DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATION IN PRODUCTION, ISOLATION, PARTIAL PURIFICATION AND SOME ANTIOXIDANT-CYTOTOXIC PROPERTIES OF PHYCOCYANIN

by

Merve KAVUT

November, 2023

İZMİR

INVESTIGATION IN PRODUCTION, ISOLATION, PARTIAL PURIFICATION AND SOME ANTIOXIDANT-CYTOTOXIC PROPERTIES OF PHYCOCYANIN

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M.SC THESIS EXAMINATION RESULT FORM

We have read the thesis entitled "**INVESTIGATION IN PRODUCTION, ISOLATION, PARTIAL PURIFICATION AND SOME ANTIOXIDANT-CYTOTOXIC PROPERTIES OF PHYCOCYANIN**" completed by **MERVE KAVUT** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

In this thesis, in mixotrophic and heterotrophic cultivations to optimize the production conditions to obtain maximum phycocyanin (C-PC) from *Arthrospira platensis-M2*; carbon (crude/technical glycerol) and nitrogen (urea/sodium nitrate) sources were examined. C-PC optimum production conditions were determined on the 15^{th} day in mixotrophic cultivation (1mM crude glycerol) as 3.75±0.12mg/g. Bioactive components like total protein, carbohydrate, lipid and carotenoid, chlorophyll-a/b, total phenolic, flavonoid and tannin, lipid peroxidation, pyruvate, and proline were also examined under optimum conditions. Then, to optimize C-PC extraction, wet, lyophilized, oven-dried, and frozen samples were mixed with ultrapure water/buffer at 4-different concentrations; and mixer mill, freeze-thaw, homogenization, and ultrasound-assisted extraction (UAE) were applied. C-PC extraction conditions were determined as 30min at 80kHz in the simple and environmentally/friendly UAE of a 5 percent ultrapure water sample. Subsequently, ammonium sulfate (AS), organic solvent, and polyethylene glycol precipitation methods were applied. The best result was determined as AS precipitation at 50 percent saturation. After the sample was applied to DEAE-Sepharose column chromatography, the best fractions collected were combined and passed through the ultrafiltrate. Thus, C-PC with 2.3 reagent purity was obtained. Then, antioxidant ($DPPH^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $ABTS^{\cdot+}$, $O_2^{\cdot-}$, total reducing power, FRAP, and metal chelation) and cytotoxic (OE-33 and HeLa cancer cell lines) properties were determined. Additionally, UV-Vis spectrum, FT-IR, and TGA characterizations of C-PC were performed. To the results, it appears that partially purified C-PC, with its economical production in the presence of crude glycerol, can be considered a value-added bioactive product with potential for use in various industrial fields.

Keywords: *A. platensis M2,* phycocyanin, mixotrophy, heterotrophy, extraction, partial purification, antioxidant, cytotoxic, characterization

FIKOSİYANİNİN ÜRETİMİ, İZOLASYONU, KISMİ SAFLAŞTIRILMASI VE BAZI ANTİOKSİDAN-SİTOTOKSİK ÖZELLİKLERİNİN ARAŞTIRILMASI

ÖZ

Bu tez çalışmasında *Arthrospira platensis*-M2'den maksimum fikosiyanin (C-PC) eldesi için üretim koşullarının optimizasyonu amacıyla miksotrofik ve heterotrofik kültivasyonlarda; karbon kaynağı (ham/teknik gliserol) ve azot kaynağı (üre/sodyum nitrat) incelenmiştir. C-PC optimum üretim koşulları 1mM ham gliserolde miksotrofik kültivasyonda 15. günde 3,75±0,12 mg/g olarak belirlenmiştir. Optimum koşullarda total protein, karbohidrat, lipid ve karotenoid, klorofil a ve b, total fenolik, flavonoid ve tanen, lipid peroksidasyon, pirüvat ve prolin gibi biyoaktif bileşenler de incelenmiştir. Ardından C-PC ekstraksiyonunun optimizasyonu için yaş, liyofilize, etüvde kurutulmuş ve donmuş örneklerin ultrasaf su/tamponla 4 farklı derişimdeki örneklerinde; mixer mill, dondurma-çözme, homojenizasyon ve ultrason destekli ekstraksiyon (UDE) uygulandı. C-PC ekstraksiyon koşulları, yüzde 5 ultrasaf su örneğinin basit ve çevre dostu olan UDE'da 80 kHz'de 30 dk olarak belirlendi. Devamında, amonyum sülfat (AS), organik çözgen ve polietilenglikol çöktürme yöntemleri uygulandı. En iyi sonuç yüzde 50 doygunlukta AS çöktürme olarak belirlendi. Örnek DEAE-Sepharose kolon kromatografisine uygulandıktan sonra toplanan en iyi fraksiyonlar birleştirilerek ultrafiltrattan geçirildi. Kısmi saflaştırma işlemlerinin sonunda 2,3 reaktif saflıkta C-PC elde edildi. Ardından antioksidan ($DPPH^{\cdot}$, $HO^{\cdot}$, NO., $ABTS^{\cdot+}$, $O_2^{\cdot-}$, toplam indirgeme gücü, FRAP ve metal şelatlama) ve sitotoksik (OE-33 ve HeLa kanser hücre hatları) özellikleri belirlendi. Ayrıca C-PC nin UV-Vis spektrum, FT-IR, ve TGA karakterizasyonları gerçekleştirildi. Sonuçlara göre ham gliserol varlığında ekonomik üretimiyle kısmi saflaştırılmış C-PC'nin, çeşitli endüstriyel alanda kullanım potansiyelinde katma değerli biyoaktif bir ürün olarak değerlendirilebileceği görülmektedir.

Anahtar kelimeler: *A. platensis M2*, fikosiyanin, miksotrofi, heterotrofi, ekstraksiyon, kısmi saflaştırma, antioksidan, sitotoksik, karakterizasyon

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

ABBREVIATIONS

$ABTS^{\cdot+}$	: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
APC	: Allophycocyanin
A. platensis	: *Arthrospira platensis*
AS	: Ammonium Sulfate
BHA	: Butylated Hydroxyanisole
BHT	: Butylated Hydroxytoluene
Chl a	: Chlorophyll a
Chl b	: Chlorophyll b
CC	: Column Chromatography
C-PC	: Phycocyanin
C-PEC	: Phycoerythrocyanin
Cys	: Cysteine
DEAE	: Diethylaminoethyl
DMSO	: Dimethyl Sulfoxide
DNA	: Deoksiribonucleic Acid
DPPH·	:1,1-Diphenyl-2-picrylhydrazyl radical
EDTA	: Etylene Diamine Tetra Asetic Acid
FB	: Frozen Biomass
FRAP	: Ferric Reducing Antioxidant Power
FT-IR	: Fourier Transform Infrared
GAE	: Gallic Acid Equivalent
$HO^{\cdot}$	: Hydroxyl Radical

IC_{50}	: Half-Maximal Inhibitory Concentration
LPO	: Lipid Peroxidation
PBPs	: Phycobiliproteins
LB	: Lyophilized Biomass
MDA	: Malondialdehyde
MTT	: (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NAPDH	: Nicotinamide adenine dinucleotide phosphate hydrid
$NO^{\cdot}$	: Nitric Oxide Radical
$O_2^{\cdot -}$	: Superoxide Anion Radical
OD	: Optical Density
ODB	: Oven-Dried Biomass
OS	: Organic Solvent
PE	: Phycoerythrin
PEG	: Polyethylene Glycol
PUFA	: Polyunsaturated Fatty Acids
QE	: Quercetin Equivalent
ROS	: Oxygen-Derived Reactive Species
TAE	: Tannic Acid Equivalent
TBA	: Thiobarbituric Acid
TCA	: Tricloroacetic Acid
TFC	: Total Flavonoid Content
TGA	: Thermogravimetric Analysis

TPC	: Total Phenolic Content
UAE	: Ultrasound Assisted Extraction
UPW	: Ultrapure Water
UV-Vis	: Ultraviole Visible
VCE	: Vitamin C Equivalent
WB	: Wet Microalgal Biomass

CHAPTER ONE
INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria are photosynthetic and prokaryotic organisms that meet 32% of the world's oxygen needs and form the first step of the food chain, and their cell walls have a peptidoglycan structure similar to gram-negative bacteria (Priyadarshani, Sahu, & Rath, 2012). Cyanobacteria, which can be consumed as food by humans and animals, are also a source of edible energy, green chemistry standards, and rich bioactive components. It is one of the most primitive life forms on earth (Singh, Kate & Banerjee, 2005). Although its cellular structure is prokaryotic, it photosynthesizes like plants but does not have plant cell walls. On the other hand, it contains glycogen, which is a complex sugar in cell membranes. In this respect, the classification of cyanobacteria is still a matter of debate today. Their inclusion in edible species makes them a raw food source, and they contain bioactive components, that differ according to the environmental conditions in which they are grown. Due to the properties of the pigments they contain, they are called blue-green algae because they are photosynthetic and aquatic (Ananya & Ahmad, 2014). Although their ancestors are known to be multicellular, most cyanobacteria are unicellular and reproduce in colonies or filaments depending on environmental conditions (Whitton, 2012). In addition, the main physiological/biochemical features that distinguish cyanobacteria from all other prokaryotes are the use of H_2O as a photo-reductant and the ability to study with a dual photosystem that allows O_2 to be released as a result (Castenholz, 2015).

Cyanobacteria, also called microalgae, contain proteins, chlorophylls, carotenoids, polyunsaturated fatty acids, vitamins, vitamin precursors, minerals, and many bioactive components in their structure (Hayes et al., 2017). In this regard, it is valuable when used in the diet in terms of the daily amount required. With the revealing of the nutraceutical, pharmaceutical, cosmetic, and therapeutic effects of the valuable components they contain, their usage areas have increased, and it has gained an increase in the world market (Raisa et al., 2016). Considering factors such as the

rise in the world population, the increase in the average life expectancy, and the decrease due to the unfavorable use of natural resources, it is shown as one of the sustainable foods of the future by many researchers (Wang et al., 2023). It has taken its status among the current research topics with its feature of reducing greenhouse gases with a low carbon footprint and which can be grown anywhere with its high adaptation ability without the need for agricultural lands. Microalgae, which is the source of many bioactive components, ensures the realization of the bioreduction process by not needing fertile agricultural land and growing it using water that is harmful to human health (Mata, Martins & Caetano, 2010). The fact that it is suitable for cultivation in conditions such as clean water shortage caused by global warming and its rich content makes microalgae more popular day by day (Esquivel-Hernández et al., 2017). The market value of microalgae is thought to be $6.5 billion (Mobin & Alam, 2017).

Cyanobacteria selection is important in research. The types of cyanobacteria, and cultivation vary depending on the bioactive component planned to be obtained (Wan et al., 2011).. For example, *Nannochloropsis oculata, Dunaliella salina, Chlorella sorokiniana, Arthrospira platensis* (*A. platensis),* and *Scenedesmus obliquus* are microalgae known to have high biomass productivity in mixotrophic cultivation in the presence of glucose (Mandal & Mallick, 2009). Lipid productivity was found to be high in the presence of glycerol in *Nannochloropsis* sp. and glucose in *N. oculata, D. salina, C. sorokiniana* and *C. vulgaris* in mixotrophic cultivation. One of the most common species of cyanobacteria, which are photosynthetic and prokaryotic organisms, is *Arthrospira* (Guschina & Harwood, 2006). *A. platensis* (formerly known as *Spirulina platensis*) has a multicellular structure and a high ability to adapt to extreme growth conditions. It has filamentous structures called trichomes, shown in Figure 1.1 (Khan et al., 2005). The basic morphological property of *Arthrospira* is that its multicellular cylindrical trichomes have an open spiral structure with prominent cross walls. In contrast, *Spirulina* has a trichome, which is usually nearly closed, with screw-like structures of uniform and narrow diameter (0.5-3 mm), whose cross walls are generally invisible in the light microscope, without gas vacuoles, and with various granules (Whitton, 2012). Single or multi-celled outward extensions of the epidermis are called trichomes (hairs). Generally, trichomes (hairs) have a lower part embedded

in the epidermis and an upper part extending from the surface. Trichome protects plants from various harmful effects such as UV light, insects, freezing intolerance, and transpiration. Some trichomes also secrete some important secretions. Trichomes are formed when epidermis cells divide, change, grow, and protrude. The simplest hair occurs when the epidermis cell grows outward (Whitton, 2012).

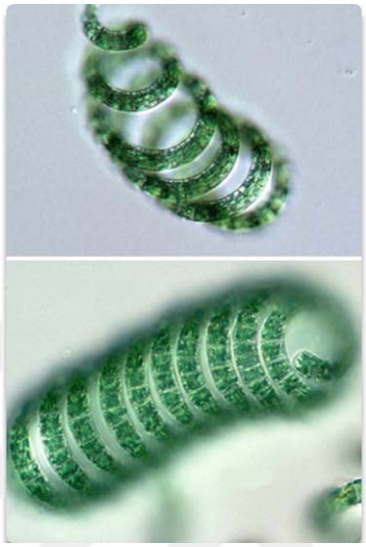

Figure 1.1 *A. platensis* trichome structure (Whitton, 2012).

1.2 Bioactive Compounds of Cyanobacteria

Cyanobacteria contain chlorophyll a and b, carotenoids, fatty acids such as ω-3, ω-6, B-group vitamins (B_{12}, B_6, B_2, B_1), various minerals (iron, zinc, selenium, calcium, magnesium), phytochemicals (phenolic compounds and tocopherols), that are essential for human nutrition (De Jesús et al., 2016). These cyanobacterial bioactive components contain various activities like antimicrobial, anticancer, antioxidant, and anti-inflammatory and are of great importance in this respect (Afreen & Fatma, 2018).

Whole photosynthetic living includes three main pigments to absorb light (Richmond, 2004): Chlorophylls, which are green pigments, and carotenoids, which are yellow-orange, and are lipophilic, while phycobilins are hydrophilic characters. Chlorophylls consist of a tetrapyrrole ring with a central magnesium atom and a long-chain alcohol. There are varieties called a, b, c, and d according to their structurally changing side groups. Microalgae are also rich in chlorophylls with high antioxidant capacity (Zepka, Jacob-Lopes, & Roca, 2019). Chlorophyll a (Chl a), carotenoids,

phycobiliproteins, which are the main components of *A. platensis*, have many benefits. Chlorophyll is a pigment that is very similar to human hemoglobin. It is seen that the chlorophyll content of *A. platensis* is 3-times higher when compared to terrestrial plants (Abd El-Baky, El Baz, & El-Baroty, 2008). Chl a is found as part of the nucleus and pigment-protein structure in all oxygenated photoautotrophic organisms (Richmond, 2004). Chl b supports Chl a in light-harvesting antennas. Chl molecules, called light-harvesting accessory pigments, help expand the range of light absorption.

The other pigments, carotenoids consisting of β-carotene, myxo xanthophyll, zeaxanthin, astaxanthin, and xanthophyll are fat-soluble pigments generated by plants and certain cyanobacteria and are effective in the formation of foods of various colors (Öztürk Ürek & Kerimoğlu, 2019). Additionally, they are responsible for plant cultivation and maturation as photosynthetic pigments and hormone precursors. Carotenoids, which have two subclasses, carotenes, and xanthophylls, also take part in metabolism as vitamin precursors (Rivera & Canela-Garayoa, 2012). The structures of β-carotene as an example of carotene and astaxanthin as an example of xanthophyll are shown in Figure 1.2. This component, which shows antioxidant and photoprotective properties in the organism, is also the source of colors such as red, yellow, and orange in animals and plants (Chekanov, 2023). There are approximately 50 types of carotenes in nature (Britton, Liaaen-Jensen & Pfander, 2004). α-carotene, β-carotene, and lycopene are examples of carotene type. On the other hand, lutein, zeaxanthin, and astaxanthin are examples of xanthophylls. The structures of xanthophylls show marked diversity. Approximately 800 types of xanthophylls have been reported in nature (Maoka, 2009).

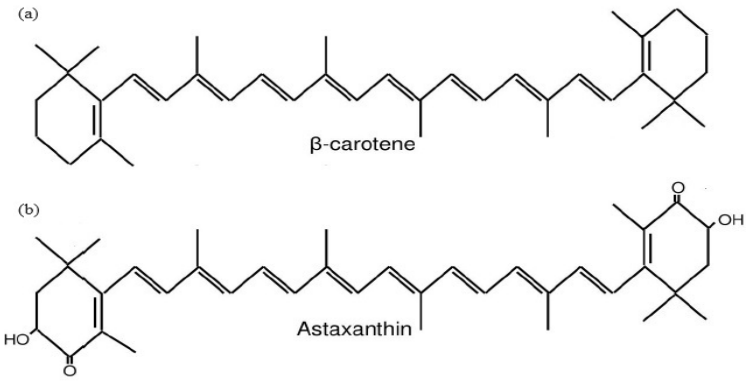

Figure 1.2 Examples of carotenoid types (a) carotene, (b) xanthophylls (Chekanov, 2023).

Carotenoids have had an important status in the human diet from the past to the present. It is included in many studies that it plays a function in lowering the risk of disorders like cancer, cardiovascular disorders, and age-related skin and eye diseases (Meléndez-Martínez et al., 2021). It has also been reported that carotenoids inhibit low-density lipoprotein (Eggersdorfer & Wyss, 2018). Additionally, these carotenoids have various roles such as being a hormone precursor, cancer chemoprotective, and prevention of cardiovascular diseases, and chronic and degenerative diseases (Igual et al., 2022). Carotenoids have role in the prevention of cardiovascular diseases, and it has been reported with their anti-inflammatory, hepatoprotective, neuroprotective, photoprotective, anti-inflammatory, and anticancer effects (Singh et al., 2021). Apart from its use in medicine, it is also widely used in many sectors such as industry, cosmetics, and food.

One of the main components contained in cyanobacteria is lipid. The lipid content of the cyanobacterial cell varies between 6-13% (Vonshak, 1997). Half of this amount consists of fatty acids. When mono- and poly-unsaturated fats and saturated fat contents were compared, it was observed that the contents of α-linoleic acid and γ-linoleic acid were mostly high (Vonshak, 1997).

Vitamins are organic compounds that cannot be produced in the body. The vitamin requests of metabolism are unraveled with foods and food supplements. It consists of two classes: water soluble (B, C) and oil soluble (A, D, E, K). *A. platensis* stands out because it contains many vitamins and vitamin precursors. While the β-carotene is a precursor of vitamin A; thiamine B_1, riboflavin B_2, and nicotinic acid are precursors of vitamin B_3.

Table 1.1 Vitamin and mineral contents of cyanobacteria (El-Moataaz et al., 2019)

Vitamins	**mg /100 g**	**Minerals**	**mg /100 g**
Nicotinic acid	12.20	Calsium	363.70
β-carotene	70	Phosphorus	123.10
Thiamine	3	Zinc	2.60
Vitamin E	60	Iron	12.40
Riboflavin	3.70	Sodium	216.70
		Potassium	170

Proteins constitute 60% of the dry weight of cyanobacteria (Jung vd., 2021). It has a rich amino acid content along with its high protein amount. Amino acids that the body cannot produce and must be obtained from food are called essential amino acids. Amino acids that can be synthesized in the metabolism and do not have to be taken with food are called non-essential amino acids. Including cyanobacteria in the diet allows the daily essential amino acid needs to be met to a large extent (Elango, 2023).

