephadex G-25 and DEAE-Sephadex columns, and the final purities of 1.15, 2.44, 2.94 and 4.14 were achieved respectively (Khazi et al., 2018). For 32 days old culture of *P. ceylanium* in BG-11 medium, the extraction with freeze-thaw cycling in 1M Tris-HCl buffer (pH=8.1) followed by ultrafiltration and AEC, the purity ratio was increased to 4.15 from 1.05 with a 63.5% recovery yield (Singh et al., 2009). Based on the results reported in this study and literature, both lyophilized and wet biomass samples of *Phormidium* sp. could result in high purity PC and pretreatment with mortar-pestle, freeze-thaw cycling, and sonication were concluded as effective techniques for this species. Anion exchange column chromatography was also recommended to obtain highest grade of PC from *Phormidium* sp.

As *G. sulphuraria*, *Scytonema* sp. is advantageous as being thermotolerant cyanobacteria because their PC is also resistant to temperature as themselves (Pumas et al., 2011). However, the PC production from it is not extensively studied in the literature. In this study, none of the methods were significantly different than the other for concentration measurements (one-way ANOVA, df = 6, F = 0.900, p = 0.522). Therefore, the aim to find an efficient extraction method could not have been achieved by this study (Figure 4.6). When the purities were compared, none of the methods were also significantly different than the other (one-way ANOVA, df = 6, F = 0.439, p = 0.841). In purity, it was the only species which a higher average purity was observed for the sonication of wet biomass (0.319±0.080) than the other methods. For dry biomass, the highest average purity was achieved with mortar and pestle (0.360±0.068) whereas the lowest average purity was obtained from the sonication (0.161±0.082). The crude PC extract obtained from *Scytonema* sp. was not purified further since obtained results were fairly low in both concentration and purity with respect to *Phormidium* sp. and *Synechocystis* sp. and the results had high standard deviations.

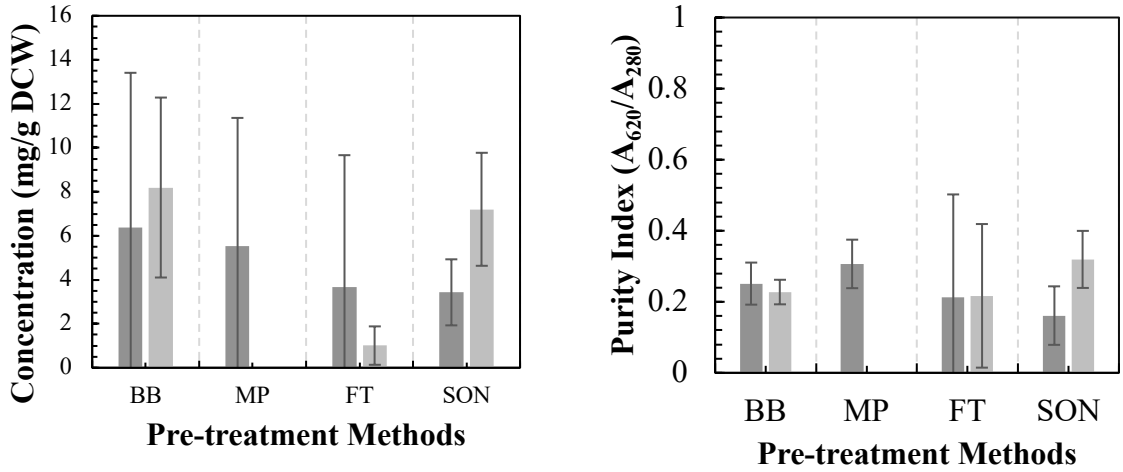


Figure 4.6. Results of the crude extract of *Scytonema* sp. (dark colored ones are freeze-dried biomass and light-colored ones are wet biomass).

Pumas et al. (2011) found that the PC is the dominant of the PBPs in *Scytonema* sp. TP40 after the extraction from the wet biomass with sonication. In another study, liquid nitrogen dried *Scytonema* isolates (40 unicyanobacterial isolates from terrestrial habitats) were treated with sonication and obtained PC amount was reported between 1.98 to 9.55 as mg PBP/mg chlorophyll a (Asencio and Hoffmann, 2013).

Synechocystis sp. was the species that the highest concentration of the PC was obtained in this study and only the lyophilized biomass was treated (Figure 4.7). The concentration values obtained after each treatment method for both wet and lyophilized biomass samples were compared (one-way ANOVA, df = 3, F = 108.841, p = 0.000). With the bead-beating, the CE concentration was 182.393±9.533 mg/g DCW which is significantly higher than the mortar and pestle, freeze-thaw cycling and sonication treatments (Tukey's post-hoc, p = 0.000 for all). Except bead-beating, the methods were not significantly different from each other. It is also the only species studied that higher than food grade purity was achieved for the crude extracts with bead-beating (1.148±0.031) and mortar-pestle (0.871±0.832). When the purities achieved after each treatment were compared, the analysis did not generate any significant difference among methods (one-way ANOVA, df = 3, F = 5.147, p = 0.028). However, after excluding the mortar and pestle which has the highest standard deviation (one-way ANOVA, df = 2, F = 820.140, p = 0.000), the purity obtained after the extraction of freeze-dried biomass by bead-beating was significantly higher than the freeze-thaw cycling and the sonication methods (Tukey's post-hoc, p = 0.000). The sonication (0.091±0.054) and freeze-thaw cycling (0.076±0.014) methods did not give desirable purity. In a comparable study by Eriksen, the highest purity ratio of 6.69 was obtained from *Spirulina platensis* by two-phase aqueous extraction followed ion-exchange chromatography (Eriksen, 2008). In this study, one of the biological replicates

of the *Synechocystis* sp. resulted in much better purity ratio (7) the elution with 0.25M NaCl from DEAE-Sepharose CL-6B column (Table 4.1). Despite the outlier, mean purity level was still above 4, indicating a strong potential for commercial production of PC from *Synechocystis* sp.

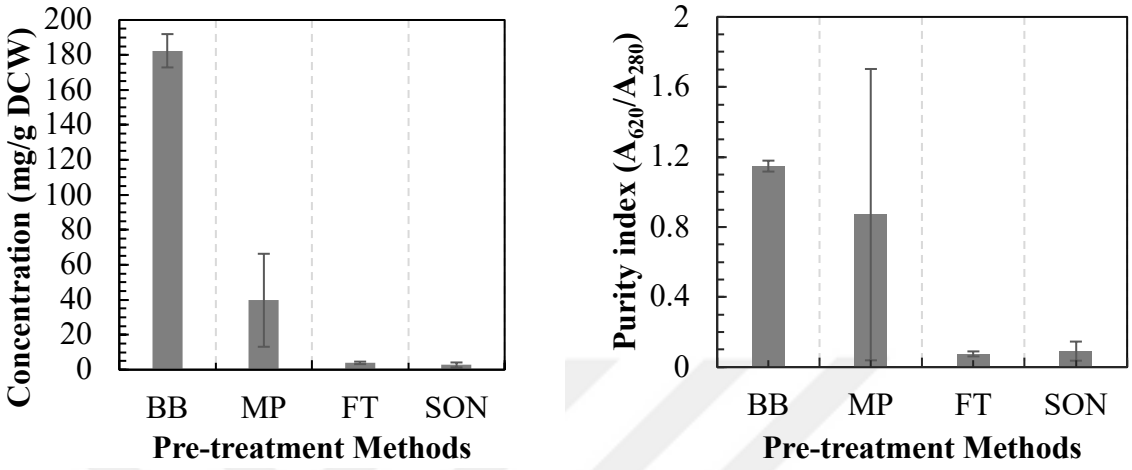


Figure 4.7. Results of the crude extract of *Synechocystis* sp.

4.1.3. Further Purification by Salt Precipitation and AEC

Crude extracts from the five cyanobacteria and one alga were further purified with ammonium sulfate precipitation (ASP) and AEC methods. This step was not applied for all of the conditions, only the higher purity and concentration yielding extractions were followed for further purification. During ammonium sulfate precipitation, two-step salting out was performed. This method is beneficial by having low cost and not being toxic for further usage of the obtained PC. The rationale behind two step application was to remove PE and other contaminant proteins from the system by low ionic concentration (20%). Then APC was separated from the PC at 50% ammonium sulfate saturation. After salting out, acetate buffer was applied as a second purification step. It facilitates the removal of linker proteins from the solution by precipitating them due to pI of such proteins. To load back onto column system which is stabilized with Na-phosphate buffer and to further separate PC, supernatant after acetate buffer application was brought to 50% saturation with crude ammonium sulfate addition. The pellet was resuspended in Na-phosphate buffer. To remove the salt from the system before the sample loading onto the column, dialysis step was applied. Rational behind the dialysis is the removal of water by diffusion from less concentrated part to higher concentration. The diffusion continues until concentration of each part becomes equal. Hence, by buffer chance in between, salt removal near to 0.01 M Na-phosphate buffer concentration was aimed. After the dialysis, samples were loaded onto the column. For the elution, increasing NaCl concentrations in Na-phosphate buffer were applied. To remove air bubble from the column system and prevent it to

occur during the separation, all solutions used were filtered through 0.45 μm syringe filters. To prevent bubbling, the equilibration of the temperature of all solutions and column is also an important step. So, prior to operation, all solutions were brought to room temperature. In addition to ionic strength, chromatography also facilitates the separation depending on the size.

Purity results, purity enhancements and recoveries from the crude extract were represented in Table 4.1. For the elution profile, the increasing ionic strength was performed. In a study by Bermejo et al. (2006), pH gradient as elution method for the separation of PC from APC was not successful for *A. platensis*. As can be seen, the recovery after column purification was very low for all species. This indicates that column procedure could be improved by using either pre-packed columns, or by using a smaller column for self-packing. Also, application of elution solvent to the system via peristaltic pump can improve the elution profile.

When sonication was applied, a few of the species resulted in lower PC yield. This might be resulted from the increased temperature as the sonication continues. Even though samples were rest on ice in between cycles, the length of the sonication can be reduced by optimization studies. As another solution, sonication can be performed in an ice bath. Overall, the highest standard deviations were observed in mortar and pestle method because it is subjected to higher human error. In addition, mortar-pestle that used in this study leaves some of the biomass as stacked to the bottom. That might also lead to deviation among replicates. Higher standard deviation among wet biomass samples might be improved by homogenizing wet biomass prior to use. Also, for the filamentous cyanobacteria, filtering after harvesting can be an improvement to reduce the variations among water contents in samples. However, a higher percentage of the deviations mainly results because of applying biological replicates.

The highest purity enhancement was observed in the mortar-pestle treated lyophilized biomass of *Phormidium* sp. after AEC whereas the lowest recovery was observed when the PC obtained from *D. tharense* was purified. This indicates that prior's resistance to pH and temperature changes is lower with respect to others. For *Phormidium* sp., the highest purity increase was achieved for the mortar-pestle treated dry biomass. Since it was the highest purity giving method for crude extract preparation, this was expected. It also yielded the highest average recovery. Except *D. tharense,* the purity was increased above food grade (0.7) for all species after salt precipitation. For *D. tharense*, the only pre-treatment yielded in purity above the food grade after salt precipitation was mortar-pestle treated lyophilized biomass. *Synechocystis* sp. was the only species that its PC is purified above the analytical

grade purity (4.0). Because the recovery after salt precipitation was very low for mortar and pestle treated lyophilized biomass, it was not purified with column chromatography.

The differences among purity enhancements might be resulted from the differences among the initial concentrations of the crude extracts. Hence it might be an improvement to bring final concentrations equal to continue with further purification. In addition, depending on the species the characteristics of the extracted PC is changed. Therefore, observing different purity enhancements and recovery yields are expected. Since recovery of PC in between different steps is higher for *Phormidium* sp. with respect to *Synechocystis* sp. and *D. tharense*, it can also be a good candidate for commercial PC production. Even though the PC amount was lower than *Synechocystis* sp. and *D. tharense*, the good stability for longer times make it beneficial for food applications as a food-colorant. Also, *Phormidium* sp. gave the highest average PC concentration and a comparable purity ratio with freeze-thaw cycling method which is very easy to process and cheap. Hence by adjusting growth parameters, the PC content of *Phormidium* sp. might become compatible with others.

Table 4.1. Salt precipitation and Anion Exchange Chromatography (AEC) results for species.

Species	Biomass type	Pretreatment method[1]	Purification method[2]	Concentration (mg mL^{-1})	Purity (A_{620}/A_{280})	Improvement in purity (% increase)	Recovery (%)
Phormidium sp.	Lyophilized	BB	Salt precip.	0.10±0.04	1.10±0.23	279%	73.89±79.67
	Lyophilized	MP	Salt precip.	0.25±0.20	1.72±0.96	341%	79.26±6.84
	Lyophilized	MP	AEC	0.02±0.01	2.33±2.31	497%	13.40±2.43
	Lyophilized	FT	Salt precip.	0.36±0.25	1.46±0.53	247%	61.70±13.26
	Lyophilized*	FT	AEC	0.03±0.01	2.17±1.18	48%	8.11±2.18
	Wet	FT	Salt precip.	0.16±0.03	1.11±0.23	164%	69.17±26.71
	Wet	SON	Salt precip.	0.08±0.07	0.70±0.39	84%	33.76±23.62
G. sulphuraria	Lyophilized	BB	Salt precip.	0.05±0.04	0.71±0.32	169%	70.37±72.23
Synechocystis sp.	Lyophilized	MP	Salt precip.	0.09±0.11	1.14±0.54	31%	16.04±14.12
	Lyophilized	BB	Salt precip.	1.29±0.04	1.95±0.41	71%	47.24±3.00
	Lyophilized	BB	AEC	0.08±0.04	4.84±1.87	324%	9.28±1.08
D. tharense	Lyophilized	BB	Salt precip.	0.07±0.10	0.61±0.25	56%	13.35±16.44
	Wet	BB	Salt precip.	0.08±0.05	0.51±0.15	54%	25.14±9.91
	Lyophilized*	MP	Salt precip.	0.08±0.00	0.80±0.01	166%	38.44±3.23
Nostoc sp.	Lyophilized	FT	Salt precip.	0.03±0.01	0.84±0.16	298%	63.60±9.54

[1] *BB: Bead-beating; MP: Mortar-pestle; FT: Freeze-thaw cycling; SON: Sonication*
[2] *Salt precip.: Ammonium sulphate precipitation; AEC: Anion-exchange chromatography*
**Two replicates instead of three.*

4.2. Extraction from Stress Applied *Synechocystis* sp. Biomass Samples

4.2.1. Growth Parameters of Reactors

Synechocystis sp. was selected for the stress application study since it resulted in the highest crude PC concentration among other species. For the stress conditions, addition of salt as in the form of NaCl, hydrogen peroxide addition and the enhancement of the light intensity were tried. In the stress application part, absorbance, cell number and pH changes over time were represented in Figure 4.8, 4.9 and 4.10 respectively. As can be seen from the graphs, the addition of salt was inhibited the growth whereas the other stress conditions did not generate differences in the growth regime. In Figure 4.11, the reactor set ups can be seen for this part of the study.

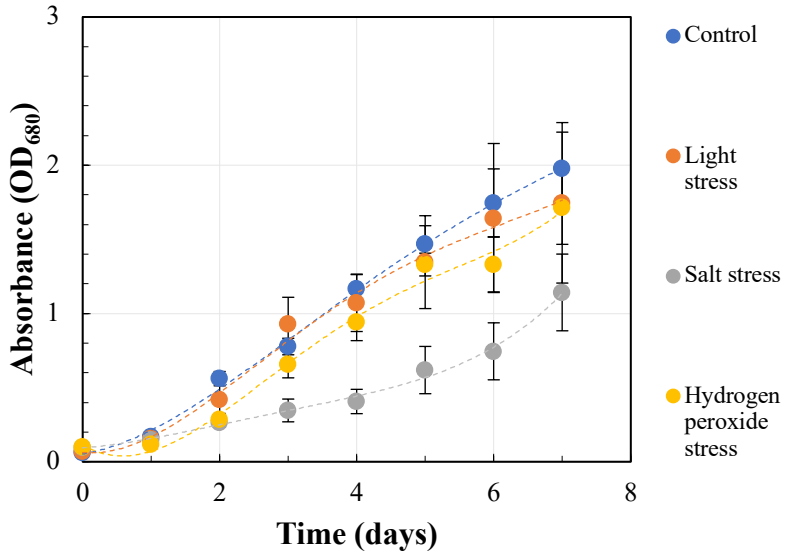


Figure 4.8. Absorbance over time graph for stress reactors.

