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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

NON-CHLOROPHYLL ACCESSORY PIGMENTS IN CYANOBACTERIA: SIMULTANEOUS EXTRACTION, SEPARATION, AND MAXIMIZATION

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Signature

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Dedicated to my family

and my friends

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LIST OF SYMBOLS

α	Alfa
β	Beta
C	Concentration
°C	Degree Celsius
ddH2O	Double-Distilled Water
dH2O	Distilled Water
γ	Gamma
g	Gram
L	Litre
µ	Micro
µl	Mcrolitre
µm	Micrometre
µmol	Micromol
mg	Miligram
ml	Mililitre
M	Molar
nm	Nanometre

LIST OF ABBREVIATIONS

A	Absorbance
ALP	Allophycocyanin
BCA	Bicinchoninic acid
BG-11	Blue-Green Medium
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EPS	Extracellular polysaccharide
FT-IR	Fourier Transform Infrared Spectroscopy
FW	Freshwater
HPLC	High-Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
M.O.	Microorganisms
OD	Optical density
PBPs	Phycobiliproteins (PBPs)
PC	Phycocyanin
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
pH	Hydrogen number
REPS	Released Exopolymeric Substances
RP-HPLC	Reversed Phase-High-Performance Liquid Chromatography
SPC	Specific conductivity
TDS	Total dissolved solids
WSCP	Water-soluble chlorophyll protein

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ABSTRACT

Non-Chlorophyll Accessory Pigments in Cyanobacteria: Simultaneous Extraction, Separation, and Maximization

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Department of Chemistry

Doctor of Philosophy Thesis

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Cyanobacteria are considered as a sustainable feedstock for the production of biochemically active compounds such as phycobiliproteins (PBPs). In this study, 12 different cyanobacteria isolated from hot-spring water resources in Ankara province were evaluated for their PBP production efficiency. Morphological analysis and Sanger sequencing revealed that 5 of the strains belong to *Nostoc* genus, 3 of them belong to *Anabaena*, and others belong to genus *Trichormus, Nodularia, Chlorogloeopsis, and Nodosilinea.*

Isolated strains have been maintained in nitrogen lacking BG-11 growth medium during the thesis study. Total PBP content of *Trichormus sp.* IMU26 and *Anabaena variabilis IMU8* were found as 23.2% and 17.3% while it was lower than 15% in all other strains studied.

The following studies were carried out to see the impact of nitrogen and

phosphorus availability on PBP production on diazotrophic cyanobacteria isolated from hot-spring water resources. For this aim, the strains *Trichormus sp. IMU26* and *Anabaena variabilis IMU8* were incubated in N-replete, N-lacking, and N-P-lacking BG-11 growth medium. Results showed that nitrogen supply resulted in higher soluble protein and saccharide content but a lower growth rate and PBP production in *Trichormus sp. IMU26* and *Anabaena variabilis IMU8*. Short term (6 days) N-P-deprivation induced PBP production with no clear change in growth while growth and PBP content decreased in the longer incubation period (12 days). Fourier transform infrared spectroscopy results refer that membrane-bound oligosaccharides may have regulatory roles for PBP production in *A.variabilis IMU8* during long term diazotrophic cultivation. Moreover, rapid induction of zeaxanthin and β-carotene production and a slight reduction of echinenone and canthaxanthin levels might be associated with increased PBP levels in short term N-P-deprivation of *Trichormus sp. IMU26.*

In conclusion, the thermal diazotrophic cyanobacteria *Trichormus sp. IMU26* and *Anabaena variabilis IMU8* were introduced to the literature as potential candidates for pilot scale PBP production. Isolated from hot-spring water resources, which differ from sea and spring waters with their unique mineralization and temperature levels, these two cyanobacteria grow well in N-lacking growth medium under room temperature and the contamination risk is fairly low.

Keywords: *Anabaena,* Cyanobacteria, Nitrogen, Phosphorus, *Trichormus*, Phycobiliproteins.

ÖZET

Siyanobakterilerde Klorofil Olmayan Aksesuar Pigmentler: Eş Zamanlı Ekstraksiyon, Ayırma ve Maksimizasyon

Mohammed Fadhil Hameed HADDAD

Kimya Bölümü (İngilizce)

Doktora Tezi

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Siyanobakteriler, fikobiliproteinler (PBP) gibi biyokimyasal olarak aktif bileşiklerin üretimi için başlıca sürdürülebilir kaynak olarak değerlendirilmektedirler. Bu çalışmada, Ankara il sınırları içerisinde bulunan bazı sıcak su kaynaklarından izole edilen 12 farklı diazotrofik siyanobakteri türü PBP üretimleri bakımından değerlendirilmiştir. Saf olarak elde edilen siyanobakteriler morfolojik analizlerinin yanı sıra Sanger dizileme ile de karakterize edilmiş, türlerden 5'inin *Nostoc* cinsine, 3'ünün *Anabaena* cinsine ve diğerlerinin *Trichormus*, *Nodularia*, *Chlorogloeopsis* ve *Nodosilinea* cinslerine ait türler olduğu belirlenmiştir.

Saf olarak elde edilen diazotrofik siyanobakteriler tez çalışması boyunca içerisinde azot kaynağı bulunmayan BG-11 büyütme ortamında kültüre alınmışlardır. Çalışılan türlerin toplam PBP içeriği *Trichormus sp. IMU26* ve *Anabaena variabilis IMU8* türlerinde sırasıyla yaklaşık % 23.2 ve 17.3 ile en

yüksek düzeylerde belirlenmiş, diğer türlerde ise %15'in altında kaydedilmiştir.

Takip eden çalışmalarda sıcak su kaynaklarından izole edilen diazotrofik siyanobakterilerin FBP üretimleri ve ilgili parametreleri üzerine azot (N) ve fosfor (P) varlığının etkilerinin belirlenmesi amacıyla *Trichormus sp. IMU26* ve *Anabaena variabilis IMU8* türleri içerisinde N-bulunan, N-bulunmayan ve N-P-bulunmayan BG-11 büyütme ortamında kültüre alınmışlardır.

Yapılan çalışmalarda *Trichormus sp. IMU26* ve *Anabaena variabilis IMU8* siyanobakterilerinin büyüme ortamına azot kaynağı ilave edildiğinde bu türlerin toplam protein ve karbohidrat içeriklerinde artış ile birlikte büyüme hızında ve PBP üretiminde azalma kaydedilmiştir. Kısa süreli (6 gün) N-P-açlığına bırakılan türlerin büyümelerinde önemli bir değişim olmasa da PBP üretimi artmış, ancak uzun vadede (12 gün) siyanobakterilerin büyüme hızları ve PBP içerikleri dikkate değer düzeyde düşmüştür. Fourier Transform Infrared Spektroskopi analizi ile elde edilen veriler diazotrofik koşullarda kültüre alınan siyanobakterilerde membran-bağlı oligosakkaritlerin PBP üretiminde kayda değer rol üstlenebileceklerini göstermiştir. Öte yandan özellikle *Trichormus sp. IMU26* türünde kısa süreli N-P-açlığı ile artan PBP üretiminin yükselen zeaksantin ve β-karoten üretimi ile birlikte ekinenon ve canthaxanthin düzeylerindeki düşüşler ile de ilişkili olabileceği kaydedilmiştir.

Sonuç olarak, bu tez çalışması ile pilot ölçekli FBP üretimi için iki farklı termal diazotrofik siyanobakteri türü; *Trichormus sp. IMU26* ve *Anabaena variabilis IMU8* siyanobakterileri tanımlanmıştır. Yüksek sıcaklık ve mineralizasyon içeriği bakımından tatlı su ve deniz suyundan oldukça farklı özellikler sergileyen termal sulardan izole edilen bu türler oda şartlarında da rahatlıkla kültüre alınabilmekte, diazotrofik yapıda olduklarından N-eksik büyüme ortamında yüksek büyüme hızı ve PBP üretimi sunmaktadırlar ve ayrıca kontaminasyon riski de oldukça düşüktür.

Anahtar kelimeler: *Anabaena*, Azot, Fikobiliprotein, Fosfor, Siyanobakteri, *Trichormus*

1
Introduction

1.1 Literature Review

Cyanobacteria are known as the simplest, smallest and most ancient members of the photosynthetic organisms on Earth, some of which were found in ancient fossils. Cyanobacteria live in a self-sustaining living, and few live as a part of some other species of algae. Some are found in harsh thermal conditions, some are seen in hot springs, and others are observed in low temperatures. Also, some of them live in cooperative with some fungi, lichens [1]. Modern-day cyanobacteria include more than 2000 species in 150 genera, with a great variety of shapes and sizes. Cyanobacteria are oxygenic photosynthetic (Photoautotrophic) gram-negative prokaryotes micro-organisms that show a connective link between plastid eukaryotes autotrophs and prokaryotes. They are an excellent source of peptides, trans-fatty acid, amino acid, vitamins, minerals and pigments like chlorophyll a, carotenoids as phycobiliprotein, which exhibit different color [2]. Cyanobacteria are common in lakes, ponds, springs, wetlands, streams, and rivers, and they play a major role in the nitrogen, carbon, and oxygen dynamics of many aquatic environments [3], [4], [5].

This characterization included some cyanobacteria strains, their type, DNA sequence, living pressure, dissolved oxygen (DO) concentration, conductivity, total dissolved solids (TDS), pH, temperature, salinity, NH4+, NO3-[6]. Also in the literature, other factors were applied to further characterize the obtained cyanobacteria (green algae) from the hot-springs (Figure 1.1), which included phycobiliprotein (PC, PE, ALC), growth measurements, total protein level, saccharide level, vitamin content [7], [8].

Figure 1.1 Landscape illustrates the forms of hot springs in isolated areas.

1.2 Objective of the Thesis

The impartial objective of this study is to characterize, using the same criteria mentioned above, the cyanobacteria species located in areas around Ankara hot-springs. Then, morphology studies have been applied to identify cyanobacteria strains. After the specific identification, other measurements took place to specifically characterize these species according to some recent studies. After these characterization, we identified which ones were recorded in gene bank and which ones were not. The non-recorded species were further investigated and recorded to the gene bank as they are considered novel.

1.3 Hypothesis

Cyanobacteria get energy through prokaryotes that receive sunlight as a source of energy using chlorophyll and various dyes. The presence of essential elements such as oxygen, nitrogen and carbon play a crucial role in aquatic environments. Until today, the picture remains unclear about the emergence of microbes like

cyanobacteria on Earth, and many researchers verified their current diversity more than two billion years ago. Cyanobacteria is the new revolution in modern science and caught the attention of a lot of scientists in the fields such as biotechnology, pharmacology, food industry. This attention was due to the unusual and specific conditions of living that these species were shown. Also, most of them are not yet reported in the literature. In this study, the idea is to collect some of these algae species from the some hot-spring areas in Ankara province/Turkey and characterize them specifically using methods obtained from other studies. This characterization will give us information about specific conditions. This study will also contribute development to the using of cyanobacteria in the areas mentioned above.

2
Genaral Information

2.1 Hot Spring Water

The development of new and modern technologies has made it possible for us to know and study microorganisms (M.O.) in harsh environments and to know their types, forms, find new sustainable methods and techniques for human food, health and their environment.

Most cyanobacteria represent a variety of M.O that can be found in diverse environments and leaders of photosynthesis of oxygen and combine to form a thick microbial mat even in harsh environments. Studies of M.O found in hot water have shown that these organisms can be exploited to generate many valuable products in technological applications, especially in the agricultural, pharmaceutical, and industrial and food fields, where the cyanobacteria represent microscopic communities found in high-temperature ecosystems. Figure 2.1

explains that places' collected samples from it [9].

Figure 2.1 Cyanobacteria (green algae) in hot springs water.

Most natural resources, hot spots or volcanic environments and hydrothermal vents are located below the surface of the soil or in the deep sea and are without any living organisms or life forms. There are heat-loving M.O that have the ability to live in such places or harsh thermal environments. These microorganisms are able to grow, live, and reproduce, so such organisms withstand harsh environmental conditions, high temperatures, high pH pearls, and high salt concentrations [10].

Cyanobacteria are among the most abundant algae on travertine around the world; they are mostly located on the surface of these rocks or deep into the sea. They are also growing in travertine covered with highly turbulent water, again partly in response to low competition with rooted plants damaged by the strong flow with high water temperature [11].

Most of the microbiological research carried out on hot springs that have extreme or gradual temperatures in addition to the distinct chemical composition of these hot springs as well as their isolated geographic locations, all of which should prevent them from exchanging species but have developed modern and unique microbial communities. To date, global knowledge about the biodiversity of hot springs, particularly regarding the second-generation sequence, remains unclear [10], [12], [13], [14].

Most studies of hot spring regions around the world on cyanobacterial areas and their composition are often missing due to the mysterious composition of these small cellular creatures and their different types, as sequencing remains inevitable to identify any new isolation and allows for a realistic assessment. Some researchers in the past dealt with blue bacteria as a whole group, and some researchers were concerned with the diversity of temperatures for springs that exist in it.

Many types of blue bacteria were worked to explore in North America, in Japan, and other types of them isolated from hot springs were worked to explore in Jordan, Algeria, Thailand, India, Chile, and Russia. However, in islands, where the presence of cyanobacteria at high water temperatures in their initial production stages is high, their presence can be observed easily by the naked eye [12].

2.2 Cyanobacteria

Cyanobacteria are known as the simplest, smallest and most ancient members of the photosynthetic organisms on Earth, some of which were found in ancient fossils. Cyanobacteria live in different environments, in freshwater, salt, wet soil, even on the surface of rocks or within a depth of up to 1 m under the sea. The vast majority of green cyanobacteria live in a self-sustaining living, and few live in part on some other species of algae. Some are found in harsh of thermal conditions, some are seen in hot springs, and other are observed in low temperatures, some of them live in cooperative with some fungi, lichens [15].

Figure 2.2 explains the cyanobacteria are oxidized photosynthetic and are found everywhere in the Earth's illuminated aquatic biosphere. Cyanobacteria from hot spring water have been recorded for nearly 200 years ago. There is a close association between these prokaryotic algae, but the differences were observed and recorded as variable indicators between hot springs that are meters or some kilometers away from each other due to chemical composition, temperature, solar radiation, or local variations of the area. Figure 2.2 indicates potential changes are due to geographical isolation, which observed between widely separated springs and to development restrictions [16].

Fixed nitrogen is highly available in the atmosphere as the Earth's atmosphere contains about 78% of nitrogen, but it has been proven to be directly used by a very limited number of organisms. This fact has led to much research on the availability of nitrogen in diverse environments. There is a whole range of these organisms that act to reduce the amount of primary nitrogen into more reactive compounds for use in cellular metabolism. However, the nitrogen provided by these primary stabilizers can be transferred to other organisms and can eventually

be recycled across the biosphere of any other organism. Although recycling and loss of the natural system would permit a stable nitrogen status within the biosphere, the recent influence and intervention by man have been the cause of some fluctuations from normal [17].

However, to avoid any risk of cyanobacteria to human health, it is useful to understand the basics and behavior of natural ecosystems and environmental conditions that can support the growth of certain types of cyanobacteria from other species and what capabilities and support their propagation in systems water and its whereabouts [18].

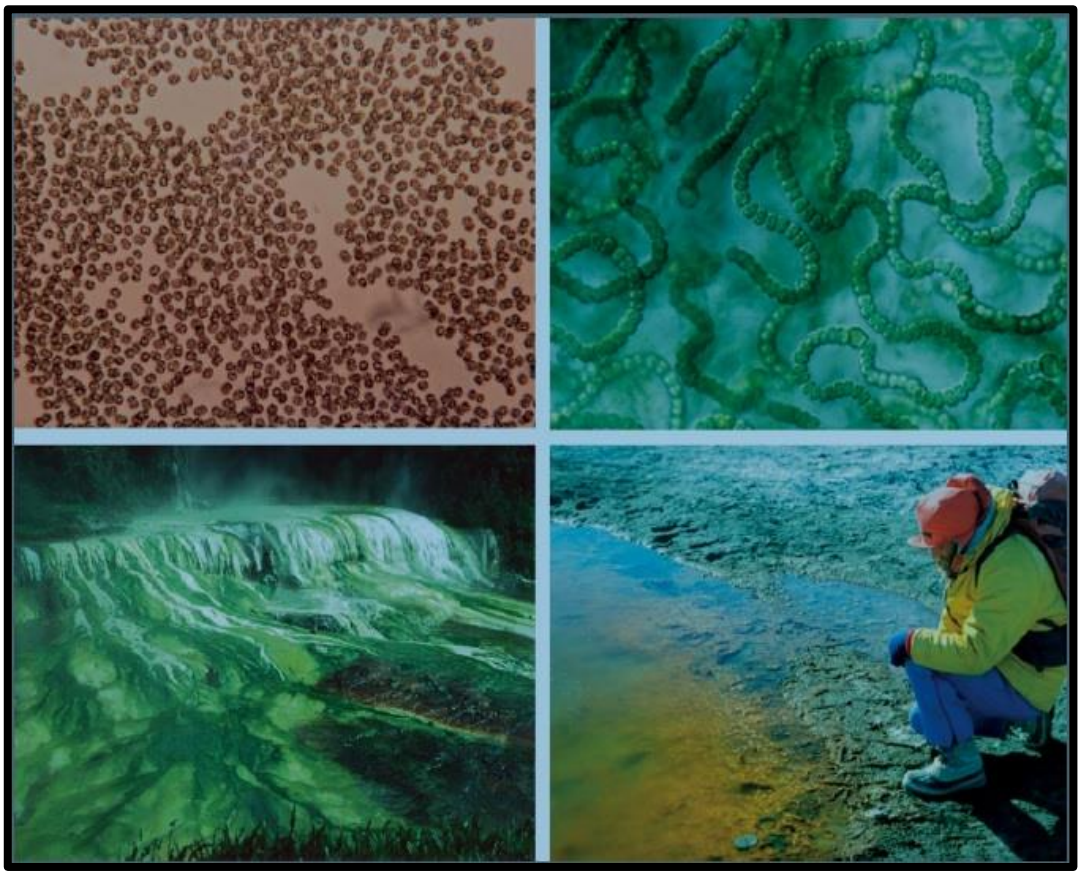


Figure 2.2 Cyanobacteria in island water ecosystems. Top left: A microscopic image of the cysts from a planted lake, the bright areas within each cell are caused by the dispersion of light by gas gaps. Top right: A microscopic image of *Nostoc* from a high Arctic lake. The largest spherical cells are heterogeneous cells, nitrogen fixation sites. Bottom right: Rich in carotenoids, bacterial mat in a pond on the McMurdo Ice Shelf, Antarctica. Bottom left: Bacterial mats in thermal spring, New Zealand [19].

Most of the leading ideas show that comparative studies of the genetic composition of cyanobacteria will contribute to reviewing their classification. The appropriate classification reflects as closely as possible the evolutionary relationships as coded, for example, coded in the 16S or 23S rRNA sequence data [20]. The integration of phenotypic, genetic, and histological information makes it possible to classify as polyphase classification [21].

Modern-day cyanobacteria include more than 2000 species in 150 genera, with a great variety of shapes and sizes. Ecologically, there are three main groups in the aquatic environment hangings forming the type of species, which form periphytic biofilms above rocks, sediments, and submerged plants. Bloom-formers, which create a wide range of water quality problems and that is mostly combined, in nutrient-rich (eutrophic) lakes, and picocyanobacteria, which are microscopic cells (less than 3 mm in diameter) that are predominantly abundant in very clear water lakes. Additional groups contain colonial non-bloom-formers, which are common in a variety of aquatic habitats, including mesotrophic lakes, wetlands, and saline waters. Metaphysical species that form aggregates are loosely associated with emergent macrophytes (water plants such as rushes and reeds that expand out of the water into the air above) and a certain type of species that grow as periphyton but that can also enter the plankton; and various symbiotic associations [22].

Cyanobacteria are oxygenic photosynthetic (Photoautotrophic) gram-negative prokaryotes M.O. that show connecting link between plastid of eukaryotes autotrophs and prokaryotes. They are an excellent source of peptides, trans-fatty acid, amino acid, vitamins, minerals and pigments like chlorophyll a, carotenoids as phycobiliprotein, which exhibit different color [23]. It is estimated to be more than 5.2 billion years old, depending on the dependence of sedimentary rocks in the Late Archaeological Period [24]. The fossils showed that their origin is as old as 3.5 billion years old, and similar fossils are the current wave, which is to rely slowly on development [25].

2.3 Characteristics of Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that pick up sunlight for energy using chlorophyll a and various accessory pigments. In addition to their remarkably long

persistence as free-living organisms (as mentioned before), cyanobacteria also form symbiotic associations with more complex biota; for example, the nitrogen-fixing species *Anabaena azollae* (or *Nostoc*) forms a symbiotic association with the floating fern *Azolla,* which is widely distributed in ponds and flooded soils. The chloroplasts in plants and algae appear to be originally derived from an endosymbiosis in which a cyanobacterium was engulfed and retained within a colorless eukaryotic cell [23].