Table 1.2 Amino acid contents of cyanobacteria (El-Moataaz et al., 2019)

Essential aminoacids	**mg/100 g**	**Non-essential aminoacids**	**mg/100 g**
Leucine	29.11	Arginine	44.91
Lysine	19.10	Aspartic acid	36.69
Phenylalanine	23.78	Cysteine	3.30
Isoleucine	14.12	Serine	18.43
Valine	18.40	Tyrosine	19.74
Threonine	13.59	Glycine	15
Methionine	5.31	Proline	14.88
		Glutamic acid	47.03

1.2.1 Phycobiliproteins (PBPs)

Phycobiliproteins (PBPs) known as pigment-protein complexes and found in phycobilisomes on the outer surface of the thylakoid membrane have linear tetrapyrrole prosthetic groups. Phycobilisomes, which are special structures located in the part of the thylakoid membrane facing the cytoplasm in cyanobacteria and red algae, are auxiliary pigments containing PBPs (Sun et al., 2003). Phycobilisomes consist of water-soluble pigment-bearing proteins and binding polypeptides known as PBPs. Phycobilins are formed by the combination of non-protein compounds such as phycoerythrobilin and phycocyanobilin with proteins (Tunail, 2009). PBPs consist of phycocyanins (75%), allophycocyanins (12%), phycoerythrins (12%), and non-pigmented polypeptides. PBPs commonly consist of hetero-monomers (two type subunits, α and β). The subunits of α/β exist in equimolar stoichiometry (αβ) and diverge in molecular weight, aminoacid sequence, and chromophore component. The basic form of the PBP structure is a stable trimer $(\alpha\beta)_3$ (Sun et al., 2003). PBPs, which make up 60% of the soluble protein, are composed of phycocyanin (C-PC),

allophycocyanin (APC), phycoerythrin (PE), and phycoerythrocyanin (C-PEC) (Kovač et al., 2017). Most cyanobacteria contain C-PC and APC, which give the cells their characteristic blue-green color (Figure 1.3) (Yu et al., 2017).

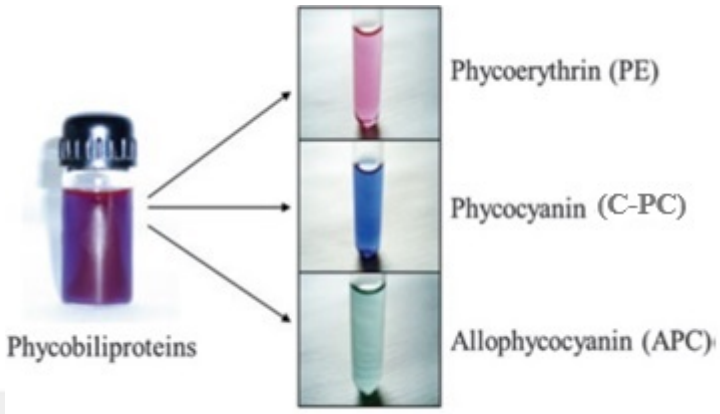

Figure 1.3 PBPs color compositions (Kannaujiya et al., 2017).

C-PC, APC, PE, C-PEC are capable of lights absorbing orange at 620 nm, red at 652 nm, yellow green at 562 nm, and yellow at 571 nm, respectively (Table 1.3).

Table 1.3 Spectral and physical properties of PBPs (Ghosh et al., 2015)

PBPs	**Max Abs (nm)**	**Fluorescence Emission (nm)**	**Molecular Weight (kDa)**	**Absorption Capacity ($L\ g^{-1}cm^{-1}$)**	**Molar Absorption Capacity $(M\text{-}cm)^{-1}$**
C-PC	620	647	108	7.0	1.54
C-PE	562	617	55	8.0	0.44
APC	652	660	100	7.3	0.73

The outermost PBPs in the phycobilisome structure capture light and transmit it toward the photosystem reaction center (Figure 1.4).

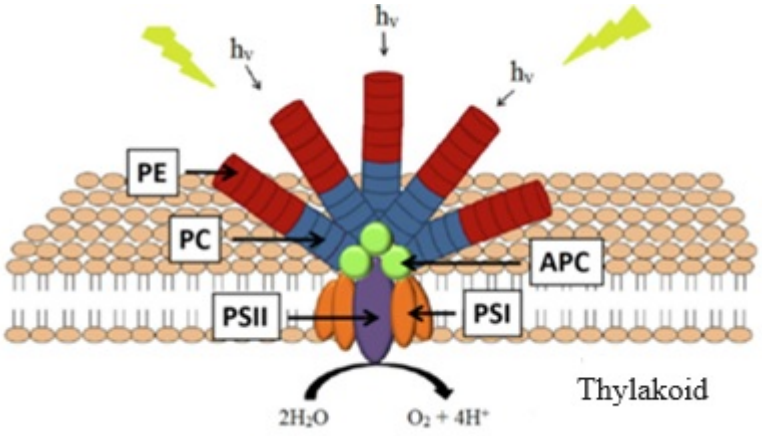

Figure 1.4 *A. platensis* phycobilisome structure (Jaeschke et al., 2021).

PBPs vary devolving on the type of organism and environmental states (Chu, 2012). These protein complex structures constitute an important part of cyanobacterial biocomponents (Tarko et al., 2012). Microalgae are organisms that can meet their nutritional and energy requires on their own under photoautotrophic conditions. The biochemical structure of microalgae can be managed by changing culture environment and physical stress to lead them to make high levels of the target biometabolite (da Silva et al., 2016).

1.2.1.1 Phycocyanin (C-PC)

A. platensis contains chlorophyll a as the main photosynthetic pigment and PBPs with complex protein structures in their phycobilisomes. C-PCs vary in color from purple to dark blue (R-PC to C-PC) and are produced by cyanobacteria, red algae, and cryptomonads (Sun et al., 2009). Generally, C-PC shows a peak around 610-620 nm in the absorption spectrum, while R-PC is characterized by two peaks at 550 nm (from phycoerythrobilin) and at 615 nm (from phycocyanobilin) (Ashaolu et al., 2021). C-PC is a water soluble, fluorescent pigment-protein with linear prosthetic groups containing specific cysteine (Cys) residues. The open chain tetrapyrrole ring gives it an intense blue color (Figure 1.5) (Jaeschke et al., 2021). C-PC consists of the apoprotein of phycocyanobilin and a chromophore group. In the α-subunit of *A. platensis* C-PC (molecular mass: 17.61 kDa and 162 amino acids) a single phycocyanobilin chromophore is linked to Cys-residue 84, while in the β-subunit (molecular mass: 18.1 kDa and 172 aminoacids) 2 chromophores are coupled to Cys

82 and Cys 153 (Wang et al., 2001). The α- and β-subunits of C-PC do not share any important see is homology. A heterodimer constitute of an α- and β- polypeptide structure is the main unit. Each of the monomeric subunits comprises of 9 α-helical domains split by strecthy loops (Scheer & Zhao, 2008). Three dimers form a disc-shaped $(\alpha\beta)_3$ trimer. Finally a $(\alpha\beta)_6$ hexamer indicates the strict connection of 2 trimers and contains in total 18 phycocyanobilin molecules (Ihssen et al., 2014). The α-subunit of C-PC may provide to the provide for the C-PC trimeric structure.

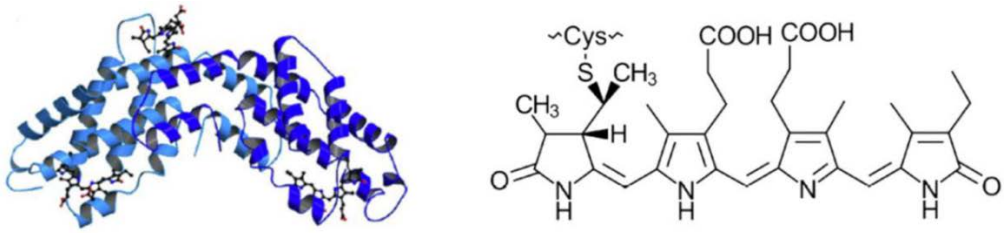

Figure 1.5 The helical structure and molecular structure of C-PC (Jaeschke et al., 2021).

PBPs need to be isolated at different purity levels, depending on their use. C-PC purity is evaluated spectrophotometrically based on the ratio betwixt the C-PC absorbance at 620 nm (A_{620}) and the absorbance values of aromatic aminoacids in whole proteins at 280 nm (A_{280}). C-PC samples with an A_{620}/A_{280} ratio less than 0.7 are noted food grade, A_{620}/A_{280} ratios between 0.7 and 3.9 are considered reagent grade, and A_{620}/A_{280} ratios better than 4.0 are noted analytical grade (Fabre et al., 2022).

There are intensive studies on the use of C-PC, a nontoxic, watersoluble pigment-protein, in various biotechnological and industrial areas such as food, cosmetics, medical, nutraceutical, and pharmaceutical applications according to different purity levels (Kathiravan et al., 2022). Fluorescence properties allow it to be used in scientific research and diagnostic applications in medicine (Fabre et al., 2022).

Currently, people prefer natural colorants instead of additives and artificial colorants because they are more conscious of the products use (Jaeschke, Teixeira, Marczak & Mercali, 2021). By preventing the consumption of chemicals that do not

benefit the body, the use of C-PC, which has many biotechnological potentials such as anticancer, antioxidant, and anti-inflammatory, in these products provides various benefit. C-PC has been noticed that it affects as a hepatoprotective, neuroprotective, antiinflammatory, antioxidant, and anti-cancer agent and has therapeutic effects against disorders like hypercholesterolemia (Jiang et al., 2017). These properties are due to its bioactive components such as C-PC and polysaccharides. As the usage areas of C-PC increase, its market share in the world also increases. Its annual market part is around 100 million dollars, and its economic production is of great significance due to the high costs (Hsieh-Lo et al., 2019).

Marquez et al. (1993) reported that the specific growth rate of mixotrophic cultures using glucose corresponds to the total of the photoautotrophic and heterotrophic specific growth rates. Studies have shown that mixotrophy shows rapidly growth and rised maximum biomass levels in comparison to photoautotrophic cultures (Marquez et al., 1993). Glucose has been shown to have little effect on the specific C-PC level in *A. platensis* but correlated with high biomass and C-PC productivity (Chen et al., 1996). While most *Arthrospira* species can be grown heterotrophically in the dark using glucose and fructose, heterotrophic cultivation for *A. platensis* has been reported to have limited effect on C-PC production (Mühling et al., 2005). Specific growth rates and produced pigment levels in heterotrophic culture of *Arthrospira* species have been shown to be relatively low (Chojnacka & Noworyta, 2004). In their study by Tabassum et al., the amount they found for C-PC production was 0.7 mg/g (Tabassum et al., 2012).

Since *A. platensis* has a resistant cell wall despite its high protein and rich bioactive content, it is necessary to apply an extraction process in order to increase the access and bioavailability of these components (Hayes et al., 2017). After determining the type of cultivation in which the biomass is increased with maximum yield, the extraction and purification of the produced metabolite is another step. Extraction is basically a method of separating the target component from the organism by using certain methods, and single or two-stage methods are used for the extraction of C-PC.

For the extraction processes of bioactive components, green extraction processes come to the fore instead of traditional extraction processes (Rombaut, Tixier, Bily & Chemat, 2014). A study in which wastes harmful to the environment is reduced by the use of green solvents, while energy saving, and efficiency rates are increased by the use of advanced extraction methods benefits nature and science in every respect. Studies have shown that the ultrasonic-assisted extraction (UAE) method to extract PBPs from *A. platensis* is an environmentally friendly method with its environmental footprint, cost, and feasibility properties (Tavanandi & Raghavarao, 2020). Therefore, depending on the biomass volume, considering its environmental friendliness and high added value, ultrasonically assisted C-PC extraction is also more economical than other methods for industrial applications (Berrouane et al., 2022). Since purification procedures are generally time-consuming and costly, advantageous methods come to the fore against conventional technologies that prevent thermal denaturation and deactivation of C-PC, especially under mild operating conditions (Nistico et al., 2022).

Freezing-thawing, UAE, glass bead, desalination, and dual-phase aqueous systems are among the extraction methods used (Fabre et al., 2022). Fabre et al (2022), in their study, determined that the C-PC had a purity level of 1-1.8 by extraction in phosphate buffer, and it was reported that more mechanical methods rarely gave results with higher purity. C-PC purity is determined by the ratio of C-PC absorbance to total protein absorbance. A good purity of C-PC should be greater than 0.7 (Fabre et al., 2022). Salt precipitation is an effective method that is often used to increase C-PC purity. The most commonly used salt in this method is ammonium sulfate. In studies with *A. platensis*, the purity was obtained from 0.9 to 1.3 and from 1.8 to 2.2 by increasing the salt concentration from 39% to 50% (Sobiechowska-Sasim et al., 2014; Marzorati et al., 2020). Higher purity levels have been achieved by using salt concentration gradients (Zhang & Chen, 1999). The aqueous two-phase purification from *A. platensis* has been shown that C-PC purity is increased from 1.18 to 3.52 by testing discrete molecular weight of polyethylene glycol (PEG), different salts, pH, and volume ratios with phase diagrams (Patil & Raghavarao, 2007).

Various methods are used in the purification of C-PC produced from *A. platensis*. Boussiba and Richmond (1979) reported that the purity fold obtained as a result of

hydroxyapatite and ion exchange chromatography after precipitation with ammonium sulfate was 4.15. In another study conducted by Zhang and Chen (1999), ion exchange and gel chromatography were applied after ammonium sulfate fractionation, and the purity of C-PC was shown to be 5.06. In a previous study, the purity level was determined as 3.6 using expanded bed adsorption and anion exchange chromatography as purification steps, and it was reported that an 8.7% yield was achieved from C-PC isolated from low-quality biomass (Niu et al., 2007). In other study, it was shown that in the C-PC purification process using a repeated two-phase aqueous system and ultrafiltration, a purity of 4.05 times and an efficiency of 85% was reached (Patil & Raghavarao, 2007). The C-PC content of *A. platensis* was 48.88 mg/g biomass, with a purity of 0.47 (Khandual et al., 2021). In this study, the optimum extraction condition was determined as a 1/50 biomass/solvent ratio for dry biomass, and the freezing/thawing method was repeated twice for 20 min and shaken at 120 rpm for 24 h. Moreover, C-PC with almost 82% of yield, 6.17 mg/mL of level, and 1.07 of the purity ratio (2-times increase) were acquired from *Spirulina* sp (Chaiklahan et al., 2011). In this study, the best purity rate of C-PC extract was acquired when wet biomass was utilized as crude material with fresh, sun-dried, and oven-dried samples, and then the raw extract was purified by membrane-process using microfiltration and ultrafiltration.

1.3 *Arthrospira platensis*

Cyanobacteria have been the focus of studies in recently as producers of bioactive components and PBPs with medicinal and functional food features (Kovač et al., 2017). *A. platensis* is one of the cyanobacterial species with high adaptability, which has a filamentous, multicellular structure, and can survive in extreme aquatic conditions with features such as bitterness-saltness and high pH (Esen & Öztürk Ürek, 2015). It is a valuable resource of bioactive components like essential fatty acids, aminoacids, and C-PC, and has been defined by the World Health Organization (WHO) as "one of the best superfoods in the world" (Khan et al., 2005). While the world population was determined as 7.67 billion in 2019, this number is expected to be 9.7 billion in 2050 (Jung et al., 2021). Therefore, it is one of the important sources of nutrition for the world population (Table 1.4).

Table 1.4 *A. platensis* nutritional content (De Jesús et al., 2016).

Content	**%**
Protein	57
Carbohydrate	13.60
Lipid	8.33
Moisture	6.98
Fiber	4.25
Ash	10.05

In addition, its growth rate is high, and it is grown without the need for agricultural land. Since it is a rich food in nutritional value, it can be used as feed. Since time immemorial, people have discovered its effects by observing migratory birds feeding on algae swimming in the lake and seeing them regain their strength after long journeys (El-Baky, Rezk & Amara, 2023). From ancient Egypt to the present day, farmers have harvested algae to feed their animals. One of these algae is *A. platensis.* As a result of the research, it was established that *A. platensis* was collected from Lake Chad in Africa and Lake Texcoco in Mexico, and its use as a part of daily nutrition dates back to the 16th century (Whitton, 2012). It has now been observed that farmers in Ethiopia give their cattle once a month water containing *A. platensis* from soda lakes in the area. Researches have indicated that *A. platensis* is the dominant species in the environment by increasing the alkalinity and salinity of the medium (Shimamatsu, 2004; Amara & Steinbüchel, 2013).

Table 1.5 The protein content of some foods (Jung et al., 2021).

Nutrition	**Protein Content**
Spirulina	57%
Soy beans	35%
Peanuts	25%
Cereals	9%

One of the reasons why *A. platensis* is widely used, is that it contains approximately twice as much protein as foods known to be rich in protein (De Oliveira et al., 2021). Thanks to this feature, it is an ideal source to add to beverages and snacks in order to obtain a food with high nutritional value (Table 1.5). Regulation of production

conditions is of important in order to high efficiency and economic production of C-PC, one of the bioactive components contained in *A. platensis*. The production cost also depends on the production, extraction, and isolation costs of C-PC from *A. platensis* (Nisticò et al., 2022). Today, one of the current research topics is the production of economical C-PC, and for this purpose, especially mixotrophic cultivation comes to the front. The biomass remaining from the C-PC extraction from *A. platensis* can be used in fields such as animal feed, fertilizer, biofuel, and bio-oil because it contains many valuable components (Ho et., 2019).

1.4 Cultivation Types

In photoautotrophic cultures, microalgae use light as an energy source and CO_2 as a carbon source in the photosynthetic metabolic pathway (Gonzalez-Toril & Pereto´, 2011). Generally, microalgae are produced by photoautotrophic cultivation. Although this type of cultivation is an easily applicable method and does not require an extra carbon source, it is hard to achieve high-density microalgae biomass due to annoyed light in the culture medium (Hamza et al., 2013, Kovač et al., 2017). As a solution to this problem experienced in photoautotrophic cultures, heterotrophic and mixotrophic cultures have come to the fore. In heterotrophic cultures, microalgae production is carried out using organic carbon sources like carbohydrates and organic acids without light (Chen et al., 2011). This type of cultivation ensures high biomass production. In addition, it was determined that microalgal cell concentration and volumetric efficiency increased, while the photoinhibition effect decreased in mixotrophic cultures performed in environments with light and external carbon sources (Chojnacka & Noworyta, 2004; Hamza et al., 2013).

In heterotrophic and mixotrophic cultures, glucose, galactose, mannose, fructose, sucrose, and lactose are usually used organic carbon sources (Abreu et al., 2012). While the energy obtained from organic carbon is used in the synthesis in the cell, the chemical energy converted from light energy is stored (Kim et al., 2013). The basic advantages of these cultures are rapid growth rates and increased biomass quality (Kamalanathan et al., 2018; Li et al., 2020). In the selection of organic carbon sources to be used in heterotrophic and mixotrophic culture media, the cost and mechanism of microalgae uptake and assimilation should be considered. In this respect, glucose is

frequently preferred due to its intracellular transport and assimilation mechanism and its higher energy content per mole compared to other substrates (Hamza et al., 2013).