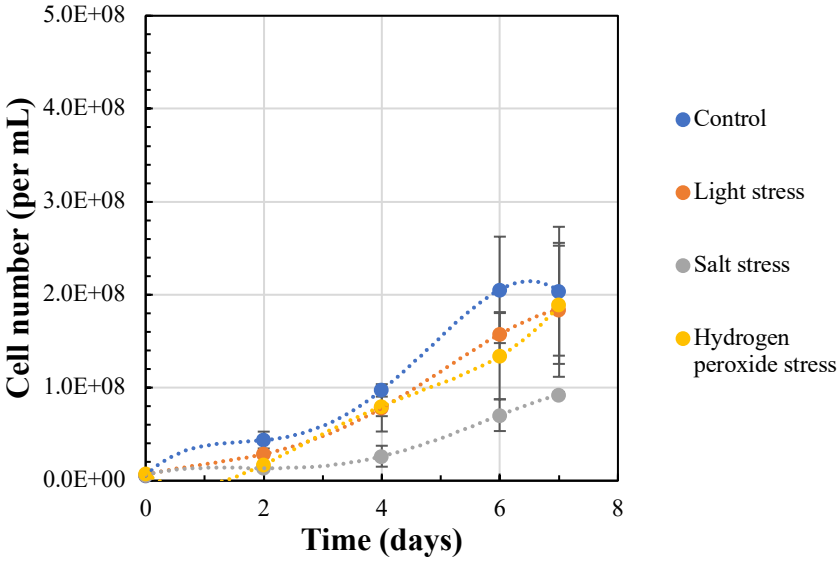


Figure 4.9. The change in cell number over time for stress applied reactors.

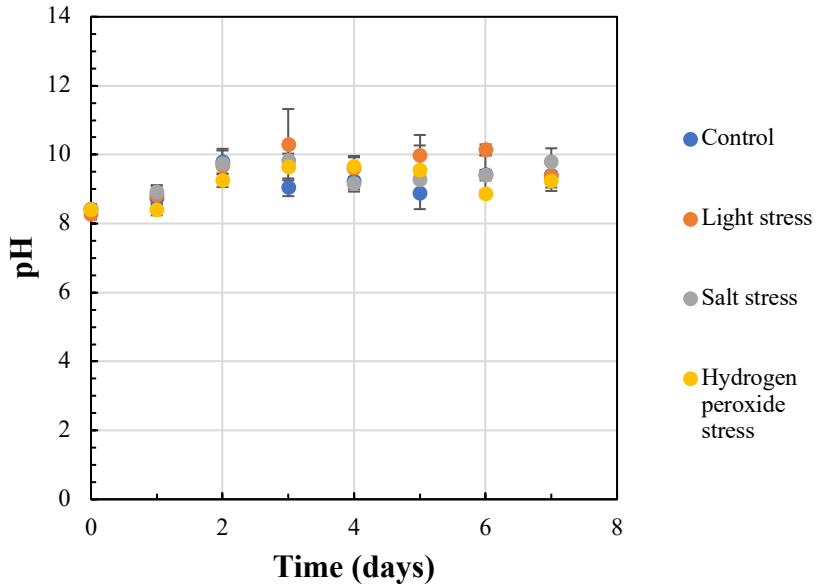


Figure 4.10. The change in pH over time for stress applied reactors.

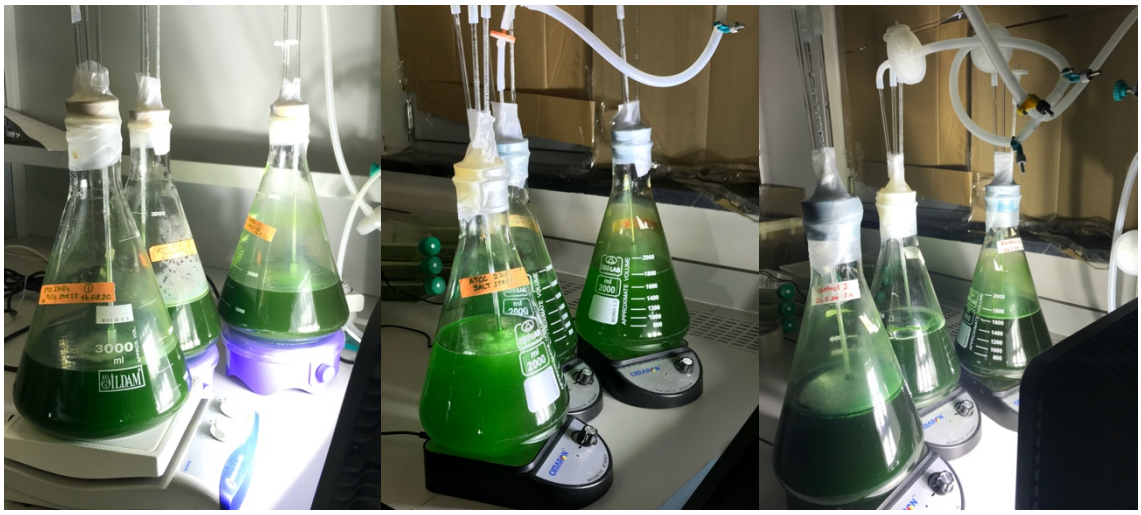


Figure 4.11. The growing of the stress reactors with the control group (left: hydrogen peroxide stress; middle: salt stress; right: control).

4.2.2. Extraction of Crude PC from Reactors

Since the highest PC was extracted from *Synechocystis* sp., it was selected as the model organism to test the effects of the stress parameters on the PC production in these cyanobacteria. When the crude extracts were compared, there was no significant difference between the control and the stress reactors. Even though CE purities were above the food grade for all biomass samples, the highest average purity was achieved for the CE obtained from the salt stress biomasses. There needs to be further exploration, but it was anticipated to be the result of the selection for phycocyanin over the production of the other proteins.

Table 4.2. The phycocyanin amount and initial purity obtained from the control and the stress reactors.

Stress condition	Reactor productivity (g/L)	Dry cell weight (g/L)	PC Concentration (mg/mL)	Purity of the CE
Control	0.75±0.08	0.51±0.07	1.79±0.21	0.86±0.04
Light stress	0.68±0.21	0.52±0.17	1.28±0.53	0.71±0.21
Salt stress	0.73±0.06	0.26±0.06	1.78±0.07	0.89±0.07
H_2O_2 stress	0.48±0.07	0.46±0.02	1.70±0.06	0.83±0.00

In a study by Schubert et al. (1993), the growth at 0.342 M NaCl concentration of *Synechocystis* sp. PCC 6803 resulted in the highest PC productivity with the highest photosynthesis per cell volume were obtained . In another study, the gene expression analysis after 0.25 mM H_2O_2 application to *Synechocystis* sp. PCC 6803 showed that 118 genes including the ones that are important for stress neutralization are affected (Kanesaki et al., 2007).

4.2.3. Purification of The Crude Extracts of Biomass Samples Exposed to Stress Conditions

The phycocyanin extracted from the stress and control groups were divided into two aliquots for two different purification steps (Table 4.3). In a step, salting out was performed for purification. After 50% saturation with ammonium sulfate, the purity index for the hydrogen peroxide stress samples were raised to 1.41± 0.02 with a recovery yield of 65.50± 2.00%. The purity index for salt stress after the same treatment was raised to 1.29± 0.09 with a 78.18± 5.26% recovery yield. The highest average purity with 50% ammonium sulfate saturation was achieved from CEs of the control reactors (1.46±0.09) with the highest average recovery yield (85.47±2.46%). In another step, activated carbon with chitosan was applied, and purer PC was obtained with one step purification. To remove remaining impurities, this method was more successful than usually applied salting out and the final extracts were brighter blue in color than salting out applied samples. Also, it is a shorter, easier and cheaper method to apply. Chitosan is a pH responsive polymer, when pH is below 6.5, it is highly soluble and it binds to negatively charged proteins at pH>6.5 (Fekrat et al., 2019). When it was controlled the pH of the extracts, the pH ranged from 5.77 to 6.06 which are below 6.5. However, the chitosan in its soluble form does not contribute to the absorbance at 620 or 650 nm. The stability of the PC was found pH 5 to 8 (Fekrat et al., 2019; Patil and Raghavarao, 2007) whereas, in another study, the stability is the highest from pH 5 to 6 (Wu et al., 2016). Moreover, the removal of the smaller impurities can be achieved by activated charcoal because it has a high surface area (Safaei et al., 2019). In addition, higher recovery rate at high purities can be achieved than column purification. For the large-scale applications, it could save time and money during the course of obtaining PC at higher than analytical grade purity. Additionally, this study is the first report of chitosan purification of the PC from *Synechocystis* sp. For the salt stress samples, the purity index of 3.50±0.23 was achieved with a recovery of 73.85±1.33%. The purity index of hydrogen peroxide samples after treatment was 4.00±0.15 with 60.85±1.85% recovery. The average purity for light stress biomasses were raised to 3.80±0.52 after the crude extract was treated with activated charcoal and chitosan. Even though the average obtained was below analytical grade, one of the replicates resulted in 4.23 for purity index which is above the analytical grade. The recovery was lower than the other treatments with 57.86±14.77%. The lower recovery might be resulted from the fact that one of the replicates yielded highly low PC concentration. Therefore, its treatment might lead to loss of PC conformity. Only the control reactor biomasses gave purity lower than 3 in average (2.97±0.48). However, the average recovery was higher when compared to other conditions (76.20±4.50%).

Table 4.3. The purity, yield and recovery results for ASP and AC/CS treatments.

Crude Extracts	Purification Method		Concentration (mg/mL)	Purity index	Recovery (%)
Control	ASP	20%	1.62±0.19	0.92±0.04	90.45±1.41
		50%	1.53±0.20	1.46±0.09	85.47±2.46
	AC/CS		1.37±0.23	2.97±0.48	76.20±4.50
Light stress	ASP	20%	1.15±0.48	0.76±0.22	89.24±2.73
		50%	1.01±0.42	1.24±0.33	77.99±1.74
	AC/CS		0.80±0.45	3.80±0.52	57.86±14.77
Salt stress	ASP	20%	1.63±0.09	0.93±0.08	91.40±2.29
		50%	1.39±0.14	1.29±0.09	78.18±5.26
	AC/CS		1.31±0.05	3.50±0.23	73.85±1.33
Hydrogen peroxide stress	ASP	20%	1.44±0.01	0.87±0.01	84.75±2.98
		50%	1.11±0.05	1.41±0.02	65.50±2.00
	AC/CS		1.03±0.00	4.00±0.15	60.85±1.85

In a work by Liao et al. (2011), PC from *Arthrospira platensis* was purified with activated charcoal and chitosan. At 0.29% (w/w) chitosan concentration, the purity was increased to 1.52. To further increase the purity to 2.78, activated charcoal at 80g/L concentration was added, and the final yield was 85%. It was also noted in this study that the extracts were bright blue in color. In a study by Fekrat et al. (2019), the amount of the chitosan and activated charcoal was optimized with response surface methodology. They found the optimum conditions as 0.24% w/v chitosan, 8.4% w/v activated charcoal with 10.2 min stirring time. The purity was increased to 3.14±0.12 which was 1.67-fold higher than ASP with 79% recovery (Fekrat et al., 2019). In a similar study by Gupta and Sainis, PC of *Anacystis nidulans* BD1 was purified with activate charcoal and chitosan treatment (Gupta and Sainis, 2010). Ten mg activated charcoal and 15 µl of 2% chitosan solution were used per ml of the extraction buffer. After 4 h at 4 °C with interval gentle vortexing, the purity ratio was enhanced to 4.72 whereas the CE purity was 2.18 with a recovery yield of 90.1%. In another study by Gantar et al. (2012), PC from *Limnothrix* sp. was purified with 1% (w/v) activated charcoal and 0.01 g/L chitosan was performed. After 15 min stirring and centrifugation, the purity ratio was increased to 3.6 from 2.0. In this study, ammonium sulfate precipitation was applied as an additional purification step, and the final purity reached was 4.2 (Gantar et al., 2012). In this study, Gupta and Sainis method was applied with slight modifications. After 20 mg/ml activated charcoal and 0.02% (w/v) chitosan addition and mixing on each hour at 4 °C for four hours, the purity ratios were raised above of the analytical grade.

4.3. Antioxidant Activity Measurement by DPPH Assay

The ammonium sulfate purified samples and the samples purified with activated charcoal and chitosan treatment were subjected to DPPH activity assay. The rapid color change from deep purple to pale yellow correlated with the L-ascorbic acid concentration was observed whereas the color change for the PC sample added wells were observed after 30 min incubation at room temperature (Figure 4.12). 100 µM DPPH in absolute MeOH was prepared fresh every two days and preserved at -20 °C. One mg/mL L-ascorbic acid stock was prepared in ultrapure water, stored at 4 °C and the same stock was used for 8 days long activity test process. Phycocyanin dilutions, on the other hand, were prepared in 0.01 M Na-phosphate buffer (pH=7.0) for 350 µL volume to be able to repeat the experiments if the results are not correct and to minimize errors due to pipetting. To prevent bubbling and evaporation during the preparation period, the mixing was performed by adding 50 µL volume into wells first and then adding the 250 µL volume. Before each run with spectrophotometer (SpectraMax, i3), 20 seconds linear shake at medium strength was applied. The cloudy color formation was observed when 100 µM DPPH dissolved in MeOH or MeOH was added into the 100 and 150 µg/mL concentrations of salting out samples. This caused high SD in the measurements. As can be seen from the R^2 values, the activity enhanced with increased concentration. The percent scavenging activity versus concentration graphs were drawn to 50 µg/mL for L-ascorbic acid because it reached the plateau after 50 µg/mL as can be verified from the bar charts. The absorbance reduction depending on the different concentrations of L-ascorbic acid and PC samples can be found in Appendix F.

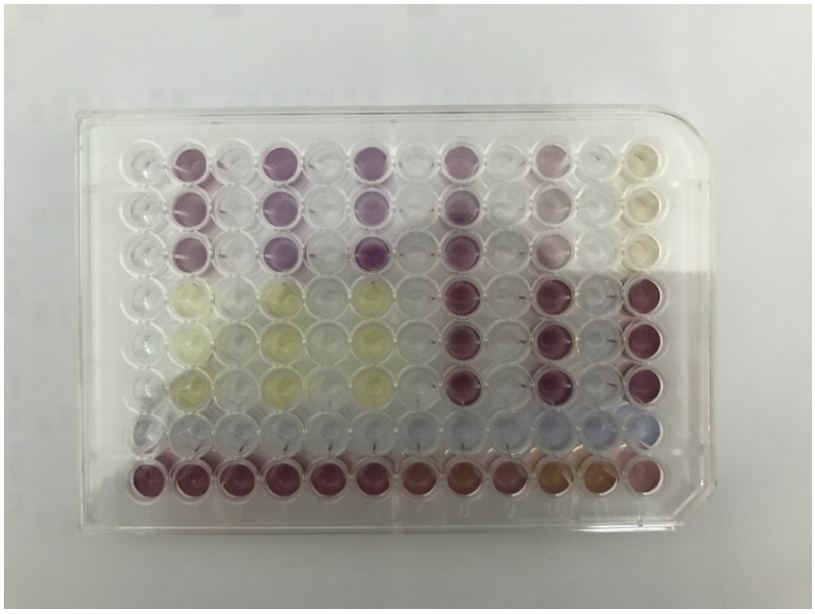


Figure 4.12. The 96-well plate design for DPPH assay.

From the activated charcoal and chitosan purified PC of the light stress reactors, the maximum scavenging activity (59.37±9.65%) was achieved at the highest concentration 150 µg/mL and its activity at concentrations from 10 to 150 µg/mL was compared with the same concentrations of the L-ascorbic acid as shown in Figure 4.13. From the equations of the Figure 4.14, the IC_{50} values for PC and L-ascorbic acid were calculated as 138.87 µg/mL and 28.49 µg/mL respectively.

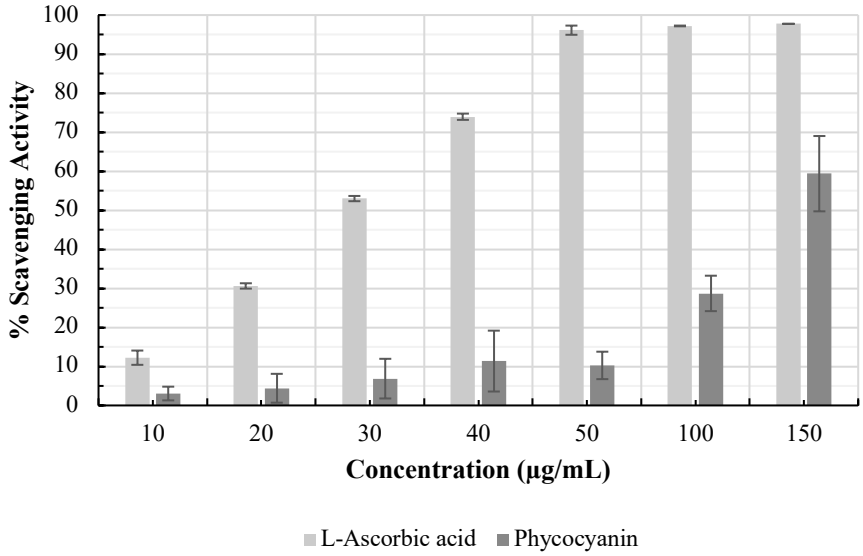


Figure 4.13. The scavenging activity of the PC obtained from the light stress and purified with the AC/CS method (L-ascorbic acid is used as control).