Cyanobacteria have a number of special characteristics that determine their relative importance in phytoplankton communities. However, the behavior of different types of green microbes in nature is not homogeneous because their ecological surroundings differ. Their response to environmental factors is essential to define water management objectives because some of them exhibit similar ecological and physiological characteristics. Their behavior can group them in plankton ecosystems as an "ecology" that usually inhabits the different aquatic ecosystem niches [26].

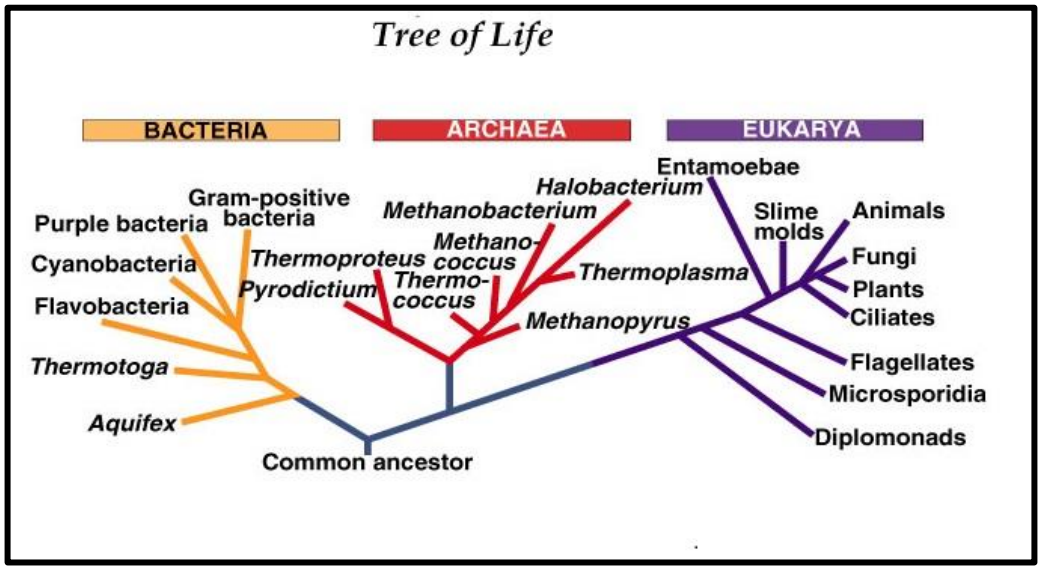
Cyanobacteria were previously classified as blue-green algae because of their algae-like appearance, containing chlorophyll instead of bacteriochlorophyll — the production of oxygen through the photosynthetic production from dual-system processes such as in algae and higher plants. Infrastructure studies of cyanobacteria have clearly shown that prokaryotic cyanobacteria lack nuclei and other organelles and have a typical cell wall of peptidoglycan for gram-negative

bacteria. It also has many advantages that distinguish it from other bacteria, especially photosynthesis and oxygen production. The term "blue-green algae" is still widely used by the media and in water quality management. The current taxonomic separation of species, especially irregular or heterogeneous varieties, is thought to be artificial and does not reflect evolutionary development. Relationships are likely to be heavily revised, as more genetic data becomes [19].

In current years, cyanobacteria have been in the spotlight due to their useful effects, among which are their high nutrient levels and their ability to produce biofuel and assimilate carbon and nitrogen. As their global biomass also increases due to climate change, there is a need to characterize potential hazards associated with this phylum properly. In a study, researchers studied the allergenic properties of cyanobacteria, thriving within different habitats and ecological niches. They observed distinct IgE-binding patterns for the studied freshwater cyanobacteria. The analysis of the corresponding bands revealed phycobilisome-related proteins, previously identified as IgE-binding proteins from *M. aeruginosa* and *Spirulina*. IgE binding was also found for the standard C-PC proteins, in contrast to the study, where no IgE-reactive bands had been [27].

2.4 Classification of Cyanobacteria

Figure 2.3 explains the classification of cyanobacteria, the cyanobacteria (Blue-green algae), aside from the fact that blue bacteria are classified as bacteria (whose nuclei are free of membranes), they have a light effect and are included in algae clusters. Blue bacteria had a crucial role in raising the level of free oxygen in the early earth's atmosphere; cyanobacteria are classified within the Monira Kingdom



(prokaryotic), Division Eubacteria, class cyanobacteria [28].

Figure 2.3 Classification of cyanobacteria [29].

The classification and regulation of blue bacteria have undergone major changes in the past two decades. In the past, blue bacteria were placed in three vegetarian orders, and their number has changed with respect to the status of the investigation of morphological variability and the species-environment [30].

The bacterial approach classifies five subgroups of cyanobacteria, corresponding to *Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales,* and *Stigonematales,* with respect to cell proliferation, cell differentiation, and molecular/biochemical characteristics [31].

The systematic classification at a higher level of cyanobacteria needs further examination based on revised genera. A complete review of the morphological description of natural populations should include microscopy of light and electron, characterization of habitat, molecular analysis of 16S RNA and other markers such as the Internal Transcribed Spacer (ITS) region and information on stored strains or DNA. In addition, an important consideration should be given to biochemical/organic data, for example, the composition of fatty acids of the bacterial cell wall, which seems to be applicable to species identification [32].

2.5 Composition of Cyanobacteria

Figure 2.4 explains the composition of cyanobacteria, the mass can be made up of a single cell, but cells often assemble and stick together in colonies of different shapes. The cell structure is simple; protoplast is free of characteristic nuclei, mitochondria, green plastids, and no endoplasmic network. Protoplast is usually composed of two parts, the exterior is colored and is known as the chromoplast, and the interior is not colored and is known as the central body. The body contains DNA, RNA in the process of proliferation. The color of the chromoplast is due to the presence of dye-based membranes scattered in the cytoplasm. These pigments are phycocyanins, chlorophyll, carotene, xanthophyll, and phycoerythrins [22].

Protoplasts, usually, are free of pituitary cavities, and these gaps may be formed in older cells. Moreover, the absence of pitched gaps is one of the reasons why the cell is resistant to drought and high osmotic pressures. In some species, pseudovacoules are thought to contain gases that result from the breathing process and help to float algae; the food stored in the form of oil droplets and granules is a distinctive starch for this type of algae. It is known as the green cyanophycean starch, which is closely related to the glycogen. Other stored substances, which are found in reproductive cells, gradually disappear during periods of growth and activity [17].

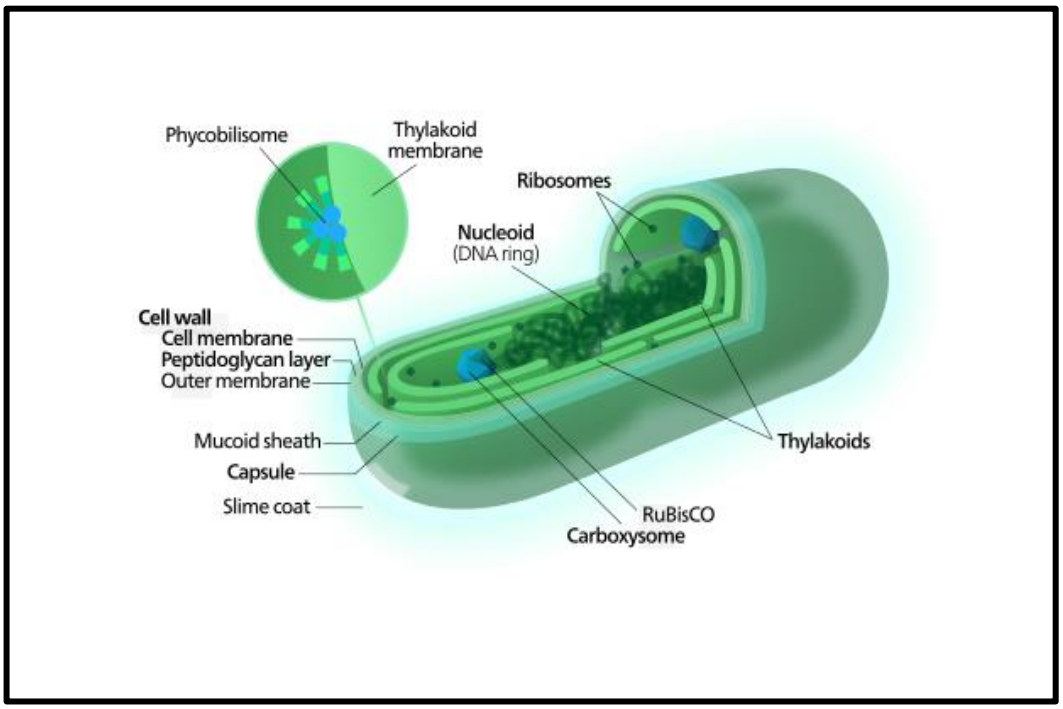


Figure 2.4 Composition of cyanobacteria.

A thick wall surrounds protoplast made up of two parts, the outer hemispheric and inner, made up of murrain, similar to that of gram-positive bacteria. A bacterium gelatin envelope also surrounds the wall. The wall may be colorless transparent and may be painted in different colors, including yellow, red, purple, and brown. The color of the algae is caused by the color group in chromoplast and the color of the outer wall. Lennon and the intensity of light falling on the moss affect the color

of the algae. Members of these algae do not have whips but may move a sliding motion on wet surfaces, and helps the secretions of the layer gelatin-coated to them [33].

2.6 Division and Reproduction

Cyanobacteria or greenish algae cells divide by simple division. Few of them are separated immediately after the division of cells, but in most species, the cells resulting from the division remain connected to each other's colonies and on the basis of the number of levels of division which is determined by the shape of the resulting colony. If the split occurs at one level, the resulting colonies are threaded, and sometimes the algae strands branch. If the division occurs at two perpendicular levels, the resulting colony has a flat class shape. If the division occurs at three levels, the resulting colony is cubic. If the number of levels exceeds three resulting in irregular colony shape, the colony is often surrounded by a gelatinous layer produced from its cells [34].

The colony cannot grow indefinitely. However, if the envelope is thick, the colony grows larger in size before splitting up. The fragmentation of the colony is due to the death of some cells or the poor connection between some cells or the feeding of an animal on part of it. In the algae, there are usually larger and denser cells than ordinary cells, known as heterocyst, which are less colored than other cells. The cell has two lateral holes in its contact areas with adjacent vegetative cells, through which micro plasmodesmata pass through it. These holes are matured, and the different vesicles are the centers of stabilization of atmospheric nitrogen. The alveolar sacs usually separate the lines because they are less attached to neighboring cells. The vegetative algae cells are located between two different ones known as the hormogonium. When the strands are separated at the different vesicles, the hormones, which escape the mother's thread, are separated and grow again [35].

Some algae are immobile stationary alginates known as akinetes. They are produced by some hormone cells, are rich in their food content, have more dense

walls than other cells, and are resistant to inappropriate environmental conditions. Germs germinate when conditions improve and are green. In some races, bacteria are formed by the splitting of a protoplast into a vegetative cell composed of numerous small endospores, and non-sexual reproduction is unknown between blue-green algae [36].

2.7 Thermophilic Cyanobacteria

Photosynthetic cyanobacteria are divided into two main categories based on cell size and composition, namely, macroalgae and microalgae in which their cells are made up of structures that resemble roots, stems, and leaves [36].

Hot springs, volcanic environments, deep-water vents environments, and geysers are usually the main sources of most M.O. and marine, usually inhospitable for most life forms; however, thermal M.O. that tolerate high temperatures can grow and live in such harsh environments, including man-made habitats. Optimum growth at temperatures more than 55°C and less than 85°C respectively, usually these organisms can tolerate harmful temperatures, high acidity, and high concentrations of salts [10].

In the last few years, thermophilic M.O. has been widely used for their biotechnological potential, particularly in industrial processes. Biotechnology allows the production of a large amount of biochemical, polymers and biofuels. It depends on conventional, bioremediation of explicit, materials and request, bioreactors made of stainless steel, complex sterilization techniques, and expensive downstream processing [38].

The microalgae that live in thermal ecosystems are heavily involved in the total biomass and productivity of the ecosystem of most of the communities in which they are present; this presence creates a platform for carbon-free production. The activity of converting carbon dioxide into biomass and fat to produce biofuels is an emerging area of interest. For example, two types of thermal microscopic algae *Chlorella* sorokiniana and *Asterarcys* quadricellulare are isolated from water bodies in and around a steel plant in India. They were examined to withstand high temperatures and light along with high carbon dioxide and NO levels [39].

High amounts of carbohydrates are collected when exposed to a high level of carbon dioxide. High amounts of fat are collected when exposed to NO_2 with carbon dioxide, which can also be used as feedstocks to produce ethanol and biodiesel, respectively. Environmental waste from industrial waste along with a large number of value-added chemicals such as refractory pigments and enzymes have been synthesized from these refractory M.O., and some studies have recently been carried out to improve the performance of electrochemical bio-systems by increasing the thermal stability of frozen proteins [21].

Some researchers worked on isolating and diagnosing strains of thermophilic cyanobacterial that were able to grow well in the middle of BG 11 of hot springs where the biological accumulation of reactive dye of these strains under temperature-loving conditions was studied. *Synechococcus sp*. *Phormidium sp.* which have the optimal hydrogen number for growth among these M.O. in the different temperatures, was studied and the possibility of absorption of these strains to different pigment concentrations and the possibility of using these strains of the organisms live to remove the dye from the effluents from sewage under thermal conditions [40].

Some researchers and previous studies found the fecundity and yield of food and fiber crops are mainly determined by efficient CO_2 fixation mechanism in the chloroplast. Although the CO_2 fixation mechanism is influenced by a number of genetic and environmental factors, it is mainly dependent on the efficiency of Rubisco or the concentration of CO_2 in the vicinity of Rubisco (an enzyme found in plant chloroplasts, stabilizes carbon dioxide in the atmosphere during photosynthesis and in grants to the resulting compound during photosynthesis of oxygen). Based on this literature review, it is highly probable that the Rubisco activity, RCA activity, and net photosynthesis are strictly correlated [41].

2.8 Biotechnological Use of Cyanobacteria

Most microalgae, bacteria, and yeasts are M.O. that are considered within traditional biotechnology in our modern studies and future research. Countries

like Japan and Taiwan have already succeeded in using them as healthy food, and the idea of using M.O. or microalgae to treat wastewater had been launched. Microscopic algae are an important source of energy through their large photosynthesis capacity, play a major role in solar biotechnology nowadays, producing large quantities of up to 200 tons of the dry weights of microscopic algae, as well as it is possible to increase the production of any crop up to 60%. Also, the production increases 20 - 35 times of this protein in any crop, this percentage is usually more than the percentage in soybeans and more than 50 times in rice, wheat or corn for the same region. It is important that microalgae can be cultivated throughout the year and harvested continuously and that consumption of water in small quantities [42].

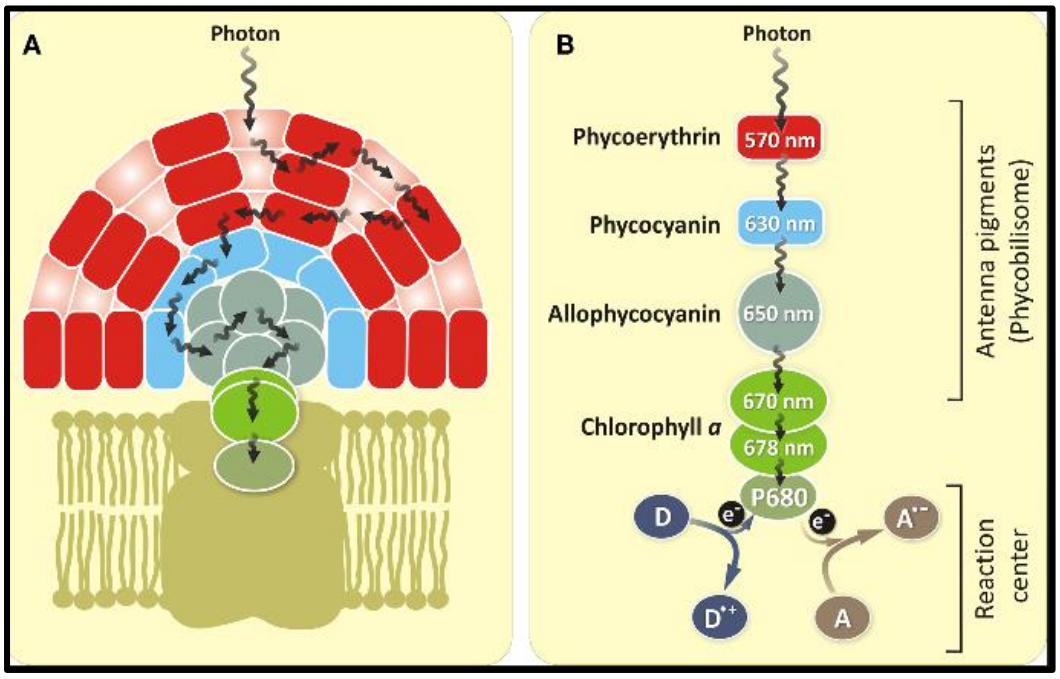
The traditional application of carbohydrate-producing cyanobacteria as a source of nitrogen in rice paddies was one of the best ways to increase the production of cyanobacteria that determine nitrogen, which in turn increases rice yields and the possibility of applying them as fertilizer or a source that can be used in biochemical applications. Lack of expensive chemical fertilizers is one of the main problems facing the agricultural sector in developing countries, so the need to stimulate the search for alternative nitrogen sources for rice cultivation is imperative. Rice fields are constantly subject to environmental changes during the maturation of rice plants [43].

The cyanobacteria are considered one of the most important sources of lipoproteins, sugars, and protein. The significance of these M.O. and the maximum possibilities to take advantage of them, their mass production and the ways to employ them for modern biotechnology are limitless [44].

Some researchers found manufactured biology methods permit the rational development of novel metabolic pathways that are based on the combination of many enzymatic activities of various origins. In addition, the manipulation of whole metabolic systems by CRISPR-based and sRNA-based technologies with many parallel targets will further excite the use of cyanobacteria for diverse applications in basic research and biotechnology [45].

2.9 Phycobiliproteins

Figure 2.5 explains the general exemplification of a phycobilisome structure. Phycobiliproteins are compounds able to be soluble in water it already exists in cyanobacteria and algae (Rhodophytes, Cryptomonads, Glaucocystophytes) and they can capture light energy and then transfer it to chlorophyll during photosynthesis. Phycobiliproteins are brilliantly colored, high-fluorescent components for photovoltaic harvesting antenna complexes of cyanobacteria (Bluish-green algae, Red algae and Cryptomonads). Phycobilisomes have a function in light-harvesting and energy migration. When the energy absorbed by the bromine (biliproteins) of the biocobelesomes reaches the reaction centers of the second photosynthesis system, a shift in excitation energy to chemical energy occurs. Bipolar proteins absorb in areas of the visible spectrum, while chlorophyll a has low absorbency. Pecobelicomas are found in Procreatiococcal Cyanobacteria

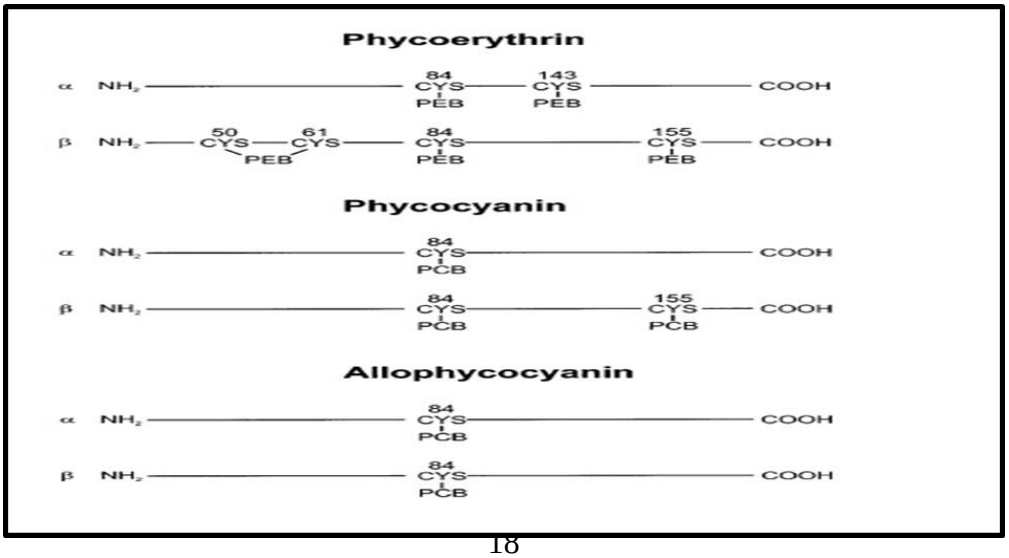


and Eukaryotic Red Algae [46].