Although microalgae cells need the lag (adaptation) phase to develop specific transport systems before the uptake of carbon sources other than glucose, it has as well been determined that the production potential of bioactive components is higher (Hamza et al., 2013). Although it is thought that the use of external carbon sources in production in heterotrophic and mixotrophic environments will increase the cost, not using light in heterotrophic environments and low light requirements of cells in mixotrophic cultures increase the economic aspect of production (Lin & Wu, 2015; Tosuner & Urek, 2021; 2022). When carbon sources were compared in mixotrophic cultivation, it was observed that sources such as glucose and glycerol were more effective than xylose (Lin & Wu, 2015). The reason for this is that glycerol and glucose transform into glyceraldehyde-3-phosphate intermediate products in the pentose phosphate pathway (Lewin, 1974). Nevertheless, almost equal concentrations of dried biomass were acquired from samples grown with glucose and glycerol under mixotrophic cultivation. PBPs are optionally degraded when cells are subjected to nitrogen starvation (Sloth et al., 2006). Because of, PBPs have acquired a secondary function as intracellular nitrogen storage components that are mobilized for other aims in nitrogen deficiency.

In studies on heterotrophic production of some microalgae with glycerol, it was determined that biomass, growth rate, and PUFA (Polyunsaturated Fatty Acids) content increased (Lin & Wu, 2015). In the previous study, the biomass concentration of *Anabaena* sp. was investigated under three culturing conditions: photoautotrophic, mixotrophic, and heterotrophic (Khoobkar & Amrei, 2021). According to the results, during the first week, an increase in biomass concentration was detected in the photoautotrophic medium, whereas the growth rate was slow in the first week in the mixotrophic medium, but rised in the second week and passed the level obtained in the photoautotrophy. The biomass amount in the heterotrophic cultivation protected from light and receiving only glucose, remained unchanged. In addition to light, microalgae used glucose as an additional energy source in the mixotrophic culture, and therefore biomass production has been reported to increase significantly compared to other types of cultivation (Khoobkar & Amrei, 2021).

Recently, one of the current research topics is the production of economical C-PC, and for this purpose, especially mixotrophic cultivation comes to the fore. Mixotrophic cultivation is achieved in the being of organic carbon resources at a lower light intensity, and heterotrophic cultivation is achieved in a dark condition. Mixotrophy is a sum of phototrophy and heterotrophy, and both organic and inorganic carbons can be used as carbon source (Figure 1.6). In this cultivation, the energy obtained from organic carbon is used in cell synthesis, while the chemical energy converted from light energy is stored (Kim et al., 2013).

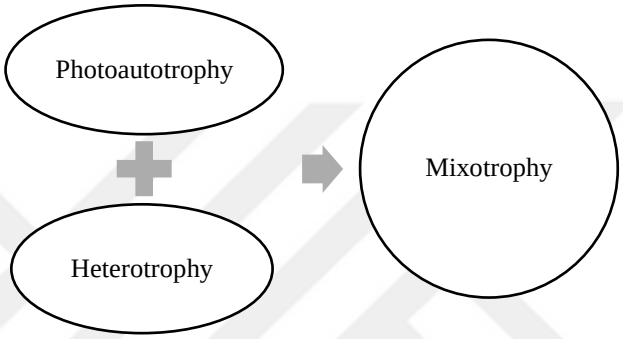

Figure 1.6 Scheme of cultivation

Mixotrophic cultivation is a great alternative method developed to come through the limitations of former cultivation methods and at the same time support high growth rate and biochemical production. The main benefits of mixotrophic cultivation are:

I. Better growth rate and biomass productivity,

II. Better lipid and carbohydrate productivity,

III. Easy scaling process,

IV. It is a long-term logarithmic growth phase (Meng et al., 2020).

Various methods are available for the extraction of C-PC obtained by mixotrophic cultivation. Because of the multilayered cell wall of *A. platensis* cell, C-PC extraction is directly linked to the lysis of the cell and is one of the significant parameters affecting cell lysis method, solvent type, solvent-biomass ratio, extraction time, extraction efficiency, and cost (Silveira et al., 2007; Moraes et al., 2010; İlter et al., 2018). The length of time in cell lysis results in the creation of fine cell debris, which can cause various problems in subsequent processing steps (Tavanandi et al., 2019). It

is important to regulate cell lysis conditions so that, rather than lysis of the cell, it can inhibit the liberation of unwanted proteins from other cell organelles, only to ensure the permeability of the membrane (Tavanandi et al., 2020). Nowadays, the extraction of C-PC with high efficiency, environmentally friendly, cheap, simple, and convenient extraction methods is of great importance. Isolated/purified C-PC finds use in different industries like food, cosmetics, pharmaceuticals, and nutraceuticals (Eriksen, 2008). C-PC, which constitutes 20% of the dry biomass of *A. plantensis* and is one of the most abundant PBPs, finds use in food and cosmetics fields as a coloring agent (Macário et al., 2022).

However, the biocompatibility of commercial C-PC from *Arthrospira* with human cells has been reported to prevent wrinkle formation by adding to the ingredients of moisturizing creams, helping the skin to repair its physical barrier role, and inhibiting oxidative stress (Gheda et al., 2023). A normal cell has a pro-oxidant-antioxidant balance against oxidative stress. However, when the production of oxygen species is greatly increased or antioxidant levels are reduced, this balance may shift towards the pro-oxidant formation, resulting in oxidative stress.

1.5 Free Radicals, Antioxidant, and Anticancer Properties

Free radicals are high-energy atoms or molecules carrying unpaired electrons. Unpaired electrons in their structure make them unstable and they can quickly react with other molecules (Halliwell & Gutteridge, 2015). The production of oxygen-derived reactive species (ROS) in aerobic organisms is tried to be kept in balance with the antioxidant defense system (Sies, 2019). The reasons why antioxidants keep reactive species in a certain balance instead of eliminating them are as follows:

- Excessive production of antioxidants costs more energy compared to cell repair.
- The antioxidant defense mechanism is insufficient, especially against some reactive species such as ROS. In this case, repair or replacement of damaged molecules is preferred.

- Reactive species have a regulatory role *in vivo*. In the regulation of cellular metabolism, the redox state affects the phosphorylation and dephosphorylation mechanisms.

In some cases, this balance is disrupted, and ROS-induced damage occurs. Accumulation of free radicals in the cell or insufficient endogenous defense mechanism disrupts this balance and causes oxidative stress. As a result of oxidation, essential compounds such as DNA, lipid, protein, carbohydrates, and enzymes are adversely affected (Sies, 2019).

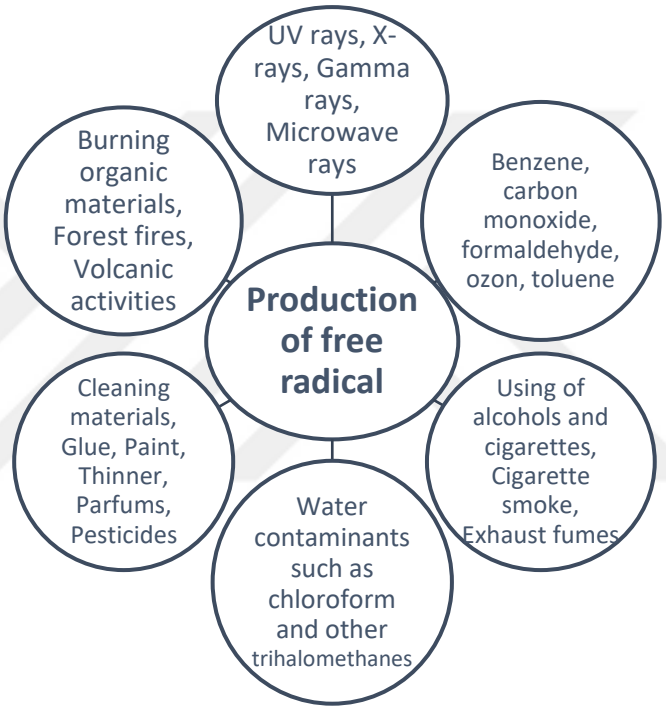

Figure 1.7 Production of free radicals (Phaniendra, Jestadi & Periyasamy, 2015)

Most oxygen-derived free radicals are produced in low amounts over normal aerobic metabolism, such as exposure to radiation and environmental pollution (Figure 1.7). Accordingly, there is damage in the cell that must be constantly corrected by the antioxidant system. Excessive amounts and effects of reactive species over existing antioxidants cause oxidative stress. Oxidative stress can cause cellular consequences such as proliferation, adaptation, migration, adhesion, cell injury, senescence, and death (Halliwell & Gutteridge, 2015). Oxygen and nitrogen-derived radicals begin to form in biological systems, causing cellular damage. To prevent this damage,

antioxidant defense mechanisms come into play in metabolism. Antioxidants are substances that quench reactive species and significantly delay or prevent their effects on their target molecules. There is a delicate balance betwixt the levels of free radicals and antioxidants in cells. Disruption of this balance works in favor of oxidative stress and causes various pathological conditions such as cell damage in metabolism. The level and content of the antioxidant defense mechanism may vary from tissue to tissue, cell type (cell to cell of the same type in a particular tissue), and different subcellular organelles. There may also be an increase in antioxidant defenses after the organism is exposed to high levels of reactive species (Yalçın, 1998).

Antioxidants can be classified in various ways as follow (Figure 1.8) (Aziz, Diab & Mohammed, 2019):

1. By their mechanism of enzymes and non-enzymes actions; antioxidant enzymes convert harmful oxidation products first into H_2O_2 and then into water. Various metals, like iron, copper, selenium, zinc, and iron, are used to be cofactors in these reactions. Plant polyphenols, carotenoids, and glutathione, as well as vitamin C and vitamin E, are non-enzyme antioxidants. These prevent free radical chain reactions.

2. According to their solubility; vitamins soluble in water and lipid. Vitamin C is a water soluble antioxidant situated in the cytoplasm and other cellular fluids.

3. By size (small molecules and large molecules); small molecules have a scavenging effect and neutralize reactive species. The main molecules in this group are vitamins C and E, carotenoids, and glutathione. Large molecules include enzymes such as CAT, GPx, and SOD, and proteins like albumin that scavenge radicals and prevent attacks on other proteins.

4. Accoding to the natural and synthetic antioxidants; minerals with antioxidant effects (like zinc, selenium, manganese, iron, and copper) serve to be cofactors of antioxidant enzymes. Vitamins B, C, and E, among the antioxidant vitamins, are important in performing many body functions. Many of the natural antioxidants are phenolic structures. These act as chain-breaking antioxidants, transforming radicals into more stable structures. The main ones known as phytochemicals are flavonoids, catechins, carotenoids, lycopene, curcumin, and their derivatives. Synthetic antioxidants are

phenolic compounds that scavenge radicals and stop their chain reactions. Major examples are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, and EDTA, a metal chelating agent.

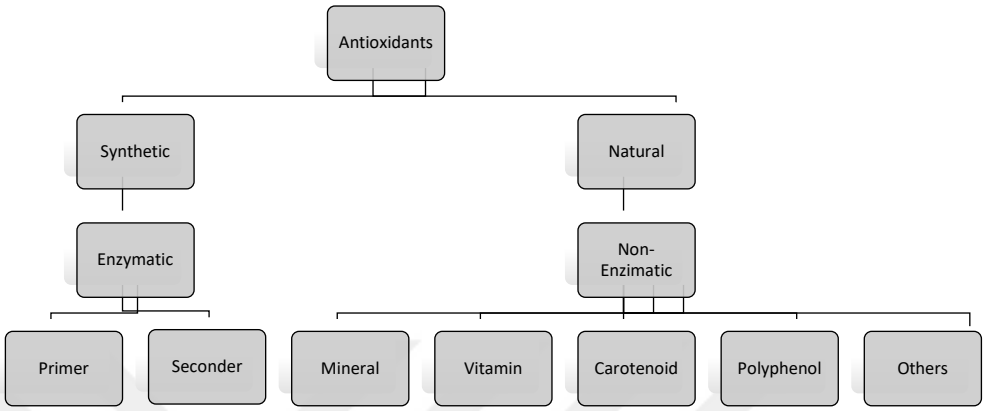

Figure 1.8 Classified of antioxidants (Aziz, Diab & Mohammed, 2019).

Radicals are highly reactive groups, their excess in metabolism causes oxidative stress. The damaging effect of radicals, when immune cells become overactive and toxic to healthy cells, causes many autoimmune diseases (Gulcin, 2020). Antioxidants are structures that prevent oxidative degradation in food and pharmaceutical products and their protective roles in the body and pathological processes mediated by oxidative stress (Figure 1.9). C-PC is one of the main antioxidant components for microalgae and inhibits cell damage, DNA oxidation, and DNA damage by quenching free radicals (Yu et al., 2017). In the previous study, it was reported that C-PC obtained from *Anabaena cylindrical* showed 40% quenching activity of $ABTS^{\cdot+}$ (Macário et al., 2022). In addition, the IC_{50} values of PE purified from *Michrochaete* were determined as 43, 23, and 553 μg/mL, respectively, as $DPPH^{\cdot}$, $ABTS^{\cdot+}$, $O_2^{\cdot-}$ scavenging activities (Afreen & Fatma, 2018).

Antioxidant's important function in delaying or protecting hepatotoxicity, cancer, and heart disease. BHA and BHT used as antioxidants in foods are artificial antioxidants that have potential health risks and toxicities. The utilize of chemical antioxidants has decreased due to their carcinogenic structure, and replacing them with

new, innocuous natural antioxidants has become an important research topic (Afreen & Fatma, 2018). According to the World Health Organization (WHO), cancer is a disease known as metastasis, in which cells grow uncontrollably/rapidly and spread to tissues and organs near the area where they are located (WHO, 2019).

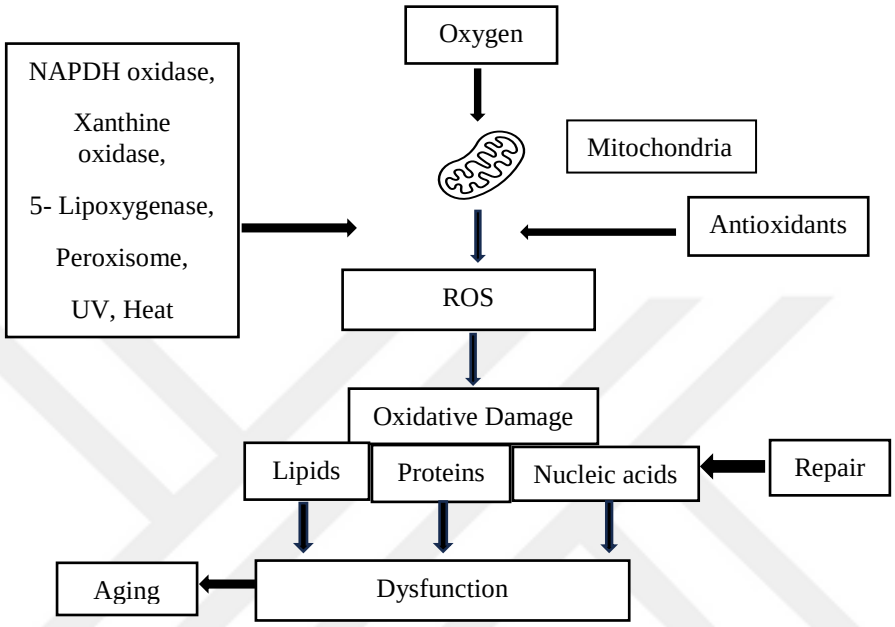

Figure 1.9 Reactive species, oxidative damage, and the aging process (Selman et al., 2012)

Excessive oxidative stress occures cancer production and eventually the death of cells. It is observed that cell death mostly occurs as a result of two basic mechanisms: Apoptosis and necrosis. However, death often occurs through mechanisms that have characteristics of both pathways (Deshpande & Zou, 2020). While mM levels of H_2O_2 cause necrosis in mammalian cells, much lower concentrations of H_2O_2 initiate apoptotic cell death. Activation of ion channels with H_2O_2 and increase in intracellular Ca^{2+} are important in determining which cell death mechanism will be chosen. In cell death with necrosis, swelling of cell organelles, loss of mitochondrial integrity, complete disruption of the structure of the peroxisomal and lysosomal membranes as well as the plasma membrane, and extrusion of cellular content occur. In this process, antioxidant enzymes and pro-oxidants such as heme, iron, and copper, which can negatively affect the surrounding cells, are released. Apoptosis is a form of cell death that has no effect on surrounding cells (Battistelli, Malatesta & Meschini, 2016). The

earliest changes seen in apoptosis are cell condensation, and chromatin fragmentation. This condition is usually associated with disruption of the DNA double helix structure. Other changes include disruption of the cytoskeletal structure, nuclear fragmentation, and ultimately the transformation of the entire cell content into apoptotic bodies containing organelle fragments without disrupting the membrane structure. Apoptotic bodies are phagocytosed by macrophages. Oxidative stress may be the cause of apoptosis. Mitochondrial permeability, which leads to the secretion of pro-apoptotic factors, induces the formation of the transition pore (Harris & DeNicola, 2020). Another mechanism is excessive consumption of GSH. –SH groups of proteins exposed to oxidative agents during the process of apoptosis can be inactivated by reactive species. High levels of reactive species can delay or stop apoptosis. In addition to facilitating the onset and development of cancer by acting as mutagens, ROS also support the proliferation, survival, and metastasis of cancer cells as signal transmission molecules. For example, H_2O_2 is the most stable and membrane-permeable ROS. These properties support the secondary messenger potential of H_2O_2 in cellular communication. This molecule is vital for the activation and maintenance of pro-tumorigenic signaling cascades. Excess H_2O_2, which participates in signal transmission pathways important for tumor development, can participate in Fenton reactions and form $HO^{\bullet}$ radicals. The $HO^{\bullet}$ radical formed also causes lipid peroxidation (LPO) reactions, increasing the cytotoxic lipid hydroperoxide concentration in the cell. For this reason, cancer cells must keep their antioxidant capacity high (Davies, 2016).

Cyanobacteria are one of the natural resources which indicate features in anti-carcinogenesis. Basically, the adverse beneficial properties of extracts from cyanobacteria are because of their phytochemical profile. Production of secondary metabolites by microalgae is perfect chemotherapeutic and antioxidant agents that are simply reachable, cheap, and safe (Romano et al., 2017).

Researches have indicated that C-PC, which has biological activity, has *in vitro* chemotherapy sensitivity, anticancer activity, and tumor-suppressive properties (Wang et al., 2001; Pardhasaradhi et al., 2003; Jaiswal et al., 2008). Wang et al. (2001) reported that C-PC inhibited the growth of HeLa cancer cells, and the rate of inhibition increased depending on the concentration. In addition, it has been determined that it has an antiproliferative effect against HepG2 cells (Roy et al., 2007).