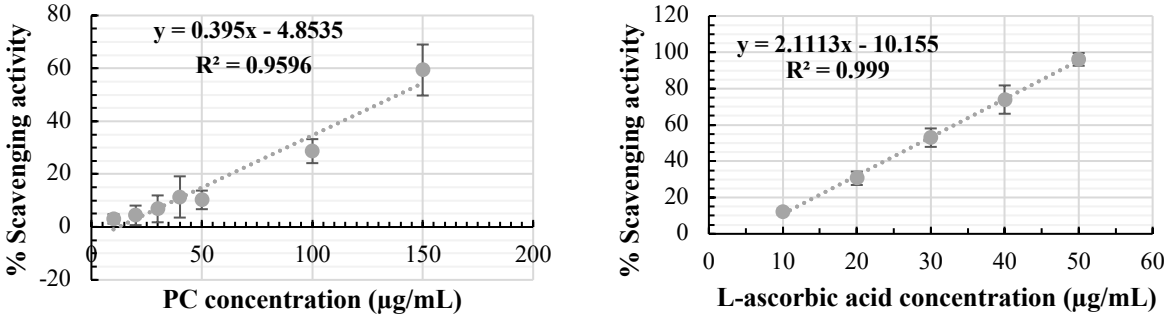


Figure 4.14. The individual graphs for the scavenging activity result of the AC/CS treated PC samples of light stress (left) and L-ascorbic acid control (right) for IC_{50} calculations.

In Figure 4.15, the scavenging activity corresponding to each concentration for ASP treated PC extracts of the light stress reactors was shown; however, due to the low CE concentration of the first reactor of light stress triplicates, the 100 and 150 µg/mL concentrations were run as duplicates. The maximum scavenging activity was 79.94±5.45% at 150 µg/mL concentration. IC_{50} values for L-ascorbic acid and PC were 28.14 µg/mL and 99.18 µg/mL respectively (Figure 4.16).

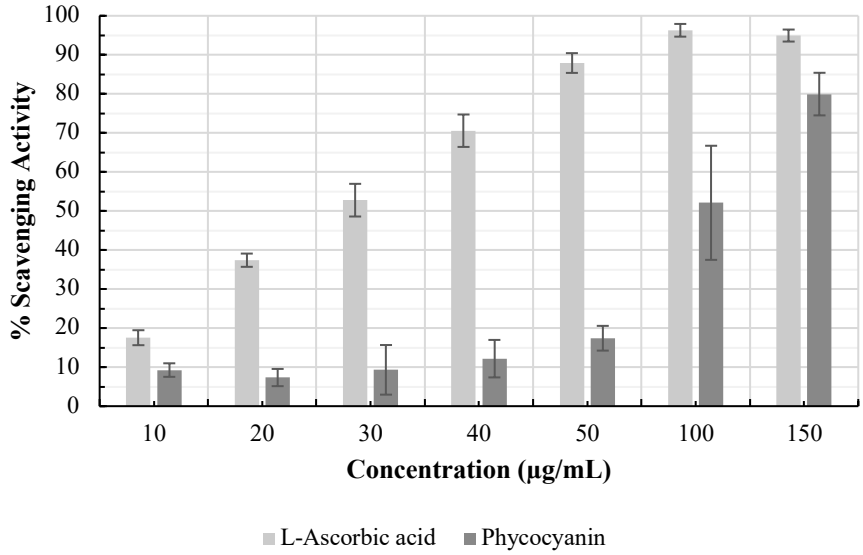


Figure 4.15. The scavenging activity of the PC obtained from the light stress and purified with the ASP method (L-ascorbic acid is used as control).

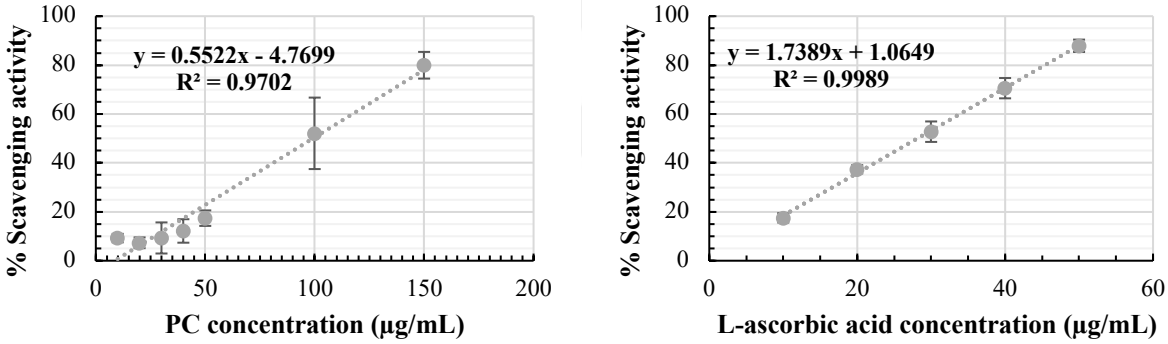


Figure 4.16. The individual graphs for the scavenging activity result of the ASP treated PC samples of light stress (left) and L-ascorbic acid control (right) for IC_{50} calculations.

When DPPH scavenging activity was calculated, the maximum scavenging activity from the AC/CS treated control samples was 42.53±0.94% at the highest 150 µg/mL concentration (Figure 4.17). The activity was lower which is correlated with the lower purity index obtained after the treatment. The IC_{50} for PC was found 183.51 µg/mL whereas it was 27.98 µg/mL for L-ascorbic acid (Figure 4.18).

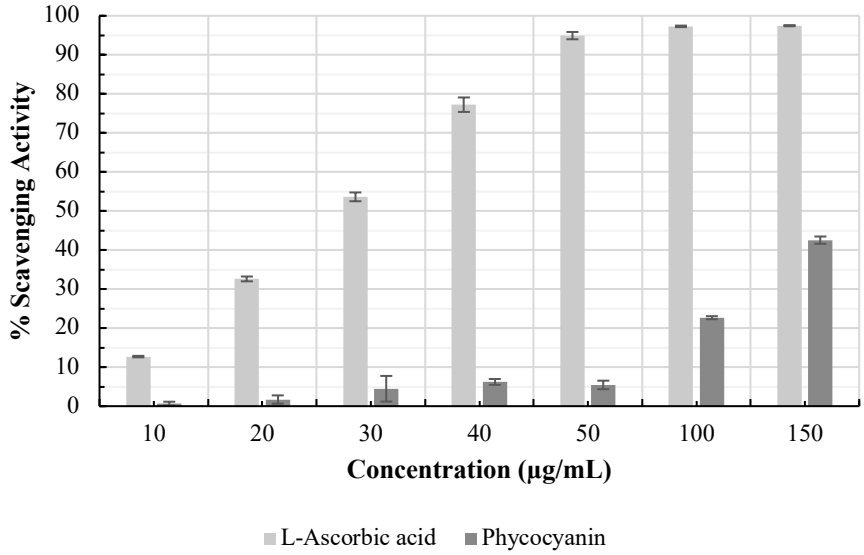


Figure 4.17. The scavenging activity of the PC obtained from the control group and purified with the AC/CS method (L-ascorbic acid is used as control).

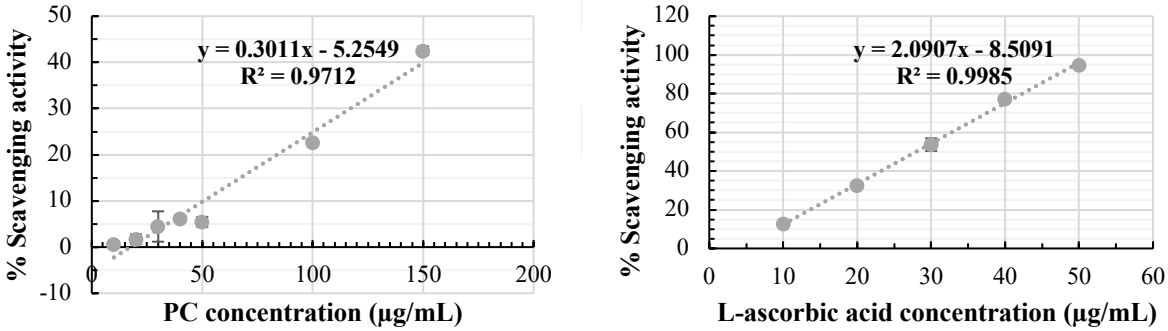


Figure 4.18. The individual graphs for the scavenging activity result of AC/CS treated PC samples of control group (left) and L-ascorbic acid control (right) for IC_{50} calculations.

After 30 min incubation with DPPH, the maximum scavenging activity from the ASP treated control samples was 70.48±11.28% at the highest 150 µg/mL concentration (Figure 4.19). The activity was lower than others except salt stress extracts. The IC_{50} for PC was found 109.67 µg/mL whereas it was 28.21 µg/mL for L-ascorbic acid (Figure 4.20).

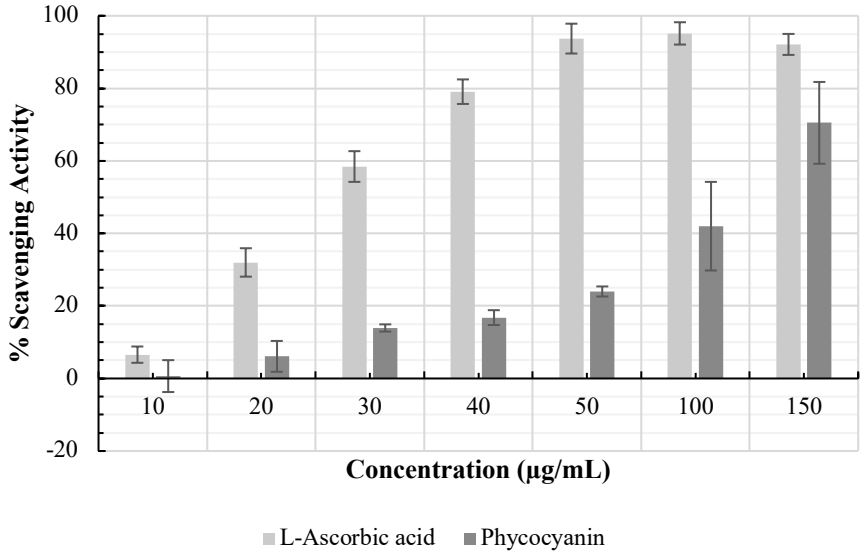


Figure 4.19. The scavenging activity of the PC obtained from the control group and purified with the ASP method (L-ascorbic acid is used as control).

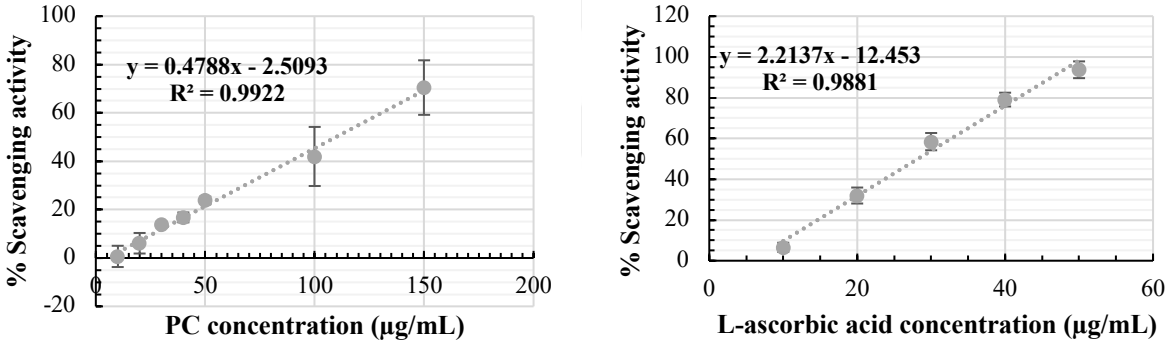


Figure 4.20. The individual graphs for the scavenging activity result ASP treated PC samples of control group (left) and L-ascorbic acid control (right) for IC_{50} calculations.

The maximum scavenging activity from the AC/CS purified hydrogen peroxide biomass samples was obtained at the highest 150 µg/mL concentration (63.76±4.46%) (Figure 4.21). The treatment resulted in the highest average purity factor among other groups which might be correlated with the highest activity. The IC_{50} for PC was found 129.04 µg/mL whereas it was 25.03 µg/mL for L-ascorbic acid (Figure 4.22).

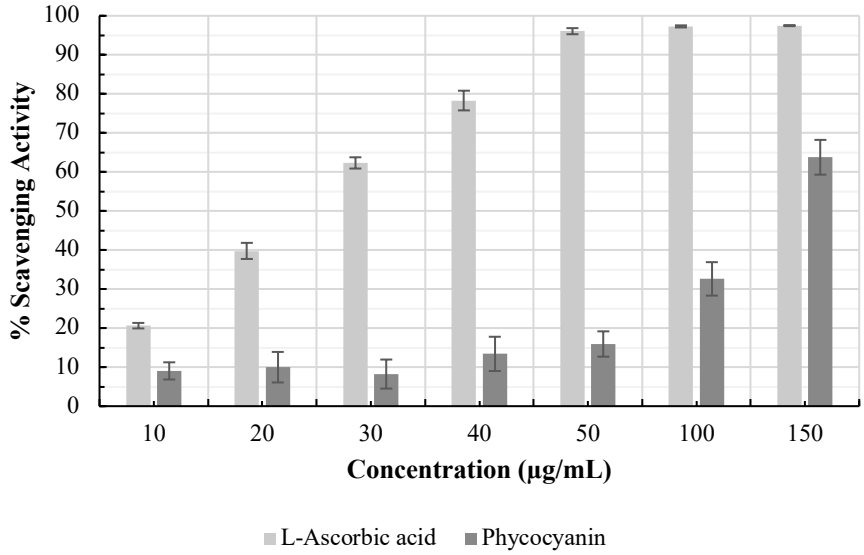


Figure 4.21. The scavenging activity of the PC obtained from the hydrogen peroxide stress and purified with the AC/CS method (L-ascorbic acid is used as control).

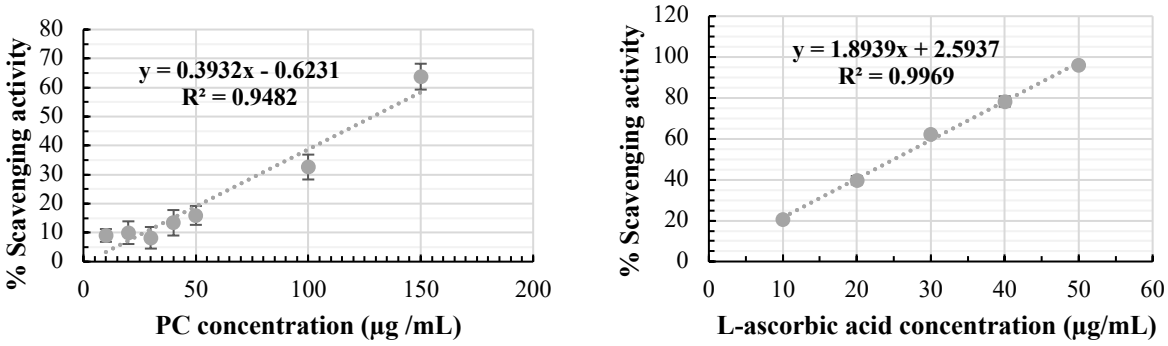


Figure 4.22. The individual graphs for the scavenging activity result of the AC/CS purified PC samples (left) of hydrogen peroxide stress and L-ascorbic acid control (right) for IC_{50} calculations.

The activity of the PC treated with ASP at the highest concentration (150 µg/mL) was 77.08±21.08% which is lower in average than the activity of the L-ascorbic acid at the same concentration (Figure 4.23). The IC_{50} for PC was found 95.66 µg/mL whereas it was 29.10 µg/mL for L-ascorbic acid (Figure 4.24).

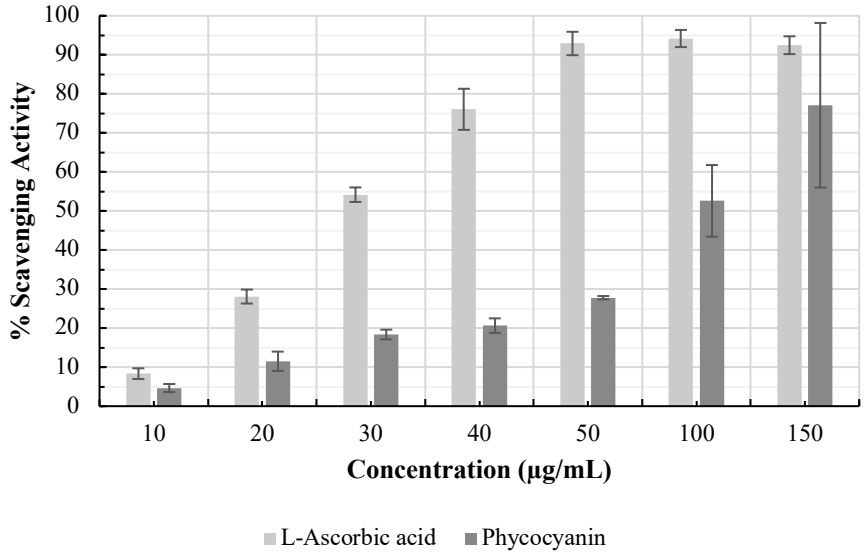


Figure 4.23. The scavenging activity of the PC obtained from the hydrogen peroxide stress and purified with the ASP method (L-ascorbic acid is used as control).