Figure 2.5 General exemplification of a phycobilisome structure [47].

Phycobiliproteins are natural additives in food manufacture and essential fluorescent inquiry in biotechnology. Various algal species may be suitable for their extraction; therefore, it is important to study different seaweeds to determine the highest species, the process of harvest light from the cyanobacterial phycobilisomes and that was the main reason to the migration of energy usually across photosystem II centers. The transfer of energy may occur from phycobilisomes to the photosystem directly under certain light conditions [48].

The phycobiliproteins are classified into three types based on their absorption spectra: phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC). They are all composed of hetero-subunits α and β and commonly exist in trimer 3(αβ) or hexamer 6(αβ) made up of equimolar monomer (αβ). One monomer contains two to five chromophore phycobilins. In cyanobacteria and red algae, phycobiliproteins assemble a macromolecular particle, phycobilisome. Efficient excitation energy coupling among the chromophores in the phycobiliprotein trimer/hexamer and among the phycobiliproteins in the phycobilisome gives them some special spectroscopic properties superior to organic fluorescent dyes. These properties make the phycobiliproteins become promising fluorescent probes used in various



fields of biological investigation [49].

Figure 2.6. Structures of types of phycobiliproteins (phycoerythrins PC or phycoerythrocyanin PE, allophycocyanins APC).

Phobobilisomes are largely composed of phycobiliproteins, and in cyanobacteria, these proteins can represent up to 50% of total cellular protein. Fatty proteins of phycobiliproteins can be divided into four main chapters based on maximum long-wave absorption, phycocyanin phycobiliprotein, which is the most abundant protein in cyanobacteria, followed by phycoerythrin and allophycocyanin. Figure 2.6 explains the structures of types of phycobiliproteins, cyanobacterial phycobiliproteins and their maxima light absorption peak are as follows: Allophycocyanin 650–660nm, Phycocyanin 610–625nm, Phycoerythrin 490–570nm [47].

2.10 Biotechnological Use of Phycobiliproteins

The natural sources of biologically active compounds promote human health. Increased interest in these sources for the creation of active, modern, and inexpensive biochemical materials obtained from traditional and inexpensive sources is justified by ongoing research on cyanobacteria. Phycobiliprotein is a high natural, which has great potential to be used in biotechnological applications such as the field of food and feed industries in addition to the field of pharmaceutical industries was used in the field of cosmetics industry [50,51,52].

Phycobiliproteins, especially phycocyanin, from different cyanobacterial species have been related to exhibit a variety of pharmacological activities, which is like antioxidant, anticancerous, neuroprotective, anti-inflammatory, hepatoprotective and hypocholesterolemic [52].

Pigments, and especially the ones present in the phycobilisome, have been considered more intensely since they performed a myriad of bioactivities. Although phycobiliproteins and phycobilins have previously shown a significant

potential of applicability in the industry because of their wide array of bioactivities, part of this potential is not yet covered due to limitations in the purifying and characterization of these molecules. It is assumed that in the next few years, more studies about the bioactivities of phycobiliproteins and phycobilins could be carried out and that the

potential application of these compounds can be achieved. It is possible that an additional screening of cyanobacteria strains for the presence of phycobilins with desirable characteristics will result in additional discoveries beyond the fast-growing strains already used in the market. In terms of the biotechnological processes of production, extraction and purification of phycobiliproteins, a wide range of conditions, parameters and protocols have been carried into account. However, the ideal or optimal method still depends of the strain and also on the application (in terms of purity levels and possible techniques). Knowing the factors to take into account to obtain phycobiliproteins from cyanobacteria, and also the potential of employment (bioactivities), it is expected, in the near future, other uses and further studies about these interesting compounds [53].

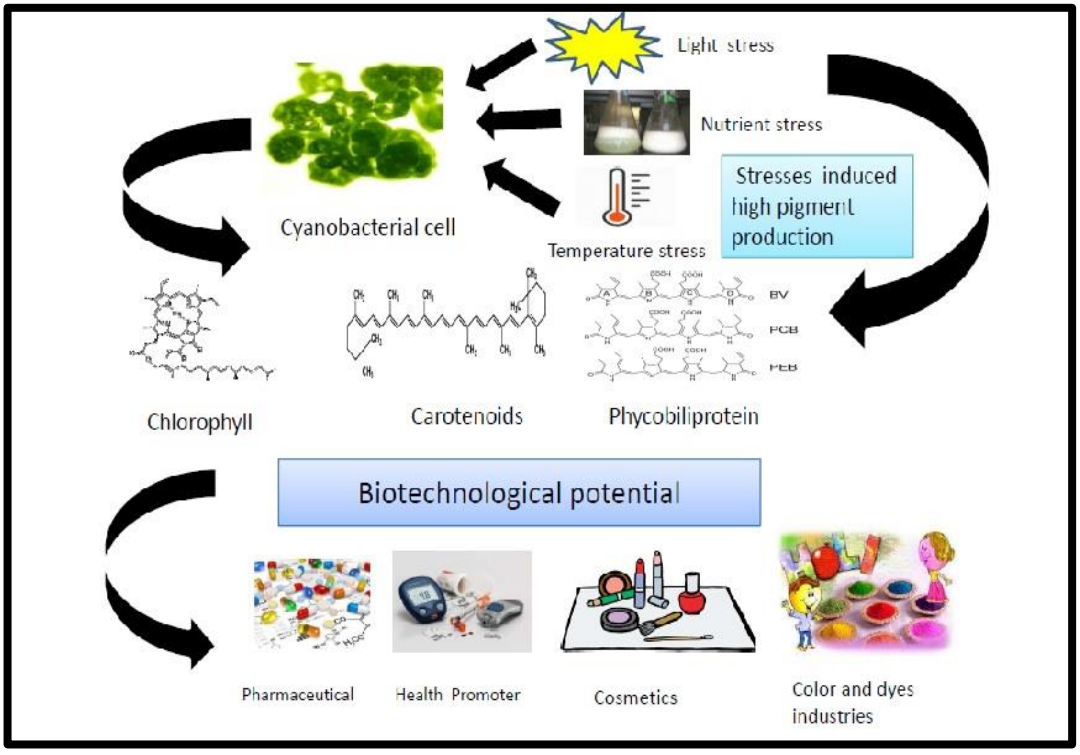


Figure 2.7. Diagram of the potential of biotechnology (chlorophyll, carotenoids, phycobiliprotein) [54].

Figure 2.7 explains the type of cyanobacteria can create a wide limit of colored pigments. Study of pigment-producing cyanobacteria, their biosynthetic pathways, and use of genetic engineering can produce the future of the cyanobacterial pigment giving most promising and attractive. Metabolic engineering has been practiced and can further be exploited in a variety of microalgae or cyanobacteria for the enhancement of pigment production. Advances in the gene level study of cyanobacteria provide knowledge about the compound metabolic system of pigment production, which allows us to utilize different new technique to produce high pigment producing species. The impact of light on the natural cyanobacterial growth has not been efficiently studied by the scientific community which expects more insight both physiologically and genetically [54].

The improvement in the field of DNA sequencing has resulted in solving more information about the complex metabolic system of the pigment-producing cyanobacteria and will help us to easily engineer these organisms for large production of the pigment molecules. While other researches in the extraction give as cost of the high-value pigment can be minimized by applying new metabolic approaches, the amount of extraction can be decreased by designing species which will dye and other practical product simultaneously, For this determination, new type and novative thinking is needed for scientific intervention. In the result, we can say that pigment production through cyanobacteria has a bright and beautiful future in the field of cosmetics, pharmaceutical, and many coloring applications. Cyanobacterial pigments require studies for their stability, congeniality and reduction of their toxicological effect to improve their commercial value in the global sale [54].

3
Materials and Methods

3.1 Materials

3.1.1 Chemical Materials

In this study, different types of chemical materials were used as shown in Table 3.1.

Table 3.1 Chemical materials used in this study

Name	Chemical structure	Company
Dipotassium hydrogen phosphate	K_2HPO_4	Merck
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	Merck
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	Alfa Aesar
Citric acid	$HOC(COOH)(CH_2COOH)_2$	Labochem International
Ammonium ferric citrate green	$C_6H_8O_7 \cdot xFe_3 + yNH_3$	Sigma
Ethylene diamine tetraacetic acid disodium salt dihydrate	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	Alfa
Sodium carbonate	Na_2CO_3	Sigma
Boric acid	H_3BO_3	VWR
Manganese(II) chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	Alfa Aesar
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	Alfa Aesar

Sodium molybdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	Abcr
Copper(II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	Labochem International
Cobaltous nitrate hexahydrate	$Co(NO_3)_2 \cdot 6H_2O$	Alfa Aesar
Hydrochloric acid	HCl	Sigma
Sodium hydroxide	NaOH	VWR
Agar	----------	Sigma
Ethylene diamine tetra acetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	Alfa
Iron(II) sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	Sigma
Molybdenum trioxide	MoO_3	Sigma
Ammonium acetate	$C_2H_7NO_2$	Acros Organics
Ammonium nitrate	NH_4NO_3	Merck
Ammonium sulfate	$(NH_4)_2SO_4$	Merck
Ammonium vanadate	NH_4VO_3	Sigma
Chromium potassium sulfate dodecahydrate	$KCr(SO_4)_2 \cdot 12H_2O$	Sigma
Nickel (II) sulfate heptahydrate	$NiSO_4 \cdot 7H_2O$	Merck
Chloroform	$CHCl_3$	Sigma
Tungstic acid sodium salt dihydrate	$Na_2WO_4 \cdot 2H_2O$	Merck
Titanium(III) sulfate	$Ti_2(SO_4)_3$	Sigma

CTAB buffer cetyl trimethylammonium bromide	An antiseptic agent also used in DNA extraction	Merck
Phenol	C_6H_5OH	Thermo
Isopropanol or isopropyl alcohol	$CH_3CHOHCH_3$	Sigma
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}NaSO_4$	Sigma
Agarose	For electrophoresis	Sigma
Ethidium bromide	For electrophoresis	Sigma
T10E1 Buffer, 1 ml of 1 M Tris base	----------	Bio-Life
Sucrose	$C_{12}H_{22}O_{11}$	Sigma
Bromophenol blue (3′,3″,5′,5″-tetrabromo-phenolsulfonphthalein, BPB	$C_{19}H_{10}Br_4O_5S$	Sigma
Sulfuric acid	H_2SO_4	Sigma
Nile red stains	$C_{20}H_{10}N_2O_2$	Sigma
n-Hexane	C_6H_{14}	Merck
DMSO dimethyl sulfoxide	$(CH_3)_2SO$	AppliChem GmbH
Sodium azide	NaN_3	Sigma
Sodium acetate	CH_3COONa	VWR
Phosphoric acid	H_3PO_4	BOTTLE
Methanol	CH_3OH	Merck

MyTaq™ DNA Polymerase	BIO-21105	Bioline
MyTaq™ Reaction Buffer	BIO-37111	Bioline

3.1.2 Kits Used in the Experiment

My TaqTM DNA Polymerase: Bioline meridian life science company.

Gel electrophoresis: Hyper Ladder™ company.

3.1.3 Tools and Devices

Types of equipment used in this study were shown in Table 3.2.

Table 3.2 Equipments used in this study

Name	Company
Autoclave	Nuve ot 90L , Multiskan GO User Manual
Automatic pipettes	Eppendorf (0.5-10µl , 0-20 µl ,0-200 µl ,0-1000 µl,0-5000 µl)
Block thermostat/ Nitrogen evaporator	Teknosem (TAB-24 Nitrogen evaporator)
Cooling centrifuge	Allegra X-15R Benchtop from Beckman Colter Life Sciences
Eppendorf microcentrifuge	5418 r speed of up to 16,873 × g allows for standard molecular biology applications
Fast prep homogenizers	Mp biomedicals fast prep-24tm 5g instrument
Florescence Microscope	NIKON H600L
Fluorescent microscope	Zeiss axiovert A1 fluorescent microscope

Freeze Dryer	Alpha 1-2 LD plus Entry Freeze Dryer Package
Freezer	Haier Biomedical's standard temperature -86°C ULT Freezer
Freezer (-10°C/ -22°C)	Inoksan (EMP.70.95.02-40)
FT-IR Spectroscopy	PerkinElmer (FT-IR_Spectroscopy)
Gel electrophoresis	The Azure c200 Gel Documentation
Hotplate	Stirrer Hotplate Stirring Stir Hot Plate VWR IKA VMS-C7 S1
HPLC (high-performance liquid chromatography)	HPLC -Shimadzu CTO-10A VP Column Oven HPLC
Ice maker	Scotsman - AF 80
Incubator	Thermo Scientific 390-0467
Microscope	Nikon Eclipse 100 LED Binocular
Nitrogen evaporator	Nitrogen evaporator Teknosem -Tab-24 WEL
pH- meter	Mettler Toledo
Rotatif wise mix	Agitateur rotatif Wise Mix RT - Cloup
Shaker	VWR Advanced Orbital Shaker Model 5000
Shaker for microtubes	Thermo-Shaker for microtubes and PCR plates
Spectrophotometer	Shimadzu balance ATX224 , DeNovix DS11 FX+
Thermo scientific easy	Thermo Scientific Easy Cast B1A Mini Gel Electrophoresis systems with Power source: Consort EV265

Thermocycler, PCR machine	Bio-Rad T100 PCR Thermal Cycler, TS-100
UV-Vis spectrophotometers	UV-Vis spectrophotometers: UV-2600 and UV

3.1.4 The Sampling Resources

The strains were isolated from 8 different hot spring water resources located in Ankara Province in Turkey. As shown in the location map (Figure 3.1), the samplings were done in Kızılcahamam-Seyhamamı (39°50'44.2"N 27°10'32.3"E), Çağa-Mülk (40°07'36.0"N 32°12'47.4"E), Çubuk-Melikşah (40°09'50.1"N 32°56'49.7"E), Beypazarı-Dutlu (40°09'12.9"N 31°54'15.4"E), Haymana (39°25'25.5"N 32°29'41.0"E), Malıköy-Uyuz thermal spring (40°34'25"N 32°39'59"E), Mürted (39°55'44.9"N 32°52'12.3"E), and Polatlı (39°53'36.2"N 32°49'02.2"E).

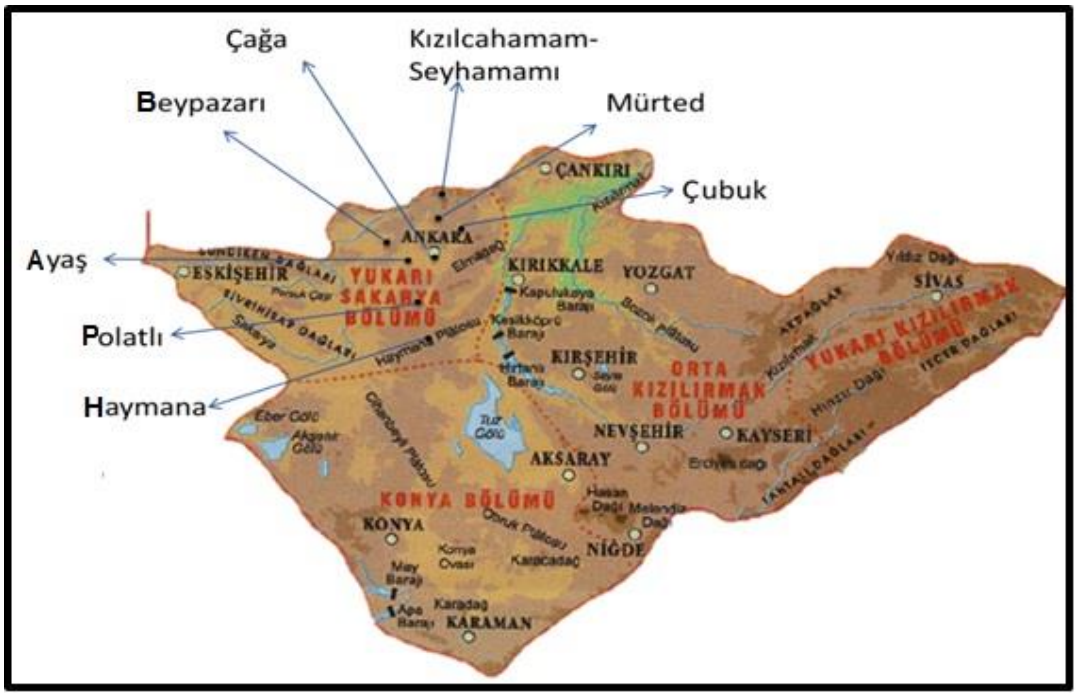


Figure 3.1 Map of strains isolated from 8 different hot spring water resources located in Ankara.

According to the General Directorate of Mineral Research, Turkey hosts approximately 600 geothermal water sources that reaches the ground at a temperature between 25-103^{o}C.

According to the data reported in Turkey Geothermal Resources Inventory published in 2005, Central Anatolia region is the region with the highest geothermal water resources. Ankara is among the richest province in terms of geothermal resources in Turkey. In different areas of Ankara like; Kızılcahamam-Seyhamamı, Çubuk-Melikşah, Beypazarı-Dutlu-Ayaş, Çağa-Mülk, Haymana, Mürted and Polatlı, all these regions are the region with the highest geothermal water resources.

There are 18 natural water outlets in 7 geothermal fields with more than 25° C fluid temperature and about 40 hot water springs opened by drilling as shown (Table 3.3). Ankara province with very different temperature and water chemistry with warm water resources reflects all the many features of the hot springs in Turkey. For this reason, the province of Ankara is an excellent field of study in order to investigate the evaluation of microorganisms in hot water resources [55].

Table 3.3 Temperature, pH, radioactivity plan and water chemistry of one of the existing water resources in Ankara.

	GEOTHERMAL AREA NAME	HOT WATER NATURAL OUTPUT NAME	WATER CHEMISTRY	TEMPERATURE OF WATER (oC)
ANKARA	Kızılcahamam - Seyhamamı	Kızılcahamam, Seyhamamı	Bicarbonate, Sodium, Chlorine, Carbon dioxide, Arsenic, Silicate	50-51
		Seyhamamı	Bicarbonate, Sodium, Calcium, Carbon dioxide, Fluoride	43
	Çubuk-Melihşah	Melikşah Kaynağı	Bicarbonate, Sodium, Magnesium	31-34
	Beypazarı-Dutlu-Ayaş	Dutlu	Chloride, Sulfate, Sodium, Calcium, Carbon Dioxide, Arsenic, Gallium	42-51
		Karakaya,	Bicarbonate, Magnesium, Calcium, Carbon dioxide	31
		Çoban	Strong Salt, Alkali, Bicarbonate, Carbon Dioxide, High mineralization	52
		Ayaş içmeceleri	Strong Salt, Alkali, Bicarbonate, Carbon Dioxide, High mineralization	51
		Feruz	Saline, Alkali, Bicarbonate, Carbon Dioxide	26
	Çağa-Mülk	Merkez	Bicarbonate, Sodium, Carbon Dioxide, Sulfate, Lithium, Beryllium	37
	Haymana	Haymana, Uyuz (Seyran)	Bicarbonate, Alkali and Earth Alkali metals, Carbon dioxide	45
		Uyuz (Seyran)	Bicarbonate, Alkali and Earth Alkali metals, Carbon dioxide	34
	Mürted	Karalar	Saline, Bicarbonate, Carbon dioxide, Sulphated acidic water	28
	Polatlı	Malıköy-Ilıcapınar (uyuz)	Sodium, Carbon Dioxide, Sulfur, Chloride, Soda cold, oligometallic	28.5
		Karahamzalı-Sabanca	Sodium, Bicarbonate, Carbon dioxide, Sulphated acidic water	28
		Sanoba	Bicarbonate, Alkali and Earth Alkali metals, Carbon dioxide	30-33
		Karacaahmet	Sodium sulfate, Calcium carbonate, Carbon dioxide	38
		, Kürttaciri (Özyurt)	Bicarbonate, Carbon dioxide, Sulphated acidic water	26
		Özhamamı	Sodium, Bicarbonate, Carbon dioxide, Alkali metals, Arsenic	32

3.1.4.1 Fields of Study and Sample Collection

Samples from the selected stations were taken directly in glass bottles from the areas where the hot water flows and from the rocks. The colonized microalgae samples were isolated and scraped with care from the outer layer of the rocks and stored in glass bottles. Care was taken in the transport of the microalgae samples to the laboratory. Water samples which contain the microalgae have been extracted from the glass bottles and examined directly under the microscope. After that, samples in the bottle left to settle in the dark for a while, the liquid on the sediment was removed, and the precipitate was taken to the Petri dishes. After that, the 22x22 mm Petri dishes were covered by using parafilm and the liquid was removed in the sunlight for a while. For algae adhering to the lamellas by phototaxis, a portion of the lamellae was washed with the selected liquid medium for isolation, and the other lamellae were made into transient preparations using glycerin and were used for the diagnosis of algae [56].