1.6 Aim of the Study

Regulation of production conditions is of great importance in terms of high efficiency and economic production of C-PC, one of the bioactive components from *A. platensis*. The production cost of *A. platensis* also depends on the production, extraction, and isolation costs of C-PC. High-efficiency extraction, high degree of purification, long-term storage and waste minimization of C-PC stand out as limiting factors in applications. Therefore, in the presented master thesis, it is aimed to increase the economic C-PC production with various cultivation types, isolate with the highest efficiency by different simple-environmental-friendly methods such as freeze-thaw, homogenization, ultrasonic-assisted and extraction with glass beads in a mixer mill, and partial purification of *A. platensis* by using some precipation methods and column chromatography. Then, biological activities of C-PC were determined as five different radical scavenging activities such as $DPPH^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $ABTS^{\cdot+}$, $O_2^{\cdot-}$, total reducing power, antioxidant capacities such as FRAP and metal chelation, and cytotoxic properties against HeLa, and OE-33 cancer cell lines. In this thesis;

- Investigation different cultivation types (mixotrophy and heterotrophy) depending on carbon and nitrogen sources/concentrations in order to achieve maximum C-PC production economically from *A. platensis*,
- Examination of some bioactive metabolites (total carbohydrate and lipid, Chl a, Chl b, total carotenoids, APC, total phenolic, total flavonoid, and total tannin, proline, pyruvate, LPO) levels under optimized growth condition,
- Isolation of the produced C-PC from *A. platensis* by various simple and environmentally friendly extraction methods (Freeze-Thaw, UAE, Homogenization, and Mixer Mill),
- Examination of C-PC with different precipitation methods (AS, Organic Solvent, PEG),
- Partial purification of C-PC with DEAE-Sepharose column chromatography, after precipitation,
- Investigation of antioxidant ($DPPH^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $ABTS^{\cdot+}$, $O_2^{\cdot-}$, total reducing power, FRAP and metal chelation) and cytotoxic (OE-33 and HeLa cancer cell lines) properties of partially purified C-PC and,

- Characterization of C-PC with UV-Vis, FT-IR, and TGA were aimed.

CHAPTER TWO

MATERIAL AND METHODS

2.1 Microorganism and Culture Conditions

The cyanobacterium is *A. platensis* M2 was utilized in this thesis. The microorganism was ensured from Adana Çukurova University, Faculty of Aquaculture.

Figure 2.1 *A. platensis* M2 (Personal archive, 2023)

To ensure the continuity of *A. platensis* M2 culture (Figure 2.1), Zarrouk's medium was used (Zarrouk, 1966) (Table 2.1). While preparing the Zarrouk's medium, 1 mL of trace element solution was included to 1 liter. The pH of the growth medium setted to 9.0±0.2 and then sterilized in an autoclave at 121˚C for 20 min. At 30˚C, in the presence of 2500 lux (30 µmol photons/m^2sec) light intensity, sterile air supply (1vvm) is performed by inoculating 750 mL growth medium in 1000 mL borosilicate glass, transparent, flat-bottomed bottles with an initial OD_{600} = 0.200.

Table 2.1. Zarrouk's medium components (Zarrouk, 1966)

Solution 1	**g/L**	**Solution 2 (Trace Elements)**	**g/L (50 mL)**
$NaNO_3$	2.5	H_3BO_3	0.143
K_2HPO_4	0.5	$MnCl_2.4H_2O$	0.00711
$CaCl_2.2H_2O$	0.04	$ZnSO_4.7H_2O$	0.011
$MgSO_4.7H_2O$	0.2	$CuSO_4.5H_2O$	0.004
NaCl	1.0	$Co(NO_3)_2.6H_2O$	0.002
K_2SO_4	1.0	Na_2MoO_4	0.000885
$FeSO_4.7H_2O$	0.01		
EDTA	0.08		
$NaHCO_3$	16.8		

Batch cultivation was performed in a 250 mL Erlenmayer with 100 mL of growth medium at 100 rpm. While white fluorescent lamps (1500 lux = 20.25 μmol photons m^{-2} s^{-2}) were used for continuous light in mixotrophic cultures, heterotrophic cultures were carried out in complete darkness (Figure 2.2). All experiments were performed in triplicate.

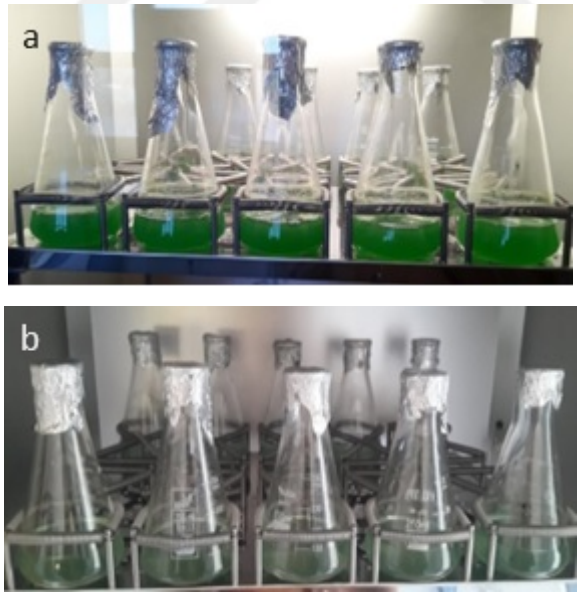

Figure 2.2 Mixotrophic cultivation (a), heterotrophic cultivation (b) of *A. platensis* (Personal archive, 2023).

2.2 Regulation of Microalgae Growth Condition for Maximum Phycocyanin Production in Mixotrophic and Heterotrophic Cultivations

2.2.1 Optimization of Carbon Source

It was studied at concentrations of 1;2.5;5 mM by using crude and technical glycerol, which is a by-product of biodiesel, in mixotrophic and heterotrophic cultivations as carbon source. The contents of crude and technical glycerol used are shown in the Table 2.2. OD_{600}, pH, dry biomass, and C-PC levels were examined depending on the incubation period.

Table 2.2 Crude and technical glycerol compositions (DB Tarımsal Enerji, 2021)

Contents	**Crude glycerol**	**Technical glycerol**
Glycerol	54.35 %	82.5 %
Water	34.81 %	7.7 %
Soap	6.52 %	6.6 %
NaOH	3.53 %	2.4 %
NaCl	0.64 %	0.5 %
Methanol	55 ppm	102 ppm

2.2.2 Optimization of Nitrogen Source

In mixotrophic and heterotrophic cultivations, $NaNO_3$ and urea as nitrogen sources were used at nitrogen deficiency (0 mM), nitrogen starvation (10 mM), and saturated nitrogen (29.4 mM) concentrations for maximum C-PC production. OD_{600}, pH, dry biomass, and C-PC levels were examined depending on the incubation period.

2.3 Methods

2.3.1 Preparation of Crude Extract

After cell collection, ultrapure water (UPW) at a concentration of 5% (w/v) was included and the cell suspension was extracted at 80 kHz for 30 min in an ultrasound-assisted sonicator to obtain the crude extract (Khoigani et al., 2017).

2.3.2 Analyzes Based on the Incubation Period

2.3.2.1 Measuring Optical Density (OD)

The growth curve of the cell was obtained by measuring the optical density of 1 mL of cell suspension in the quartz cuvette at 600 nm (Esen & Öztürk Ürek, 2014).

2.3.2.2 Measuring pH

The pH level was determined by measuring with a pH meter throughout the incubation period (Esen & Öztürk Ürek, 2014).

2.3.2.3 Measuring Dry Biomass

1 mL of cell suspension was placed in a tared watch glass and dried at 105˚C until constant weight. After waiting for it to cool in the desiccator, the dry biomass amount was calculated (Esen & Öztürk Ürek, 2014).

2.3.2.4 Determination of Total Protein Content

Total protein determination was detected to the Bradford method (Bradford, 1976).

Bradford reagent; Coomassie Brilliant Blue G-250 (25 mg) was prepared by dissolving 12.5 mL of ethanol and adding 85% phosphoric acid (25 mL) and making the total volume up to 250 mL with ultra pure water.

For analysis, absorbance was measured at 595 nm 2 min after 100 µL sample was mixed with 900 µL reagent. Bovine serum albumin (0-10 µg/mL) was used as a standard ($y = 0.0457x$; $R^2 = 0.9967$).

2.3.2.5 Determination of Chlorophyll a/b and Total Carotenoid Contents

5 mL of microalgae sample was centrifuged at 5000 rpm for 10 min to precipitate the cell (Lichtenthaler & Wellburn, 1983). The cell pellet was homogenized in 5 mL of 95% ethanol at 8000 rpm for 1 min, and at 9500 rpm for 1 min at intervals of 30 seconds. After centrifugation of the cell solution at 5000 rpm for 10 min, absorbance was measured at 470 nm, 648.6 nm, and 664.2 nm. Calculations are shown as it below:

$$\text{Chl a } (\mu g/mL): 13.36 \times A_{664.2} - 5.19 \times A_{648.6} \quad (2.1)$$

$$\text{Chl b } (\mu g/mL): 27.43 \times A_{648.6} - 8.12 \times A_{664.2} \quad (2.2)$$

$$\text{Total Carotenoids } (\mu g/mL): (1000 \times A_{470} - 2.13 \times \text{Chl a} - 97.64 \times \text{Chl b})/209 \quad (2.3)$$

The obtained results were divided cell weight and the unit was shown as μg/g.

2.3.2.6 Determination of PBP (C-PC, APC) Contents

The absorbance values of the crude extract at 562 nm, 620 nm, and 652 nm were measured to determine the PBP content (Tarko, Duda-Chodak, & Kobus, 2012). Calculations are shown as it below:

$$\text{C-PC } (mg/g): [(A_{620} - 0.474 \times A_{652}) /5.34] / \text{g wt} \quad (2.4)$$

$$\text{APC } (mg/g): [(A_{652} - 0.208 \times A_{620}) /5.09] / \text{g wt} \quad (2.5)$$

The purity of the C-PC was determined by the A_{620}/A_{280} ratio.

2.3.2.7 Determination of Total Carbohydrate Content

Total carbohydrate content was determined by the phenol-sulfuric acid method (Dubois et al. 1956). 1 mL of the prepared microalgal crude extract, 1 mL 5% (w/v) phenol, and 5 mL concentrated H_2SO_4 were added in a test tube, vortexed, and kept for 20 min. The absorbance of the content was read at 470 nm. Total carbohydrate content was calculated from the calibration curve obtained utilizing glucose monohydrate (0–250 μg/mL) as the standard ($y = 0.00762x$; $R^2 = 0.998$).

2.3.2.8 Determination of Total Lipid Content

Phospho-vanillin reagent; Vanillin (0.6 g) was mixed in ethanol (10 mL) and ultra pure water (90 mL) and then phosphoric acid (400 mL) were added.

100 μL of microalgae sample and 2 mL of concentrated H_2SO_4 were mixed and kept at 100°C for 10 min (Mishra et al., 2014). After incubation for 5 min in an ice bath, 5 mL of phospho-vanillin reagent were included to the mixture and the absorbance was read at 530 nm after 15 min of incubation at 37°C. Total lipid (0-100 μg/mL) was calculated with the sunflower oil standard ($y = 0.00453x$; $R^2 = 0.993$).

2.3.3 Analyzes at Maximum Phycocyanin Production Conditions

2.3.3.1 Determination of Proline Content

Proline level of the obtained cells was estimated by the method of Bates, Waldren and Teare (1973). After centrifugation, it was mixed with 1 g of cells and 10 mL of 3% (w/v) sulphosalicylic acid. After being homogenized at 80 kHz for 30 min, the upper phase (1 mL) was separated by centrifugation (12000 rpm, 10 min, 4 ˚C) and mixed with 1 mL of 2.5% (w/v) ninhydrin solution and 1 mL acetic acid (Esen & Öztürk Ürek, 2014). The mixture was incubated in a boiling water bath for 1 h and then directly transferred to an ice bath. After adding 2 mL of toluene, it was mixed for 15 sec and left at room temperature until phase separation occurred (approximately 30 min). The absorbance level of the upper toluene phase was read at 520 nm. The amount was calculated utilizing L-proline (0-80 µg/mL) as the standard ($y = 0.0241x$; $R^2 = 0.986$). Calculations are shown as it below:

[Proline (µmol/g)] = [(µg proline/mL * toluene) / 115.5 µg /µmol] / [(g sample) /5] (2.6)

2.3.3.2 Determination of Pyruvate Content

The pyruvate content of the obtained cells was detected according to the method of Friedeman and Haugen (1943).

2,4-dinitrophenylhydrazine reagent; 0.1 g 2,4-dinitrophenylhydrazine was mixed in 100 mL 2N HCI.

After mixing 0.6 mL of the prepared microalgal crude extract with 1 mL 2,4-dinitrophenylhydrazine reagent, it was left for 5 min, and 1.2 mL 2N NaOH was added and vortexed again. The absorbance value of the sample was read at 520 nm. Pyruvate level was calculated from the calibration curve obtained using pyruvic acid (1-25 µg/mL) as the standard ($y = 0.0325x$; $R^2 = 0.9977$).

2.3.3.3 Determination of Total Phenolic Content (TPC)

Reaction mixture contains Folin Ciocalteu reagent, sodium carbonate (7%, w/v) and crude microalgae extract (Singleton, Orthofer & Lamuela-Raventós, 1999). After 30 min of incubation at room temperature, the absorbance value of the mixture was

determined at 765 nm and gallic acid (0-20 µg/mL) was used as the standard (y = 0.0831x; R^2=0.9909). The TPC was quantified in milligrams of gallic acid equivalent per gram of cell (mgGAE/g).

2.3.3.4 Determination of Total Flavonoids Content (TFC)

The reaction medium containing aluminum chloride (10%, w/v), sodium acetate (1 M), and crude microalgal extract was incubated in the dark for 30 min (Sakanaka, Tachibana & Okada, 2005). At the end of the incubation, the absorbance value of the samples was detected at 415 nm. The total content of flavonoids for sample was expressed as quercetin equivalent using quercetin (0–20 µg/mL) as the standard (y = 0.0469x; R^2 = 0.9967). The total flavonoid content (TFC) was measured in milligrams of quercetin equivalent per gram of cell (mgQE/g).

2.3.3.5 Determination of Total Tannin Content (TTC)

Total tannin content (TTC) was detected utilizing the method identified by Broadhurst and Jones (1978). For this purpose, the mixture containing the prepared microalgal extract, vanillin solution (4%, w/v), and concentrated HCl was vortexed and incubated at 25˚C in the dark for 20 min. After incubation, absorbance value was monitored at 500 nm and the results were shown as tannic acid equivalent using the tannic acid (0-100 µg/mL) calibration curve (y = 0.0171x; R^2 = 0.971). TTC was quantified as milligrams of tannic acid equivalents per gram of cell (mgTAE/g).

2.3.3.6 Determination of Lipid Peroxidation (LPO)

LPO value was estimated according to Ohkawa, Ohishi, and Yagi (1979). Centrifugally separated cells (0.1 g) were mixed with 1.5 mL of 5% (w/v) trichloroacetic acid (TCA) and extracted. Equal volumes of supernatant and 0.5% (w/v) thiobarbituric acid (TBA) were added. The mixture was kept in a water bath (95˚C) for 30 min and then putted to an ice bath. The absorbances of the samples (532 and 600 nm) were measured and the LPO value was calculated as malondialdehyde (MDA) equivalent according to the following equation:

$$MDA\ (\frac{nmol}{g}) = \frac{(A532-A600)\ x\ volume\ of\ \ extraction}{155\ x\ g\ sample} \quad (2.7)$$

2.4 Optimization of Extraction of C-PC

2.4.1 Mixer Mill Extraction

Oven-dried (ODB), lyophilized (LB), frozen (FB) (at -20˚C for 24 h), and wet microalgal biomass (WB) samples were prepared at concentrations of 1; 2.5; 5; 8 % (w/v) with UPW and also 10 mM phosphate buffer (pH = 7.0). In the 1 mL of cell suspension, cell lysis was carried out at 4˚C and 25˚C for 5 and 10 min by placing them in 2 mL round-bottom plastic vials containing 0.5 g (0.5- and 0.25-mm diameter) glass beads (Schütte & Kula, 1988). Then, protein and C-PC levels were investigated in the supernatants after centrifugation at 12000 rpm, 10 min, and 4°C.

2.4.2 Ultrasound-Assisted Extraction (UAE)

ODB, LB, FB (at -20˚C for 24 h), and WB samples were prepared at concentrations of 1; 2.5; 5; 8 % (w/v) with UPW and also 10 mM phosphate buffer (pH = 7.0). Cell suspensions were extracted for 10 min in the ultrasonicator at a constant temperature, depending on the frequency of 37 and 80 kHz (Khoigani et al., 2017). The extract obtained at the end of the extraction was centrifuged at 5000 rpm for 10 min and the amounts of C-PC and protein in the supernatants were determined. The frequency was determined according to the findings in which the highest amounts of C-PC and protein were obtained, and then the process was repeated at constant temperature for 5 and 30 min. The process conditions in the ultrasonicator were optimized by determining the amounts of C-PC and protein.

2.4.3 Homogenization

ODB, LB, FB (at -20˚C for 24 h), and WB samples were made at concentrations of 1; 2.5; 5; 8 % (w/v) with UPW and also 10 mM phosphate buffer (pH = 7.0). After centrifuging the prepared suspensions using a homogenizer for different times and speeds (1 min at 8000 rpm, 1 min at 9500 rpm, 1 min at 13500 rpm) at 4˚C, protein, and C-PC levels were investigated in the supernatants (Esen & Öztürk Ürek, 2015).

2.4.4 Freeze-Thaw Method

The samples of ODB, LB, FB (at -20˚C for 24 h), and WB were made at concentrations of 1; 2.5; 5; 8 % (w/v) with UPW and also 10 mM phosphate buffer (pH = 7.0). Each cycle consists of freezing cell suspensions at − 20˚C and then thawing them at room temperature (~ 20˚C). Protein and C-PC analyses were performed after centrifugation in double and triple cycles to determine the optimal condition (Berrouane et al., 2022).

2.5 Isolation and Partial Purification of C-PC

2.5.1 Precipitation with Ammonium Sulfate (AS)

After the determined optimal C-PC extraction method, the C-PC extract obtained with the best yield and purity was subjected to a protein precipitation process with ammonium sulfate at 25, 50, 75% (w/v) saturation at 4˚C. The precipitate obtained after centrifugation was dissolved in UPW (Su et al., 2010). C-PC and protein levels were estimated in the samples prepared in the upper and lower phases.

2.5.2 Precipitation with Organic Solvent (OS)

The crude C-PC extract was treated with organic solvents (ethanol and methanol) in different ratios (1/1, 1/2, 1/3 (v/v)) at 4˚C and protein precipitation was performed. The precipitate obtained after centrifugation was dissolved in UPW. C-PC and protein levels were estimated in the samples prepared in the upper and lower phases (Novák & Havlíček, 2016).

2.5.3 Precipitation with Polyethylene Glycol (PEG)

The crude C-PC extract was treated with PEG (2000 and 5000) of different molecular weights at 4˚C and protein precipitation was performed. The precipitate obtained after centrifugation was dissolved in UPW. C-PC and protein levels were estimated in the samples prepared in the upper and lower phases (Hönig & Kula, 1976).