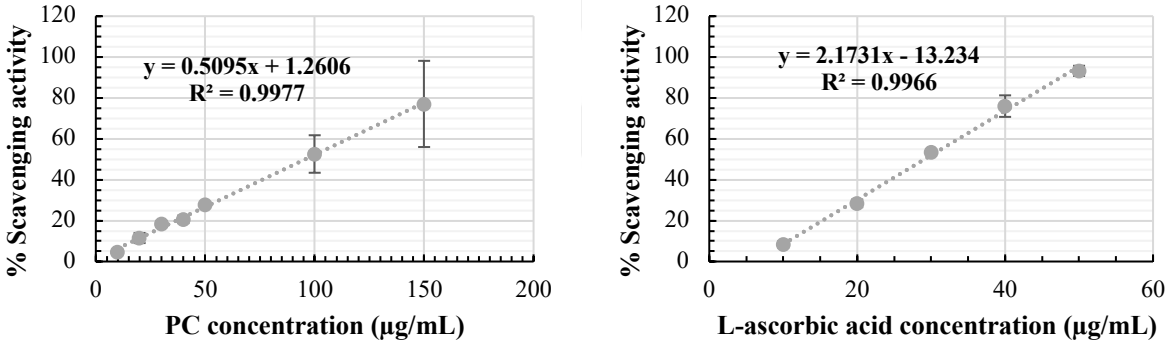


Figure 4.24. The individual graphs for the scavenging activity result of the ASP purified PC samples (left) of hydrogen peroxide stress and L-ascorbic acid control (right) for IC_{50} calculations.

In Figures 4.25 and 4.26, the percent scavenging activity results for AC/CS applied salt stress extracts were represented. For the 40 µg/mL concentration of L-ascorbic acid, duplicate value was used because one of the sample blanks were missed to add and there was no sample to try once more. The maximum scavenging activity were 47.54±3.02% at the highest 150 µg/mL concentration (Figure 4.25). The IC_{50} for PC was found 164.04 µg/mL whereas it was 23.90 µg/mL for L-ascorbic acid (Figure 4.26).

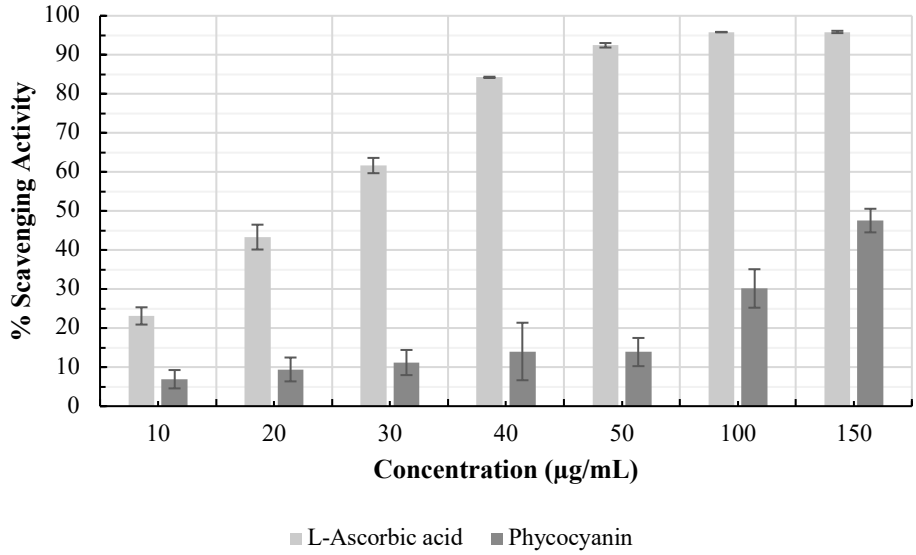


Figure 4.25. The scavenging activity of the PC obtained from the salt stress and purified with the AC/CS method (L-ascorbic acid is used as control).

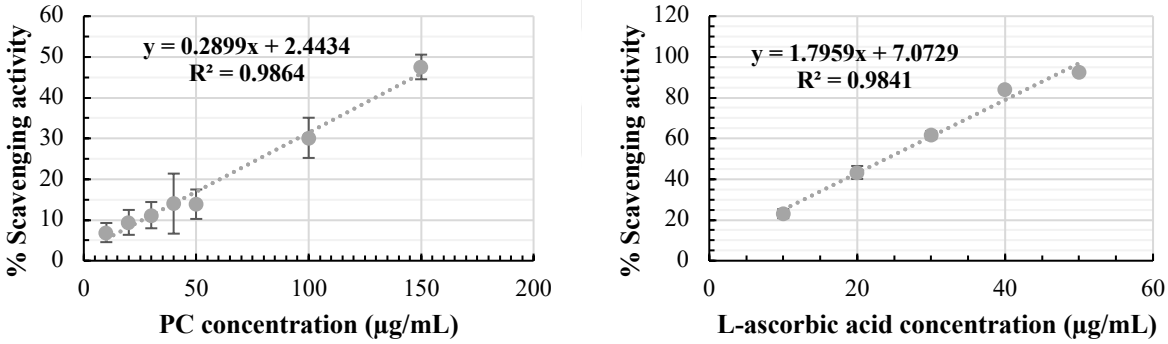


Figure 4.26. The individual graphs for the scavenging activity result of the AC/CS purified PC samples (left) of salt stress and L-ascorbic acid control (right) for IC_{50} calculations.

In Figure 4.27, the percent scavenging activities of the PC and the control compound L-ascorbic acid at different concentrations were shown. The maximum scavenging activity from the ASP treated salt stress extracts were 58.80±23.06% at the highest 150 µg/mL concentration. The IC_{50} for PC was found 124.96 µg/mL whereas it was 26.78 µg/mL for L-ascorbic acid (Figure 4.28).

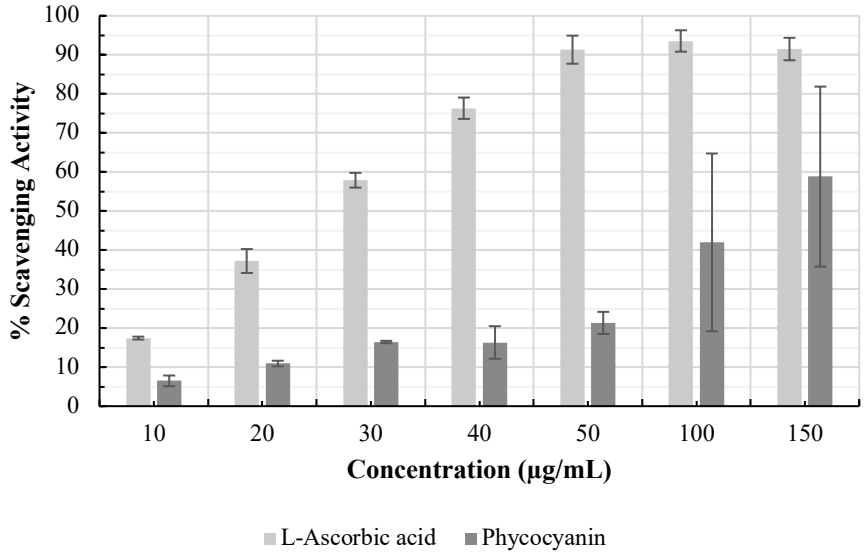


Figure 4.27. The scavenging activity of the PC obtained from the salt stress and purified with the ASP method (L-ascorbic acid is used as control).

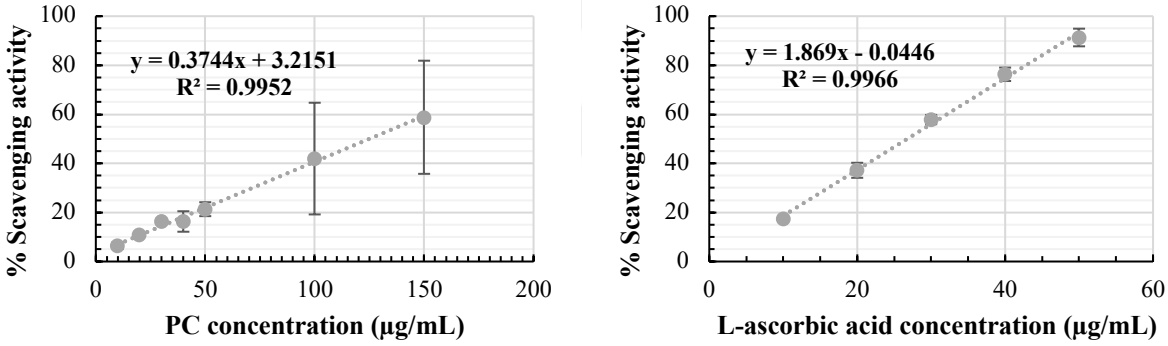


Figure 4.28. The individual graphs for the scavenging activity result of the ASP purified PC samples (left) of salt stress and L-ascorbic acid control (right) for IC_{50} calculations.

As a summary, Table 4.4 and 4.5 were generated showing the IC_{50} and % scavenging activity values for each group after each purification technique. IC_{50} value represents the concentration required for the 50 % inhibition and it is inversely correlated with the strength of the extracted PC. As can be seen from the tables, IC_{50} values for L-ascorbic acid ranged in from 23.90 to 29.10 µg/mL. Since range is small, it can be said that the assay conditions were comparable. The highest % scavenging activity with the lowest , IC_{50} value was obtained from the H_2O_2 stress group after AC/CS treatment. It was correlated with the achievement of the highest average purity index from the group. In addition, all of the stress conditions were resulted in higher activities in average than the control group after their PCs were purified with AC/CS application. This might indicate that applied stress factor might be increasing the final bioactivity of the obtained PC. As can be seen from the Table 4.5, the highest average % scavenging activity was achieved from the light stress group after ASP. Even

though the highest average purity was obtained from the control group after the ASP, the control group did not give the highest average % scavenging activity. This might also support that stress factors might be affecting the final bioactivity of the extracted PC positively.

Table 4.4. The % scavenging activity and IC_{50} results for each group after AC/CS purification.

AC/CS	**Control Group**		**Light Stress Group**		**Salt Stress Group**		**H_2O_2 Stress Group**	
	PC	L-Ascorbic acid	PC	L-Ascorbic acid	PC	L-Ascorbic acid	PC	L-Ascorbic acid
IC_{50} (µg/mL)	183.51	27.98	138.87	28.49	164.04	23.90	129.04	25.03
Scavenging (%) at 150 (µg/mL)	42.53 ±0.94	97.46 ±0.14	59.37 ±9.65	97.75 ±0.02	47.54 ±3.02	95.86 ±0.29	63.76 ±4.46	97.48 ±0.13

Table 4.5. The % scavenging activity and IC_{50} results for each group after ASP purification.

ASP	**Control Group**		**Light Stress Group**		**Salt Stress Group**		**H_2O_2 Stress Group**	
	PC	L-Ascorbic acid	PC	L-Ascorbic acid	PC	L-Ascorbic acid	PC	L-Ascorbic acid
IC_{50} (µg/mL)	109.67	28.21	99.18	28.14	124.96	26.78	95.66	29.10
Scavenging (%) at 150 (µg/mL)	70.48± 11.28	92.11± 2.89	79.94± 5.45	94.94± 1.54	58.80± 23.06	91.48± 2.88	77.08± 21.08	92.47± 2.28

In a study by Fekrat et al. (2019), ASP applied PC extracts of *A. platensis* gave 68.5±2.1% DPPH scavenging activity at 100 µg/mL whereas AC/CS treated PC samples showed 78.4±1.9% DPPH scavenging activity at the same concentration . In another study by Renugadevi et al. (2018), the PC extracted in acetate buffer by FT, and purified with dialysis after 62% ammonium sulfate precipitation gave 78.75% DPPH scavenging activity at 200 µg/mL concentration. Interestingly, the concentration versus percent scavenging activity was not linear in this study because 68.75% scavenging activity was achieved at the minimum tested concentration 5 µg/mL. In a work by Wu et al. (2016), the IC_{50} value for the DPPH activity of food grade PC obtained by ASP combined with dialysis was found 1.86±0.18 mg/mL. So, it can be said that the values obtained in this study is comparable with the literature. However, the ASP method gave higher percent scavenging activity than AC/CS

purification which was not expected. This might have been resulted from the fact that the pH of the AC/CS purified samples were not stabilized around pH 7 and their pH ranged from 5.77 to 6.06. Even though the PC is stable in the pH range studied, it might not be as active as it is in the pH 7 solution.

5. CONCLUSIONS

Phycocyanin is a water soluble, light absorbing pigment with the absorption maxima at 615-625 nm. It takes part in phycobilisomes with the other PBPs and enhances the light spectrum that algae can absorb beyond the chlorophyll. With diverse beneficial effects on cancer, inflammation, Alzheimer's and Parkinson diseases, the interest on PC is growing tremendously. For the time being, *Spirulina* is the most commercialized source whereas there are numerous other algae that can be a competitive source to *Spirulina* species. Competition can be classified by PC production, extraction efficiency and initial purity. In this study, the food grade PC was obtained from all studied algae and cyanobacteria species except *Scytonema* sp. after purification steps. Meanwhile, obtained purity of the crude extract was above the food grade level for *Synechocystis* sp. Also, the highest purity was achieved with further purification with column chromatography. Hence, it can be safely concluded as a potential PC source for commercialization.

Additionally, thermophilic and thermo acidophilic algae as a source of PC is also gaining much importance since the stability of PC during various processes is also critical on the commercialization procedure. It was shown in literature that PC obtained from these species is more resistant to denaturation conditions. Even though, PC yield was low for *G. sulphuraria*, its thermophilic PC and acidic growth conditions which eliminates the possibility of contamination during the cultivation make it a suitable candidate for PC production. Therefore, the optimization for PC production can be examined by changing its growth conditions with the changes in the medium composition.

Notably, it is the first and most comprehensive study performed on *D. tharense,* a local isolate from Denizli Region in Turkey. When its initial PC concentrations were compared to other species such as *Phormidium* sp., *G. sulphuraria*, and *Synechocystis* sp., it looks highly promising as a new PC source. In addition, even though there is no exact information about its nitrogen fixation capability, during the isolation stage, nitrogen free medium was tried to eliminate the growth of the other contaminating species in the local isolate culture and growth was observed. Hence, nitrogen fixation from the air is beneficial to reduce the cost of PC production from *D. tharense* by eliminating the need for additional nitrogen in the medium. For further studies, its growth conditions should be optimized to obtain the highest possible PC production from it. In addition, the optimization of the extraction process can be studied to get the highest yield.

As huge differences in PC content among stress conditions were not observed in this study, it can be concluded that less process control might be required for the commercial production of PC from *Synechocystis* sp. For example, during summer times when higher light intensities exist, the similar yields can be obtained.

When the pretreatment methods were compared, the most successful one was bead-beating. It is a fully automated system, so it is less prone to human error than mortar and pestle which is the second best among pretreatment techniques. This automatization also reduces the differences among replicates as biomass tend to stick to mortar and pestle surfaces. In addition, freeze-dried biomass gave higher PC yield for most of the tested conditions. Even though using dry biomass increases the production cost, the ability to be stored for longer times and not being susceptible to bacteriological degradation makes it advantageous over using wet biomass as starting material.

Lastly, the phycocyanin showed lower antioxidant activity than vitamin C after ASP and AC/CS applications. However, ASP treated PC resulted in higher average % scavenging activities than AC/CS treated PC. The antioxidant activity measurements also revealed that the stress factors that applied in this study to trigger oxidative stress mechanism in the cyanobacteria might be beneficial to increase the final bioactivity of the extracted phycocyanin.

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APPENDIX A: PHYLOGENY INFORMATION

Table A.1. Phylogeny information and morphological characteristics of the species used in the study.

Empire	Kingdom	Subkingdom	Phylum	Class	Subclass	Order	Family	Genus and Species	Strain	Morphology
Prokaryota	Eubacteria	Negibacteria	Cyanobacteria	Cyanophyceae	Oscillatoriophycidae	Oscillatoriales	*Oscillatoriaceae*	*Phormidium* sp.	SAG 14.92	Filamentous
Eukaryota	Plantae	Biliphyta	Rhodophyta	Cyanidiophyceae	Cyanidiophytina	Cyanidiales	*Galdieriaceae*	*G. sulphuraria*	SAG 21.92	Unicellular
Prokaryota	Eubacteria	Negibacteria	Cyanobacteria	Cyanophyceae	Synechococcophycidae	Synechococcales	*Merismopediaceae*	*Synechocystis* sp.	ATCC 27183	Unicellular
Prokaryota	Eubacteria	Negibacteria	Cyanobacteria	Cyanophyceae	Oscillatoriophycidae	Oscillatoriales	*Desertifilaceae*	*Desertifilum tharense*	Local isolate	Filamentous
Prokaryota	Eubacteria	Negibacteria	Cyanobacteria	Cyanophyceae	Nostocophycidae	Nostocales	*Scytonemataceae*	*Scytonema* sp.	UTEX LB 1163	Filamentous
Prokaryota	Eubacteria	Negibacteria	Cyanobacteria	Cyanophyceae	Nostocophycidae	Nostocales	*Nostocaceae*	*Nostoc* sp.	UTEX B EE5	Filamentous

APPENDIX B: BUFFER PREPARATIONS AND MEDIA RECIPIES

Buffer preparations (www.aatbio.com)

Acetate buffer (0.1 M at pH=4.5):

- Prepare 800 mL dH_2O
- Add 3.691 g of sodium acetate (anhydrous)
- Add 3.302 g of acetic acid (1.693 mL)
- Adjust pH
- Complete volume to 1L.