Dynamic parameters such as temperature, salinity, pH, ammonium content, nitrate content, dissolved oxygen, conductivity and total dissolved solid levels of the water resources were measured by means of a multi-parameter reader (YSI ProPlus, USA) on-site when the isolation was accomplished in July 2016 [57].

3.1.4.2 Isolation, Purification, and Culturing of Cyanobacteria

Microscopy of the samples, BG-11, Allen, FW pre-enrichment types of media were prepared and incubated for 5 days. At the end of the incubation, the microscopical examination of the cultures was prepared and the species were identified. The isolation of cyanobacteria was carried out according to the serial dilution method. Dilution processes are usually a successful method in the isolation of microalgae that can grow well in the BG-11 medium. However, this method is not enough to get rid of the bacteria contamination for the production of axenic cultures. Methods such as antibiotic treatment have also been used for the species to be determined by microscopical examination in serial dilution, some nutrients were be placed in some

diluted flasks or incubation were tried under different growth conditions. In general, the dilution method was summarized as follows: 2 ml of the 10 ml of the sample containing media were transferred into other flasks which contain 20 ml of sterile medium solution and left inside a shaker with a light source to be mixed.

It was then transferred to the next flasks by taking 2 ml from the flasks which contain 20 ml and transferred to another new 20 ml flasks under aseptic conditions.

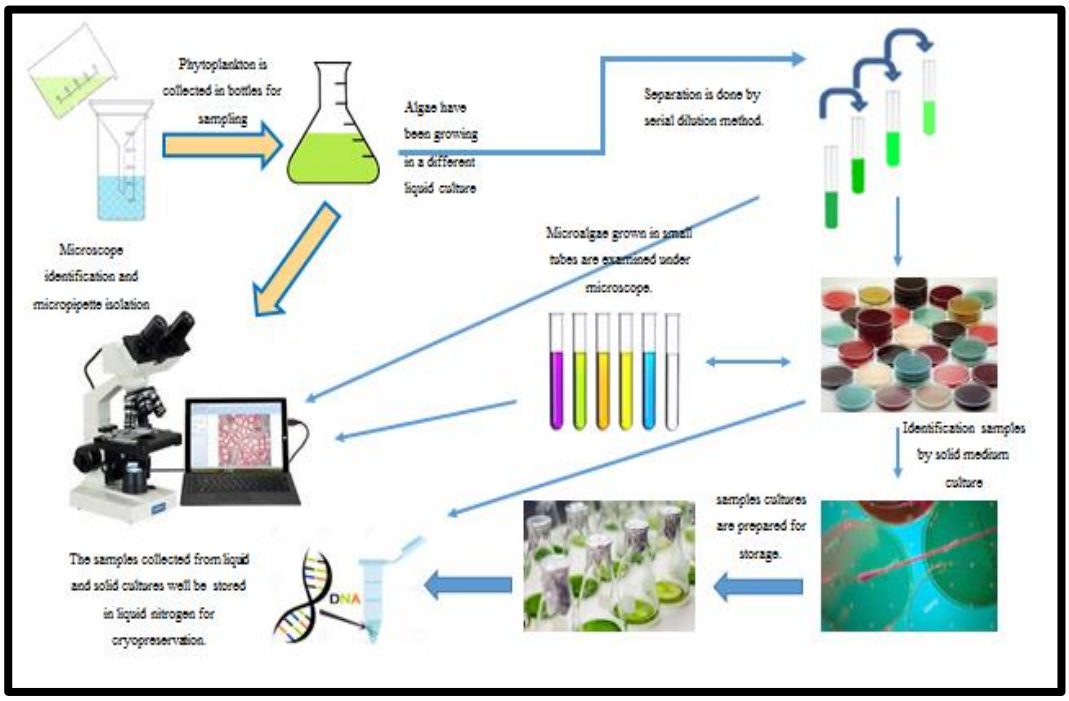


Figure 3.2 Diagram illustrates the microalgae's extracting procedure

A clear method flow chart was very complex. The flow chart illustrates the extraction procedure used in this study (Figure 3.2). This process was repeated and incubated for 10 days. At the end of the incubation period and at an appropriate temperature and light, these flasks were allowed to incubate with shaking, and the microscopical study was performed after 15 days. After that, samples were taken and examined under the microscope to identify the cyanobacteria colonies produced and the purity of the product. After the identification, pure cells were then transferred to a new flask by selecting a single

colony and allowed to be incubated in solid and liquid media. Isolation methods often do not follow a specific method,

the most appropriate method was decided and applied according to the need for observation and quantitative analysis.

3.1.5 Types of Media

Initially, this study started by using four different culture media was used to determine which medium was better and more suitable for the culturing of cyanobacteria.

3.1.5.1 Preparation of BG11 (Blue-Green Medium)

The 8 different stocks solution were separately prepared by dissolving 75.0 g of $NaNO_3$, 2.0 g K_2HPO_4, 3.75 g $MgSO_4 \cdot 7H_2O$, 1.80 g $CaCl_2 \cdot 2H_2O$, 0.30 g citric acid, 0.30 g ammonium ferric citrate green, 0.05 g $EDTANa_2$, and 1.00 g Na_2CO_3 were separately dissolved in 500 ml of distilled water. Then, the trace metal stock solution was prepared in 1 liter distilled water by dissolving 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$ and 0.05 g $Co(NO_3)_2 \cdot 6H_2O$. Totaly 9 stock solutions were prepared. 10 ml was taken from each stock solution and 1ml was taken from the trace metal stock solution. Total volume was 81 ml. As a final step for preparing BG11 medium, 81 ml will be transferred to a new 1L bottle and the remaining volume will be compensated with distilled water [58].

BG11-N and BG11-NP solutions were prepared using the same procedure but with small differences. In BG11-N, $NaNO_3$ was not added. In BG11-NP, $NaNO_3$ and K_2HPO_4 were not added. These solutions were prepared as solid and liquid media and the pH was adjusted to 7.1 with 1M NaOH or HCl. To prepare the agar (solid media), 15.0 g of agar was added to the 1 L prepared solution (Oxoid L11) and autoclaved at 15 psi for 15 minutes [59].

3.1.5.2 Preparation of Z8 Medium

Preparing Z8 medium dispends on three stocks solutions.

- **Stock solution I**

Stock solution I was prepared as shown in Table 3.4.

Table 3.4 Preparation of stock solution I for the production of Z8 media

Chemical structure	**Amount**
$MgSO_4 \cdot 7H_2O$	0.25 g
$NaNO_3$	0.467 g
$Ca(NO_3)_2 \cdot 4H_2O$	59 mg
Na_2CO_3	31 mg
NH_4Cl	0.02 g
FeEDTA solution	10 mL
Gaffron micronutrients	1.0 mL
The resulted media was dissolved separately in ddH_2O, gathered together in 1L bottle diluted to 1 liter.	

- **Stock solution II**

Fe EDTA solution: Fe EDTA solution was made by using two types of solutions, the first solution (A) prepared by dissolving 2.8 g of $FeCl_3$ in 100 ml of 0.1N HCl, while, the second solution (B) was prepared by dissolving 3.9 g $EDTANa_2$ in 100 ml of 0.1 N NaOH. After that 10 ml of solution (A) and 9.5 ml of solution (B) were added in a new 1L bottle, then diluted with distilled water to complete 1 L.

- **Stock solution III**

Stock solution III was prepared as shown in Table 3.5.

Table 3.5 Preparation of stock solution III for the production of Z8 media

Chemical structure	Amount (g)
$ZnSO_4 \cdot 7H_2O$	0.22
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.088
$Co(NO_3)_2 \cdot 6H_2O$	0.146
$VOSO_4 \cdot 6H_2O$	0.054
$Al_2(SO_4)_3K_2SO_4 \cdot 2H_2O$	0.474
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.198
$Cd(NO_3)_2 \cdot 4H_2O$	0.154
$Cr(NO_3)_3 \cdot 7H_2O$	0.037
$Na_2WO_4 \cdot 2H_2O$	0.033
KBr	0.119
KI	0.083
The resulted media was dissolved separately in dH_2O, gathered together in 1L bottle diluted to 1 liter.	

Finally, 10 ml of stock solution I and II were added to 1 mL of stock solution III, and the mixture was then diluted to 1 liter with dH_2O. The diluted mixture was autoclaved at 15 psi for 15 min, and the pH of the resulted media was fixed until 6-7 degree was reached.

To prepare the agar (solid media), 15g of agar was added to 1L of the prepared solution [60].

3.1.5.3 Preparation of Freshwater Medium (Fw)

The freshwater medium was obtained by preparing and mixing 10 stock solutions according to the quantities mentioned in Table 3.6 and 3.7.

Stock Solution (SL)	Volume (ml)	Chemicals	Concentration (g / 100 ml)
SL1	20	$Ca(NO_3)_2.4H_2O$	0.2
SL2	10	K_2HPO_4	0.1
SL3	25	$MgSO_4.7H_2O$	0.1
SL4	20	Na_2CO_3	0.1
SL5	50	$Na_2SiO_3.5H_2O$	0.1
SL6	10	Ferric-citrate	0.1
SL7	10	Citric acid	0.1
SL8*	5	Micronutrient solution*	
SL9**	30	Soil extract**	
	820	Distilled water	

Table 3.6 Freshwater media preparation

*For SL8 micronutrient solution was prepared according to Table 3.7. The solutions (I and II) were separately prepared, autoclaved, cooled down and then pooled together.

Table 3.7 Preparation of SL8 solution

Solution	(ml, g)	Solution, Chemicals
Solution I	881 mL	Distilled water
	1 mL	0.1 g $ZnSO_4 \cdot 7H_2O$ / 100 mL
	2 mL	0.0758 g $MnSO_4 \cdot 7H_2O$ / 100 mL
	5 mL	0.2 g H_3BO_3 / 100 ml
	5 mL	0.02 g $Co(NO_3)_2 . 6H_2O$ / 100 mL
	5 mL	0.02 g $Na_2MoO_4 . 2H_2O$ / 100 mL
	1 mL	0.0005 g $CuSO_4 \cdot 5H_2O$ / 100 mL
	0.4 g	EDTA(TriplexIII)
Solution II	100 mL	0.7 g $FeSO_4 \cdot 7H_2O$ + 0.4 g EDTA(TriplexIII) / 1000 ml

** SL 9 solution is a soil extract solution, prepared as follows: 50 g of soil was boiled (e.g. unfertilized garden soil) in 500 mL dd-H_2O for 30min. The resulted solution was centrifuged at 5500 rpm for 15 min. The supernatant was isolated and filtered by using 3 μm filter until the purity was reached. After that, the solution was autoclaved twice at 121°C for 35 min. to kill any fungal spores.

***SL 10 is a mix of vitamins as shown in Table 3.8, as a stimulator for the bacteria growth. 1ml of SL 10 solution was added to the autoclaved solution by using 0.2 μm sterilized filter.

Table 3.8 Stock solution of SL 10

Solution	Volume	Chemicals
SL 10	1 mL	(0.1 g Vit. B_1 + 0.025 mg Vit. H + 0.015 mg Vit. B_{12}) / 100 mL

After mixing all of the solutions together (SL 1-10) liquid freshwater medium was prepared. Solid freshwater medium was prepared by adding 1.0-1.3 % agar and the resulted media was autoclaved [61].

3.1.5.4 Preparation of Allen Medium

To prepare the Allen medium, first, we need to prepare a solution as demonstrated in Table 3.9.

Component	Amount ml	Stock Solution Concentration
HEPES buffer	2.3 g / L	
$NaNO_3$	1.5 g / L	
P-IV metal solution *	1 ml / L	
$K_2HPO_4 \cdot 7H_2O$	5 ml / L	1.5 g / 200 ml
$MgSO_4 \cdot 7H_2O$	5 ml / L	1.5 g / 200 ml
Na_2CO_3	5 ml / L	0.8 g / 200 ml
$CaCl_2 \cdot 2H_2O$	10 ml / L	0.5 g / 200 ml
$Na_2SiO_3 \cdot 9H_2O$	10 ml / L	1.16 g / 200 ml
Citric acid·H_2O	1 ml / L	1.2 g / 200 ml
* P-IV metal solution was prepared according to Table 3.10		

Table 3.9 Solution concentration of Allen medium

Table 3.10 P-IV metal stock solution concentration

Component	Amount (g/L)
$Na_2EDTA{\cdot}2H_2O$	0.75
$FeCl_3 \cdot 6H_2O$	0.097
$MnCl_2 \cdot 4H_2O$	0.041
$ZnCl_2$	0.005
$CoC_2 \cdot 6H_2O$	0.002
$Na_2MoO_4 \cdot 2H_2O$	0.004

50 mL of a resulted mixture from Table 3.9 was added to 950 dH_2O and the pH will be adjusted to 7.8 using 1M NaOH or HCl, then the mixture will be autoclaved at 121°C for 20 min.

The solid media will be prepared by adding 13g of agar to 1L of Allen media, and the mixture was autoclaved with the same conditions used above.

3.2 DNA Experiments

3.2.1 DNA Extraction

Initially, we added 400 µl of (pre-warmed to 60 ^oC) extraction buffer on 100mg of microalga biomass and vortex for 1 min at maximum speed. After that, we added 40 µl of SDS (10%) + 8 µl of proteinase K (20mg/ml) vortex for the 30s at maximum speed and incubate at 60 ^oC for 1h by using thermos shaker (the speed

was adjusted to low speed (100rpm) during incubation). 0.2% β-mercaptoethanol or 1% PVP can be substituted with SDS if necessary. After that, the microtube was inverted upside-down 3 times, and 400 µl phenol: chloroform (1:1) was added and mixed by

inversion for 2 min (the rotator was set at 20 rpm speed). Centrifugation was applied then to all the tubes at 14000 g for 10 min at 4 ^{o}C, the mixture was left to settle for 1 min at 4 ^{o}C so that the resulted layers were not mixed. There must be 3 layers; a lower layer which is the organic phase distinguished by green pigment; a little-interface layer which must be white, if not, cells are not lysed; and an upper-aqueous phase which contains DNA. We transferred the upper-aqueous phase to a new microfuge tube and then added 300 µl isopropanol. After that the tubes were mixed by inversion for 3 min at 20 rpm speed, and incubated for at least for 1h (it may be 2h or more, DNA must be visible after incubation) at RT or overnight, then the solution was spin at 14000 g for 15 min at 4 ^{o}C, and the supernatant was removed carefully. The pellet which represents the DNA was washed with 750 µl ice-cold EtOH (70%) and rotated it for 2 min at 20 rpm speed. Following this step, centrifugation was applied to the tube at 14000 g for 1 min at room temperature. The supernatant was discarded and the washed pellet was taken. The pellet (DNA) was left to dry by leaving the tube open sterile cabinet for appx. 10 minutes. Then 300 µl sterile T10E1 (Tris, 10mM +EDTA, 1mM; pH 8.0) was added (or ddH_2O can be added as a replacement of T10E1). To measure the concentration of the purified DNA, nanodrop was used for spectrophotometric quantification at a wavelength between (260-280 nm). If the concentration was high a serial dilution of DNA solution must be made according to the settings (1, 1:1, 1:4, and 1:8 DNA:ddH_2O) to see optimal dilution ratio for the quantification. After the quantification, a 50µl of DNA was taken in an aliquot and stored at -20 ^{o}C for further analysis [62], [63], [64].

3.2.2 Gel Electrophoresis

The gel electrophoresis was used twice in this study. The first time was to know the molecular weight band gives us a good indicated quality of DNA before the PCR. The second gel electrophoresis was used after PCR to know the amplification efficiency of DNA.

Agarose gel electrophoresis technique for the DNA will help show the purity of the isolated DNA, while spectrophotometry will give an indication of the concentration. Initially, we prepared 1 % solution of agarose by dissolving 1 g of agarose in 100 mL of 0.5x TBE buffer in a microwave for approximately 2 min, then allowed to cool for

a couple of minutes. For the polymerization of agarose solution, we added 2.5 µl of ethidium bromide to the beaker, mixed it well and poured the hot mixture in the tank to be slowly cooled until the solution transforms into a gel (a comb-like plastic was inserted to form the wells). To assure the complete polymerization, the mixture was left to settle for 20 min at room temperature and the comb was removed soon after. The sample was loaded to the separated wells as following; 10 µl 1kb ladder + 5 µl sample + 5 µl water + 2 µl 6x loading buffer, then the gel was run for 30 min at 100 volts. After that, the gel was exposed to UV light and a picture was taken to confirm the isolated DNA quality. If the bands were thick and dark it means that the molecular weight is high and dilution is needed (the same procedure is repeated for the diluted solution) and the presence of a high molecular weight band indicated good quality DNA. However, the presence of a smeared band indicated DNA degradation [65].

3.2.3 Polymerase Chain Reaction (PCR)

Out of 10 primers (Table 3.11) provided by the literature from previous studies, we wanted to choose the best primer for the amplification of the DNA of cyanobacteria. To do that we need to try these 10 primers with the isolated DNA of

the cyanobacteria. PCR was carried out in this process, and after amplification, gel electrophoresis was performed to confirm the efficiency of these primers [74].

10 µl was taken from all 10 primers in a new clean Eppendorf, and 190 µl of ddH_2O was added to each tube, this process was made to dilute the primers. Then, to a new Eppendorf, 10 µl of the isolated DNA was added to 20 µl of the diluted primer. From the last mixture (DNA + diluted primer), we took 1 µl in a new PCR tube. Taq DNA polymerase and the other PCR solutions were added according to Table 3.12. PCR settings were made according to Table 3.13 by using 30 cycles. Gel electrophoresis of the DNA showed the efficiency of the primers.

Table 3.11 Primer pairs used studies.

Primer name	**Primer sequences**	**Primer Kind**
pA	5'- AGA GTTTGATCC TGG CTC AG - 3'	Forward
B23S	5'- CTT CGC CTC TGTGTGCCT AGGT- 3'	Reverse
CYA108F	5'- ACG GGT GAG TAA CRC GTR A- 3'	Forward
CYA16SSCYR	5'- CTT CAY GYA GGC GAG TTG CAGC- 3'	Reverse
16S545R	5'- ATT CCG GAT AAC GCT TGC - 3'	Reverse
CYA359F	5'- GGG GAA TCT TCC GCA ATG GG- 3'	Forward
CYA781R	5'- GAC TAC AGG GGT ATC TAA TCC- 3'	Reverse
27F	5'- AGA GTT TGA TCM TGG CTC - 3'	Forward
27FR	5'- TTG GGC GTA AAG CGT AG- 3'	Forward, Reverse
809R	5'- GCT TCG GCA CGG CTC GGG TCG ATA- 3'	Reverse

Table 3.12 Polymerase chain reaction

Name	**Volume (µl)**
Master mix	5

Forward primer	1
Reverse primer	1
Double-distilled Water	12
DNA	5

PCR Program Sitting :

Table 3.13 PCR program sitting.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	94	94	50	72
Time (min.)	5	10	1.30	2
30 cycles were done.				

After confirming the PCR results by the gel electrophoresis, the 10 tubes (DNA+10 primers) were sent for sequencing to know which primer is the most suitable for cyanobacteria's DNA amplification. The results obtained by the sequencing confirmed that Forward 27F :(AGA GTT TGA TCM TGG CTC) and Reverse 809R :(GCT TCG GCA CGG CTC GGG TCG ATA) of the 10 primers were the best for DNA amplification.

3.2.4 Sequence Information of Cyanobacteria Species

The selected primers were then applied to 12 types of DNA sequences obtained from 12 different cyanobacteria species. The same procedure above was performed for the 12 DNA types with the selected primers followed by PCR and gel

electrophoresis. The samples including PCR products were sent for sequencing to Sentromer DNA Technologies in Istanbul Turkey (https://www.sentromer.com/). The same primers used at PCR were also used for Sanger sequencing. Twelve nucleotide sequences are analyzed on the NCBI database, and finally, BLAST results are used for identification of the strain. After that the results were compared with the data from the gene bank program. Genomic results of diazotrophic cyanobacteria are summarized in the following charts (Figure 4.6).

3.3 Extraction and Estimation of Phycobiliproteins

The cyanobacteria samples were cultured and in beakers, and 5 ml of each sample was isolated in tubes and centrifuged at 4000 xg for 5 minutes. The supernatant was discarded and 5 ml of sodium phosphate buffer (0.1M, pH7.0, containing 1mM sodium azide) was added to the pellet to prepare it for sonication (200W, 30 kHz) for 2 min. The samples were left to freeze at -86 °C for 20 min. Then the samples were left around 10 minutes at room temperature. Centrifugation is followed for the samples at 4000 xg for 5 minutes and the clear supernatant was collected for absorbance measurement.