2.5.4 Column Chromatography (CC)

After determining the precipitation method with the best purity, the concentrated C-PC sample was performed to a DEAE Sepharose column (1.6×20 cm) balanced with 20 mM phosphate buffer (pH = 7.0) (Roy & Pabbi, 2022). C-PC elution was achieved

and finally performed with 20 mM phosphate buffer (pH=7.0) including 1 M and 2 M NaCl. The flow rate was 0.6 mL min^{-1} and 2.5 mL fractionated. The absorbance value of the fractions was monitored at 620 and 280 nm. The purity of the fractions was estimated by the A_{620}/A_{280} ratio. Fractions with the highest purity were concentrated by ultrafiltration.

2.5.5 Ultrafiltration

The obtained C-PC sample was concentrated using ultrafiltrates of 10 kDa (Amicon cell equipped with a YM10 cellulose membrane). Thus, the purity ratio (A_{620}/A_{280}) of C-PC was increased (Martelli et al., 2014).

2.6 Characterization of Partially Purified C-PC

2.6.1 Determination of Antioxidant Properties

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH·): This method is based on scavenging DPPH· in the presence of a hydrogen-donating antioxidant source (Shimada et al., 1992). $DPPH^{\cdot}$ solution (1 mM) was prepared in methanol and varying concentrations of partially purified C-PC were mixed. The reaction mixture was kept for 30 min at room temperature and in the dark, and then its absorbance value was read at 517 nm. Vitamin C was used for the standard (y=2.6707x; R^2 = 0.995). The absorbance value of the $DPPH^{\cdot}$ solution at 517 nm was taken as A_0 and IC_{50} values, which are the amount that achieves 50% scavenging, were detected. DPPH· quenching activity was determined by the following formula.

$$\%Scavenging = \frac{A_0 - A_{sample}}{A_0} \quad (2.8)$$

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$): ABTS is oxidized by oxidants to a colored compound, $ABTS^{\cdot+}$, and its quenching activity is determined as the ability to directly react with the ABTS radical. In the method, ABTS solution and potassium persulfate (final concentration 2.45 mM) were added and kept in the dark for 16 h (Nenadis et al., 2004). The radical cation solution formed as a result of the reaction was diluted to an initial absorbance value of 1.00±0.02 at 734 nm and partially purified C-PC at different concentrations was applied. The absorbance values

of the resulting reaction mixture at 0 and 6 min were measured at 734 nm and then detected IC_{50} values. Vitamin C was used as positive control (y=53.58x; R^2 = 0.9717).

Hydroxyl Radical ($HO^{\cdot}$) Scavenging: In the method, the reaction mixture was contained partially purified C-PC at varying concentrations, $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (100 µM), vitamin C (100 µM), deoxyribose (2.8 mM), phosphate buffer (20 mM, pH 7.4). After the mixture was kept at 37˚C for 60 min, 2% (w/v) TCA and 1% (w/v) TBA were mixed to the medium and kept in a boiling water bath for 15 min (Halliwell et al., 1987). Absorbance was read at 535 nm against the mixture containing buffer instead of deoxyribose. A_0 was prepared by adding phosphate buffer to the reaction medium instead of the sample and IC_{50} values, which are the amount that achieves 50% scavenging, were determined. The percent $HO^{\cdot}$ quenching activity was determined by the following formula:

$$\%Scavenging = \frac{A_0 - A_{sample}}{A_0} \quad (2.9)$$

Nitric Oxide Radical ($NO^{\cdot}$) Scavenging: In this method, the reaction medium was contained sodium nitroprusside (10 mM), phosphate-salt buffer (20 mM, pH 7.4) and partially purified C-PC in varying concentrations (Jaiswal et al., 2010). The mixture was kept for 150 min at 25˚C. Thereafter, Griess reagent (A+B, A: 2% (w/v) sulfanilamide, B: 0.2% (w/v) naphthyl ethylenediamine dihydrochloride) was included to the medium and kept for 30 min at 25˚C. A_0 was prepared by adding phosphate buffer to the reaction medium instead of the sample and IC_{50} values, which are the amount that achieves 50% scavenging, were determined. The absorbances of samples were read at 546 nm. The percent $NO^{\cdot}$ quenching activity was determined by the following formula:

$$\%Scavenging = \frac{A_0 - A_{sample}}{A_0} \quad (2.10)$$

Superoxide Anion Radical ($O_2^{\cdot -}$) Scavenging: This method is based on the formation of a colored complex of the stable product as a result of oxidation, reduction of superoxide, or binding of the radical to an indicator. The reaction medium was prepared by mixing with superoxide radical was formed with nicotinamide adenine dinucleotide (78 µM) (reduced form), nitro blue tetrazolium (50 µM), phenazine methosulfate (10 µM) in Tris-HCl buffer (16 mM, pH 8.0) and partially purified C-PC

in varying concentrations (Liu et al., 1997). The absorbances of the formed colored complex of the sample and blank were read at 560 nm and IC_{50} values, which are the amount that achieves 50% scavenging, were determined. The percent $O_2^{\cdot -}$ quenching activity was determined according to the following formula:

$$\% \, Scavenging = \left[1 - \frac{A_{sample}}{A_{blank}}\right] \times 100 \qquad (2.11)$$

Total Reducing Power: Reaction mixture was contained $K_3Fe\,(CN)_6$ (1%, w/v), phosphate buffer (0.2 M, pH 6.6), and varying concentrations of partially purified C-PC. After the reaction medium was kept for 30 min at 50˚C, TCA (10%, w/v) was included, and then the mixture was centrifuged (Oyaizu, 1986). Samples taken from the upper phase of the solution were mixed with $FeCl_3$ (0.1%, w/v) and their absorbance value was monitored at 700 nm. Increasing absorbance value is an indicator of increasing total reducing power. Using vitamin C as the standard ($y = 0.0957x$; $R^2 = 0.9864$), total reducing power was explained as milligrams of vitamin C equivalents per gram of cell (mgVCE/g).

Ferric Reducing Antioxidant Power (FRAP): This method is based on a typical electron transfer that measures the reduction of Fe^{3+} ions by antioxidants in an acidic environment to an intense blue complex of Fe^{2+} ions. The reaction medium included varying concentrations of partially purified C-PC, acetate buffer (300 mM, pH 3.6), 2,4,6-Tris (2-pyridyl)-s-triazine (10 mM) and $FeCl_3$ (20 mM) was kept for 30 min at 37 ˚C in a dark environment (Benzie & Strain, 1996; Thaipong et al., 2006). Then, absorbances were read at 593 nm. The FRAP value of the sample was calculated as $FeSO_4$ equivalent ($y=0.0545x$; $R^2 = 0.9857$). It was explained as milligrams of $FeSO_4$ equivalents per gram of cell.

Metal Chelating: Metal chelating capacity is detected by measuring the creation of the Fe^{2+} ferrozine complex. In the method, $FeCl_2$ (1 mM) and varying concentrations of partially purified C-PC, were kept at room temperature for 30 min and then ferrozine (5 mM) was included to the mixture (Carter, 1971). The absorbance of the colored complex formed by the binding of $Fe^{2+}$to ferrozine was read at 562 nm. EDTA was utilized as a positive control ($y=9.352x$; $R^2= 0.9911$). IC_{50} values, which are the amount that achieves 50% quenching, were determined for the sample.

2.6.2 Determination of Cytotoxic Properties

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the transformation of MTT into blue formazan crystals by living cells (Mosmann, 1983). Cancer cell lines as HeLa (ATCC® CCL-2), and OE-33 (DSMZ ACC706) were inoculated at 1×10^5 cells per well in 96-well plates and grown in a humidified atmosphere of 5% CO_2 at 37˚C. The cancer cell lines were incubated with different concentrations of the C-PC in 10% DMSO for 24 and 48 h. After incubation, MTT (5 mg/mL) was added to each well and the plates were held for about 4 h at 37˚C. The formazan crystals that formed were subsequently mixed in DMSO and absorbance was monitored at 570 nm utilizing a microplate reader. The percent inhibition values of the C-PC against HeLa, and OE-33 cells were calculated, and then cytotoxic effects of C-PC were determined.

2.6.3 Characterization of C-PC

2.6.3.1 Ultraviolet-Visible Spectroscopy (UV-Vis)

After 5 mL of microalgal sample was centrifuged, homogenized by adding 20 mM phosphate buffer (pH 7.0) at a rate of 12.5 mL per gram. Then it was centrifuged again and the UV-Vis spectrum of the supernatant between 200-800 nm was measured.

*2.6.3.2 Fourer Transform Infrared Spectroscopy (FT-IR***)**

After 10 mg KBr was dried, it was turned into a pellet before measurement, and the measurement was made after dropping the C-PC extract. Measurements were made with the Perkin Elmer Spectrum BX FT-IR System device.

2.6.3.3 Thermogravimetric Analysis (TGA)

The C-PC sample, which was dried by lyophilization, was weighed 10 mg in powder form and analyzed in a nitrogen environment at 600˚C. Measurements were made with a Perkin Elmer STA 6000 DTA / TG device.

2.7 Statical Analysis

The statistical analyses were conducted in triplicate and the results were reported as the mean ± standard deviation. Statistical significance was evaluated through the application of the ANOVA Students t-test. A p-value of less than 0.05 was deemed to be statistically significant.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Regulation of Microalgae Growth Condition for Maximum C-PC Production

3.1.1 Effect of Carbon Source /Concentration and Cultivation Type on the Production of C-PC

Generally, microalgae are produced by photoautotrophic cultivation, and they utilize CO_2 like the carbon resource and light like an energy resource in the photosynthetic metabolic pathway (Gonzalez-Toril & Pereto´, 2011). Although this type of cultivation is an easily applicable method and does not require an extra carbon source, it is hard to achieve high-density microalgae biomass due to the annoyed light inside of culture medium (Hamza et al., 2013). To solve this problem experienced in photoautotrophic cultures, mixotrophic and heterotrophic cultures have come to the fore (Kovač et al., 2017). In heterotrophic cultures, microalgae production is carried out using organic carbon sources like carbohydrates and organic acids without of light (Chen et al., 2011). This type of cultivation ensures high biomass production. In addition, it has been determined that microalgal cell concentration and volumetric efficiency increase and the photoinhibition effect decreases in mixotrophic cultures performed in environments with light and external carbon sources (Chojnacka & Noworyta, 2004; Hamza et al., 2013).

In the study, the OD_{600} values of *A. platensis* depending on the crude glycerol carbon source and concentration were shown in Figure 3.1. When the growth curves of *A. platensis* depending on crude glycerol concentration are examined, it is seen that as the concentration increases, it follows a lower trend until the stationary phase. Then the growth curves show a parallel trend depending on the crude glycerol concentration in the stationary phase.

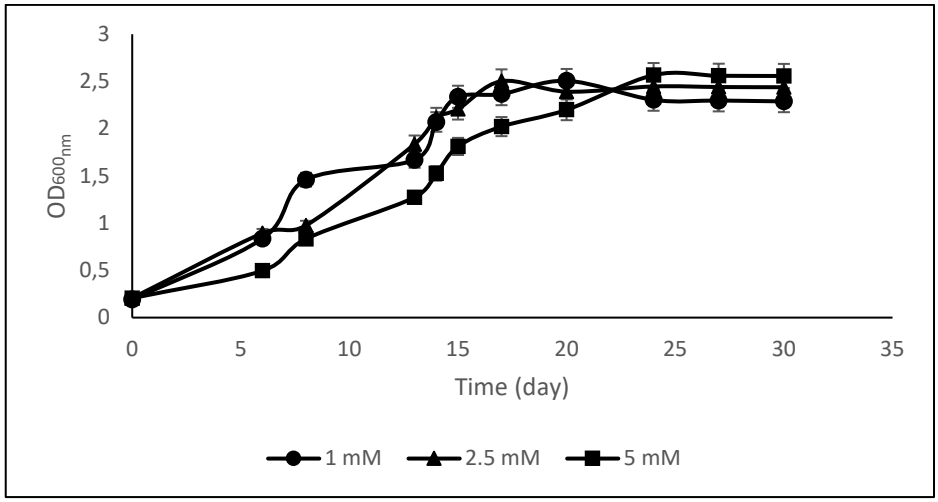

Figure 3.1 *A. platensis* growth curves depending on the incubation period and crude glycerol concentration in mixotrophic cultivation at 30˚C and 100 rpm. Results were expressed as mean±standard deviation of three independent replicate.

When C-PC production dependent on crude glycerol concentration was examined, the best C-PC in the presence of 1 mM crude glycerol was found to be 3.75±0.12 mg/g on day 15 (Figure 3. 2 and Table 3.1). Since it was observed that C-PC production decreased as the concentration of crude glycerol increased, the optimal glycerol concentration was determined as 1 mM.

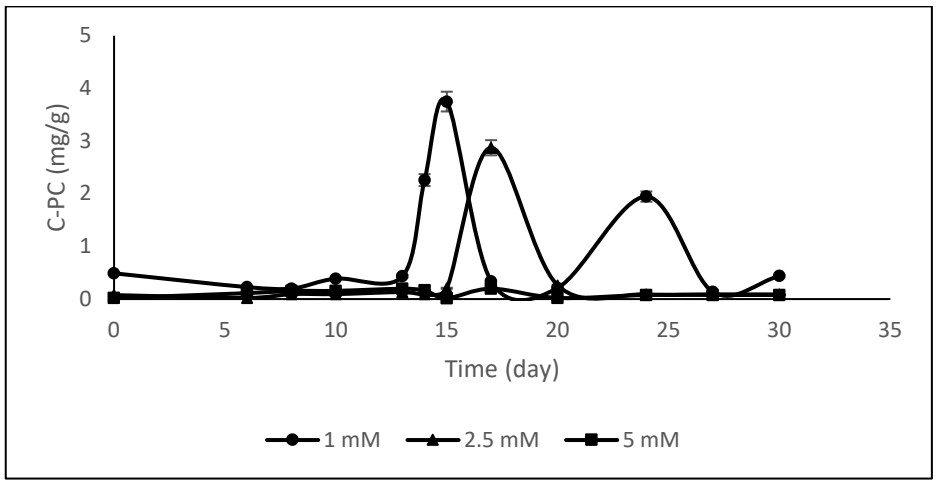

Figure 3.2 C-PC production dependent on the incubation period in mixotrophic cultivation including crude glycerol. Results were expressed as mean±standard deviation of three independent replicate.

Table 3.1 Maximum C-PC contents produced in mixotrophic cultivation including crude glycerol. Results were expressed as mean±standard deviation of three independent replicate.

Crude glycerol concentration	**1 mM**	**2.5 mM**	**5 mM**
Incubation days	15	17	17
Maximum amount (mg/g)	3.75±0.12	2.87±0.17	0.20±0.01

As in the cultivation of the entity of crude glycerol, mixotrophic cultivation was accomplished in the addition of 1-, 2.5-, and 5-mM technical glycerol. The best C-PC production was determined as 1.61±0.11 on the 15th day in mixotrophic cultivation at 1 mM technical glycerol. When equated to the maximum C-PC production in the inside of crude glycerol, it was determined that the obtained value was around 49% ($p<0.05$).

A. platensis, which lives in a basic environment, was cultivated in a medium adjusted to pH 9.0. The pH continued to increase as the cell grew and into the stationary phase when maximum C-PC production was reached. Then in the death phase, pH decreases were observed as the cell wall broke down and intracellular materials were released (Figure 3.3).

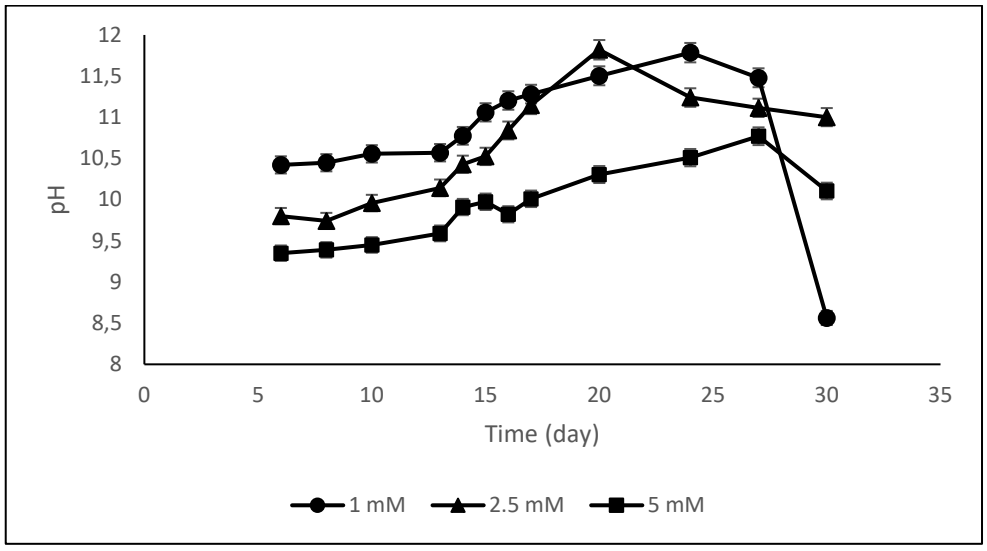

Figure 3.3 pH variation on the production of C-PC dependent on the incubation period in mixotrophic cultivation including crude glycerol. Results were expressed as mean±standard deviation of three independent replicate.

In mixotrophic cultivation, while the dry biomass of *A. platensis* increases with the growth of the cell, a decrease was noted with the death phase. The increase in the concentration of crude glycerol as a carbon resource inside of growth medium also caused an rise in dry biomass (Figure 3.4).

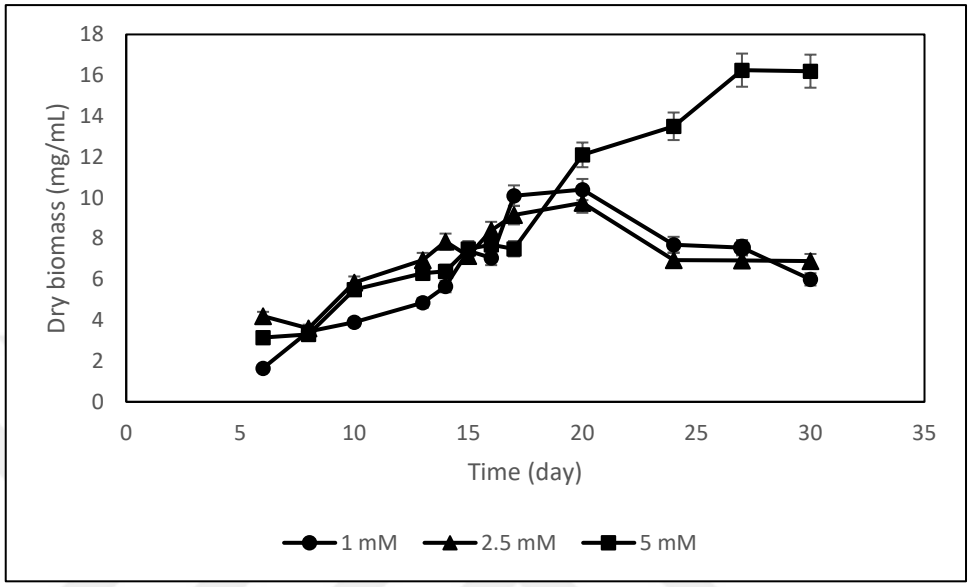

Figure 3.4 Dry biomass variation on the production of C-PC dependent on the incubation period in mixotrophic cultivation including crude glycerol. Results were expressed as mean±standard deviation of three independent replicate.