Na-Phosphate buffer (0.05M at pH=7.0):

- Prepare 800 mL dH_2O
- Add 7.742 g of Na-phosphate dibasic heptahydrate
- Add 2.914 g of Na-phosphate monobasic monohydrate
- Adjust pH
- Complete volume to 1L.

Media Preparations:

In the preparation of culture media, ultrapure water was used. All the chemicals added were prepared in stocks and autoclaved at 121 ℃ for 15 minutes. Additions from the stocks were performed in the biological hood to preserve their sterility. Metal solutions were not autoclaved. Vitamin additions into the media were performed after autoclave from the previously prepared and filter-sterilized aliquots which are preserved at -20℃. For solid media, 15 g/L agar was added before autoclave. Soil extract was prepared by pasteurization.

BG-11 Medium (UTEX)

Compound	Final Concentration
$NaNO_3$	17.6 mM
K_2HPO_4	0.22 mM
$MgSO_4.7H_2O$	0.30 mM
$CaCl_2.2H_2O$	0.20 mM
Citric Acid.H_2O	0.03 mM
Ferric Ammonium Citrate	0.02 mM
$Na_2EDTA.2H_2O$	0.002 mM
Na_2CO_3	0.18 mM
BG-11 Trace Metals Solution	1 mL/L

BG-11 Trace Metal Solution (UTEX)

Compound	Final Concentration
H_3BO_3	46 mM
$MnCl_2.4H_2O$	9 mM
$ZnSO_4.7H_2O$	0.77 mM
$Na_2MoO_4.2H_2O$	1.6 mM
$CuSO_4.5H_2O$	0.3 mM
$Co(NO_3)_2.6H_2O$	0.17 mM

Cyanidium Medium (=Acid) (SAG)

Compound	Final Concentration
$(NH_4)_2SO_4$	7.56 mM
K_2HPO_4	0.11 mM
$MgSO_47H_2O$	0.08 mM
Soil extract	30 mL/L
Glucose	27 g/L
Thiamin	1.1 g/L
Vitamin B_{12}	0.135 g/L

Seawater Medium (=SWESS “Seewasser+ Erddekokt+ Salze”) (SAG)

Compound	Final Concentration
KNO_3	1.98 mM
K_2HPO_4	0.11 mM
$MgSO_47H_2O$	0.08 mM
Soil extract	30 mL/L
SAG Micronutrient Solution	5 mL/L
Filtered Sea Water	Prepare in it
Vitamin B_{12}	0.135 g/L

SAG micronutrient solution (SAG)

Compound	Final Concentration
$ZnSO_4.7H_2O$	3.47 μM
$MnSO_4.4H_2O$	8.96 μM
H_3BO_3	0.16 mM
$Co(NO_3)_2.6H_2O$	0.003 mM
$Na_2MoO_4.2H_2O$	0.004 mM
$CuSO_4.5H_2O$	0.02 μM
$FeSO_4.7H_2O$	2.52 mM
EDTA	1.92 mM

MB3N Medium (UTEX)

Compound	Final Concentration
$NaNO_3$	8.82 mM
K_2HPO_4	0.43 mM
$MgSO_4.7H_2O$	0.30 mM
$CaCl_2.2H_2O$	0.17 mM
KH_2PO_4	1.29 mM
NaCl	0.43 mM
P IV Metal Solution	6 mL/L
Vitamin B_{12}	1.35 g/L
Biotin	1 mL/L
Thiamine	300 μl/L

P IV Metal Solution (UTEX)

Compound	**Final Concentration**
$Na_2EDTA.2H_2O$	2 mM
$FeCl_3.6H_2O$	0.36 mM
$MnCl_2.4H_2O$	0.21 mM
$ZnCl_2$	0.037 mM
$CoCl_2.6H_2O$	0.0084 mM
$Na_2MoO_4.2H_2O$	0.017 mM

APPENDIX C: ANOVA RESULTS

Table C. 1. The analysis of the concentration results for *D. tharense* in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	13.33576	4.91081	.143	-3.1593	29.8308
	mp-dry	22.53845*	4.91081	.006	6.0434	39.0335
	ft-dry	29.86667*	4.91081	.001	13.3717	46.3617
	son-wet	32.25563*	4.91081	.000	15.7606	48.7506
	son-dry	29.94607*	4.91081	.001	13.4511	46.4411
bb-wet	bb-dry	-13.33576	4.91081	.143	-29.8308	3.1593
	mp-dry	9.20269	4.91081	.460	-7.2923	25.6977
	ft-dry	16.53091*	4.91081	.049	.0359	33.0259
	son-wet	18.91987*	4.91081	.022	2.4249	35.4149
	son-dry	16.61031*	4.91081	.048	.1153	33.1053
mp-dry	bb-dry	-22.53845*	4.91081	.006	-39.0335	-6.0434
	bb-wet	-9.20269	4.91081	.460	-25.6977	7.2923
	ft-dry	7.32821	4.91081	.675	-9.1668	23.8232
	son-wet	9.71717	4.91081	.406	-6.7778	26.2122
	son-dry	7.40762	4.91081	.666	-9.0874	23.9026
ft-dry	bb-dry	-29.86667*	4.91081	.001	-46.3617	-13.3717
	bb-wet	-16.53091*	4.91081	.049	-33.0259	-.0359
	mp-dry	-7.32821	4.91081	.675	-23.8232	9.1668
	son-wet	2.38896	4.91081	.996	-14.1061	18.8840
	son-dry	.07940	4.91081	1.000	-16.4156	16.5744
son-wet	bb-dry	-32.25563*	4.91081	.000	-48.7506	-15.7606
	bb-wet	-18.91987*	4.91081	.022	-35.4149	-2.4249
	mp-dry	-9.71717	4.91081	.406	-26.2122	6.7778
	ft-dry	-2.38896	4.91081	.996	-18.8840	14.1061
	son-dry	-2.30956	4.91081	.996	-18.8046	14.1855
son-dry	bb-dry	-29.94607*	4.91081	.001	-46.4411	-13.4511
	bb-wet	-16.61031*	4.91081	.048	-33.1053	-.1153
	mp-dry	-7.40762	4.91081	.666	-23.9026	9.0874
	ft-dry	-.07940	4.91081	1.000	-16.5744	16.4156
	son-wet	2.30956	4.91081	.996	-14.1855	18.8046
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 2. The analysis of the concentration results for *G. sulphuraria* in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-.89344	1.14707	.983	-4.8102	3.0233
	mp-dry	2.09400	1.14707	.554	-1.8228	6.0108
	ft-wet	4.42546*	1.14707	.022	.5087	8.3422
	ft-dry	4.33567*	1.14707	.026	.4189	8.2524
	son-wet	5.41161*	1.14707	.005	1.4948	9.3284
	son-dry	3.45773	1.14707	.100	-.4590	7.3745
bb-wet	bb-dry	.89344	1.14707	.983	-3.0233	4.8102
	mp-dry	2.98744	1.14707	.196	-.9293	6.9042
	ft-wet	5.31891*	1.14707	.005	1.4021	9.2357
	ft-dry	5.22911*	1.14707	.006	1.3123	9.1459
	son-wet	6.30505*	1.14707	.001	2.3883	10.2218
	son-dry	4.35118*	1.14707	.025	.4344	8.2680
mp-dry	bb-dry	-2.09400	1.14707	.554	-6.0108	1.8228
	bb-wet	-2.98744	1.14707	.196	-6.9042	.9293
	ft-wet	2.33146	1.14707	.438	-1.5853	6.2482
	ft-dry	2.24167	1.14707	.480	-1.6751	6.1584
	son-wet	3.31761	1.14707	.123	-.5992	7.2344
	son-dry	1.36373	1.14707	.887	-2.5530	5.2805
ft-wet	bb-dry	-4.42546*	1.14707	.022	-8.3422	-.5087
	bb-wet	-5.31891*	1.14707	.005	-9.2357	-1.4021
	mp-dry	-2.33146	1.14707	.438	-6.2482	1.5853
	ft-dry	-.08980	1.14707	1.000	-4.0066	3.8270
	son-wet	.98615	1.14707	.973	-2.9306	4.9029
	son-dry	-.96773	1.14707	.975	-4.8845	2.9490
ft-dry	bb-dry	-4.33567*	1.14707	.026	-8.2524	-.4189
	bb-wet	-5.22911*	1.14707	.006	-9.1459	-1.3123
	mp-dry	-2.24167	1.14707	.480	-6.1584	1.6751
	ft-wet	.08980	1.14707	1.000	-3.8270	4.0066
	son-wet	1.07594	1.14707	.959	-2.8408	4.9927
	son-dry	-.87793	1.14707	.985	-4.7947	3.0388
son-wet	bb-dry	-5.41161*	1.14707	.005	-9.3284	-1.4948
	bb-wet	-6.30505*	1.14707	.001	-10.2218	-2.3883
	mp-dry	-3.31761	1.14707	.123	-7.2344	.5992
	ft-wet	-.98615	1.14707	.973	-4.9029	2.9306
	ft-dry	-1.07594	1.14707	.959	-4.9927	2.8408

Table C. 2. (continued)

son-wet	son-dry	-1.95388	1.14707	.625	-5.8707	1.9629
son-dry	bb-dry	-3.45773	1.14707	.100	-7.3745	.4590
	bb-wet	-4.35118*	1.14707	.025	-8.2680	-.4344
	mp-dry	-1.36373	1.14707	.887	-5.2805	2.5530
	ft-wet	.96773	1.14707	.975	-2.9490	4.8845
	ft-dry	.87793	1.14707	.985	-3.0388	4.7947
	son-wet	1.95388	1.14707	.625	-1.9629	5.8707
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 3. The analysis of the concentration results for *Phormidium* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	2.35118	10.25812	1.000	-32.6761	37.3784
	mp-dry	-1.07890	10.25812	1.000	-36.1061	33.9483
	ft-wet	2.96029	10.25812	1.000	-32.0670	37.9875
	ft-dry	-15.63071	10.25812	.728	-50.6580	19.3965
	son-wet	3.84579	10.25812	1.000	-31.1815	38.8730
	son-dry	2.46941	10.25812	1.000	-32.5578	37.4967
bb-wet	bb-dry	-2.35118	10.25812	1.000	-37.3784	32.6761
	mp-dry	-3.43008	10.25812	1.000	-38.4573	31.5972
	ft-wet	.60911	10.25812	1.000	-34.4181	35.6364
	ft-dry	-17.98189	10.25812	.596	-53.0091	17.0454
	son-wet	1.49461	10.25812	1.000	-33.5326	36.5219
	son-dry	.11824	10.25812	1.000	-34.9090	35.1455
mp-dry	bb-dry	1.07890	10.25812	1.000	-33.9483	36.1061
	bb-wet	3.43008	10.25812	1.000	-31.5972	38.4573
	ft-wet	4.03919	10.25812	1.000	-30.9881	39.0664
	ft-dry	-14.55181	10.25812	.784	-49.5791	20.4754
	son-wet	4.92469	10.25812	.999	-30.1026	39.9519
	son-dry	3.54831	10.25812	1.000	-31.4789	38.5756
ft-wet	bb-dry	-2.96029	10.25812	1.000	-37.9875	32.0670
	bb-wet	-.60911	10.25812	1.000	-35.6364	34.4181
	mp-dry	-4.03919	10.25812	1.000	-39.0664	30.9881
	ft-dry	-18.59100	10.25812	.561	-53.6182	16.4362
	son-wet	.88550	10.25812	1.000	-34.1417	35.9127
	son-dry	-.49087	10.25812	1.000	-35.5181	34.5364

Table C. 3. (continued)

ft-dry	bb-dry	15.63071	10.25812	.728	-19.3965	50.6580
	bb-wet	17.98189	10.25812	.596	-17.0454	53.0091
	mp-dry	14.55181	10.25812	.784	-20.4754	49.5791
	ft-wet	18.59100	10.25812	.561	-16.4362	53.6182
	son-wet	19.47650	10.25812	.512	-15.5507	54.5037
	son-dry	18.10012	10.25812	.589	-16.9271	53.1274
son-wet	bb-dry	-3.84579	10.25812	1.000	-38.8730	31.1815
	bb-wet	-1.49461	10.25812	1.000	-36.5219	33.5326
	mp-dry	-4.92469	10.25812	.999	-39.9519	30.1026
	ft-wet	-.88550	10.25812	1.000	-35.9127	34.1417
	ft-dry	-19.47650	10.25812	.512	-54.5037	15.5507
	son-dry	-1.37637	10.25812	1.000	-36.4036	33.6509
son-dry	bb-dry	-2.46941	10.25812	1.000	-37.4967	32.5578
	bb-wet	-.11824	10.25812	1.000	-35.1455	34.9090
	mp-dry	-3.54831	10.25812	1.000	-38.5756	31.4789
	ft-wet	.49087	10.25812	1.000	-34.5364	35.5181
	ft-dry	-18.10012	10.25812	.589	-53.1274	16.9271
	son-wet	1.37637	10.25812	1.000	-33.6509	36.4036

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 4. The analysis of the concentration results for *Scytonema* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-1.81110	3.72411	.999	-14.5274	10.9052
	mp-dry	.84107	3.72411	1.000	-11.8752	13.5574
	ft-wet	5.37379	3.72411	.771	-7.3425	18.0901
	ft-dry	2.72385	3.72411	.988	-9.9924	15.4401
	son-wet	-.82204	3.72411	1.000	-13.5383	11.8943
	son-dry	2.95293	3.72411	.982	-9.7634	15.6692
bb-wet	bb-dry	1.81110	3.72411	.999	-10.9052	14.5274
	mp-dry	2.65217	3.72411	.990	-10.0641	15.3685
	ft-wet	7.18489	3.72411	.494	-5.5314	19.9012
	ft-dry	4.53494	3.72411	.876	-8.1814	17.2512
	son-wet	.98906	3.72411	1.000	-11.7272	13.7054
	son-dry	4.76403	3.72411	.850	-7.9523	17.4803
mp-dry	bb-dry	-.84107	3.72411	1.000	-13.5574	11.8752
	bb-wet	-2.65217	3.72411	.990	-15.3685	10.0641

Table C. 4. (continued)

mp-dry	ft-wet	4.53272	3.72411	.876	-8.1836	17.2490
	ft-dry	1.88277	3.72411	.998	-10.8335	14.5991
	son-wet	-1.66311	3.72411	.999	-14.3794	11.0532
	son-dry	2.11186	3.72411	.997	-10.6044	14.8282
ft-wet	bb-dry	-5.37379	3.72411	.771	-18.0901	7.3425
	bb-wet	-7.18489	3.72411	.494	-19.9012	5.5314
	mp-dry	-4.53272	3.72411	.876	-17.2490	8.1836
	ft-dry	-2.64995	3.72411	.990	-15.3662	10.0663
	son-wet	-6.19583	3.72411	.648	-18.9121	6.5205
	son-dry	-2.42086	3.72411	.993	-15.1372	10.2954
ft-dry	bb-dry	-2.72385	3.72411	.988	-15.4401	9.9924
	bb-wet	-4.53494	3.72411	.876	-17.2512	8.1814
	mp-dry	-1.88277	3.72411	.998	-14.5991	10.8335
	ft-wet	2.64995	3.72411	.990	-10.0663	15.3662
	son-wet	-3.54588	3.72411	.957	-16.2622	9.1704
	son-dry	.22909	3.72411	1.000	-12.4872	12.9454
son-wet	bb-dry	.82204	3.72411	1.000	-11.8943	13.5383
	bb-wet	-.98906	3.72411	1.000	-13.7054	11.7272
	mp-dry	1.66311	3.72411	.999	-11.0532	14.3794
	ft-wet	6.19583	3.72411	.648	-6.5205	18.9121
	ft-dry	3.54588	3.72411	.957	-9.1704	16.2622
	son-dry	3.77497	3.72411	.942	-8.9413	16.4913
son-dry	bb-dry	-2.95293	3.72411	.982	-15.6692	9.7634
	bb-wet	-4.76403	3.72411	.850	-17.4803	7.9523
	mp-dry	-2.11186	3.72411	.997	-14.8282	10.6044
	ft-wet	2.42086	3.72411	.993	-10.2954	15.1372
	ft-dry	-.22909	3.72411	1.000	-12.9454	12.4872
	son-wet	-3.77497	3.72411	.942	-16.4913	8.9413

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 5. The analysis of the concentration results for *Synechocystis* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	mp-dry	142.69333*	11.55205	.000	105.6996	179.6871
	ft-dry	178.49667*	11.55205	.000	141.5029	215.4904
	son-dry	179.65667*	11.55205	.000	142.6629	216.6504
mp-dry	bb-dry	-142.69333*	11.55205	.000	-179.6871	-105.6996