The phycobiliproteins in the samples were measured at (OD 562, OD 620, and OD652) for the calculation of phycobiliproteins (phycocyanin- PC, Allophycocyanin –APC, Phycoerythrin –PE) according to the following equations [66].

$$PC\ (mg/\ mL) = [A620 - 0.474\ (A652)] / 5.34 \quad (3.1)$$

$$APC\ (mg/\ mL) = [A652 - 0.208\ (A620)] / 5.09 \quad (3.2)$$

$$PE\ (mg/\ mL) = [A562 - 2.41\ (PC) - 0.849\ (APC)] / 9.62 \quad (3.3)$$

3.4 Extraction of Chlorophyll a and Carotenoids

3.4.1 Harvesting Biomass

1 ml of each cyanobacteria cell was collected from the previous cultures and centrifuged at 15,000 x g at the room temperature for 7 min. The supernatant was discarded and the samples were stored at -86 °C for the extraction process.

3.4.2 Extraction of Carotenoids and Chlorophyll a

The first step includes the addition of 1 ml of pre-cooled (4 °C) pure methanol to the stored green-colored cyanobacteria. The sample was homogenized by pipetting up and down, and then each Eppendorf was covered with aluminum foil for 20 min to protect the sample from light. Centrifugation was applied for the samples at 15,000 x g, 4°C around 5 min. The color of the pellet should be purple or green-blue in order to prove that carotenoids and chlorophyll-a were extracted. If not, the procedure should be repeated until we get this color change.

3.4.3 Measurement of Carotenoids and Chlorophyll a

In this study, the concentrations of pigments were measured by using the spectrophotometer, the blank is the pure methanol alone. Then the absorbance of blank and sample were measured at 470 nm, 665 nm, and 720 nm. After that, the concentration of carotenoid and chlorophyll a content were calculated according to the equations:

$$\text{Chla [µg/ml]} = 12.9447\ (A665 - A720) \quad (3.4)$$

$$\text{Chla [µM]} = 14.4892\ (A665 - A720);\ \text{for Chla molar mass} = 893.4890\ \text{g/mol} \quad (3.5)$$

$$\text{Carotenoids [µg/ml]} = [1{,}000\ (A470 - A720) - 2.86\ (\text{Chla [µg/ml]})] / 221 \quad (3.6)$$

The measurements were repeated three times in order to ensure the obtained results and to obtain the mean and standard deviation for each pigment [67], [68], [69], [70].

3.5 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR was performed on the biomass of the cyanobacteria cells to determine the compounds that are inside these cells. To do that, cyanobacteria cells were cultured in BG11 +N, -N, -NP liquid media for 10 days, and cells were collected in the 5^{th} and 10^{th} day. The collected cells were placed in a 2mL Eppendorf tubes, centrifuged at 2000 x g for 5 min and froze in -86 ^{o}C for 24h. After that, the cells were taken out of the freezer and dried in a lyophilizer at 40 ^{o}C for 12h. FT-IR was then performed on the powdered-like cells obtained after drying with the lyophilizer. The wavelength of the FT-IR devise was from 4000-450 cm^{-1}, and 64 scanning for each sample [71].

3.6 Growth Measurements Content

3.6.1 Harvesting Culturing Biomass

1 mL Cyanobacteria sample was taken from the culture suspension, after that measurement, the absorbance of each sample was made at 680nm. Then the concentration of the pigments was measured by using spectrophotometer with slit width 1 nm and the same media was used as the blank.

3.7 Measurement of Total Protein Content

3.7.1 Extraction of Total Protein Content

From the same biomass used in FT-IR determination, frozen cell pellets were taken and resuspended (50-100mg) in 500 mL lysis buffer (50 mM Tris–HCl pH 8.0, 2% SDS,10 mM EDTA, and protease inhibitor mixed). After that the cells were frozen at –86 °C and quickly thaw them at 37 °C to allow partial cell breakage. Then, sufficient amount of glass beads was added to break the cells by vigorous vortexing in a homogenizer (10 cycles of mixing for 30 sec and cooling on ice for 30 sec). After that samples were incubated in room temperature (use rotator) for 15 minutes. The samples were then centrifuged at 5000g for 20 min at 4 ^{o}C. Then the pellet was discarded and the supernatant was saved for protein determination.

3.7.2 Protein Determination

To determine the protein concentration we need to prepare unknown samples from the obtained protein samples above. Before we started the experiment, protein samples obtained from cyanobacteria were heated at 60 ^{o}C mixed with the working reagent (1 part 4% copper sulfate + 50 parts Bicin Coninic Acid Solution) and saved in the dark at room temperature. The standard was prepared in triplets according to Table 3.14 [72].

The unknown samples were made using the same volumes in the table but only the BSA was replaced with the protein sample we need to measure. After that 200 µl of the working reagent was added and mixed in a shaker, then incubated at 60°C for 30 min and also cooled until room temperature was reached. Then the absorbance was measured at 562 nm and the protein concentration was obtained. Protein calibration curve was shown in Figure 3.3.

Table 3.14. BSA concentrations used to obtain BSA standard curve

BSA (µg/ml)	Water
-	10 µL
1.5 µL	8.5 µL
3 µL	7 µL
5 µL	5 µL
7.5 µL	2.5 µL
10 µL	-

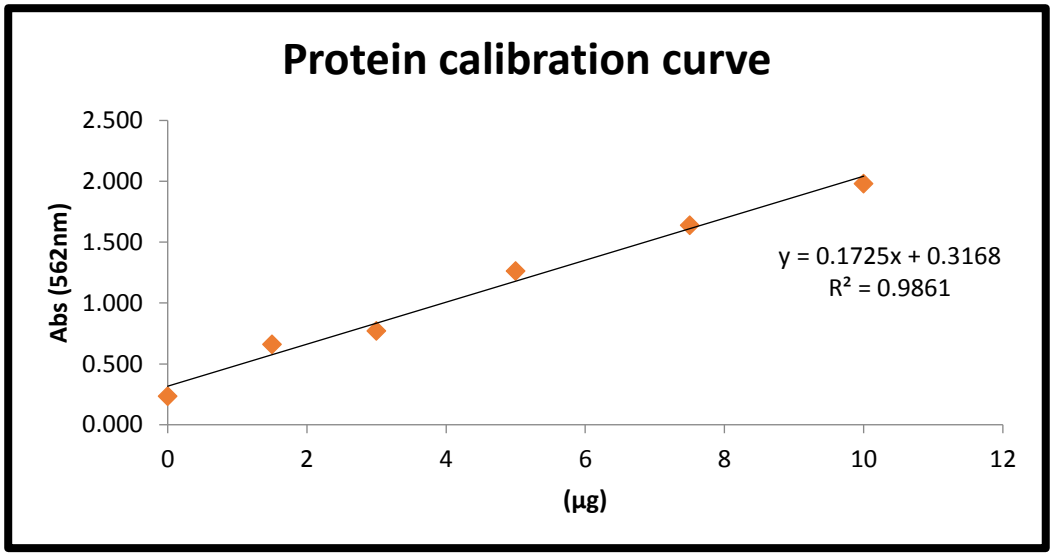


Figure 3.3. Protein calibration curve

3.8 Measurement of Total Saccharide Content

3.8.1 Harvesting Biomass

1 ml of the sample culture suspension was taken and the concentration was measured using OD730. Then the cells were centrifuged at 15,000 xg at room temperature for around 5 min and the supernatant was discarded. After that, the samples were kept at -86 °C.

3.8.2 Extraction of Saccharide

The samples stored were taken out of the freezer and placed on ice to melt. Then 1 ml of precooled methanol at 4 °C was added. The cells were homogenized with methanol by pipetting and covered with aluminum foil to prevent the light from affecting the samples. Then the pigments were extracted from the samples by allowing all cells to sit at 4 °C in a dark place for 20 min. The samples were then centrifuged at 15,000 x g, 4 °C for around 5 min, and the color of pellet checked the by the naked eye. If the pellet was purple and no green color was observed, then we can measure the saccharide concentration using the OD730. If the sample's pellet was green, then the procedure should be repeated [73].

3.8.3 Measurement of Total Saccharide

500µl distilled water was added to each sample's pellet and mix well by pipetting, and the samples were moved to a fume hood. Then phenol 5% was prepared (w/w) by dissolving 1 g of phenol into 19 ml of distilled water, the phenol is prepared to penetrate the cells. After that, 500 µl of phenol 5% was added to each pellet and 500 µl of distilled water was added to the mixture and mix well with pipetting. The samples were kept at RT for 15 min. 60 µl of each sample was transferred to a 96-well plate, then 150 µl of 96% sulfuric acid was added to each pellet. At the end, the concentration of the total saccharide was measured at 490nm using a spectrophotometer. The standard here is D-glucose.

3.8.4 Glucose Calibration

500 µg/ml D-glucose (w/w) was prepared by dissolving 5 mg of D-glucose in 9.995 ml of distilled water. Then the 500-µg/ ml D-glucose solution was diluted according to Table 3.15. Saccharide calibration curve was shown in Figure 3.4.

Table 3.15 Preparation of calibration series of D-glucose in distilled water

Eppendorf tube number	**Glucose solution (500 µg/mL)**	**Distilled water (µL)**	**Final glucose concentration (µg/mL)**
1	25 µL	475	25
2	50 µL	450	50
3	75 µL	425	75
4	100 µL	400	100
5	300 µL	200	300
6	500 µL	-	500

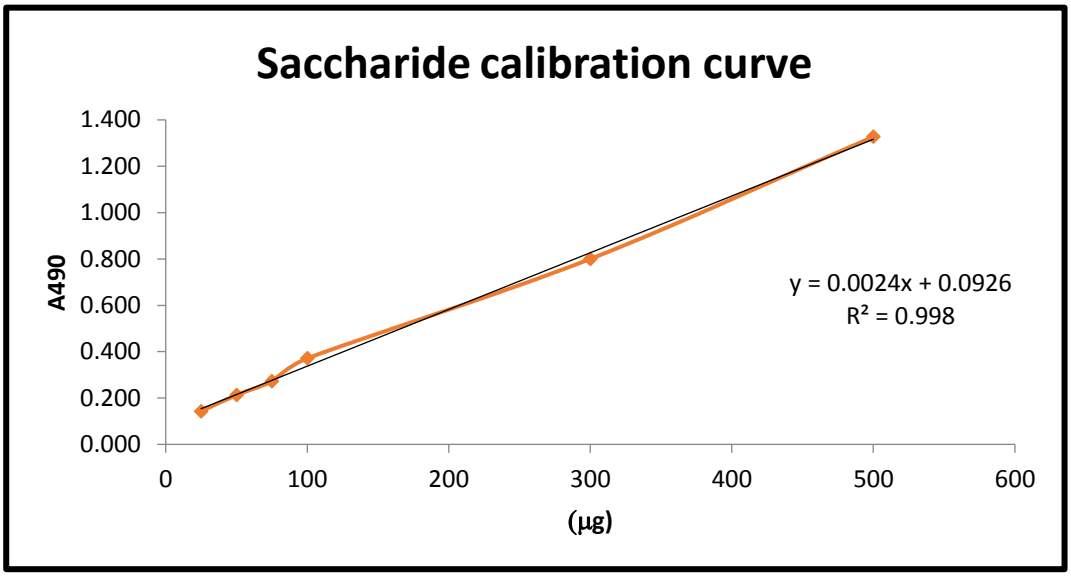


Figure 3.4. Saccharide calibration curve

3.9 Analysis in High-Performance Liquid Chromatography (HPLC)

3.9.1 Carotenoid Analysis by HPLC

2 ml extraction buffer (50% MeOH- 50% THF) was added to 0.1 g wet samples collected (lyophilized but there were moisture after taken from -20°C to room temperature) and samples were vortexed and filtered with 0.45 µm PTFE filters (45/25mm), [74].

HPLC settlements

- Mobil phase : 95% MeOH : 5% THF
- 0.8 flow rate (mL/min)
- PDA 450 nm
- First, wash column 25 min (1 run takes less than 25 min)
- Column type: C8 [74].

Calculations: The formula below was used to the term amount of carotenoid in cyanobacteria

$$x = \frac{1\times(\text{HPLC Peak})\times(\text{Dilution Ratio})}{(\text{Standard Area})\times(\text{Amount of Sample})} \times 100 \quad (3.7)$$

- Dilution Ratio: 2mL
- Amount of Sample: 0.1 g
- Standard Area: For Lutein: 320,000/ For the others :280,000

3.9.2 Vitamin Analysis (B_2) by HPLC

This experiment was started by adding 2.5 N sodium acetate and then added 0.1 N HCl. Then the buffer solution at the pH between 4.5-5.0 was prepared. 4 mL from the buffer solution was added into 0.1 g wet algae samples. Then samples were vortexed. 0.1 ml enzyme (Phosphatase acid) (0.5 mL solid enzyme in 7 mL ddH_2O) was added. After that, the samples were incubated in shaker horizontally 3 h at 37°C. 6 mL 1N HCl was added to 4 ml sample in the tube. Then samples were filtered with 0.45 µm PET filters (Chromafil) (45/25mm), [76], [77].

3.10 Fluorescence Imaging of Cyanobacterial lipids (Nile Red)

Initially, 1mL of cyanobacteria sample was collected and the absorbance was measured at OD720 nm. Then the final absorbance of the cyanobacteria solution was adjusted to 0.2-0.3 ml with respective growth media for Nile red staining of live cells. After dilution (if needed) 980 µl of cyanobacteria suspension was transferred into a new Eppendorf tube, and 250 µl of Nile Red dissolved in DMSO (1 µg/ mL) was added. The suspension was shaken for 10 min under dark conditions. After that, we used a fluorescence microscope to observe the fluorescence of samples and to determine the cyanobacteria lipid content [78].

4
Results

4.1 Fields of Studies and Sampling

The present study was carried out in the Microalgae Culture lab in Istanbul Medeniyet University, Turkey. Different cyanobacteria samples were collected from 8 different hot spring water resources located in Ankara Province of Turkey.

There are many dynamic water parameter values measured in this study, the parameters showing the dynamic water chemistry of the geothermal fields where microalgae samples were measured by a multi-parameter measuring instrument [79].

The dissolved oxygen (DO %) level enters the water by diffusion from the atmosphere as a by-product of photosynthesis by algae and plants. The concentration of dissolved oxygen level in epilimnetic waters continually equilibrates with the concentration of atmospheric oxygen to maintain 100% dissolved oxygen level saturation. The excessive algae growth can over-saturate (greater than 100% saturation) the water with dissolved oxygen level when the rate of photosynthesis is greater than the rate of oxygen diffusion to the atmosphere.

The level of dissolved oxygen in water gave a clear water, so the highest dissolved oxygen level was 95.6 % in Maliköy Uyuz Hamamı region, and on the other hand, the lowest dissolved oxygen level gave a 41.2% in Dutlu region as shown in Table 4.1.

The specific conductivity is known as a numerical expression of an aqueous solution's capacity to carry an electric current. This ability depends on the presence of ions, their total concentration, mobility, valence, relative concentrations, and the temperature of the liquid. The conductivity level gives as

the highest electric current level in Dutlu region with 9060, and the low electric current level in Melikşah region with a value of 310 (Table 4.1).

The total dissolved solids (TDS) will give us the value of organic materials in water that was collected from that area, as shown in Table 4.1. The total dissolved solids had the lowest level (304), and the DO was 83.4 % in Melikşah region, it means that the level of the organic compound is low in this area, and the water was clean. But on the other side, in Dutlu region, the total dissolved solids were around 5141 and the DO was 41.2%, showing that the organic compound's level was high, it indicates the water is so dirty, while in Maliköy Uyuz Hamamı region the (TDS) was the lowest 310, and the DO was 83.4 %, and the spc was also low in the . From another side the Pressure (N/cm^2) values were close to each other in most all regions, but the SPC was low in the Melikşahand so high in Dutlu. Consequently, in this study, the relationship between some variables measured was not as it was expected, and the other variables measured were parallel depending on the region that the sample collected from it as shown in Table 4.1.

Table 4.1 Dynamic water parameter values measured in the summer term of hot water resources.

Sampling site	Pressure (N/cm^2)	DO %	DO (mg/L)	SPC	Conductivity [S/m]	TDS (mg/L)
Beypazarı	703	71	4.1	5949	7950	4021
Dutlu	700	41.2	2.81	7830	9060	5141
Ayaş	698	84.2	7.40	6240	5925	4265
Maliköy Uyuz Hamamı	695	95.6	8.85	6305	6095	4139
Kızılca hamam	674	67.9	4.23	2890	3690	1890
Seyhamamı	665	64.5	4.16	1596	2125	1045
Melikşah	676	83.4	10.65	406	310	304

The dominant nitrogen level depends mainly on the ammonium level, and the ammonium level, in its turn, depends on the temperature, salinity of water, pH, and NO_3^- (mg/L). The areas with the highest ammonium concentrations were determined in the water samples taken from Malıköy and Seyhamamı geothermal areas in Ankara as shown in Table 4.2.

Table 4.2 Temperature, pH, salinity, ammonium and nitrate values of the hot water sources in the summer.

Sampling site	Temp (^oC)	Salinity (ppt)	pH	NH_4+ (mg/L)	NO_3^- (mg/L)
Beypazarı	41.2	3.12	7.1	8.20	6.90
Dutlu	34.9	4.14	6.9	7.50	7.47
Ayaş	21.6	3.64	7.9	7.15	6.24

Maliköy Uyuz Hamamı	26.5	3.25	6.2	12.1	7.05
Kızılcahamam	43.1	1.35	6.8	7.58	4.38
Seyhamamı	42.4	0.84	6.7	12.1	12.07
Melikşah	18.3	0.19	7.8	3.22	1.35

The temperature also was the highest (43.1 ^{o}C) in Kızılcahamam region, while in Melikşah region was (18.3 ^{o}C) the lowest when compared to other regions. On the other hand, the highest salinity (ppt) level in Dutlu area was 4.14 with the highest conductivity, 9060. It means that the ionization of water was also the highest, while the lowest salinity (ppt) level (0.19) was in Melikşah region with the lowest conductivity 310. It means that the ionization of water was lowest also in this region.

In Maliköy Uyuz Hamamı and Ayaş areas, the amount of pH was ranged between 6.2 to 7.9.

The level of ammonium and nitrate was highest NH_4^+ (12.1 mg/L) and NO_3^- (12.07 mg/L) in Seyhamamı region. While in Melikşah region had the lowest NH_4^+ (3.22 mg/L) and NO^-_3 (1.35 mg/L). In Kızılcahamam region the high level of NH_4^+ (7.58 mg/L) and the low level of NO_3^- (4.38 mg/L) with salinity (1.35 ppt) and 43.1 ^{o}C temperature were as shown in Table 4.2.

The strain list is presented below to select samples of cyanobacteria which were isolated from hot spring water resources located in Ankara where cyanobacterial biodiversity is high. Some of the different phycobiliprotein and carotenoid produced diazotrophic cyanobacteria which are already isolated from different volcanic lakes and thermal water resources of Turkey. Table 4.3 explains all the strain list used in this study (Empire: Prokaryote, Kingdom: Bacterıa, Subkingdom: Negıbacterıa, Phylum: Cyanobacterıa).

Class: Cyanophyceae, Group: Nostocales Familia: Nostocaceae / **Species: *Nostoc***	**Bey pazarı**	**Dutlu**	**Ayaş**	**Malı köy**	**Kızılca hamam**	**Sey hamamı**	**Melik şah**
Nostoc sp. IMU1						*	
Nostoc sp. IMU17	*						
Nostoc sp. IMU20					*		
Nostoc sp. IMU19			*				
Species: *Trichormus*				Malı köy			
Trichormus sp. IMU26				*			
Species: *Nodularıa*		Dutlu					
Anabaenopsis sp. IMU23		*					
Species: *Anabaena*	Bey pazarı				Kızılca hamam	Sey hama	

						mı	
Anabaena variabilis sp. IMU8						*	
Anabaena sp. IMU30					*		
Microchaete sp. IMU22	*						
Class: Cyanophyceae Group: Nostocales Familia: Chlorogloeopsidaceae Species: *Chlorogloeopsis*		Dutlu					
Chlorogloeopsis sp. IMU3		*					
Class: Cyanophyceae, Group: Nostocales Familia: Nostocaceae Species: *Nostoc*							Melik şa
Mastigocladus sp. IMU2							*
Class: Synechococcophycıdeae Group: Synechococcales Familia: Protochlorotrıacheae Species: *Nodosilinea*				Malı köy			
Nodosilinea sp. IMU5				*			

Table 4.3 List of all the strains used in the study.

4.2. Types of Media Used in the Study

Cultivation was made on liquid and solid media, which are BG11 and nitrogen deprived BG11 medium. The selected nitrogen-fixing species of cyanobacteria, which we already had at İstanbul Medeniyet University, were also used. We made

cultivation on solid plates of BG11 and nitrogen deprived BG11 media and Z8 medium to compare the growth of cyanobacterium too (Figure 4.1 and Figure 4.2). We investigated that the best medium was BG11-N for cyanobacterial growth. All species are left to grow by time, and microscopic characteristics had been made continually. Some species had been isolated from paddy fields, and the species which were selected from our culture collection used to investigate their DNA genotypically (Figure 4.1).