In heterotrophic cultivation, C-PC production was investigated inside of dark environment at 30˚C and 100 rpm in the entity of crude glycerol and technical glycerol at concentrations of 1-, 2.5- and 5- mM. To results acquired, the maximum C-PC production was obtained like 0.11 mg/g on the 13th day in heterotrophic cultivation including 1 mM crude glycerol.

As a result, in this thesis, since the highest C-PC production was acquired inside of 1 mM crude glycerol, crude glycerol and mixotrophic cultivation were decided as the carbon source and cultivation type in production of C-PC.

3.1.2 Effect of Nitrogen Source /Concentration on the Production of C-PC

After determining the cultivation type and carbon source/concentration to maximum C-PC production from *A. platensis*, the effect of nitrogen source/concentration was examined. For this purpose, $NaNO_3$, and urea were utilized

as nitrogen resources in the mixotrophic cultivation. The experiments were carried out at saturated (29.4 mM), starvation (10 mM), and deficiency (0 mM) nitrogen concentrations.

As seen in Figure 3.5, when $NaNO_3$ was used as a nitrogen source, it was observed that nitrogen deficiency and starvation conditions negatively affected C-PC production from *A. platensis*.

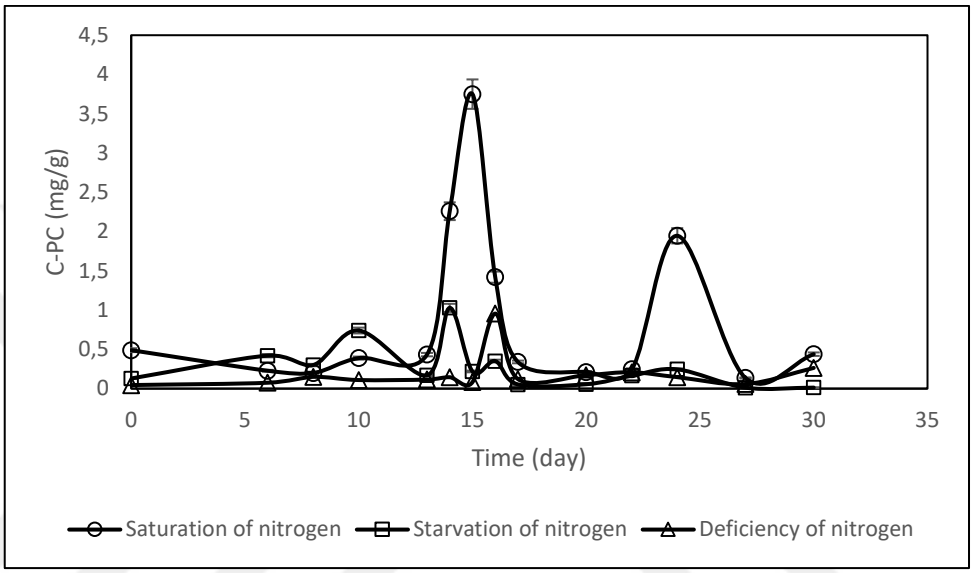

Figure 3.5 C-PC production variations depending on $NaNO_3$ concentrations in the presence of 1 mM crude glycerol. Results were expressed as mean±standard deviation of three independent replicate.

Table 3.2 Maximum C-PC contents produced in mixotrophic cultivation in the presence of varying concentrations of $NaNO_3$. Results were expressed as mean±standard deviation of three independent replicate.

$NaNO_3$ Concentration	**$NaNO_3$ deficiency**	**$NaNO_3$ starvation**	**$NaNO_3$ saturation**
Incubation days	16	14	15
Maximum amount (mg/g)	0.95±0.07	1.03±0.08	3.75±0.12

When C-PC production was examined under mixotrophic conditions at three concentrations of urea (0, 10, 29.4 mM), the maximum C-PC production was detected like 1.585±0.11 mg/g on the 16^{th} day inside of 10 mM urea (Table 3.2). This value is

54.18% higher than the C-PC obtained in the presence of $NaNO_3$ under the same conditions ($p<0.05$). However, the C-PC level obtained in the medium with saturation $NaNO_3$ concentration was determined to be 2.37 times higher ($p<0.05$). For this reason, the $NaNO_3$ and saturated nitrogen concentration was determined as optimal for maximum C-PC production from *A. platensis*. Since C-PC is a nitrogen storage compound in *A. platensis*, reaching the highest nitrogen saturation concentration is also supported by a mixotrophic study in the presence of glucose (Chen & Zhang, 1997).

Optimal conditions for maximal C-PC production from *A. platensis* in mixotrophic cultivation were determined as 1 mM crude glycerol and 29.4 mM $NaNO_3$. In this optimal condition, the purity of C-PC as A_{620}/A_{280} value was determined as 0.83.

3.1.3 Change of Biometabolites in Optimal C-PC Production Condition

The biometabolites in *A. platensis* microalgae are generally PBPs, Chl a, Chl b, total carotenoids, protein, lipids, and carbohydrates. Changes in these biometabolites under maximum C-PC production conditions were also investigated. Chl a, Chl b, total carotenoid, and APC levels from *A. platensis* were examined depending on the incubation period (Figure 3.6). The grades of Chl b, Chl a, and total carotenoid followed a similar trend depending on the incubation period. A similar trend was observed in total carotenoid, Chl b, and Chl a curves included stationary phase, especially between the 15^{th} and 27^{th} days of the incubation period. Chl a level was continued to increase up to the 15^{th} day when C-PC production is maximum.

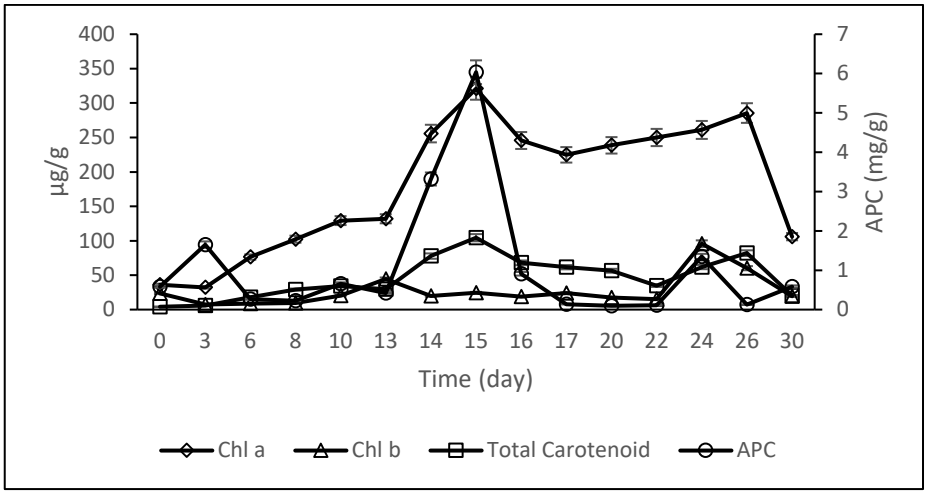

Figure 3.6 Chl a, Chl b, total carotenoid, and APC levels depending on the incubation period under C-PC optimized production conditions. Results were expressed as mean±standard deviation of three independent replicate.

While in a previous study, the maximum Chl a level was obtained as 106.3±5.2 µg/g on the 14th day, this value was calculated like 0.321±0.03 mg/g on the 15th day in this thesis. When total carotenoid levels were compared, a result was obtained that was approximately 3-times higher than the value in the previous study ($p<0.05$) (Esen & Öztürk Ürek, 2015).

While Chl a, total carotenoid and APC levels reach their maximum value on the 15th day, the Chl b level is at its maximum value on the 24th day. Chl a, total carotenoid, and APC level have its maximum value on the 15th day, directly proportional to total protein and C-PC levels. When we compare these bioactive components levels, APC has the highest, followed by Chl a, total carotenoid, and Chl b, respectively (Table 3.3).

Table 3.3 Maximum Chl a, Chl b, total carotenoid, and APC levels depending on the incubation period under C-PC optimized production conditions. Results were expressed as mean±standard deviation of three independent replicate.

Biometabolites	**Chl a**	**Chl b**	**Total Carotenoid**	**APC**
Days	15	24	15	15
Maximum amount (mg/g)	0.321±0.03	0.096±0.002	0.104±0.01	6.03±0.31

Total protein, lipid, and carbohydrate levels depending on the incubation period under C-PC optimum production conditions were given in Figure 3.7. When the total components were examined under maximum production conditions depending on the incubation period (Table 3.4), the highest level is observed in the amount of total protein on the 15th day, followed by the total lipid level on the 27th day and the total carbohydrate level on the 20th day. *A. platensis*, which is described in the literature as having a high protein amount, is supported by the experimental results (Jung et al., 2021).

According to the literature, it is known that carbohydrate synthesis in microalgae linked on nitrogen restriction in the culture condition operating in batch mode. However, in the current thesis study, since optimized conditions were obtained in a nitrogen-saturated environment, this negatively affected carbohydrate production (Menegotto et al., 2019). Total lipid level is supported by the relevant literature, alike results were acquired in the research by Rosero-Chasoy and colleagues (Rosero-Chasoy et al., 2022).

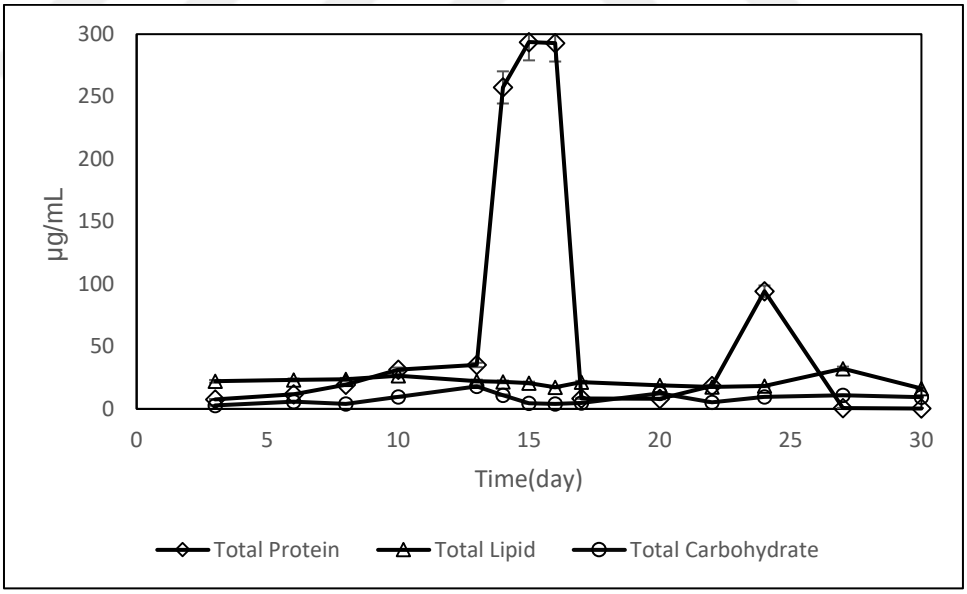

Figure 3.7 Total protein, total lipid and total carbohydrate levels depending on the incubation period under C-PC optimum production conditions. Results were expressed as mean±standard deviation of three independent replicate.

When the total protein amounts were examined in the mixotrophic cultivation of *A. platensis* at varying crude glycerol concentrations, the highest production was detected on the 15th day at 1 mM ($p<0.05$). In addition, as the crude glycerol concentration increased, lower maximum protein levels were determined on further incubation days. These results are also similar to C-PC production. In addition, in the 1 mM technical glycerol medium, where the best C-PC value was obtained, a total protein value of 55.305±4.58 µg/mL was obtained on the 15th day.

Table 3.4 Maximum total protein, lipid and carbohydrate amounts depending on the incubation period under C-PC optimum production conditions. Results were expressed as mean±standard deviation of three independent replicate.

Biometabolites	**Total Protein**	**Total Lipid**	**Total Carbohydrate**
Incubation days	15	27	20
Maximum amount (µg/mL)	293.71±12.77	32.23±2.54	12.46±1.08

Total phenolic, flavonoid, and tannin amounts, and pyruvate, proline, and LPO levels were determined by spectrophotometric methods under maximum C-PC production conditions. The results acquired were given in Table 3.5.

Table 3.5 TPC, TFC, and TTC as well as pyruvate, proline, and LPO amounts depending on the incubation time under C-PC optimum production conditions. Results were expressed as mean±standard deviation of three independent replicate.

Biometabolites	**Amount**
TPC	10.20±0.95 mg/g
TFC	95.59±2.43 µg/g
TTC	86.28±2.57 µg/g
Pyruvate	310.01±12.84 µg/g
Prolin	0.72±0.06 µmol/g
LPO	0.01±0.001 nmol/g

The TPC value found from *A. platensis* in the study by Hammad, El Desoky, and Saleh was lower than the value found in the thesis (Hammad, El Desoky & Saleh, 2023). In the research of Tarko and colleagues, the TPC value was not found inside of SAG strain of *A. platensis*, but in the thesis, this value was found to be 10.20±0.95

mg/g (Tarko, Duda-Chodak & Kobus, 2012). Also, the TPC of *A. platensis* (Gomont) Geitler (MIYE 101) was found as 12.1 mg GAE/g (Ismaiel, El-Ayouty, & Piercey-Normore, 2016).

Very high TPC, TFC, TTC and pyruvate values were obtained under optimal C-PC production conditions. Additionally, the fact that low levels of proline and LPO values were obtained can be said to indicate that *A. platensis* is not under stress.

In the selection of organic carbon sources to be used in mixotrophic culture media, the cost and mechanism of microalgae uptake and assimilation should be considered. In this respect, glucose is frequently preferred due to its intracellular transport and assimilation mechanism and its higher energy content per mole compared to other substrates (Hamza et al., 2013). Although microalgae cells need the lag (adaptation) phase to develop specific transport systems before the uptake of carbon sources other than glucose, it has also been determined that the bioactive component production potential is higher (Lin & Wu, 2015; Hamza et al., 2013). Although it is thought that the use of external carbon sources in production in heterotrophic and mixotrophic environments will increase the cost, not using light in heterotrophic environments and low light requirements of cells in mixotrophic cultures increase the economic aspect of production (Lin & Wu, 2015; Tosuner & Urek, 2021, 2022). As seen in this study, when a low-cost carbon source is desired to be used, glycerol, which is 10% of the transesterification reaction products and therefore waste, is an important alternative (Leite et al., 2015). The fact that glycerol does not have a toxic effect at high concentrations and is compatible with the enzyme and membrane structure has made it suitable for use in microalgal cultures. The level of C-PC produced in optimal mixotrophic cultivation is comparable to the literature and was determined to be 2.67 times higher than that produced from *A. platensis* (Gamont) Geitler ($p<0.05$) (Esen & Öztürk Ürek, 2014, and 2015).

3.2 C-PC Extraction from *A. platensis*

C-PC, the main pigment protein of *A. platensis*, is found in complexes with other PBPs that form phycobilisomes (Rahman, Hosano & Hosano, 2022). Obtaining the valuable metabolites in *A. platensis* during the extraction stage is a bit difficult. Methods involving heat may cause these components to deteriorate. Environmentally

friendly extraction practices are preferred both for sustainability and to minimize the loss of bioactive components (de Morais et al., 2018). For example, in the glass bead extraction method, the cell wall is broken down and the pigments in the thylakoid membrane are extracted, preventing the degradation of metabolites to a certain extent. Various extraction methods such as freeze-thaw assisted extraction, were used to increase the A_{620}/A_{280} value of C-PC, which was obtained as 0.83 in the optimal condition.

3.2.1. Freeze-Thaw Assisted Extraction

It was carried out by freezing the freeze-thaw assisted extraction process involved freezing microalgal WB, LB, ODB, and FB samples at − 20˚C and then thawing them in UPW/10 mM phosphate buffer (pH 7.0) at 1, 2.5, 5 and 8% (w/v) at room temperature. This process is done in cycles. Then, C-PC purity levels in each process were determined (Table 3.6).

Table 3.6 C-PC purity values as a result of freeze-thaw assisted extraction of WB, LB, ODB, and FB samples.

	A_{620}/A_{280}											
Sample	**WB**			**LB**			**ODB**			**FB**		
	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3
1% UPW	0.36	0.64	0.79	0.58	0.62	0.81	0.11	0.07	0.08	0.30	0.77	0.64
2.5% UPW	0.28	0.53	0.92	0.74	0.84	0.88	0.12	0.13	0.63	0.46	0.40	0.45
5% UPW	0.36	0.99	0.94	0.70	0.78	0.70	0.21	0.15	0.16	0.26	0.47	0.80
8% UPW	0.33	0.75	0.96	0.81	0.82	0.87	0.35	0.34	0.39	0.35	0.59	0.58
1% buffer	0.30	0.32	0.54	0.71	0.83	0.76	0.07	0.13	0.07	0.57	0.74	0.44
2.5% buffer	0.24	0.25	0.37	0.86	0.69	0.77	0.15	0.08	0.12	0.51	0.67	0.43
5% buffer	0.32	0.37	0.53	0.65	0.82	0.86	0.26	0.25	0.44	0.65	0.76	0.45
8% buffer	0.53	0.50	0.33	0.81	0.84	0.82	0.34	0.34	0.42	0.59	0.76	0.32

*C-1:Cycle 1; *C-2:Cycle 2; *C-3:Cycle 3

In the freeze-thaw extraction method, WB, LB, ODB, and FB was studied (Table 3.6). When the purity rates of the samples studied in this method were compared, the best values were listed as follows. 5% UPW in WB> 2.5% UPW in LB> 8% buffer in FB> 2.5% UPW in ODB.

3.2.2. Homogenization (Mechanical Cell Disruption)

The homogenization process was performed at 4˚C for different times and speeds in UPW/10 mM phosphate buffer (pH 7.0) at 1, 2.5, 5, and 8% (w/v) of microalgal WB, LB, ODB, and FB samples. Then, C-PC purity levels in each process were determined (Table 3.7).

In the homogenization extraction method, WB, LB, ODB, and FB was studied (Table 3.7). When the purity values of the samples studied in this method were compared, the best values were listed as follows; 8% buffer in WB> 2.5% buffer in LB> 5% UPW in FB> 5% UPW in ODB.

Table 3.7 C-PC purity values as a result of homogenization of WB, LB, ODB, and FB samples.