Table C. 5. (continued)

mp-dry	ft-dry	35.80333	11.55205	.058	-1.1904	72.7971
	son-dry	36.96333	11.55205	.050	-.0304	73.9571
ft-dry	bb-dry	-178.49667*	11.55205	.000	-215.4904	-141.5029
	mp-dry	-35.80333	11.55205	.058	-72.7971	1.1904
	son-dry	1.16000	11.55205	1.000	-35.8337	38.1537
son-dry	bb-dry	-179.65667*	11.55205	.000	-216.6504	-142.6629
	mp-dry	-36.96333	11.55205	.050	-73.9571	.0304
	ft-dry	-1.16000	11.55205	1.000	-38.1537	35.8337
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 6. The analysis of the concentration results for *Nostoc* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	.16667	.77309	1.000	-2.4731	2.8065
	mp-dry	-1.99333	.77309	.204	-4.6331	.6465
	ft-wet	.45667	.77309	.996	-2.1831	3.0965
	ft-dry	-2.59333	.77309	.056	-5.2331	.0465
	son-wet	-.05333	.77309	1.000	-2.6931	2.5865
	son-dry	-1.44000	.77309	.532	-4.0798	1.1998
bb-wet	bb-dry	-.16667	.77309	1.000	-2.8065	2.4731
	mp-dry	-2.16000	.77309	.145	-4.7998	.4798
	ft-wet	.29000	.77309	1.000	-2.3498	2.9298
	ft-dry	-2.76000*	.77309	.038	-5.3998	-.1202
	son-wet	-.22000	.77309	1.000	-2.8598	2.4198
	son-dry	-1.60667	.77309	.414	-4.2465	1.0331
mp-dry	bb-dry	1.99333	.77309	.204	-.6465	4.6331
	bb-wet	2.16000	.77309	.145	-.4798	4.7998
	ft-wet	2.45000	.77309	.077	-.1898	5.0898
	ft-dry	-.60000	.77309	.984	-3.2398	2.0398
	son-wet	1.94000	.77309	.227	-.6998	4.5798
	son-dry	.55333	.77309	.989	-2.0865	3.1931
ft-wet	bb-dry	-.45667	.77309	.996	-3.0965	2.1831
	bb-wet	-.29000	.77309	1.000	-2.9298	2.3498
	mp-dry	-2.45000	.77309	.077	-5.0898	.1898
	ft-dry	-3.05000*	.77309	.019	-5.6898	-.4102
	son-wet	-.51000	.77309	.993	-3.1498	2.1298

Table C. 6. (continued)

ft-wet	son-dry	-1.89667	.77309	.247	-4.5365	.7431
ft-dry	bb-dry	2.59333	.77309	.056	-.0465	5.2331
	bb-wet	2.76000*	.77309	.038	.1202	5.3998
	mp-dry	.60000	.77309	.984	-2.0398	3.2398
	ft-wet	3.05000*	.77309	.019	.4102	5.6898
	son-wet	2.54000	.77309	.063	-.0998	5.1798
	son-dry	1.15333	.77309	.745	-1.4865	3.7931
son-wet	bb-dry	.05333	.77309	1.000	-2.5865	2.6931
	bb-wet	.22000	.77309	1.000	-2.4198	2.8598
	mp-dry	-1.94000	.77309	.227	-4.5798	.6998
	ft-wet	.51000	.77309	.993	-2.1298	3.1498
	ft-dry	-2.54000	.77309	.063	-5.1798	.0998
	son-dry	-1.38667	.77309	.572	-4.0265	1.2531
son-dry	bb-dry	1.44000	.77309	.532	-1.1998	4.0798
	bb-wet	1.60667	.77309	.414	-1.0331	4.2465
	mp-dry	-.55333	.77309	.989	-3.1931	2.0865
	ft-wet	1.89667	.77309	.247	-.7431	4.5365
	ft-dry	-1.15333	.77309	.745	-3.7931	1.4865
	son-wet	1.38667	.77309	.572	-1.2531	4.0265

*. The mean difference is significant at the 0.05 level.

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 7. The analysis of the concentration results for *Nostoc* sp. in one-way ANOVA with Tukey's post-hoc (mp-dry and son-dry were excluded).

Multiple Comparisons						
Dependent Variable: PC_conc						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	.16667	.35444	.988	-.9998	1.3332
	ft-wet	.45667	.35444	.704	-.7098	1.6232
	ft-dry	-2.59333*	.35444	.000	-3.7598	-1.4268
	son-wet	-.05333	.35444	1.000	-1.2198	1.1132
bb-wet	bb-dry	-.16667	.35444	.988	-1.3332	.9998
	ft-wet	.29000	.35444	.919	-.8765	1.4565
	ft-dry	-2.76000*	.35444	.000	-3.9265	-1.5935
	son-wet	-.22000	.35444	.968	-1.3865	.9465
ft-wet	bb-dry	-.45667	.35444	.704	-1.6232	.7098
	bb-wet	-.29000	.35444	.919	-1.4565	.8765
	ft-dry	-3.05000*	.35444	.000	-4.2165	-1.8835
	son-wet	-.51000	.35444	.619	-1.6765	.6565

Table C. 7. (continued)

ft-dry	bb-dry	2.59333*	.35444	.000	1.4268	3.7598
	bb-wet	2.76000*	.35444	.000	1.5935	3.9265
	ft-wet	3.05000*	.35444	.000	1.8835	4.2165
	son-wet	2.54000*	.35444	.000	1.3735	3.7065
son-wet	bb-dry	.05333	.35444	1.000	-1.1132	1.2198
	bb-wet	.22000	.35444	.968	-.9465	1.3865
	ft-wet	.51000	.35444	.619	-.6565	1.6765
	ft-dry	-2.54000*	.35444	.000	-3.7065	-1.3735
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 8. The analysis of the purity results for *D. tharense* in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	.06764	.11187	.989	-.3081	.4434
	mp-dry	.09176	.11187	.958	-.2840	.4675
	ft-dry	.21538	.11187	.433	-.1604	.5911
	son-wet	.18159	.11187	.600	-.1942	.5573
	son-dry	.14583	.11187	.778	-.2299	.5216
bb-wet	bb-dry	-.06764	.11187	.989	-.4434	.3081
	mp-dry	.02412	.11187	1.000	-.3516	.3999
	ft-dry	.14774	.11187	.769	-.2280	.5235
	son-wet	.11395	.11187	.903	-.2618	.4897
	son-dry	.07819	.11187	.979	-.2976	.4539
mp-dry	bb-dry	-.09176	.11187	.958	-.4675	.2840
	bb-wet	-.02412	.11187	1.000	-.3999	.3516
	ft-dry	.12362	.11187	.870	-.2521	.4994
	son-wet	.08983	.11187	.962	-.2859	.4656
	son-dry	.05407	.11187	.996	-.3217	.4298
ft-dry	bb-dry	-.21538	.11187	.433	-.5911	.1604
	bb-wet	-.14774	.11187	.769	-.5235	.2280
	mp-dry	-.12362	.11187	.870	-.4994	.2521
	son-wet	-.03379	.11187	1.000	-.4095	.3420
	son-dry	-.06955	.11187	.987	-.4453	.3062
son-wet	bb-dry	-.18159	.11187	.600	-.5573	.1942
	bb-wet	-.11395	.11187	.903	-.4897	.2618
	mp-dry	-.08983	.11187	.962	-.4656	.2859

Table C. 8. (continued)

son-wet	ft-dry	.03379	.11187	1.000	-.3420	.4095
	son-dry	-.03576	.11187	.999	-.4115	.3400
son-dry	bb-dry	-.14583	.11187	.778	-.5216	.2299
	bb-wet	-.07819	.11187	.979	-.4539	.2976
	mp-dry	-.05407	.11187	.996	-.4298	.3217
	ft-dry	.06955	.11187	.987	-.3062	.4453
	son-wet	.03576	.11187	.999	-.3400	.4115

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 9. The analysis of the purity results for *G. sulphuraria* in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-.07572	.32373	1.000	-1.1811	1.0297
	mp-dry	.06186	.32373	1.000	-1.0435	1.1673
	ft-wet	.01458	.32373	1.000	-1.0908	1.1200
	ft-dry	-.38966	.32373	.882	-1.4951	.7157
	son-wet	.18877	.32373	.996	-.9166	1.2942
	son-dry	-.14210	.32373	.999	-1.2475	.9633
bb-wet	bb-dry	.07572	.32373	1.000	-1.0297	1.1811
	mp-dry	.13758	.32373	.999	-.9678	1.2430
	ft-wet	.09030	.32373	1.000	-1.0151	1.1957
	ft-dry	-.31394	.32373	.953	-1.4194	.7915
	son-wet	.26449	.32373	.979	-.8409	1.3699
	son-dry	-.06638	.32373	1.000	-1.1718	1.0390
mp-dry	bb-dry	-.06186	.32373	1.000	-1.1673	1.0435
	bb-wet	-.13758	.32373	.999	-1.2430	.9678
	ft-wet	-.04728	.32373	1.000	-1.1527	1.0581
	ft-dry	-.45153	.32373	.796	-1.5569	.6539
	son-wet	.12691	.32373	1.000	-.9785	1.2323
	son-dry	-.20397	.32373	.994	-1.3094	.9014
ft-wet	bb-dry	-.01458	.32373	1.000	-1.1200	1.0908
	bb-wet	-.09030	.32373	1.000	-1.1957	1.0151
	mp-dry	.04728	.32373	1.000	-1.0581	1.1527
	ft-dry	-.40425	.32373	.863	-1.5097	.7012
	son-wet	.17419	.32373	.998	-.9312	1.2796
	son-dry	-.15669	.32373	.999	-1.2621	.9487
ft-dry	bb-dry	.38966	.32373	.882	-.7157	1.4951

Table C. 9. (continued)

ft-dry	bb-wet	.31394	.32373	.953	-.7915	1.4194
	mp-dry	.45153	.32373	.796	-.6539	1.5569
	ft-wet	.40425	.32373	.863	-.7012	1.5097
	son-wet	.57844	.32373	.576	-.5270	1.6838
	son-dry	.24756	.32373	.985	-.8578	1.3530
son-wet	bb-dry	-.18877	.32373	.996	-1.2942	.9166
	bb-wet	-.26449	.32373	.979	-1.3699	.8409
	mp-dry	-.12691	.32373	1.000	-1.2323	.9785
	ft-wet	-.17419	.32373	.998	-1.2796	.9312
	ft-dry	-.57844	.32373	.576	-1.6838	.5270
	son-dry	-.33087	.32373	.940	-1.4363	.7745
son-dry	bb-dry	.14210	.32373	.999	-.9633	1.2475
	bb-wet	.06638	.32373	1.000	-1.0390	1.1718
	mp-dry	.20397	.32373	.994	-.9014	1.3094
	ft-wet	.15669	.32373	.999	-.9487	1.2621
	ft-dry	-.24756	.32373	.985	-1.3530	.8578
	son-wet	.33087	.32373	.940	-.7745	1.4363

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 10. The analysis of the purity results for *G. sulphuraria* in one-way ANOVA with Tukey's post-hoc after FT-dry and SON-dry were excluded.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-.07572	.04254	.434	-.2157	.0643
	mp-dry	.06186	.04254	.610	-.0781	.2019
	ft-wet	.01458	.04254	.997	-.1254	.1546
	son-wet	.18877*	.04254	.009	.0488	.3288
bb-wet	bb-dry	.07572	.04254	.434	-.0643	.2157
	mp-dry	.13758	.04254	.055	-.0024	.2776
	ft-wet	.09030	.04254	.282	-.0497	.2303
	son-wet	.26449*	.04254	.001	.1245	.4045
mp-dry	bb-dry	-.06186	.04254	.610	-.2019	.0781
	bb-wet	-.13758	.04254	.055	-.2776	.0024
	ft-wet	-.04728	.04254	.797	-.1873	.0927
	son-wet	.12691	.04254	.080	-.0131	.2669
ft-wet	bb-dry	-.01458	.04254	.997	-.1546	.1254
	bb-wet	-.09030	.04254	.282	-.2303	.0497
	mp-dry	.04728	.04254	.797	-.0927	.1873

Table C. 10. (continued)

ft-wet	son-wet	.17419*	.04254	.014	.0342	.3142
son-wet	bb-dry	-.18877*	.04254	.009	-.3288	-.0488
	bb-wet	-.26449*	.04254	.001	-.4045	-.1245
	mp-dry	-.12691	.04254	.080	-.2669	.0131
	ft-wet	-.17419*	.04254	.014	-.3142	-.0342
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 11. The analysis of the purity results for *Phormidium* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	.06537	.15708	.999	-.4710	.6017
	mp-dry	-.10120	.15708	.994	-.6375	.4351
	ft-wet	-.12706	.15708	.980	-.6634	.4093
	ft-dry	-.12468	.15708	.982	-.6610	.4117
	son-wet	-.04434	.15708	1.000	-.5807	.4920
	son-dry	-.08572	.15708	.997	-.6221	.4506
bb-wet	bb-dry	-.06537	.15708	.999	-.6017	.4710
	mp-dry	-.16657	.15708	.930	-.7029	.3698
	ft-wet	-.19243	.15708	.873	-.7288	.3439
	ft-dry	-.19005	.15708	.879	-.7264	.3463
	son-wet	-.10970	.15708	.991	-.6461	.4266
	son-dry	-.15109	.15708	.954	-.6874	.3853
mp-dry	bb-dry	.10120	.15708	.994	-.4351	.6375
	bb-wet	.16657	.15708	.930	-.3698	.7029
	ft-wet	-.02586	.15708	1.000	-.5622	.5105
	ft-dry	-.02348	.15708	1.000	-.5598	.5129
	son-wet	.05686	.15708	1.000	-.4795	.5932
	son-dry	.01548	.15708	1.000	-.5209	.5518
ft-wet	bb-dry	.12706	.15708	.980	-.4093	.6634
	bb-wet	.19243	.15708	.873	-.3439	.7288
	mp-dry	.02586	.15708	1.000	-.5105	.5622
	ft-dry	.00238	.15708	1.000	-.5340	.5387
	son-wet	.08273	.15708	.998	-.4536	.6191
	son-dry	.04134	.15708	1.000	-.4950	.5777
ft-dry	bb-dry	.12468	.15708	.982	-.4117	.6610
	bb-wet	.19005	.15708	.879	-.3463	.7264

Table C. 11. (continued)

ft-dry	mp-dry	.02348	.15708	1.000	-.5129	.5598
	ft-wet	-.00238	.15708	1.000	-.5387	.5340
	son-wet	.08035	.15708	.998	-.4560	.6167
	son-dry	.03897	.15708	1.000	-.4974	.5753
son-wet	bb-dry	.04434	.15708	1.000	-.4920	.5807
	bb-wet	.10970	.15708	.991	-.4266	.6461
	mp-dry	-.05686	.15708	1.000	-.5932	.4795
	ft-wet	-.08273	.15708	.998	-.6191	.4536
	ft-dry	-.08035	.15708	.998	-.6167	.4560
	son-dry	-.04138	.15708	1.000	-.5777	.4950
son-dry	bb-dry	.08572	.15708	.997	-.4506	.6221
	bb-wet	.15109	.15708	.954	-.3853	.6874
	mp-dry	-.01548	.15708	1.000	-.5518	.5209
	ft-wet	-.04134	.15708	1.000	-.5777	.4950
	ft-dry	-.03897	.15708	1.000	-.5753	.4974
	son-wet	.04138	.15708	1.000	-.4950	.5777

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 12. The analysis of the purity results for *Scytonema* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	.02372	.11833	1.000	-.3803	.4278
	mp-dry	-.05542	.11833	.999	-.4595	.3486
	ft-wet	.03441	.11833	1.000	-.3696	.4384
	ft-dry	.03812	.11833	1.000	-.3659	.4422
	son-wet	-.06813	.11833	.997	-.4722	.3359
	son-dry	.08999	.11833	.985	-.3140	.4940
bb-wet	bb-dry	-.02372	.11833	1.000	-.4278	.3803
	mp-dry	-.07914	.11833	.992	-.4832	.3249
	ft-wet	.01069	.11833	1.000	-.3933	.4147
	ft-dry	.01440	.11833	1.000	-.3896	.4184
	son-wet	-.09185	.11833	.984	-.4959	.3122
	son-dry	.06627	.11833	.997	-.3378	.4703
mp-dry	bb-dry	.05542	.11833	.999	-.3486	.4595
	bb-wet	.07914	.11833	.992	-.3249	.4832
	ft-wet	.08983	.11833	.985	-.3142	.4939
	ft-dry	.09353	.11833	.982	-.3105	.4976

Table C. 12. (continued)

mp-dry	son-wet	-.01271	.11833	1.000	-.4167	.3913
	son-dry	.14541	.11833	.872	-.2586	.5494
ft-wet	bb-dry	-.03441	.11833	1.000	-.4384	.3696
	bb-wet	-.01069	.11833	1.000	-.4147	.3933
	mp-dry	-.08983	.11833	.985	-.4939	.3142
	ft-dry	.00371	.11833	1.000	-.4003	.4077
	son-wet	-.10254	.11833	.972	-.5066	.3015
	son-dry	.05558	.11833	.999	-.3485	.4596
ft-dry	bb-dry	-.03812	.11833	1.000	-.4422	.3659
	bb-wet	-.01440	.11833	1.000	-.4184	.3896
	mp-dry	-.09353	.11833	.982	-.4976	.3105
	ft-wet	-.00371	.11833	1.000	-.4077	.4003
	son-wet	-.10625	.11833	.967	-.5103	.2978
	son-dry	.05187	.11833	.999	-.3522	.4559
son-wet	bb-dry	.06813	.11833	.997	-.3359	.4722
	bb-wet	.09185	.11833	.984	-.3122	.4959
	mp-dry	.01271	.11833	1.000	-.3913	.4167
	ft-wet	.10254	.11833	.972	-.3015	.5066
	ft-dry	.10625	.11833	.967	-.2978	.5103
	son-dry	.15812	.11833	.824	-.2459	.5622
son-dry	bb-dry	-.08999	.11833	.985	-.4940	.3140
	bb-wet	-.06627	.11833	.997	-.4703	.3378
	mp-dry	-.14541	.11833	.872	-.5494	.2586
	ft-wet	-.05558	.11833	.999	-.4596	.3485
	ft-dry	-.05187	.11833	.999	-.4559	.3522
	son-wet	-.15812	.11833	.824	-.5622	.2459

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 13. The analysis of the purity results for *Synechocystis* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	mp-dry	.27719	.34061	.846	-.8136	1.3680
	ft-dry	1.07235	.34061	.054	-.0184	2.1631
	son-dry	1.05658	.34061	.058	-.0342	2.1473
mp-dry	bb-dry	-.27719	.34061	.846	-1.3680	.8136
	ft-dry	.79516	.34061	.169	-.2956	1.8859
	son-dry	.77938	.34061	.180	-.3114	1.8701

Table C. 13. (continued)

ft-dry	bb-dry	-1.07235	.34061	.054	-2.1631	.0184
	mp-dry	-.79516	.34061	.169	-1.8859	.2956
	son-dry	-.01578	.34061	1.000	-1.1065	1.0750
son-dry	bb-dry	-1.05658	.34061	.058	-2.1473	.0342
	mp-dry	-.77938	.34061	.180	-1.8701	.3114
	ft-dry	.01578	.34061	1.000	-1.0750	1.1065

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 14. The analysis of the purity results for *Synechocystis* sp. in one-way ANOVA with Tukey's post-hoc (mp-dry excluded).