Figure 4.1 BG11and BG11-N agar media of cyanobacterial species.

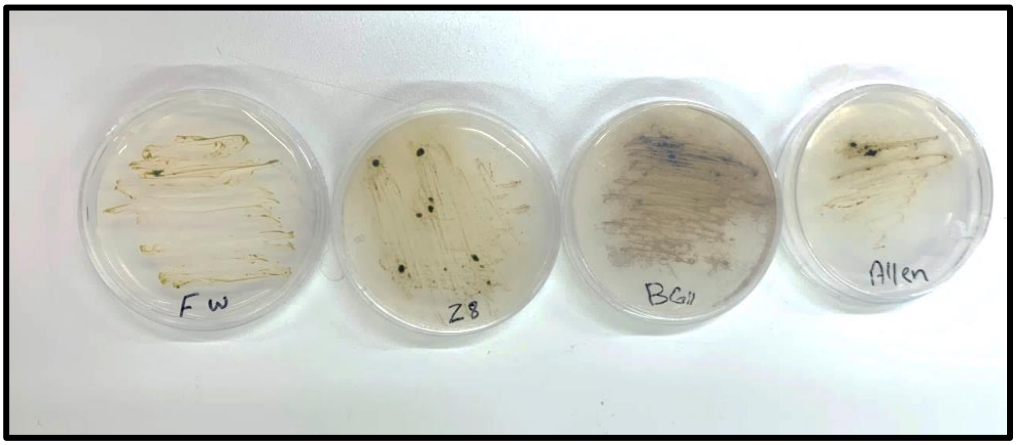


Figure 4.2 Characters of growth of cyanobacterial species on FW, Z8, BG11, and Allen media(x40).

Cyanobacteria which are isolated from hot spring water resources were cultivated in different types of media (FW, Z8, BG11, and Allen media) to compare the growth of cyanobacterium too (Figure 4.1 and Figure 4.2). We investigated that the best medium was BG11 for cyanobacterial growth. It has been confirmed that the quantity and the quality of growth that have grown in BG11-N soled better than the growth of cyanobacteria that have grown in Z8-N and Z8 and the other types of media.

4.3. Isolation, Purification, and Cultivation of Cyanobacteria

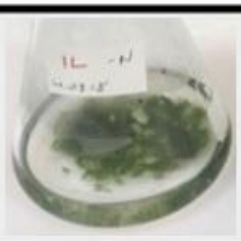
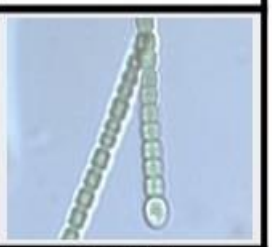
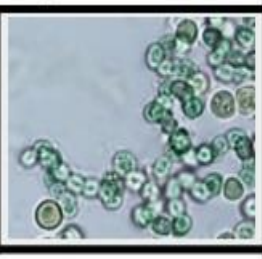
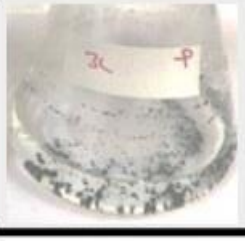
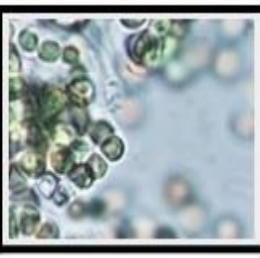
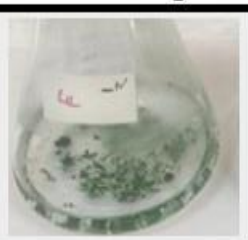
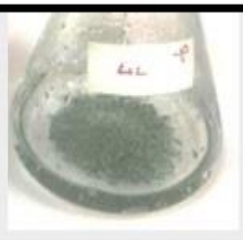
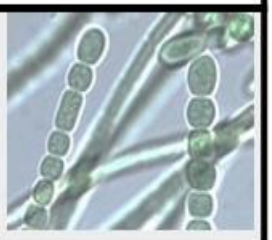
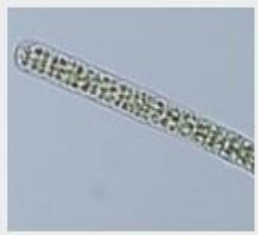
The selection of suitable algae strains was an important factor in the overall success of biofuel production from microscopic algae [82].

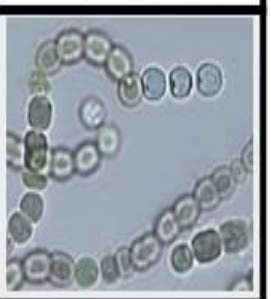
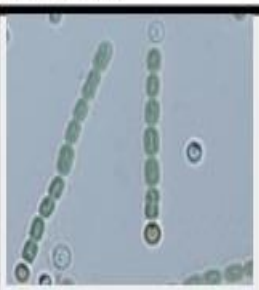
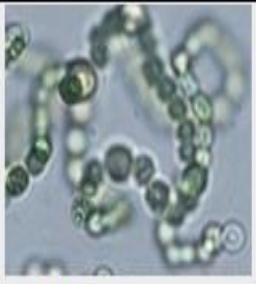
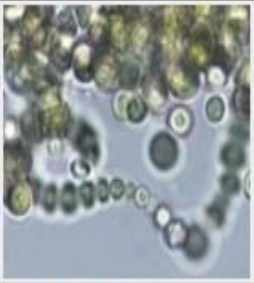
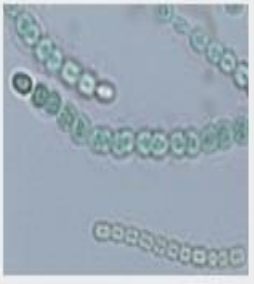
Algal species were grown in the 50 mL medium in 100 mL flasks under the continuous light intensity of 100 μE/(m^2/s) in a temperature-controlled orbital shaker with a 120 rpm speed under 25 °C temperature. BG11-N0 medium was used as control and $NaNO_3$ was not included in the growth medium for nitrogen deprivation.

Microalgae were cultivated under defined conditions for 5 days and then half of the samples centrifuged at 2000 g for 5 min and replaced in BG11-N0-P0 medium to

observe the effect of phosphorous and to maximize the carotenoid and phycobiliprotein production for 5 more days (Figure 4.3).

Light microscope imagining of the cyanobacteria which grow in BG11-N and BG11-NP mediums had been made on the 10^{th} day too (Figure 4.3), and it was found that there are no big morphologically differences between BG11-N and BG11- NP media.

Name of specius		Name of specius	
Nostoc sp. IMU1-N		*Nostoc sp.* IMU1-NP	
Culture Images	Light Microscope Imagining	Culture Images	Light Microscope Imagining
			
Chlorogloeopsis sp. IMU3-N		*Chlorogloeopsis sp.* IMU3-NP	
			
Mastigocladus sp. IMU2 -N		*Mastigocladus sp. IMU2* -NP	
			
Nodosilinea sp. IMU5-N		*Nodosilinea sp. IMU5 -NP*	
			

Name of specius		Name of specius	
Anabaena variabilis. IUM8-N		*Anabaena variabilis.* IUM8 -NP	
Culture Images	Light Microscope Imagining	Culture Images	Light Microscope Imagining
			
Nostoc sp. IMU17-N		*Nostoc sp.* IMU17-NP	
			
Nostoc sp. IMU19-N		*Nostoc sp.* IMU19-NP	
			
*Nostoc sp.*IUM20-N		*Nostoc sp.*IUM20-NP	
			

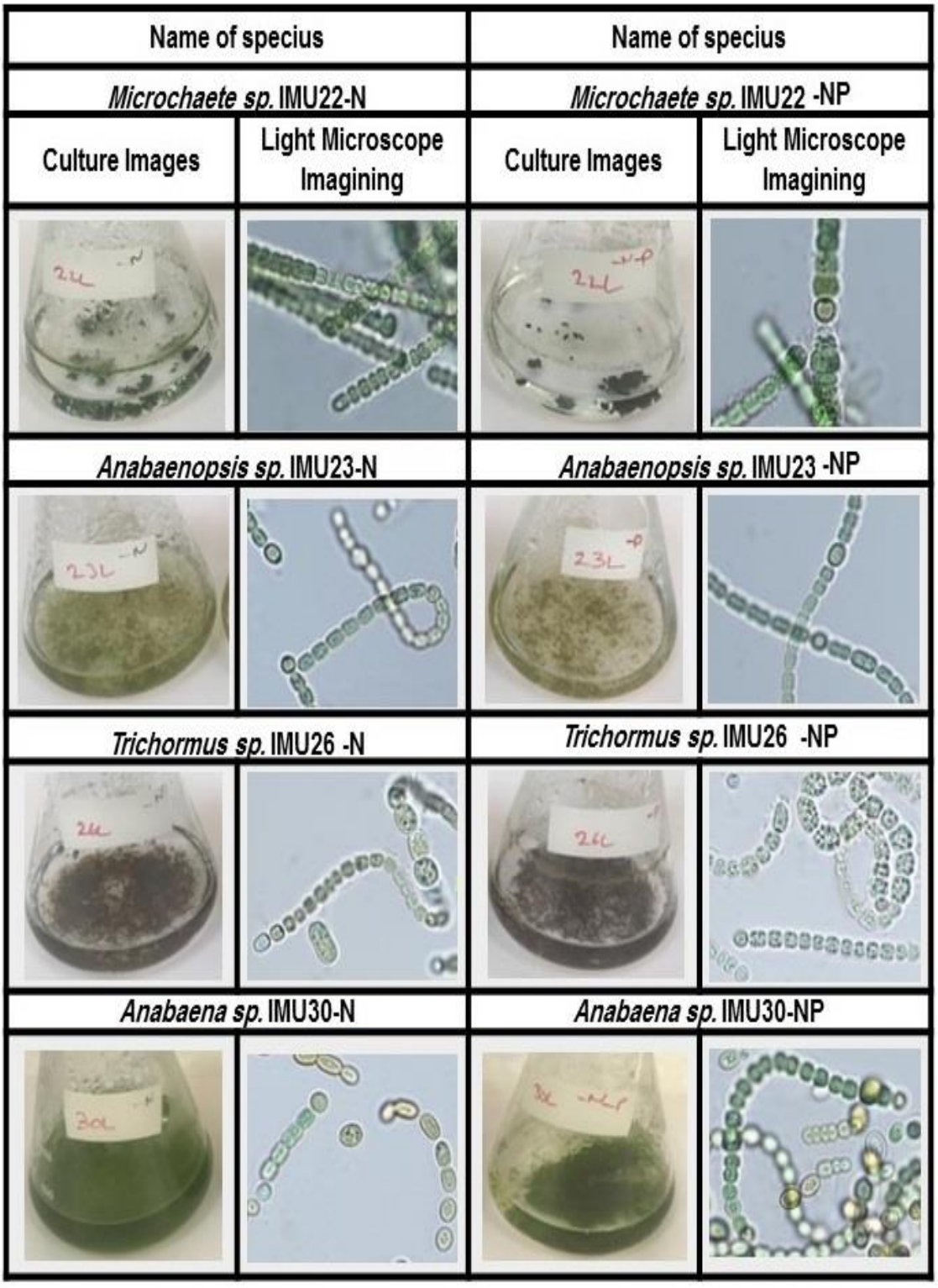


Figure 4.3 Images of cyanobacteria in BG11-N and BG11- NP mediums on the 10th day (The width of each microscope image is 50µm (x40))

4.4 Genomic Information and Genomic DNA Sequence of the Cyanobacteria Species

Diazotrophic twelve different cyanobacterial species from hot spring water were defined, selected, and extraction of their phycobiliprotein and carotenoid production were carried out. Quantitative analysis was carried out using fluorescent absorbance.

The cyanobacterial species that produce considerably high phycobiliprotein and carotenoid were exposed to several stress factors; with paying special attention to changes on growth.

The data obtained from these measurements were analyzed statistically, and probabilities for the most optimal stress factors compositions were created using a different design of experiment software. A new different set of experimental designs also run, and the most optimal stress factor composition for maximized phycobiliprotein and carotenoid production were defined. Lastly, selected cyanobacterial species were grown in photobioreactors where control and optimal stress parameters created and their production efficiencies of cells with growth kinetics and life cycle analysis were determined. The species were identified based on their microscopical and genomic information. Genomic DNA was extracted from algal species following phenol-chloroform method on a pellet obtained by centrifugation of 10 mL of algal culture at the late-log phase. DNA amplification from genomic DNA containing a partial 16S ribosomal RNA region was performed by PCR using the following primers: Forward (27F): 5' AGAGTTTGATCMTGGCTCAG3' and Reverse (809R): 5' GCTTCGGCACGGCTCGGGTCGATA3'. Same primers were used for Sanger sequencing. Sequence comparison of the 16S rRNA genes was performed using the NCBI databases with BLAS search (National Center for Biotechnology Information) and BioEdit-graphical biological sequence editor v7.0.9.

The total DNAs were extracted from all isolates in this study and were used as a template for PCR assays. The purity and concentration of genomic DNA were measured using nanodrop spectrophotometer as shown in Table 4.4.

Table 4.4 The purity and concentration of genomic DNA of cyanobacterial species

Variable		Absorbance (260/280)	Purity (ng/µl)
Cyanobacterial species	*Nostoc sp.* IMU1	1.83	12.01
	Chlorogloeopsis sp. IMU3	1.68	166.3
	Mastigocladus sp. IMU2	2.10	174.09
	Nodosilinea sp. IMU5	2.14	74.11
	Anabaena variabilis IMU8	1.98	124.42
	Nostoc sp. IMU17	2.02	175.90
	Nostoc sp. IMU19	2.09	61.31
	Nostoc sp. IMU20	1.93	15.93
	Microchaete sp. IMU22	1.98	64.64
	Anabaenopsis sp. IMU23	1.7	30.92
	Trichormus sp. IMU26	2.02	32.08
	Anabaena sp. IMU30	1.56	78.15

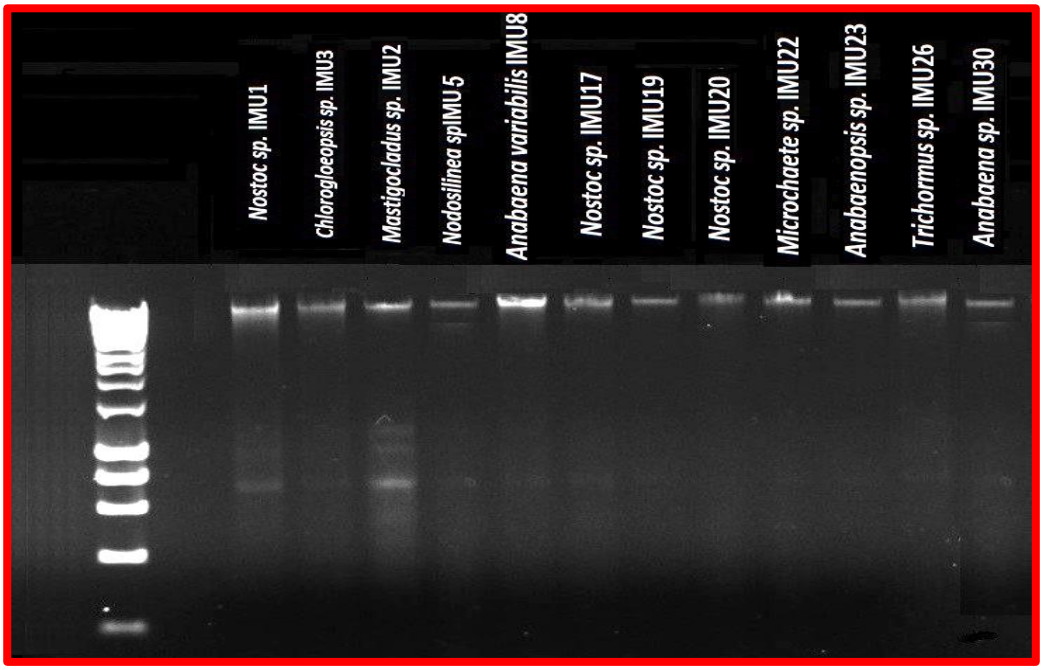


Figure 4.4 Electrophoresis of genomic DNA of the twelve different cyanobacterial species

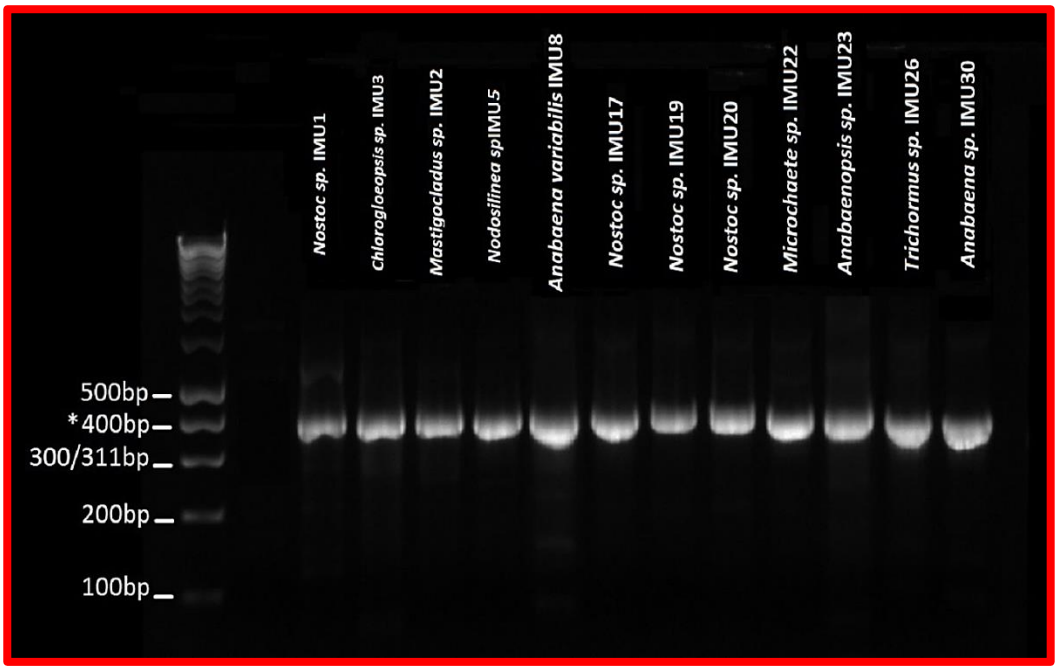
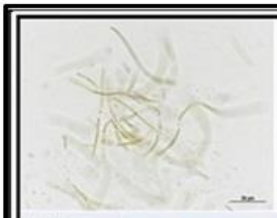


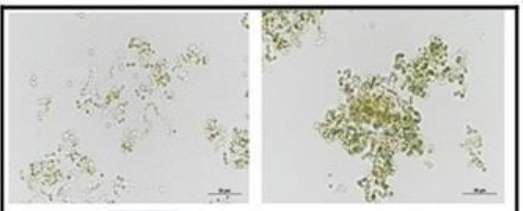
Figure 4.5 Electrophoresis of PCR products produced from genomic DNA of twelve different cyanobacterial species

4.5 Morphology and Sequence Information of Cyanobacteria Species

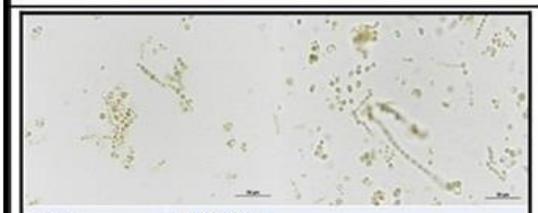
The same primers used at PCR were also used for Sanger sequencing. Twelve nucleotide sequences are analyzed on the NCBI database, and finally, BLAST results are used for identification of the strain. The samples including PCR products were sent for sequencing to Sentromer DNA Technologies in istanbul Turkey, (https://www.sentromer.com/) , after that the results were compared with the data from the gene bank program. Genomic results of diazotrophic cyanobacteria are summarized in the following charts (Figure 4.6).