	A_{620}/A_{280}											
Sample	**WB**			**LB**			**ODB**			**FB**		
	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3	*C-1	*C-2	*C-3
1% UPW	0.33	0.33	0.32	0.71	0.71	0.79	0.08	0.09	0.10	0.24	0.59	0.45
2.5% UPW	0.63	0.77	0.77	0.77	0.79	0.79	0.21	0.21	0.23	0.19	0.30	0.57
5% UPW	0.72	0.78	0.86	0.76	0.80	0.85	0.43	0.34	0.68	0.18	0.53	0.73
8% UPW	0.77	0.99	1.01	0.72	0.78	0.84	0.63	0.44	0.53	0.25	0.64	0.71
1% buffer	0.65	0.59	0.44	0.60	0.61	0.69	0.21	0.22	0.20	0.22	0.25	0.40
2.5% buffer	0.79	0.75	0.75	0.69	0.89	0.90	0.19	0.25	0.32	0.18	0.26	0.34
5% buffer	1.04	0.99	1.03	0.83	0.85	0.73	0.47	0.61	0.75	0.18	0.24	0.36
8% buffer	0.97	1.05	0.97	0.83	0.83	0.87	0.66	0.68	0.75	0.19	0.32	0.42

*C-1: 8000 rpm, *C-2: 9500 rpm, *C-3: 13500 rpm

3.2.3. Ultrasound-Assisted Extraction (UAE)

In the UAE process, samples were arranged in UPW/10 mM phosphate buffer (pH 7.0) at 1, 2.5, 5, and 8% (w/v) of microalgal WB, LB, ODB, and FB samples. Extraction was carried out in an ultrasonicator at constant temperature for 10 min, depending on the frequency of 37 and 80 kHz. The frequency was determined according to the result of obtaining the highest purity level, and then the process was repeated at constant temperature for 5 and 30 min (Khoigani et al., 2017). Then, C-PC purity levels in each process were determined (Table 3.8).

In the UAE method, WB, LB, ODB, and FB were examined (Table 3.8). When the purity values of the samples studied in this method were compared, the best values were listed as follows; 5% UPW in WB (80 kHz)> 5% buffer in LB (80 kHz)> 1% UPW in FB (37 kHz)> 5% UPW in ODB (37 kHz). Similar good results were obtained in FB samples, except for 2.5% UPW and buffer values at 37 kHz frequency.When the best results were compared with UAE and homogenization results, UAE stood out with a significant difference ($p < 0.05$).

Table 3.8 C-PC purity values by UAE with WB, and LB samples (a), ODB, and FB samples (b).

a	**A_{620}/A_{280}**											
Sample	**WB**						**LB**					
	37 kHz			80 kHz			37 kHz			80 kHz		
Time (min)	5	10	30	5	10	30	5	10	30	5	10	30
1% UPW	0.75	0.97	0.96	0.72	0.58	0.64	0.73	0.65	0.52	0.61	0.61	0.44
2.5% UPW	0.62	0.60	0.54	0.80	0.86	0.82	0.83	1.01	1.00	0.85	0.87	0.85
5% UPW	0.58	0.49	0.44	0.47	0.41	1.15	1.01	0.86	0.94	0.99	0.99	0.77
8% UPW	0.52	0.48	0.43	0.51	0.60	0.74	1.00	0.83	0.84	1.00	0.84	1.00
1% buffer	0.51	0.53	0.72	0.53	0.29	1.12	0.79	0.88	0.82	0.80	0.81	0.70
2.5% buffer	0.47	0.56	0.78	0.91	0.60	0.93	0.80	0.84	0.81	0.78	0.84	1.00
5% buffer	0.65	0.61	0.73	0.47	0.51	0.52	0.99	0.91	0.91	1.01	1.07	0.86
8% buffer	0.33	0.89	0.97	0.63	0.73	0.82	1.00	0.99	0.99	1.02	0.98	1.02

b	**A_{620}/A_{280}**											
Sample	**ODB**						**FB**					
	37 kHz			80 kHz			37 kHz			80 kHz		
Time (min)	5	10	30	5	10	30	5	10	30	5	10	30
1% UPW	0.09	0.08	0.09	0.08	0.08	0.08	0.56	0.59	0.98	0.30	0.32	0.60
2.5% UPW	0.12	0.14	0.16	0.19	0.11	0.14	0.37	0.50	0.58	0.23	0.32	0.45
5% UPW	0.28	0.24	0.62	0.25	0.22	0.25	0.25	0.47	0.93	0.20	0.59	0.71
8% UPW	0.50	0.35	0.46	0.37	0.3	0.45	0.32	0.50	0.96	0.17	0.22	0.58
1% buffer	0.08	0.08	0.08	0.07	0.07	0.07	0.64	0.74	0.97	0.29	0.44	0.56
2.5% buffer	0.13	0.11	0.15	0.13	0.13	0.13	0.68	0.81	0.85	0.30	0.28	0.41
5% buffer	0.28	0.28	0.38	0.29	0.25	0.30	0.71	0.76	0.98	0.21	0.20	0.39
8% buffer	0.44	0.39	0.45	0.42	0.34	0.44	0.66	0.82	0.92	0.27	0.14	0.29

ODB: Oven-dried biomass, FB: Freeze biomass

3.2.4. Extraction with Glass Beads in Mixer Mill

For the extraction process with glass beads in the mixer mill, the microalgal WB, LB, ODB, and FB samples were mixed with 1, 2.5, 5, and 8% (w/v) cells in UPW/10 mM phosphate buffer (pH 7.0) suspensions were prepared. 1 mL of these samples was placed in 2 mL round-bottomed plastic vials containing 0.5 g (0.5 and 0.25 mm diameter) glass beads and extraction was accomplished at room temperature for 5 and 15 min (Schütte & Kula, 1988). Then, C-PC purity levels in each process were determined (Table 3.9).

WB, LB, ODB, and FB were examined in the mixer mill extraction method (Table 3.9). When the purity values of the samples studied in this method were compared, the best values were listed as follows; 5% buffer (0.50 mm) in WB > 5% buffer (0.50 mm) in LB> 8% buffer (0.25 mm) in LB> 5% UPW (0.50 mm) in ODB.

The A_{620}/A_{280} value of C-PC produced under optimal conditions was determined as 0.83. The purpose of the extraction process is to increase this purity value. When the extraction methods used were compared, the best result was 1.15 in UAE with WB and 5% UPW sample. It is followed by a value of 1.08 in the 5% buffer sample with LB in extraction with Mixer Mill, 1.05 in the 8% buffer sample with WB in homogenization extraction, and 0.99 in the UPW sample with 5% WB in freeze-thaw assisted extraction. According to the best extraction result obtained, the purity of C-PC was rised by 38.55% ($p<0.05$). Therefore, 5% UPW in WB (80 kHz) was determined as the best C-PC extraction method.

UAE is a type of green extraction. Since it is a type of extraction that minimizes cell damage that may occur during extraction and prevents degradation due to the temperature factor, it is preferred to use it with high efficiency. For these reasons, the fact that the best result in this study was obtained in the UAE sample is supported by the literature (Berrouane et al., 2022).

Table 3.9 C-PC purity values by mixer mill with WB, and LB samples (a), ODB, and FB samples (b).

a	**A_{620}/A_{280}**							
Sample	**WB**				**LB**			
Glass bead	0.25 mm		0.50 mm		0.25 mm		0.50 mm	
Time (min)	5	15	5	15	5	15	5	15
1% UPW	0.39	0.31	0.50	0.47	0.70	0.83	0.70	0.89
2.5% UPW	0.25	0.23	0.95	0.96	0.85	0.82	0.94	0.84
5% UPW	0.23	0.24	0.95	0.94	1.03	1.03	1.03	0.99
8% UPW	0.21	0.24	0.76	0.81	1.04	1.01	1.02	1.01
1% buffer	0.28	0.24	0.77	0.78	0.69	0.74	0.69	0.70
2.5% buffer	0.25	0.20	0.83	0.80	0.93	0.79	0.80	0.93
5% buffer	0.23	0.29	1.01	1.02	1.07	1.07	1.08	1.01
8% buffer	0.27	0.28	0.99	0.99	1.02	0.98	1.01	0.99

b	**A_{620}/A_{280}**							
Sample	**ODB**				**FB**			
Glass bead	0.25 mm		0.50 mm		0.25 mm		0.50 mm	
Time (min)	5	15	5	15	5	15	5	15
1% UPW	0.13	0.37	0.10	0.53	0.17	0.26	0.46	0.41
2.5% UPW	0.18	0.28	0.23	0.64	0.27	0.41	0.62	0.62
5% UPW	0.53	0.48	0.38	0.49	0.26	0.63	0.69	0.52
8% UPW	0.62	0.73	0.82	0.47	0.64	0.67	0.51	0.53
1% buffer	0.23	0.47	0.13	0.29	0.26	0.25	0.43	0.41
2.5% buffer	0.26	0.26	0.54	0.60	0.26	0.27	0.44	0.44
5% buffer	0.44	0.51	0.33	0.43	0.29	0.56	0.62	0.51
8% buffer	0.84	0.88	0.88	0.63	0.48	0.62	0.66	0.57

3.3 Isolation and Partial Purification of C-PC

After the C-PC extracted at 80 kHz for 30 min in UAE, it was precipitated by various methods such as AS, PEG, and organic solvent. Then, partial purification of C-PC was carried out by DEAE Sepharose column chromatography.

3.3.1 Precipitation with Ammonium Sulfate (AS)

In order to precipitate C-PC extracted with UAE, saturation rates of 25, 50, and 75% (w/v) AS were applied. After the precipitation process, C-PC and purity levels in the upper and lower phases were determined (Figure 3.8).

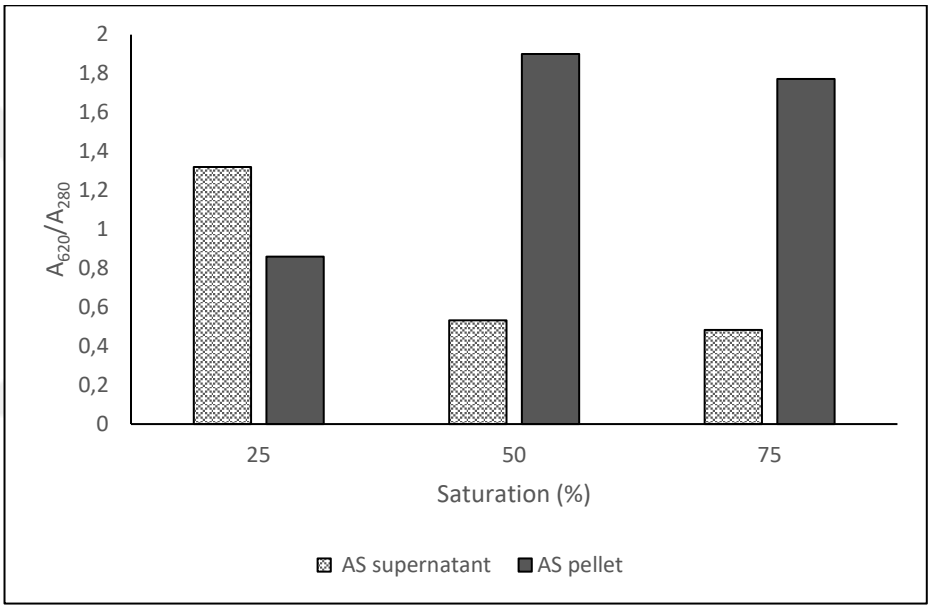

Figure 3.8 C-PC purity levels in supernatant and pellet after AS precipitation with 25, 50, and 75% (w/v) saturation.

Considering the C-PC purity values in the supernatant and pellet at 25, 50, and 75% (w/v) AS saturation rates, the best result was observed in the pellet at 50% (w/v) saturation. The purity value of C-PC was determined as 1.90.

In their study, Bhaskar and his colleagues used AS precipitation in their purification steps to obtain C-PC from *A. platensis* Geitler. In their study, the best results were obtained in AS precipitation at 50% saturation, and this supports the thesis study (Bhaskar, Gopalaswamy & Raghu, 2005). In previous research, the purity values of C-

PC from *A. platensis* were determined as 0.825 in the crude extract and 1.135 after precipitation with 40% ammonium sulfate (Safari et al., 2020).

3.3.2 Precipitation with Organic Solvent (OS)

In order to precipitate the C-PC extracted with UAE, 1/1, 1/2, and 1/3 (v/v) ratios of ethanol and methanol were applied. After the precipitation process, C-PC and purity levels in the upper and lower phases were determined (Figure 3.9).

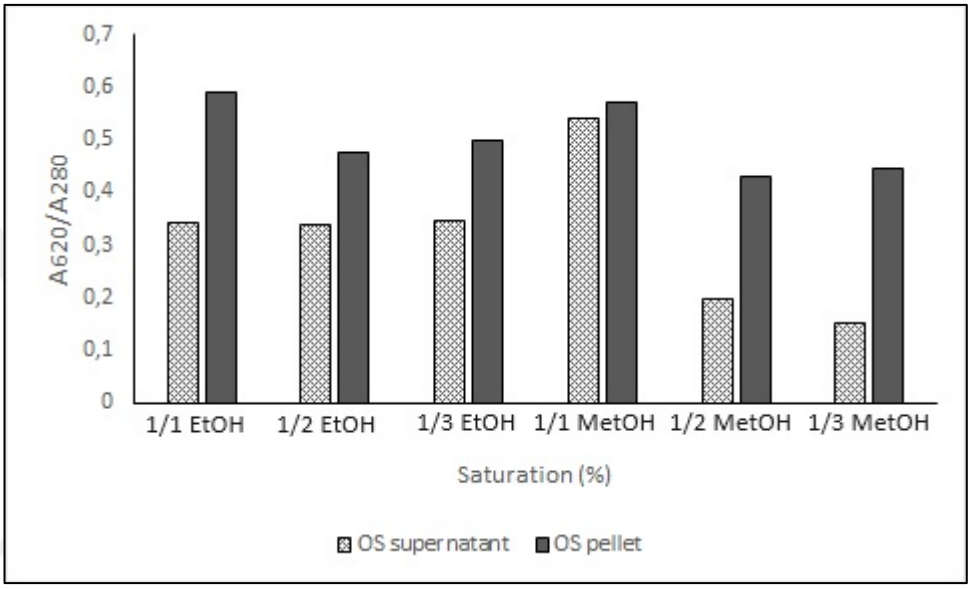

Figure 3.9 C-PC purity levels in the supernatant and pellet after precipitation with ethanol and methanol at 1/1, 1/2, and 1/3 (v/v) ratios.

When considering at the C-PC purity values in the supernatant and pellet in 1/1, 1/2, and 1/3 (v/v) ratios of ethanol and methanol, the best result was determined in the pellet with 1/1 (v/v) ethanol ratio. The purity value of C-PC was estimated as 0.59.

3.3.3 Precipitation with PEG (PEG)

PEG 2000 and PEG 5000 were applied at rates of 10 and 15% (w/v) to precipitate the C-PC, extracted with UAE. After the precipitation process, C-PC purity levels in the upper and lower phases were determined (Figure 3.10).

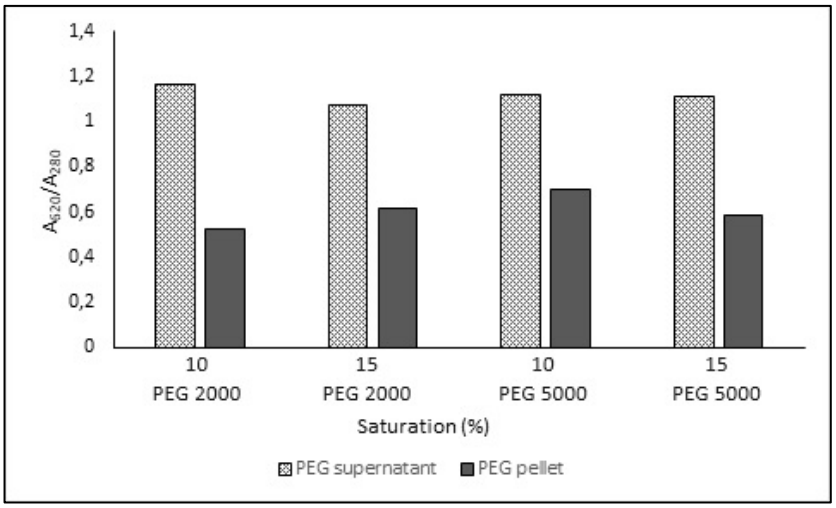

Figure 3.10 C-PC levels in the supernatant and pellet after precipitation with 10 and 15% (w/v) PEG 2000 and 5000.

Considering the C-PC purity values in the supernatant and pellet with 10 and 15% (w/v) PEG-2000 and -5000, the best result was observed in the supernatant with 10% (w/v) PEG-2000. The purity value was determined as 1.17. Considering previous studies, C-PC was observed in the upper PEG phase in the PEG precipitation extraction method. The hydrophobicity of the upper phase increases due to the increase in the affinity of the proteins in the organic phase (Antecka et al., 2022).

When the best purity C-PC levels obtained from precipitation methods were compared, it was found to be 1.90 at 50 % (w/v) AS, 1.17 in 10% PEG-2000, and 0.59 at 1/1 (v/v) rate in ethanol. When the results obtained were evaluated, the best precipitation method was determined at 50% AS saturation. In this situation, the purification value of C-PC was 65.22% greater than that of C-PC obtained from the UAE ($p<0.05$).

3.3.4 Column Chromatography (CC)

DEAE Sepharose column chromatography was applied to purify C-PC, which was extracted at 80 kHz for 30 min in UAE and then precipitated with 50% (w/v) AS. In the CC, total of 55 fractions were obtained and the purity values and C-PC concentrations were shown in Figure 3.11.

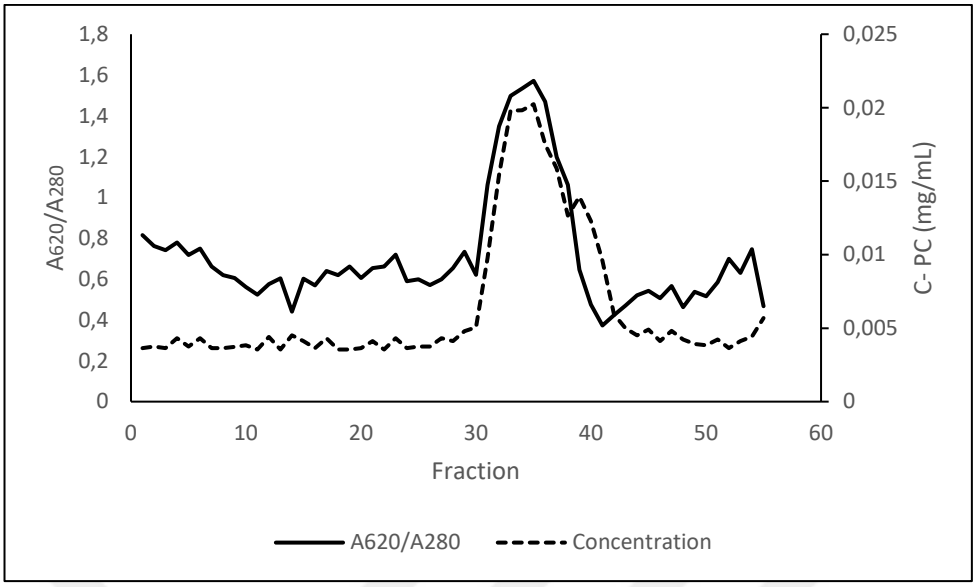

Figure 3.11 Variations of C-PC concentration and purity levels of DEAE Sepharose column chromatography fractions.