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	ft-dry	1.07235*	.03035	.000	.9792	1.1655
	son-dry	1.05658*	.03035	.000	.9635	1.1497
ft-dry	bb-dry	-1.07235*	.03035	.000	-1.1655	-.9792
	son-dry	-.01578	.03035	.865	-.1089	.0774
son-dry	bb-dry	-1.05658*	.03035	.000	-1.1497	-.9635
	ft-dry	.01578	.03035	.865	-.0774	.1089
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 15. The analysis of the purity results for *Nostoc* sp. in one-way ANOVA with Tukey's post-hoc.

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-.06922	.04298	.679	-.2160	.0775
	mp-dry	-.15690*	.04298	.033	-.3036	-.0102
	ft-wet	-.02406	.04298	.997	-.1708	.1227
	ft-dry	-.18230*	.04298	.011	-.3290	-.0356
	son-wet	-.09023	.04298	.403	-.2370	.0565
	son-dry	-.09130	.04298	.390	-.2380	.0554
bb-wet	bb-dry	.06922	.04298	.679	-.0775	.2160
	mp-dry	-.08768	.04298	.434	-.2344	.0591

Table C. 15. (continued)

bb-wet	ft-wet	.04516	.04298	.933	-.1016	.1919
	ft-dry	-.11308	.04298	.188	-.2598	.0337
	son-wet	-.02102	.04298	.999	-.1678	.1257
	son-dry	-.02208	.04298	.998	-.1688	.1247
mp-dry	bb-dry	.15690*	.04298	.033	.0102	.3036
	bb-wet	.08768	.04298	.434	-.0591	.2344
	ft-wet	.13284	.04298	.088	-.0139	.2796
	ft-dry	-.02540	.04298	.996	-.1721	.1213
	son-wet	.06667	.04298	.712	-.0801	.2134
	son-dry	.06560	.04298	.726	-.0811	.2123
ft-wet	bb-dry	.02406	.04298	.997	-.1227	.1708
	bb-wet	-.04516	.04298	.933	-.1919	.1016
	mp-dry	-.13284	.04298	.088	-.2796	.0139
	ft-dry	-.15824*	.04298	.031	-.3050	-.0115
	son-wet	-.06618	.04298	.719	-.2129	.0806
	son-dry	-.06724	.04298	.705	-.2140	.0795
ft-dry	bb-dry	.18230*	.04298	.011	.0356	.3290
	bb-wet	.11308	.04298	.188	-.0337	.2598
	mp-dry	.02540	.04298	.996	-.1213	.1721
	ft-wet	.15824*	.04298	.031	.0115	.3050
	son-wet	.09207	.04298	.381	-.0547	.2388
	son-dry	.09100	.04298	.394	-.0557	.2377
son-wet	bb-dry	.09023	.04298	.403	-.0565	.2370
	bb-wet	.02102	.04298	.999	-.1257	.1678
	mp-dry	-.06667	.04298	.712	-.2134	.0801
	ft-wet	.06618	.04298	.719	-.0806	.2129
	ft-dry	-.09207	.04298	.381	-.2388	.0547
	son-dry	-.00107	.04298	1.000	-.1478	.1457
son-dry	bb-dry	.09130	.04298	.390	-.0554	.2380
	bb-wet	.02208	.04298	.998	-.1247	.1688
	mp-dry	-.06560	.04298	.726	-.2123	.0811
	ft-wet	.06724	.04298	.705	-.0795	.2140
	ft-dry	-.09100	.04298	.394	-.2377	.0557
	son-wet	.00107	.04298	1.000	-.1457	.1478

*. The mean difference is significant at the 0.05 level.

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

Table C. 16. The analysis of the purity results for *Nostoc* sp. in one-way ANOVA with Tukey's post-hoc (son-wet and son-wet excluded).

Multiple Comparisons						
Dependent Variable: PC_purity						
Tukey HSD						
(I) pretreatment_type	(J) pretreatment_type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
bb-dry	bb-wet	-.06922	.03322	.298	-.1785	.0401
	mp-dry	-.15690*	.03322	.006	-.2662	-.0476
	ft-wet	-.02406	.03322	.946	-.1334	.0853
	ft-dry	-.18230*	.03322	.002	-.2916	-.0730
bb-wet	bb-dry	.06922	.03322	.298	-.0401	.1785
	mp-dry	-.08768	.03322	.136	-.1970	.0216
	ft-wet	.04516	.03322	.664	-.0642	.1545
	ft-dry	-.11308*	.03322	.042	-.2224	-.0038
mp-dry	bb-dry	.15690*	.03322	.006	.0476	.2662
	bb-wet	.08768	.03322	.136	-.0216	.1970
	ft-wet	.13284*	.03322	.017	.0235	.2422
	ft-dry	-.02540	.03322	.935	-.1347	.0839
ft-wet	bb-dry	.02406	.03322	.946	-.0853	.1334
	bb-wet	-.04516	.03322	.664	-.1545	.0642
	mp-dry	-.13284*	.03322	.017	-.2422	-.0235
	ft-dry	-.15824*	.03322	.005	-.2676	-.0489
ft-dry	bb-dry	.18230*	.03322	.002	.0730	.2916
	bb-wet	.11308*	.03322	.042	.0038	.2224
	mp-dry	.02540	.03322	.935	-.0839	.1347
	ft-wet	.15824*	.03322	.005	.0489	.2676
*. The mean difference is significant at the 0.05 level.						

*ft-dry: freeze-thaw lyophilized; son-dry: sonication lyophilized; mp-dry: mortar and pestle lyophilized; bb-dry: bead-beating lyophilized; ft-wet: freeze-thaw wet; son-wet: sonication wet; mp-wet: mortar and pestle wet; bb-wet: bead-beating wet

APPENDIX D: GROWTH DATA OF BATCHES

Cell Count of *G. sulphuraria* (per mL)					
Days	**0**	**2**	**4**	**6**	**7**
Flask 1	1.86E+07	1.63E+07	1.96E+07	2.11E+07	1.64E+07
Flask 2	1.14E+07	1.59E+07	1.50E+07	1.55E+07	1.84E+07
Flask 3	1.33E+07	1.35E+07	1.43E+07	1.51E+07	1.11E+07

Absorbance of *G. sulphuraria* at 680 nm								
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Flask1	0.671	0.678	0.673	0.705	0.720	0.732	0.746	0.748
Flask2	0.581	0.575	0.573	0.599	0.637	0.654	0.658	0.667
Flask3	0.594	0.601	0.613	0.679	0.717	0.758	0.759	0.864

pH of G. *sulphuraria*								
Day	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Flask 1	2.760	2.720	2.660	2.680	2.680	2.690	2.680	2.690
Flask 2	2.650	2.660	2.610	2.630	2.600	2.610	2.640	2.620
Flask 3	2.630	2.640	2.600	2.580	2.570	2.570	2.590	2.620

Dry Weights of *Scytonema* sp. (g/L)												
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Flask 1	0.170	0.120	0.130	0.110	0.130	0.180	0.130	0.100	0.120	0.280	0.220	0.330
Flask 2	0.130	0.110	0.210	0.140	0.140	0.170	0.230	0.110	0.210	0.380	0.350	0.250
Flask 3	0.110	0.130	0.150	0.130	0.180	0.240	0.200	0.140	0.140	0.220	0.150	0.410

Absorbance of *Scytonema* sp. at 680 nm												
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Flask 1	0.039	0.055	0.131	0.175	0.118	0.377	0.413	0.444	0.265	0.681	0.638	0.794
Flask 2	0.036	0.155	0.207	0.219	0.257	0.194	0.386	0.446	0.258	0.465	0.788	0.427
Flask 3	0.031	0.082	0.096	0.095	0.106	0.167	0.232	0.23	0.182	0.334	0.231	0.644

Dry Weights of *Nostoc* sp. (g/L)										
Days	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**
Flask 1	0.160	0.220	0.160	0.240	0.300	0.360	0.390	0.590	0.630	0.580
Flask 2	0.200	0.340	0.140	0.210	0.260	0.320	0.410	0.510	0.940	1.500
Flask 3	0.160	0.270	0.170	0.260	0.310	0.420	0.540	0.630	0.630	0.430

pH of *Nostoc* sp.									
Days	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**
Flask 1	7.800	7.160	7.980	7.620	8.780	9.120	9.440	9.790	9.120
Flask 2	7.950	7.910	7.970	8.460	9.260	8.540	9.890	9.750	9.600
Flask 3	7.790	8.030	8.190	8.720	9.090	9.180	9.980	9.500	9.840

Absorbance of *Nostoc* sp. at 680 nm							
Days	**6**	**8**	**10**	**12**	**14**	**16**	**18**
Flask 1	0.045	0.096	0.16	0.226	0.248	0.328	0.331
Flask 2	0.038	0.078	0.132	0.204	0.222	0.415	0.346
Flask 3	0.07	0.115	0.206	0.249	0.271	0.312	0.675

Dry Weights of *Phormidium* sp. (g/L)					
Days	**0**	**1**	**2**	**3**	**4**
Flask 1	1.440	1.490	1.860	2.300	2.240
Flask 2	1.520	1.590	1.610	1.750	1.830
Flask 3	1.420	1.360	1.580	1.610	2.060

pH of *Phormidium* sp.					
Days	**0**	**1**	**2**	**3**	**4**
Flask 1	8.550	8.610	9.000	9.020	9.480
Flask 2	8.560	8.810	9.320	9.910	9.560
Flask 3	8.450	8.550	8.550	8.850	8.910

Absorbance of *Phormidium* sp. at 680 nm					
Days	**0**	**1**	**2**	**3**	**4**
Flask 1	0.099	0.116	0.171	0.312	0.283
Flask 2	0.105	0.120	0.162	0.291	0.328
Flask 3	0.141	0.117	0.145	0.252	0.332

Absorbance of *Synechocystis* sp. at 680 nm								
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Flask 1	0.188	0.604	1.024	1.252	1.463	1.775	2.272	2.167
Flask 2	0.180	0.402	0.563	0.643	0.718	0.947	1.344	1.662
Flask 3	0.184	0.559	1.121	1.372	1.624	1.910	2.634	2.629

pH of *Synechocystis* sp.								
Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Flask 1	7.63	9.12	10.17	9.72	9.94	11.02	10.55	9.40
Flask 2	7.76	10.34	10.50	9.58	10.18	10.02	10.48	9.79
Flask 3	8.00	8.83	10.34	9.69	10.84	9.46	9.47	9.54

Cell count of *Synechocystis* sp. (per mL)					
Days	**0**	**2**	**4**	**6**	**7**
Flask 1	9.31E+06	6.58E+07	1.57E+08	2.78E+08	2.42E+08
Flask 2	1.14E+07	4.18E+07	1.02E+08	1.63E+08	1.76E+08
Flask 3	8.31E+06	8.15E+07	1.39E+08	3.47E+08	3.13E+08

APPENDIX E: THE PICTURES OF CRUDE EXTRACTS

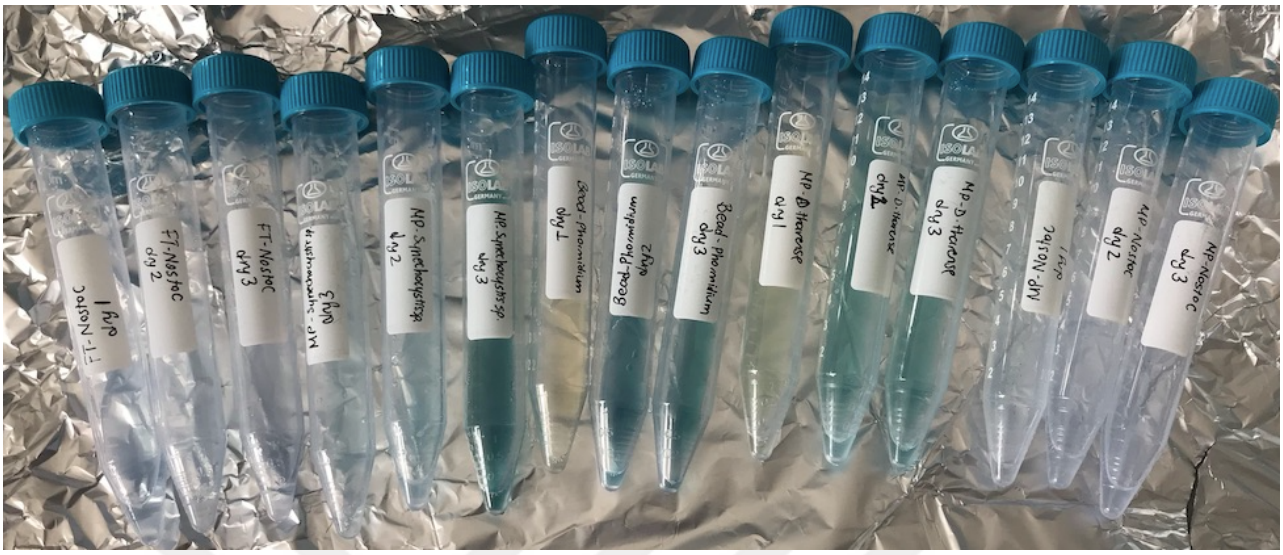


Figure D. 1. The crude extracts inside the 15 mL falcon tubes (part 1).

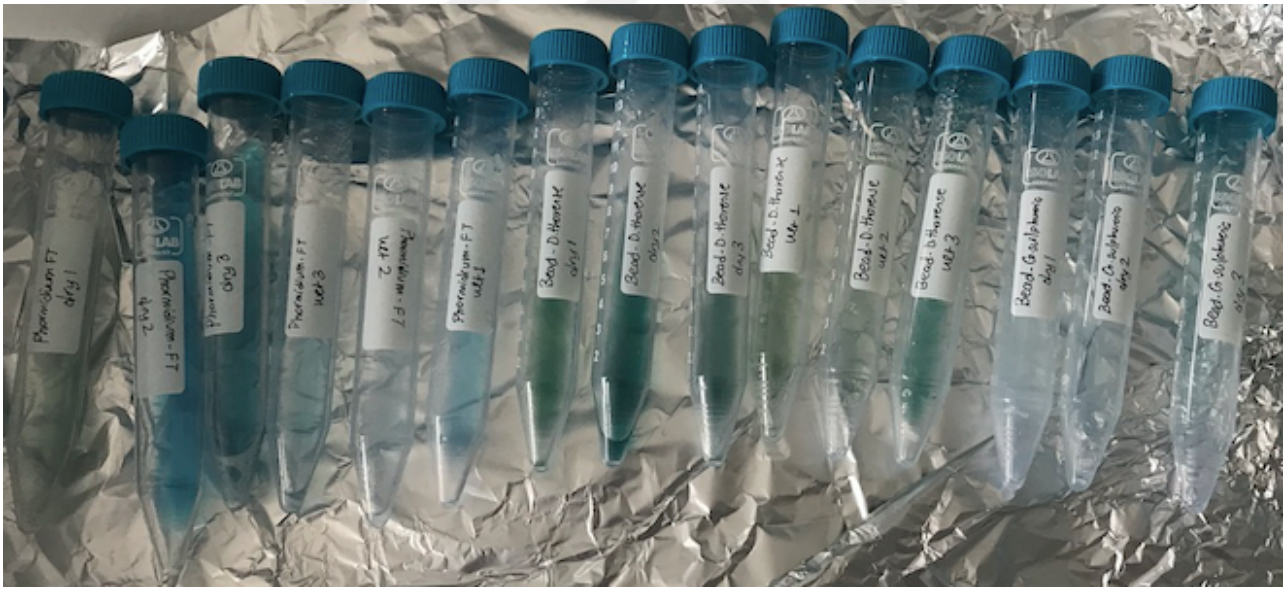


Figure D. 2. The crude extracts inside the 15 mL falcon tubes (part 2).