Location:	**Sey hamamı**	
Sequence info:	***Nostoc sp.* IMU1**	
	AGTGGCGGACGGGTGARTAACGCGTGAGAATCTARCTTCAG GTCGGGGATAATACTGGAAACGGTGGCTAATACCGGATGTG CCGAAAGGTAAAAGGCTTGCTGCCTGAAGATGARCTCGCGT CTGATTAGCTAGTTGGTGTGGTAAGAGCGCACCAAGGCGTCS ATCAGTAGCTGGTCTGARAGGATGATCAGCCACACTGGGAC TGARACACGGCCCARACTCCTACGGGAGGCAGCAGTGGGGA ATTTTCCGCAATGGGCGAAAGCCTGACGGARCAATACCGCG TGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAAG GAATAAAAAAAATGAAGGKACTTGAGGAATAAGCATCGGCTA ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCG TTATCCGGAATGATTGGGCGTAAAGCGTCCGCAGGKGGCGA TGTAAGTCTGCTGTTAAAGAGCAAAGCTTAACTTTGTAAAAG CAGTGGAAACTACATAGCTAGAGTACGTTCGGGGCARARGG AATTCCTGGTGTAGCGGTGAAATGCGTARAGATCAGGAAGA ACACCGGTGGCGAARGCGCTCTGCTAGGCCGTAACTGACAC TGAGGGACGAAAGCTAGGGGA	
EMBL / Gen Bank No	Name of the Strain	% Homology
CP003548.1	*Nostoc sp.* PCC 7107	% 98

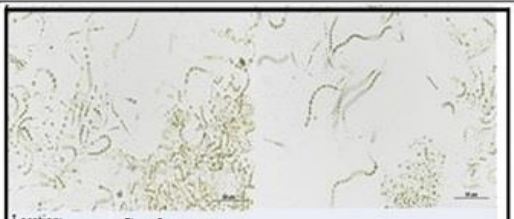


Location:	**Dutlu**	
Sequence info:	***Chlorogloeopsis sp.* IMU3**	
	TAACGSGTGAGAATCTARCTTCAGGTCSGGGACMACCACTGGA AACGGTGGCTAATACCGGATGTGCCGAAAGGTGAAAGATTTAT TGCCTGAAGATGARCTCGCGTCTGATTAGCTAGTAGGTGTGGT AAGAGCGCACCTAGGCGACGATCAGTAGCTGGTCTGARAGGAT GATCAGCCACACTGGGACTGARACACGGCCCAGACTCCTACGG GAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGA CGGARCAATACCGCGTGAGGGAGGAAGGSTCTTGGGTTGTAAA CCTCTTTTCTCAGGGAATAAAAAAATGAAGGTACCTGAGGAAT AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAR GATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCA GGTGGCAATGTAAGTCTGCTGTTAAAGAATGAGGCTCAACCTC ATACCAGCAGTGGAAACTACATAGCTAGAGTGCGTTCGGGGTA GARGGAATTYCTGGTGTAGCGGTGAAATGCGTAGATATCAGGA AGAACACCGGTGGCGAAAGCGCTCTACTAGGCCGCAACTGAC ACTGAGGGACGAAAGCTAGGGGA	
EMBL / Gen Bank No	Name of the Strain	% Homology
JN166685.1	*Chlorogloeopsis sp.* GSP606-1	%97



Location:	**Melik şah**	
Sequence info:	***Mastigocladus sp.* IMU2**	
	CGGTGCTTTCGGGCATAGTGGCGGACGGGTGAGTAACG CGTGAGAATCTAGCTTCAGGKTCGGGACAACATTGGGA AACCGATGCTAATACCGGATGTGCCGTGAGGTGAAAG GCTTGCTGCCTGAAGATGARCTCGCGTCTGATTAGCTA GTTGGTAGTGTAAGGGACTACCAAGGCGACGATCAGTA GCTGGTCTGARAGGATGATCAGCCACACTGGAACTGAR ACACGGTCCAGACTCCTACGGGAGGSAGCAGTGGGGA ATTTTCCGCAATGGGCGAAAGCCTGACGGARCAATACC GCGTGAGGGAGGAAGGSTCTTGGGTTGTAAACCTCTTT TCTCAAGGAAGAAGTTCTGACGGTACTTGAGGAATCAG CATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG ARGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGC GTCCGCAGGTGGTTGTGTGTGTCTATTGTCAAAGCATC ATGCTTAACATGATAAGGGCAGTGGAAACTACACAACT AGAGTGCGTTCGGGGCARARGGAATTCCTGGTGTAGCG GTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCG AAAGCGCTCTGCTAGGCCGTAACTGACACTGAGGGACG AAA	
EMBL / Gen Bank No	Name of the Strain	% Homology
JX316764.1	*Mastigocladus laminosus* CCAP 1447/3	%98

Location:	**Malı köy**	
Sequence info:	***Nodosilinea sp.* IMU5**	
	CTACAGGGGTATCTAATCCCTTTCGCTCCCCTAGCTTTCGTCCC TCAGCGTCAGTTGTGGCCCAGTAGAGCGCCTTCGCCACTGGTG TTCTTCCCGATATCTACGCATTTCACCGCTACACCGGGAATTCC CTCTACCCCTACCACACTCAAGTTCCCCAGTTTCCATTGSCGAT CCACAGTTGARCTGTGACCTTTGACAACAGACTTAARAAACCG CCTGSGGACGCTTTACGCCCAATAATTCCGGAWAACGCTTGSC TCCTCCGTCTTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGC TTATTCCTCTGGKACCGTCAGTTCTTCTTCCCAGARAAAARAGG TTTACAACCCTAAGGSCTTCCTCCCTCACGCGGCGTTGCTCCGT CAGGSTTGSGCCCATTGSGGAAAATTCCCCACTGCTGCCTCCCG TARGARTCTGGGCCGTGTCTCARTCCCAGTGTGGSTGAWCATC CTCTTARACCAGCTACTGATCGTCGCCTTGGTGAGCCCTTACCT CACCAACTARCTAATCAGAMGCGAGTTCATCCTCAGGSCATAA ATGTTTCACCTCTCGGCACATTGGGTATTARCCACCGTTTCCMG TGGKTGKCCCC	
EMBL / Gen Bank No	Name of the Strain	% Homology
KF246481.1	*Nodosilinea sp.* CENA51	%96



Location:	**Sey hamamı**
Sequence info:	***Anabaena variabilis* IMU8**

GGGTGARTAACGCGTGAGAATCTACATTYAGGTCGGGGACA
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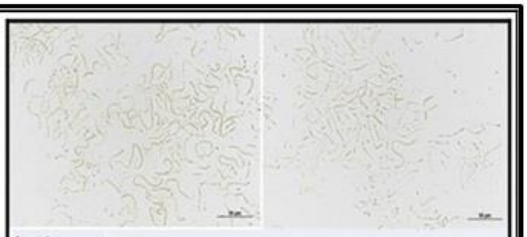
AAAAGGSTTGCCGCCTGARAATGAGCTCGCGTCTGATTAGC
TAGTTGGGGGTGTAAGARACCACCAAGGCGACGATCAGTAG
CTGGTCTGARAGGATGATCAGCCACACTGGGACTGARACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCG
CAATGGGCGAAAGCCTGACGGARCAATACCGCGTGAGGGA
GGAAGGSTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAA
AAAAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGARGATGCAAGCGTTATCC
GGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCACTGTAAG
TCTGCTGTCAAARARCAAGGSTCAACCTTGTAAAGGCAGTG
GAAACTACAGAGCTAGAGTACGTTCGGGGCAGAAGGAATT
CCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACA
CCGGTGGCGAAAGCGTTCTGCTAGGCCTGTACTGACACTGA
GGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCC

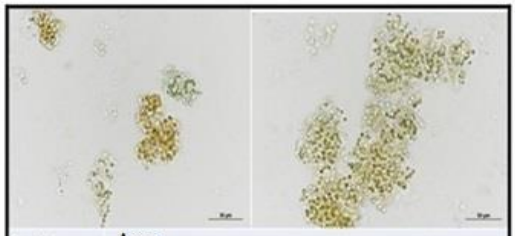
EMBL / Gen Bank No	Name of the Strain	% Homology
AY274616.1	*Anabaena variabilis* CIBNOR 23	%97



Location:	**Bey pazarı**
Sequence info:	***Nostoc sp.* IMU17**

GGGTGAGTAACGCGTGAGAATCTGGCTTCAGGTCGGGGACAAC
CACTGGAAACGGTGGCTAATACCGGATATGCCGAGAGGTGAA
AGATTAATTGCCTGAAGATGAGCTCGCGTCTGATTAGCTAGTT
GGGAGTGTAAGARACTACCAAGGSGACGATCAGTAGCTGGTCT
GARAGGATGATCAGCCACACTGGGACTGARACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGA
AAGCCTGACGGARCAATACCGCGTGAGGGAGGAAGGCTCTTG
GGTTGTAAACCTCTTTTCTCAAGGAAGAAAAAAATGACGGTAC
TTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAA
GCGTCCGCAGGTGGCTGTGAAAGTCTGCTGTTAAAGAATGAGG
CTCAACCTCATCAGARCAGTGGAAACTACACGGCTAGAGTGCG
TTCGGGGTAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAG
ATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTACTAGGCCG
CAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGAT
TAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTA

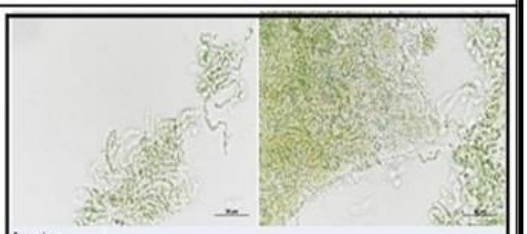
EMBL / Gen Bank No	Name of the Strain	% Homology
KM438192.1	*Nostocales* cyanobacterium NapMSlm13	99%



Location:	**Ayaş**
Sequence info:	***Nostoc sp.* IMU19**

AGTAACGCGTGAGAATCTAGCTTCAGGTCGGGGACAACCACTGG
AAACGGTGGCTAATACCGGATGTGCCGAAAGGTGAAAGATTTAT
TGCCTGAAGATGARCTCSCGTCTGATTAGCTAGTAGGTGTGGTAA
GARCGCACCTAGGCGACGATCAGTAGCTGGTCTGARAGGATGAT
CAGCCACACTGGGACTGARACACGGCCCARACTCCTACGGGAGG
CAGCAGTGGGGAATTTTCCSCAATGGGCGAAAGCCTGACGGARC
AATACCGCGTGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTT
TCTCAGGGAATAAAAAAATGAAGGTACCTGARGAATAAGCATCG
GCTAACTCCGTGSCAGCAGCCGCGGTAATACGGARGATGCAAGC
GTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCAATG
TAAGTCTGCTGTTAAAGAATGAGGCTCAACCTCATACCAGCAGT
GGAAACTACATAGCTWGAGTGCGTTCGGGGTAGAGGGAATTCCT
GGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGG
CGAAAGCGCTCTACTAGGCCGCAACTGACACTGAGGGACGAAAG
CTAGGGGAGCGAATGGGATT

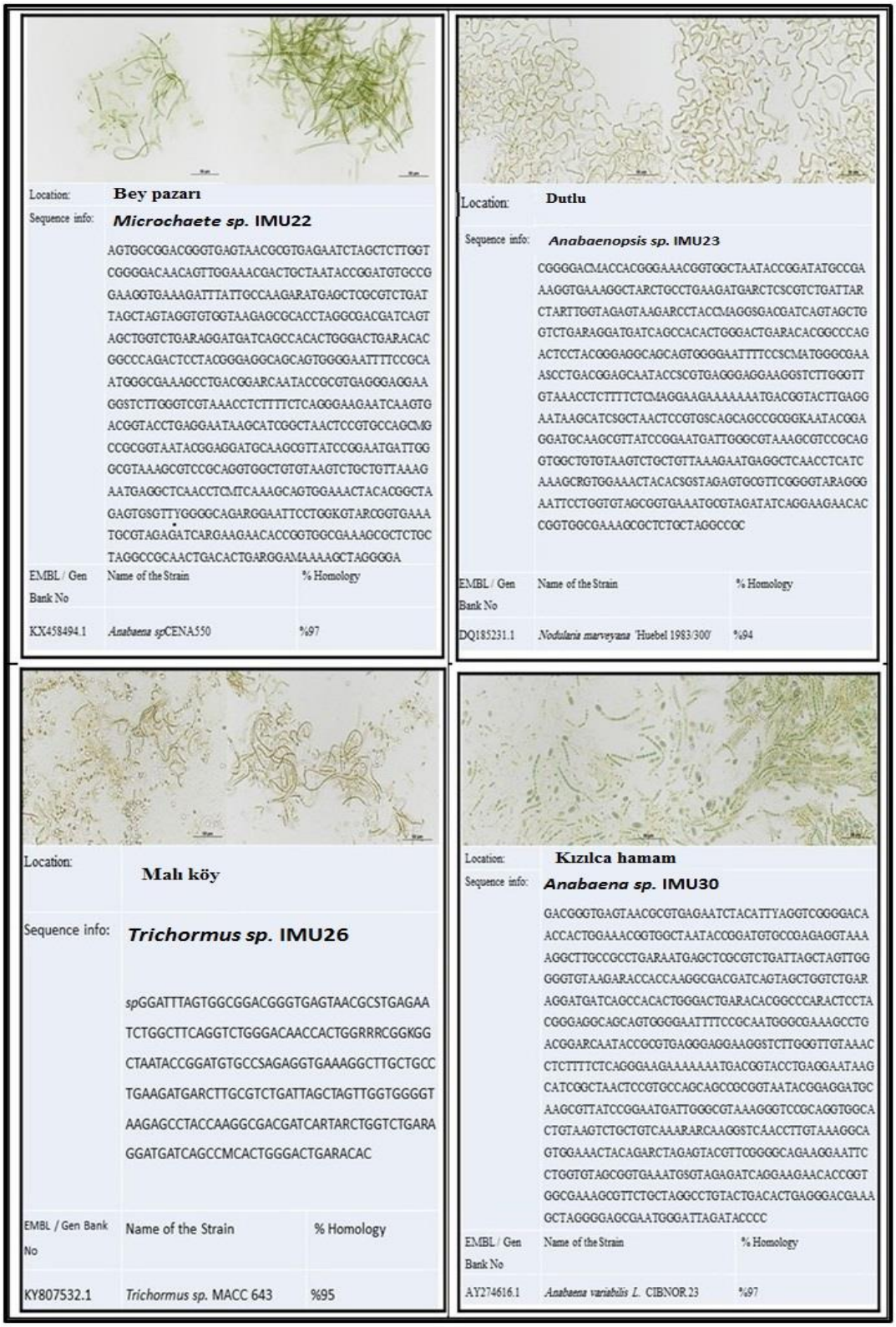
EMBL / Gen Bank No	Name of the Strain	% Homology
AP018180.1	*Nostoc carneum* NIES-2107	%97



Location:	**Kızılca hamam**
Sequence info:	***Nostoc sp.* IMU20**

ACTTAGGTACTTAGTGGCGGACGGGTGAGTAACGCGTGAGAATCT
GGCTTCAGGTCGGGGACAACCACTGGAAACGGTGGCTAATACCGG
ATGTGCCGCAAGGTAAAAGGCTTGCCGCCTGAAGATGARCTCGCG
TCTGATTAGCTAGTAGGKGGGGTAAAAGCCTACCTAGGCGACGAT
CAGTAGCTGGTCTGARAGGATGATCAGCCACACTGGGACTGARAC
ACGGCCCARACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCA
ATGGGCGAAAGCCTGACGGARCAATACCGCGTGAGGGAGGAAGG
STCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACACAATGACGG
TACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAG
CGTCCGCAGGTGGCTATGTAAGTCTGCTGTTAAAGAGTGAGGCTC
AACCTCATAAGARCAGTGGAAACTACATGGCTAGAGTGCGTTCGG
GGTAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAR
GAAGAACACCGGTGGCGAAAGCGCTCTGCTAGGCCGCAACTGAC
ACTGAGGGACGAAAGCTAGGGGAGCGAATGGGA

EMBL / Gen Bank No	Name of the Strain	% Homology
KR137578.1	*Nostoc sp.* CENA357	%99



Location:	**Bey pazarı**
Sequence info:	***Microchaete sp.* IMU22**

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CGGGGACAACAGTTGGAAACGACTGCTAATACCGGATGTGCCG
GAAGGTGAAAGATTTATTGCCAAGARATGAGCTCGCGTCTGAT
TAGCTAGTAGGTGTGGTAAGAGCGCACCTAGGCGACGATCAGT
AGCTGGTCTGARAGGATGATCAGCCACACTGGGACTGARACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCA
ATGGGCGAAAGCCTGACGGARCAATACCGCGTGAGGGAGGAA
GGSTCTTGGGTCGTAAACCTCTTTTCTCAGGGAAGAATCAAGTG
ACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCMG
CCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGG
GCGTAAAGCGTCCGCAGGTGGCTGTGTAAGTCTGCTGTTAAAG
AATGAGGCTCAACCTCMTCAAAGCAGTGGAAACTACACGGCTA
GAGTGSGTTYGGGGCAGARGGAATTCCTGGKGTARCGGTGAAA
TGCGTAGAGATCARGAAGAACACCGGTGGCGAAAGCGCTCTGC
TAGGCCGCAACTGACACTGARGGAMAAAAGCTAGGGGA

EMBL / Gen Bank No	Name of the Strain	% Homology
KX458494.1	*Anabaena sp*CENA550	%97

Location:	**Dutlu**
Sequence info:	***Anabaenopsis sp.* IMU23**

CGGGGACMACCACGGGAAACGGTGGCTAATACCGGATATGCCGA
AAGGTGAAAGGCTARCTGCCTGAAGATGARCTCSCGTCTGATTAR
CTARTTGGTAGAGTAAGARCCTACCMAGGSGACGATCAGTAGCTG
GTCTGARAGGATGATCAGCCACACTGGGACTGARACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCSCMATGGGCGAA
ASCCTGACGGAGCAATACCSCGTGAGGGAGGAAGGSTCTTGGGTT
GTAAACCTCTTTTCTCMAGGAAGAAAAAAATGACGGTACTTGAGG
AATAAGCATCSGCTAACTCCGTGSCAGCAGCCGCGGKAATACGGA
GGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTCCGCAG
GTGGCTGTGTAAGTCTGCTGTTAAAGAATGAGGCTCAACCTCATC
AAAGCRGTGGAAACTACACSGSTAGAGTGCGTTCGGGGTARAGGG
AATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACAC
CGGTGGCGAAAGCGCTCTGCTAGGCCGC

EMBL / Gen Bank No	Name of the Strain	% Homology
DQ185231.1	*Nodularia marveyana* 'Huebel 1983/300'	%94

Location:	**Malı köy**
Sequence info:	***Trichormus sp.* IMU26**

*sp*GGATTTAGTGGCGGACGGGTGAGTAACGCSTGAGAA
TCTGGCTTCAGGTCTGGGACAACCACTGGRRRCGGKGG
CTAATACCGGATGTGCCSAGAGGTGAAAGGCTTGCTGCC
TGAAGATGARCTTGCGTCTGATTAGCTAGTTGGTGGGGT
AAGAGCCTACCAAGGCGACGATCARTARCTGGTCTGARA
GGATGATCAGCCMCACTGGGACTGARACAC

EMBL / Gen Bank No	Name of the Strain	% Homology
KY807532.1	*Trichormus sp.* MACC 643	%95

Location:	**Kızılca hamam**
Sequence info:	***Anabaena sp.* IMU30**

GACGGGTGAGTAACGCGTGAGAATCTACATTYAGGTCGGGGACA
ACCACTGGAAACGGTGGCTAATACCGGATGTGCCGAGAGGTAAA
AGGCTTGCCGCCTGARAATGAGCTCGCGTCTGATTAGCTAGTTGG
GGGTGTAAGARACCACCAAGGCGACGATCAGTAGCTGGTCTGAR
AGGATGATCAGCCACACTGGGACTGARACACGGCCCARACTCCTA
CGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTG
ACGGARCAATACCGCGTGAGGGAGGAAGGSTCTTGGGTTGTAAAC
CTCTTTTCTCAGGGAAGAAAAAAATGACGGTACCTGAGGAATAAG
CATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGC
AAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCA
CTGTAAGTCTGCTGTCAAARARCAAGGSTCAACCTTGTAAAGGCA
GTGGAAACTACAGARCTAGAGTACGTTCGGGGCAGAAGGAATTC
CTGGTGTAGCGGTGAAATGSGTAGAGATCAGGAAGAACACCGGT
GGCGAAAGCGTTCTGCTAGGCCTGTACTGACACTGAGGGACGAAA
GCTAGGGGAGCGAATGGGATTAGATACCCC

EMBL / Gen Bank No	Name of the Strain	% Homology
AY274616.1	*Anabaena variabilis L.* CIBNOR 23	%97

Figure 4.6 Sequence information of cyanobacteria species

4.6 Extraction and Estimation of Phycobiliproteins of Twelve Different Cyanobacteria Species

Phycobiliproteins are water-soluble proteins found in cyanobacteria and some eukaryotic algae (Figure 4.7), (Rhoids, Cryptomonad, Glucosetopate) that capture photovoltaic energy, which then moves to chlorophyll during photosynthesis. Phycobiliproteins are mainly based on their absorption. There are three types of red phycobiliprotein pigmentations, two of which are red algae (R & B-phycoerythrin) and blue-green algae (C-phycoerythrin).

There are also three types of blue Phycocyanin pigmentation, one of them is characterized by red algae (R-phycocyanin), while the other two distinguish blue-green algae (C-phycoerythrin & allophycocyanin).