C-PC levels and purity values in the fractions collected were determined in the DEAE Sepharose column chromatography. Then, after combining the fractions with the best values, ultrafiltration was applied using 10 kDa ultrafiltrates (Amicon cell equipped with YM10 cellulose membrane). As a result, the purity value was determined as 2.30. It can be said that C-PC, produced under optimized conditions according to the production, extraction, and purification steps, was purified 2.78 times ($p<0.05$).

Table 3.10 The purity, and recovery values of C-PC in the purification stages.

Purification stages	**A_{620}/A_{280}**	**Recovery (%)**
Crude	0.827	100
UAE	1.15	48.66
AS	1.90	32.95
CC	2.3	2.84

Purity in the purification steps and recovery values at each step are given in Table 3.10. The recovery value was supported in the study conducted by Herrera et al. (1989).

3.4 Characterization of Partially Purified C-PC

Antioxidant, cytotoxic, and characterization properties of partially purified C-PC samples were examined.

3.4.1 Determination of Antioxidant Properties

The antioxidant properties of the partially purified C-PC such as $DPPH^{\cdot}$, $ABTS^{\cdot+}$, $HO^{\cdot}$, $NO^{\cdot}$, $O_2^{\cdot-}$, and metal chelating were examined and then IC_{50} levels were determined (Table 3.11).

Table 3.11 IC_{50} values and positive controls of antioxidants. Results were expressed as mean±standard deviation of three independent replicate.

Antioxidant Properties	**IC_{50} (µg/mL)**	**Positive control (IC_{50}, µg/mL)**
$DPPH^{\cdot}$ scavenging	13.42±0.82 µg/mL	BHT (26.80±1.40 µg/mL)
$ABTS^{\cdot+}$ scavenging	1.66 ±0.10 µg/mL	Vitamin C (0.94±0.01 µg/mL)
$HO^{\cdot}$ scavenging	100.56±2.74 µg/mL	BHT (5.10±0.20 µg/mL)
$NO^{\cdot}$ scavenging	75.73±1.35µg/mL	Vitamin C (36.76±1.03 µg/mL)
$O_2^{\cdot-}$ scavenging	17.02 ±0.93 µg/mL	Vitamin C (137.10±11.25 µg/mL)
Metal chelating	64.01±1.28 µg/mL	EDTA (5.50±0.30 µg/mL)

When comparing the IC_{50} concentrations of DPPH·, positive control BHT with 50% scavenging value, the concentration of C-PC is almost half of the positive control ($p<0.05$). In the previous study, the IC_{50} value of the DPPH· scavenging was determined be 47.07 µg/mL (Hammad, El Desoky & Saleh, 2023). In another study, this value was found to be 28 µg/mL in methanolic extract of *A. platensis* (Gheda et al., 2023). When compared with this literature data, the IC_{50} value found was 3.51- and 2.08-times better than the literature data, respectively ($p<0.05$). When comparing the IC_{50} concentrations of C-PC and positive control vitamin C for $ABTS^{\cdot+}$scavenging, the IC_{50} value of C-PC is almost half of the that of positive control. When IC_{50} concentrations are compared, the concentration of C-PC is 1.66±0.10 µg/mL, while the concentration of vitamin C is 0.94±0.01 µg/mL. When comparing the IC_{50} concentrations of C-PC and BHT, positive control, for the $ABTS^{\cdot+}$scavenging, the IC_{50} of C-PC is almost half of the positive control. In the study executed by Tarko et al.,

the $ABTS^{\cdot+}$ scavenging value was found to be 0.7±0.02 equivalent to Trolox (Tarko, Duda-Chodak & Kobus, 2012).

When IC_{50} concentrations are compared for $HO^{\cdot}$ scavenging, the concentration of C-PC is 100.56±2.74 µg/mL, while the value of BHT is 5.10±0.20 µg/mL. For $NO^{\cdot}$ scavenging, IC_{50} values of positive control vitamin C and C-PC were compared and the IC_{50} value of C-PC was almost 2 times that of the positive control. For scavenging $O_2^{\cdot-}$, the IC_{50} concentration of C-PC was almost 8.05-fold higher than that of the positive control, vitamin C ($p<0.05$). Additionally, IC_{50} value for metal chelating of C-PC is 64.01±1.28 µg/mL. In a previous study, the Fe^{2+}-chelating activity (%) of C-PC from *S. platensis* was determined as 40.23±1.45 % (Safari et al., 2020).

According to the results, C-PC from *A. platensis* serves as potential antioxidants by scavenging DPPH, ABTS, HO, NO, and superoxide radicals, as well as connective to stabilizing lipid peroxidation, donating hydrogen or electrons and metal ions (Gheda et al., 2023). As a defense mechanism, *A. platensis* is capable of resisting oxidative stressors by activating both non-enzymatic and enzymatic antioxidants. This antioxidant compound, C-PC has a major role in chemoprotection and regulation of antioxidants. *A. platensis* has high antioxidant activity thanks to the valuable bioactive components such as C-PC, carotenoids, vitamins, and minerals (Hammad, El Desoky & Saleh, 2023). In addition, total phenolic, flavonoid, and tannin contents are among the components that support antioxidant potential.

The total reducing power value of C-PC was guessed to be 14.00±1.06 mg VCE/g. The FRAP value of C-PC was detected to be 1.50±0.12 mg $FeSO_4$/g. In a previous study, the FRAP value of C-PC from *A. platensis* was determined as 0.051±0.01 mgTAE/g (Safari et al., 2020).

3.4.2 Determination of Cytotoxic Properties

Cytotoxicity values of different concentrations of C-PC from UAE, AS, and CC against the HeLa and OE-33 cancer cell lines were examined after 24 and 48 h incubation. The cytotoxic effect against these cancer cells was determined as % inhibition. In the experiments conducted for HeLa, the best result was a 53.41% inhibition value at 9.4±0.52 µg/mL concentration in the AS sample after 24 h incubation. No significant scavenging value was found after 48 h. The best result

against the OE-33 cell line was a 73% inhibition value at 25.1±1.37 µg/mL concentration in the colon sample after 24 h. After 48 h, the determined percent inhibition was decreased as 8%.

In the research of Li et al. (2006) an important reduction in the cell number of HeLa cells treated with C-PC was observed compared to control cells. They also completed that C-PC could stimulate characteristic apoptotic properties in HeLa cells, like degradation of genomic DNA, chromatin margination, membrane blebbing and cell shrinkage of HeLa cells. They detected that it could establish intercellular cell adhesion molecule 1 protein and the expression of Fas while inhibiting the expression of B cell lymphocytic-leukemia proto-oncogene 2 protein (Bcl-2) (Li et al., 2006). In the research achieved by Gheda and colleagues, the IC_{50} value was found to be 38.91±2.4 µg/mL against the HeLa cell line (Gheda et al., 2023). In a previous study, total phenolic components were 150.5±1.18 mg gallic acid equivalents/mg extract (Safari et al., 2020). Whole examined extracts and compounds displayed an inhibitory impact on the viability of HepG2 cells in a dose dependent attitude in the absence of cytotoxicity on normal cells. IC_{50} values of, the methanolic extract, phenolic compounds, and the aqueous extract were 1.7±0.14, 1.28±0.22, and 0.86±0.14 mg/mL, in order. The results indicated that the methanolic and aqueous extract from *A. platensis* can obstruct the proliferation of liver cancer cells (HepG2) (Akbarizare. et al., 2020). Moreover, there was a affirmative effect of *A. platensis* ethanol extract on MCF7 at 72h period with 85 µg/mL (MZ et al., 2011).

3.4.3 Characterization of C-PC

C-PC from *A. platensis* was characterized by methods such as UV-Vis, FT-IR, and TGA.

3.4.3.1 Ultraviolet-Visible Spectroscopy (UV-Vis)

As a result of the spectrum scanning between 200-800 nm in the crude extract on the 15th day, the peak observed at 620 nm indicates the entity of C-PC. Similar studies also observed that the spectrum is the same (Rastogi, Sonani & Madamwar, 2015). The UV spectrum is shown in Figure 3.12.

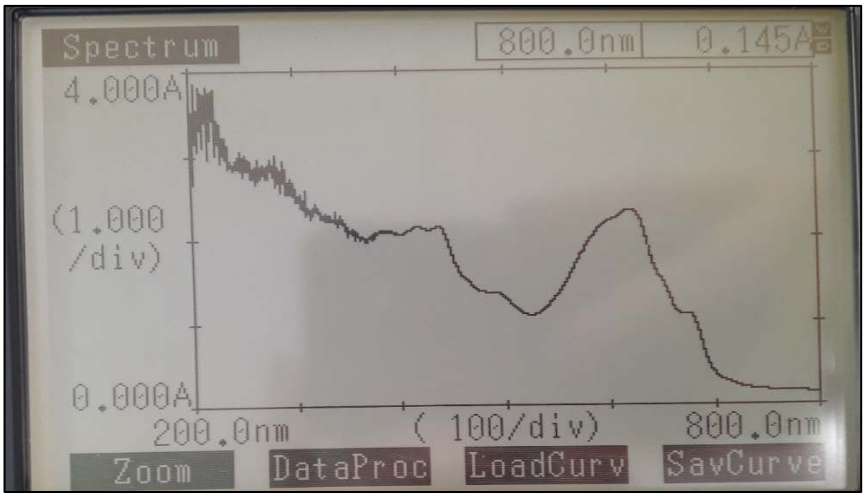

Figure 3.12 UV-Vis spectrum in the range of 200-800 nm at maximum production conditions of C-PC

3.4.3.2 Fourer Transform Infrared Spectroscopy (FT-IR)

The spectrum of C-PC was taken in the range of 400-4000 cm^{-1} (Figure 3.13). According to this spectrum, the peak at 3448 cm^{-1} indicates N-H stretching vibration. The 2352 cm^{-1} peak correlates with to the C-H stretching vibration. The 1636 cm^{-1} N-H bending vibration. The 1384 cm^{-1} CH_2 represents the bending vibration. The 609 cm^{-1} peak indicates the functional groups of C-PC (Mahendran et al., 2022).

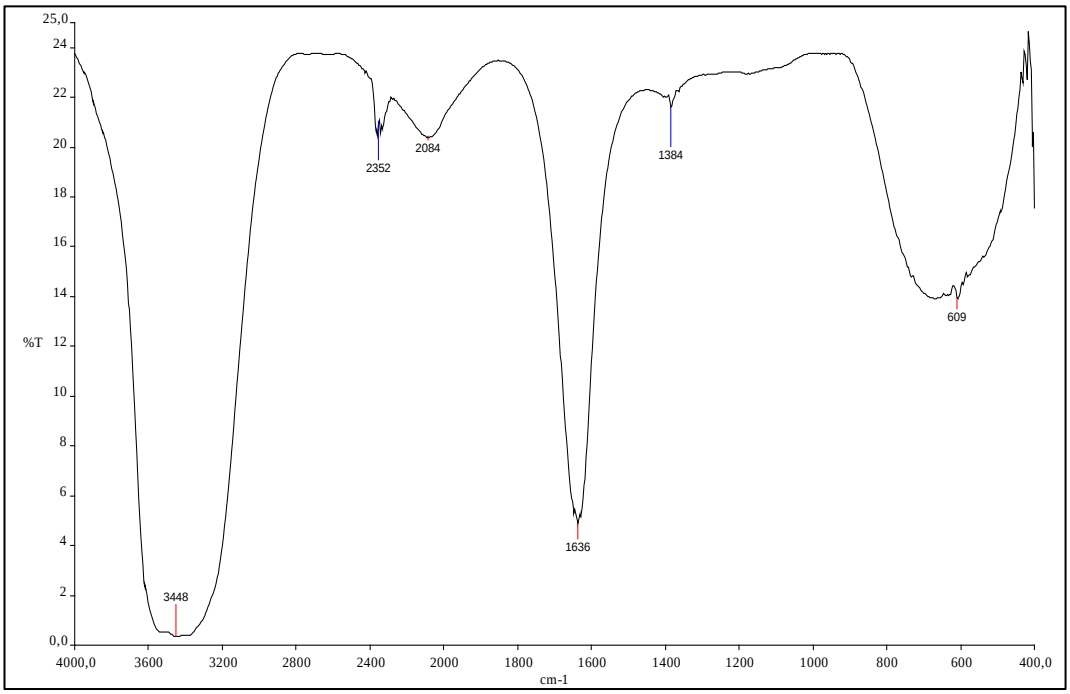

Figure 3.13 FT-IR spectrum of partially purified C-PC

3.4.3.3 Thermogravimetric Analysis (TGA)

Thermal stability is an important characteristic of application areas. The degradation steps for *A. platensis* occur in three steps (Figure 3.14). In the first step, a mass loss of 4.68% due to humidity was observed between 45-100 ˚C. Then, a mass loss of 58.87% was detected between 247-340 ˚C. The degradation temperature was detected at temperatures above 340 ˚C.

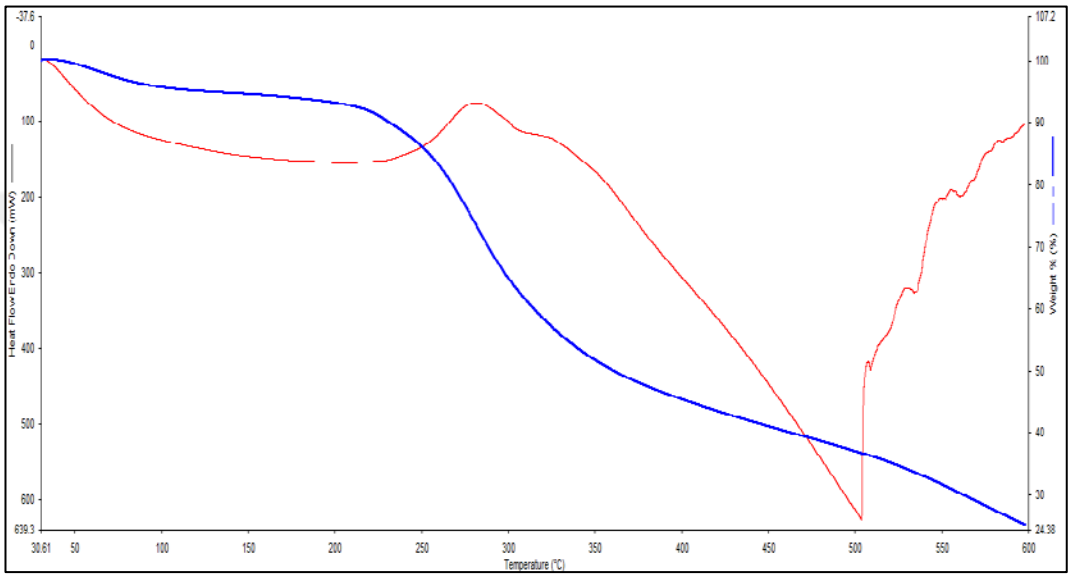

Figure 3.14 TGA spectrum of lyophilized *A. platensis*

CHAPTER FOUR

CONCLUSION

Cyanobacteria are phototrophic microorganisms with cell walls including peptidoglycan, like gram-negative bacteria. *A. platensis* is a blue green cyanobacteria has high environmental-adaptability, that can survive at high alkaline pH levels and is a resource of many bioactive components, vitamins and minerals. Between these ingredients, C-PC is being utilized in industrial process, cosmetics, food, medical and pharmaceutical with its non toxic, water soluble pigment protein construction. In the sense of great activity and economic production of C-PC from *A. platensis*, particularly mixotrophic cultivation stands out the reorganization of cultivation situation. Heterotrophic cultivation is performed in an environment in absence of light and mixotrophic cultivation is performed in the entity of organic carbon resources at lower light intensity. Mixotrophy, whichever is a composition of phototrophic and heterotrophic cultivation, can utilize both of inorganic and organic carbon like a carbon resource.

In this thesis, mixotrophic cultivation of *A. platensis* M2 was performed in media including 1; 2.5; 5 mM crude glycerol, dependent the incubation time, and the great C-PC production was detected as 3.75±0.12 mg/g on the 15^{th} day at 1mM concentration. In the presence of 1mM crude glycerol, when the effect of 0, 10; 29.4 mM concentrations of $NaNO_3$ on C-PC production was investigated, the great value was detected in the entity of saturated $NaNO_3$. In the entity of technical glycerol with the equal concentration, the maximum C-PC production was acquired at a lower value as 1.85±0.15 mg/g. With respect to acquired results, cheap C-PC production in the entity of crude glycerol is an model of the bioconversion of a waste product into a valuable bioactive produce. The fact that glycerol does not have a toxic effect at high concentrations and is compatible with the enzyme and membrane structure makes it suitable for use as a carbon source in microalgal cultures. Mixotrophic cultivation is a great alternative method developed to accomplish the limitations of former cultivation methods and also to advange a biochemical accumulation and high growth rate (Meng et al., 2020).

In this study, samples prepared in various ways for the extraction of C-PC were optimized using process such as freeze-thaw, homogenization, mixer-mill, UAE, and the best extraction method was determined to be UAE. The extracted C-PC was partially purified by DEAE Sepharose column chromatography and ultrafiltration methods after precipitation with 50% (w/v) ammonium sulfate, and the purity was determined as 2.3.

C-PC purity is appraised spectrophotometrically bottomed on the ratio between the C-PC absorbance value at 620 nm and the absorbance values of aromatic aminoacids in whole proteins at 280 nm. A ratio less than 0.7 is considered food grade C-PC preparations, a ratio betwixt 0.7 and 3.9 is considered reagent-grade, and better than 4.0 is considered analytically pure (Fabre et al., 2022). This level of purity also indicates that the produced C-PC is of reagent-grade purity. It is important that use of C-PC, a non toxic, water soluble pigment protein, in various biotechnological and industrial areas such as food, cosmetics, medical, nutraceutical, and pharmaceutical applications according to different purity levels of C-PC (Kathiravan et al., 2022).

According to the results, C-PC has antioxidant properties such as quenching $DPPH^{\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$, $ABTS^{\cdot+}$ and $O_2^{\cdot-}$ radicals, metal chelating, total reducing power, FRAP, as well as its cytotoxic effects against HeLa and OE-33 cancer cell lines. In addition, these results are comparable to literature data. It has been showed that C-PC proceeds as a hepatoprotective, neuroprotective, antiinflammatory, antioxidant, and anti-cancer agent and has therapeutic effects against diseases such as hypercholesterolemia economic production is of great importance due to the high production costs (Hsieh-Lo et al., 2019). Microalgal pigment production is one of the important topics in the field of blue biotechnology, also called water or marine biotechnology, and allows the use of natural pigments in different fields. Therefore, microalgae production is encouraged for pigment production owing to the increasing request for natural pigments (Ghosh et al., 2015). By the same token, blue biotechnology includes the utilize of freshwater and marine organisms to rise the develop compounds and food supply of commercial price (Narberhaus, 2015). However, C-PC has good potential in commercial markets, industrial, and medical and its market price is known to be round

$100 million annually. In addition, the global market share in 2027 is estimated to be $245.5 million (Ashaolu et al., 2021).

In this thesis that is an up-to-date and original study, after the production of C-PC from *A. platensis*, a natural source, by economical and high-efficiency mixotrophic cultivation, and its isolation, through simple, effective and environmentally friendly extraction, and partial purification, its antioxidant activity against 5 different radicals that have very important effects on metabolism, as well as its cytotoxic properties against 2 cancer cell lines have been demonstrated.

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