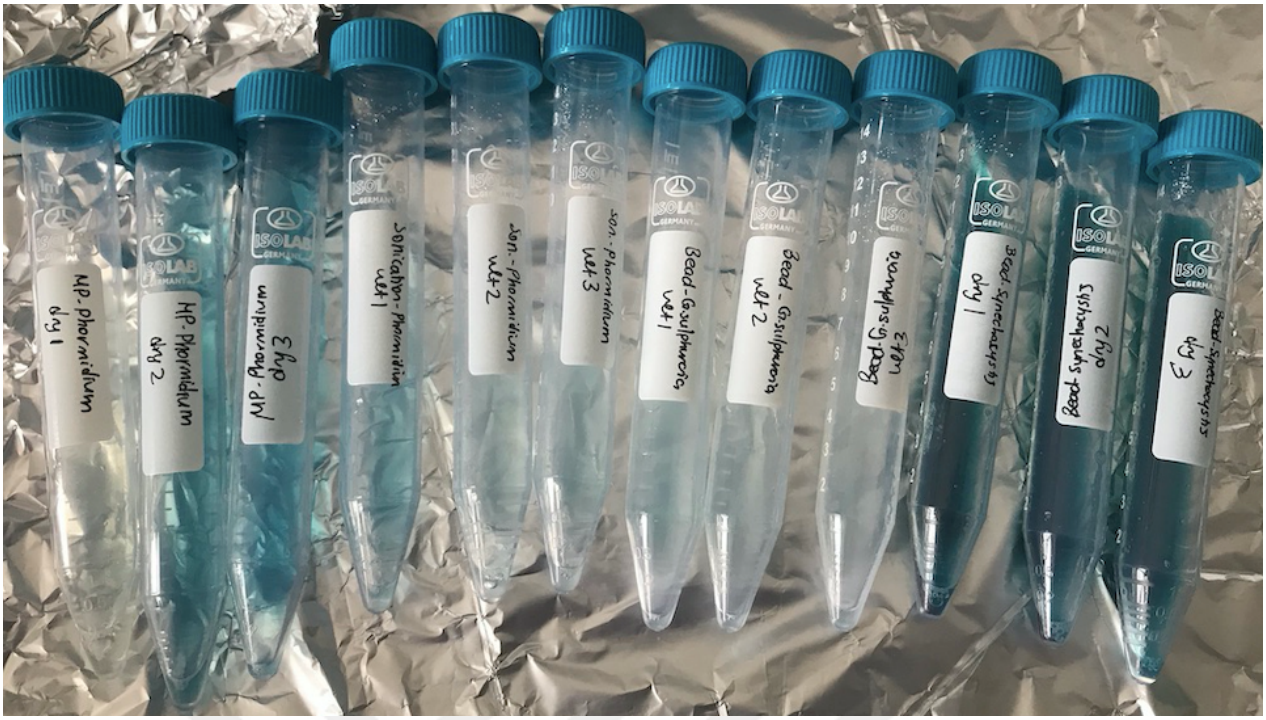


Figure D. 3. The crude extracts inside the 15 mL falcon tubes (part 3).



Figure D. 4. The crude extracts inside the 15 mL falcon tubes (part 4).

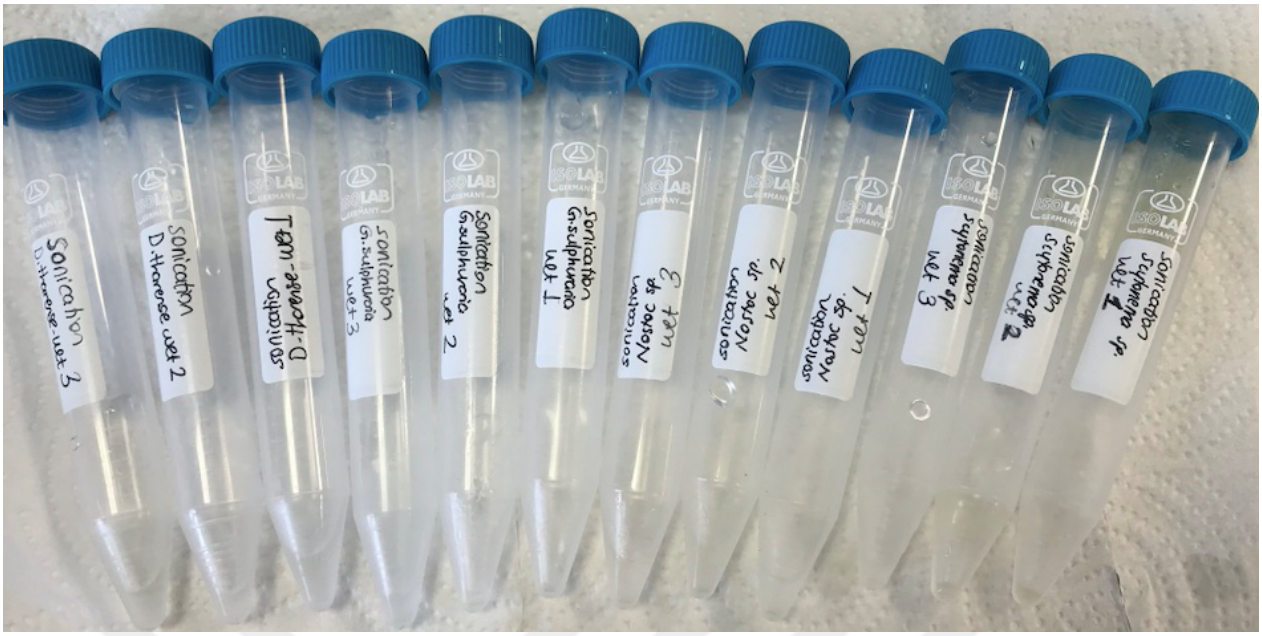


Figure D. 5. The crude extracts inside the 15 mL falcon tubes (part 5).

APPENDIX F: L-ASCORBIC ACID STANDARD CURVES

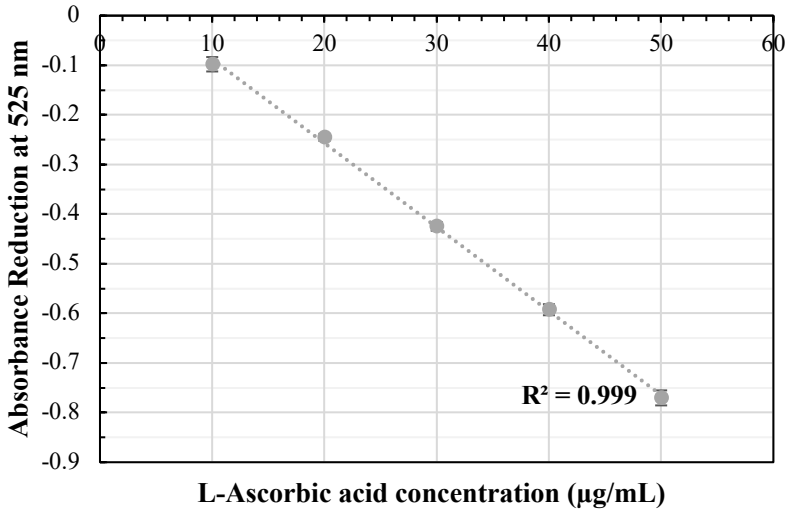


Figure E.1. Absorbance reduction versus L-ascorbic acid concentration graph for AC/CS applied PC from the light stress samples.

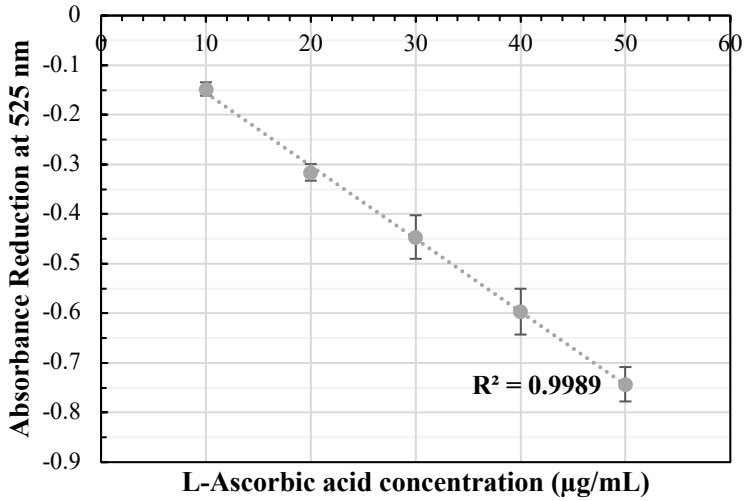


Figure E.2. Absorbance reduction versus L-ascorbic acid concentration graph for ASP applied PC from the light stress samples.

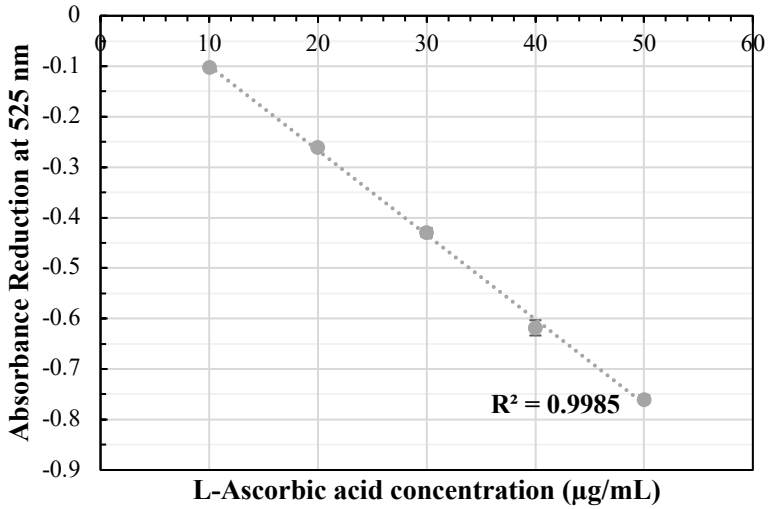


Figure E.3. Absorbance reduction versus L-ascorbic acid concentration graph for AC/CS applied PC from the control samples.

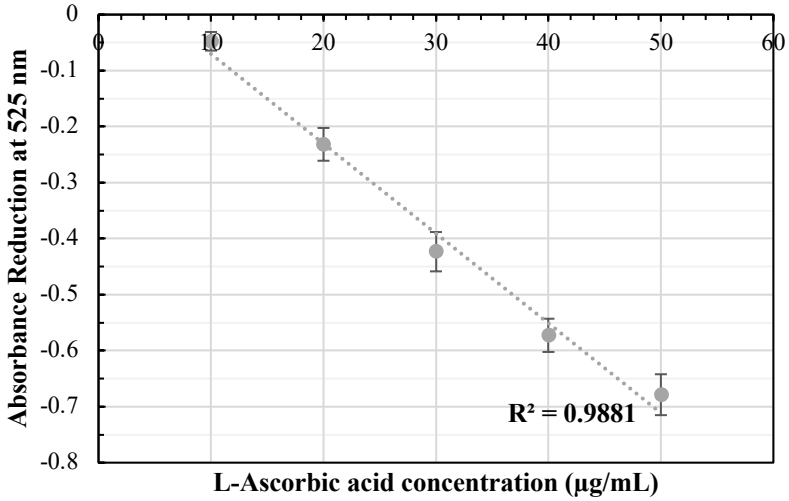


Figure E.4. Absorbance reduction versus L-ascorbic acid concentration graph for ASP applied PC from the control samples.

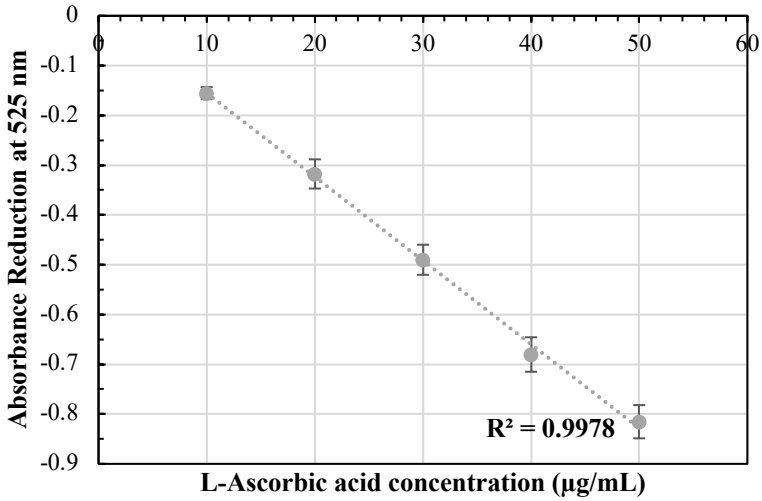


Figure E.5. Absorbance reduction versus L-ascorbic acid concentration graph for AC/CS applied PC from the hydrogen peroxide stress samples.

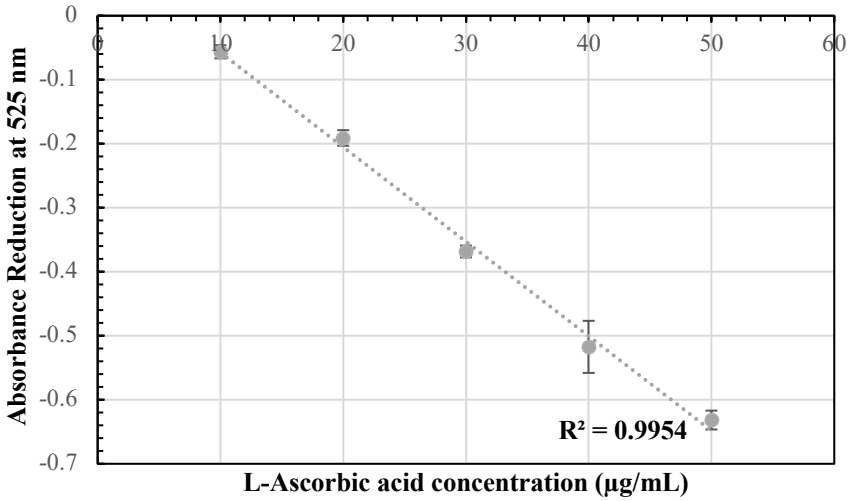


Figure E.6. Absorbance reduction versus L-ascorbic acid concentration graph for ASP applied PC from the hydrogen peroxide stress samples.

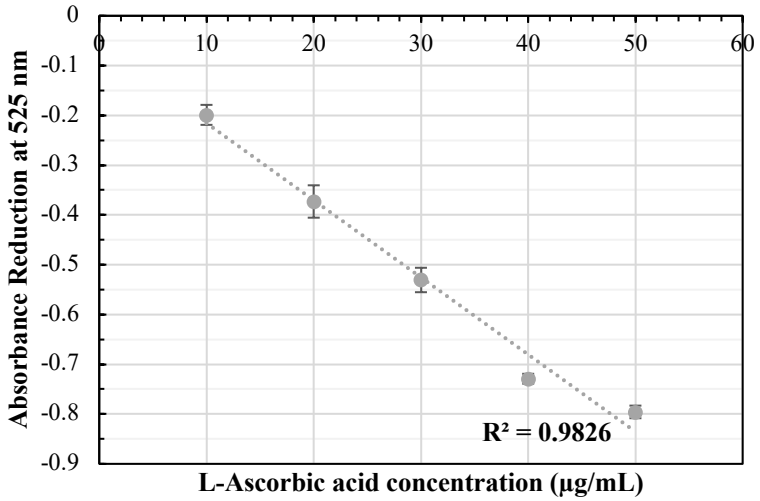


Figure E.7. Absorbance reduction versus L-ascorbic acid concentration graph for AC/CS applied PC from the salt stress samples.

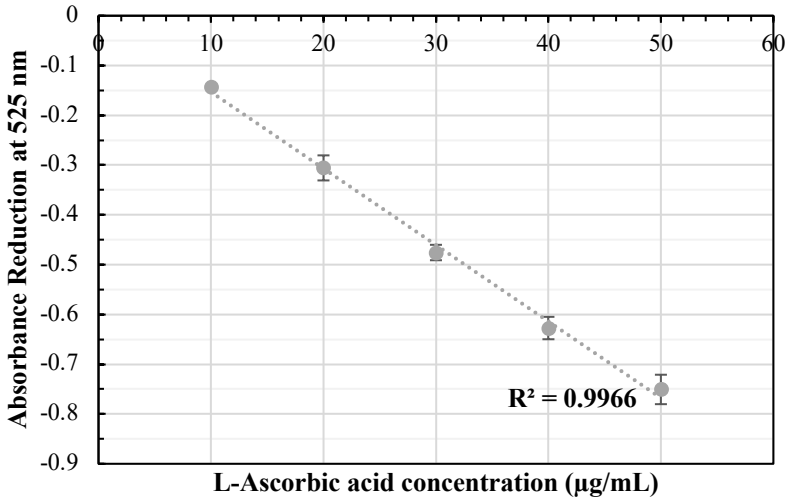


Figure E.8. Absorbance reduction versus L-ascorbic acid concentration graph for ASP applied PC from the salt stress samples.