Each algal group is characterized by future pigments such as Green algae are used as high-end plants, chlorophyll a and b. Structure, and gold algae are used as chlorophyll a and c with fucoxanthin. Blue-green and red algae are used as vicobelium compounds.

Phycobiliproteins and carotenoids are the pigment-protein complexes and have a number of health benefits when consumed. This research aims to define most optimal stress parameters in which phycobiliprotein and carotenoid production are maximized while a decrease in cell growth is considerably low for phycobiliprotein and carotenoid production by diazotrophic cyanobacteria [27].

The phycobiliproteins B-phycoerythrin (B-PE), R-phycoerythrin (R-PE) and allophycocyanin (APC) are among the preferred pigmentation for applications that require either high sensitivity or simultaneous multicolor detection.

Figure 4.7 Extraction and estimation of phycobiliproteins.

4.6.1 Phycocyanin

Phycocyanin content of cyanobacteria was analyzed (Figure 4.8). The strains of *Nostoc sp.* IMU1, *A.variabilis L.* IMU8, and *Trichormus sp.* IMU26 showed high phycocyanins content when compared to other strains under these studies. Phosphorus deprivation was induced production of phycocyanins in *Nostoc sp.* IMU1. On the other hand, *Nostoc sp.* IMU1 showed normal levels of phycocyanins on the 5th day of growth while it gave a rapid increase in phycocyanins content.

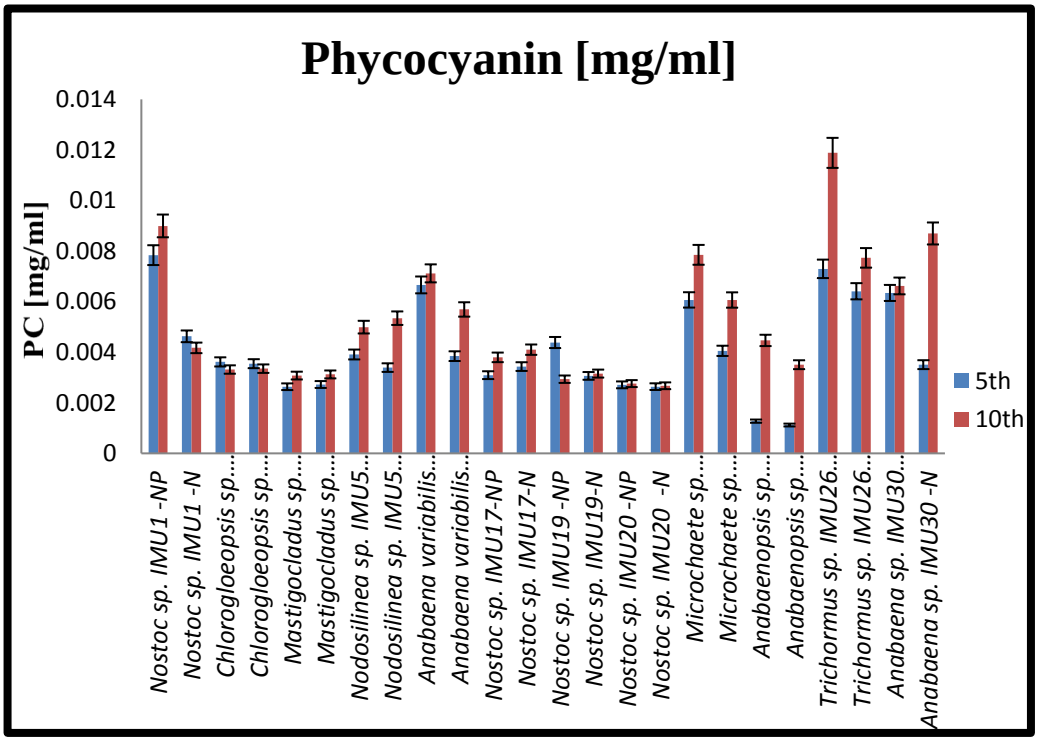


Figure 4.8 Production of phycocyanin from twelve different species of cyanobacteria in BG11 -N and -NP deprivation.

Impact of +N, -N and -NP deprivation on phycocyanin production of selected three cyanobacteria was shown in (Figure 4.9), -N deprivation was the controlled unit in this experiment. The specific data showed the phycocyanin content of *Nostoc sp.* IMU1+N was increased (12%) at the day 5th day of the incubation, while the value of *Nostoc sp. IMU1*+N was decreased on the 10th day of the incubation (73%). However, the *Nostoc sp.* IMU1-NP was increased (23.6%) at the 5th day, but the value decreased to (74%) at the 10th of the incubation. The *Anabaena variabilis*. IMU8 +N was decreased (18.6 %) at the 5th day, while this value had another decrease up to (32.4%) at the 10th day of the incubation. However, *Anabaena variabilis.* IMU8 -NP was decreased to (59%) at the 10th day of the incubation. *Trichormus sp.* IMU26 +N was also decreased around (7.3%) at the 5th and the 10th day of the incubation. While the *Trichormus sp.* IMU26 -NP was decreased (11%), (52%) at the day 5th, the 10th day of the incubation respectively.

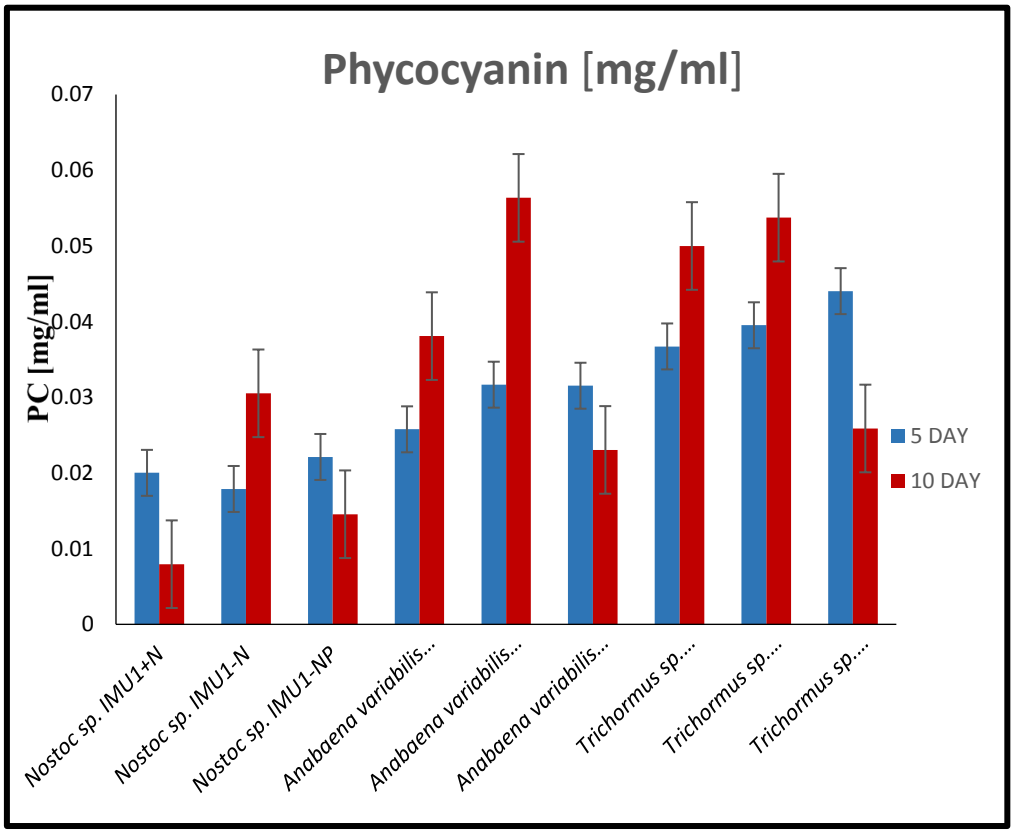


Figure 4.9 Impact of +N -N and -NP deprivation on phycocyanin production of selected three cyanobacteria.

4.6.2 Allophycocyanin

Allophycocyanins content of cyanobacteria was analyzed (Figure 4.10). The strains *Nostoc sp*. IMU1, *A.variabilis L.* IMU8, *Trichormus sp.* IMU26, showed high allophycocyanins content when compared to other strains under these studies. Phosphorus deprivation induced production of allophycocyanins in *Nostoc sp.* IMU1. On the other hand, *Nostoc sp.* IMU1 gave a rapid increase in phycocyanins content on 10th days of growth at -P deprivation.

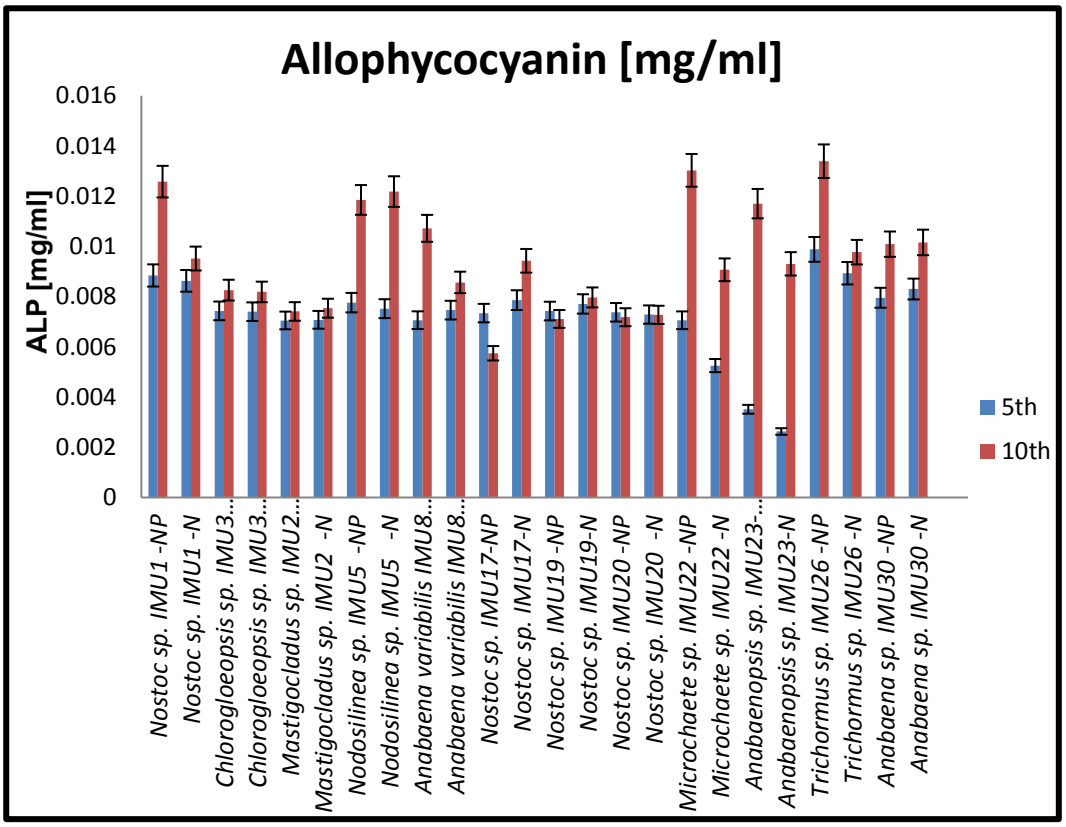


Figure 4.10 Production of allophycocyanin from twelve different species of cyanobacteria in BG11 -N and -NP deprivation.

Impact of +N, -N and -NP deprivation on allophycocyanin production of selected cyanobacteria was shown in (Figure 4.11), -N deprivation was the controlled unit in this experiment. The specific data showed the Allophycocyanin content of *Nostoc sp.* IMU1+N was increased (7%) at the day 5th day of growing, while the value of *Nostoc sp*. IMU1+N was increased to 61% at the 10th day of growing. However, the *Nostoc*

sp. IMU1-NP was increased to 15% at the 5th day, but it was decreased to 43% at the 10th day of growing. The *Anabaena variabilis.* IMU8+N was decreased (22.1 %) at the 5th day, while this value was changed to (31 %) at the 10th day of growing. However, the *Anabaena variabilis.* IMU8-NP was decreased to 59% at the 10th day of growing. The *Trichormus sp.* IMU26 +N was also (13%), (8%) at the day 5th and the 10th day of growing respectively. But The *Trichormus sp.* IMU26 -NP was increased (7%) at the 5th day of growing, and was decreased to (53%) at the 10th day of growing.

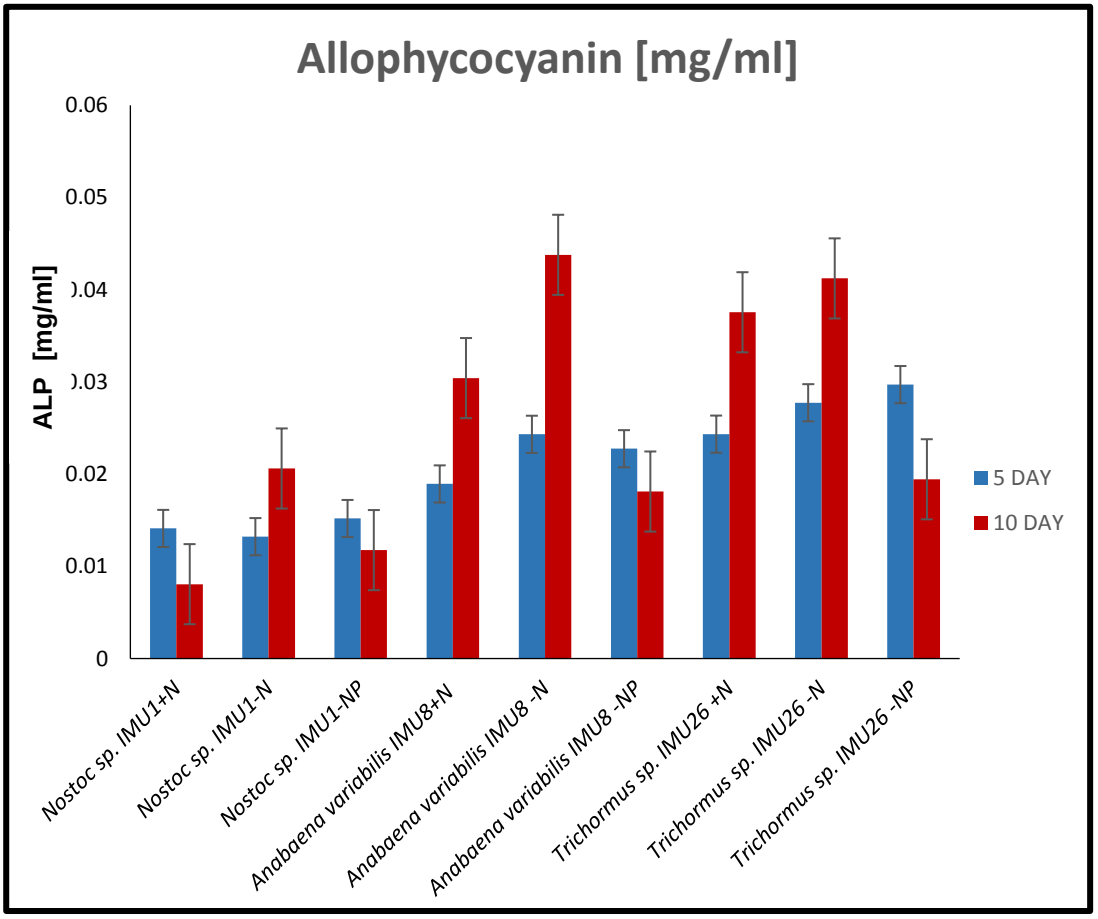


Figure 4.11 Impact of +N, -N and -NP deprivation on allophycocyanin production of three selected cyanobacteria.

4.6.3 Phycoerythrin

Phycoerythrins content of cyanobacteria was analyzed (Figure 4.12). The strains *A.variabilis L.* IMU8 and *Trichormus sp.* IMU26 showed high phycoerythrins content when compared to other strains under these studies. Phosphorus deprivation induced production of PE in *Nostoc sp.* IMU1.

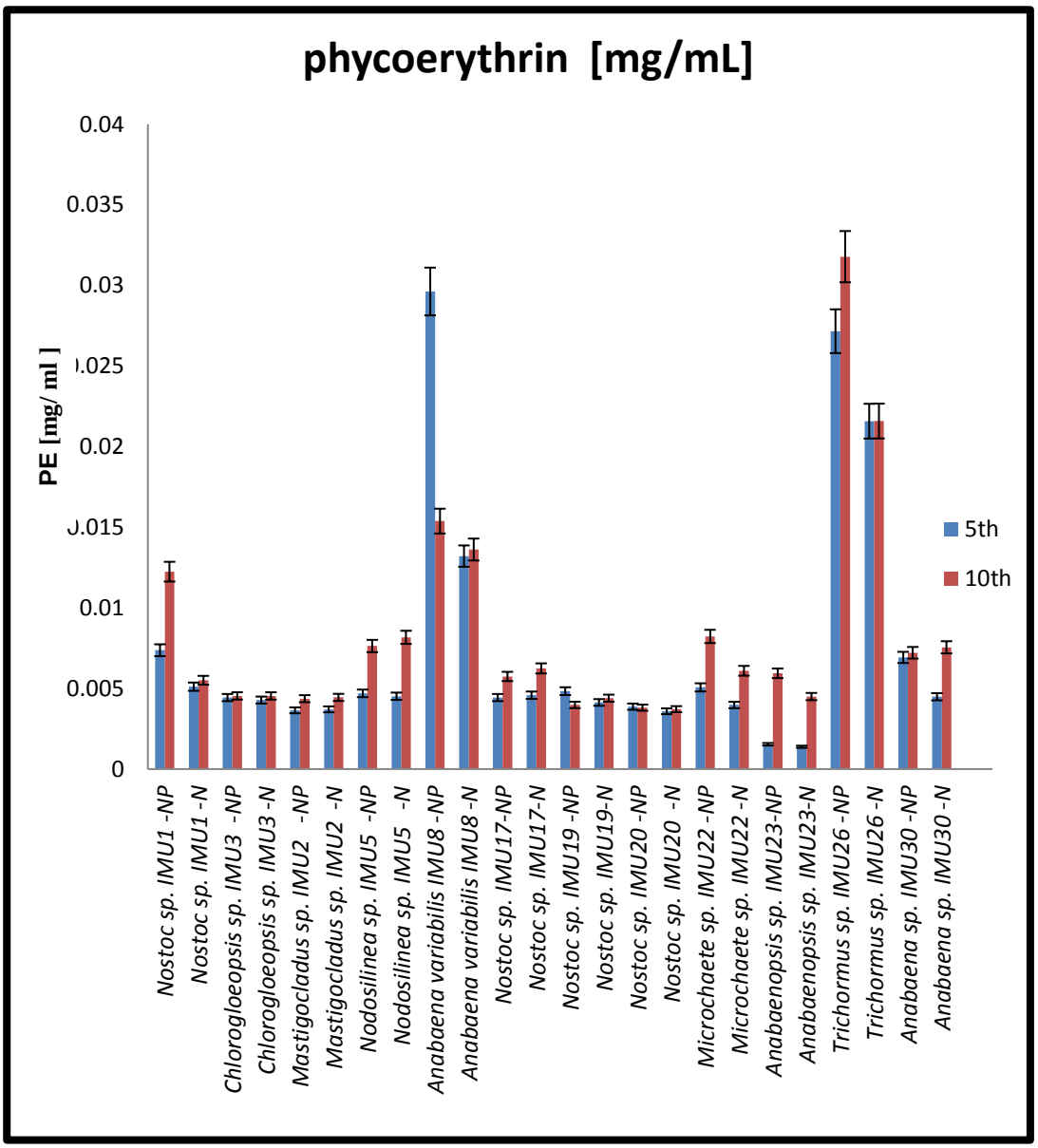


Figure 4.12 Production of phycoerythrin from different species of twelve cyanobacteria in BG11 -N and -NP deprivation.

Impact of +N, -N and -NP deprivation on phycoerythrin production of selected cyanobacteria was shown in (Figure 4.13). The specific data showed the phycoerythrin content of *Nostoc sp.* IMU1+N was increased (12%) at the 5th day of growing, while the value of *Nostoc sp.* IMU1+N was decreased to the (22%) at the 10th day of growing. However, the *Nostoc sp.* IMU1-NP was increased (20%) at the 5th day, but it was decreased to (38%) at the 10th day of growing. The *Anabaena variabilis.* IMU8+N was decreased (21%) at the day 5th day, while this value changed to (38 %) at the 10th day of growing. However, the *Anabaena variabilis.* IMU8-NP was decreased to (68%) at the 10th day of growing. The *Trichormus sp.* IMU26 +N was also (8%), (14%) at the 5th and the 10th day of growing respectively. But the *Trichormus sp.* IMU26 -NP was increased (8%) at the 5th day of growing and was decreased to (57%) at the 10th day of growing.

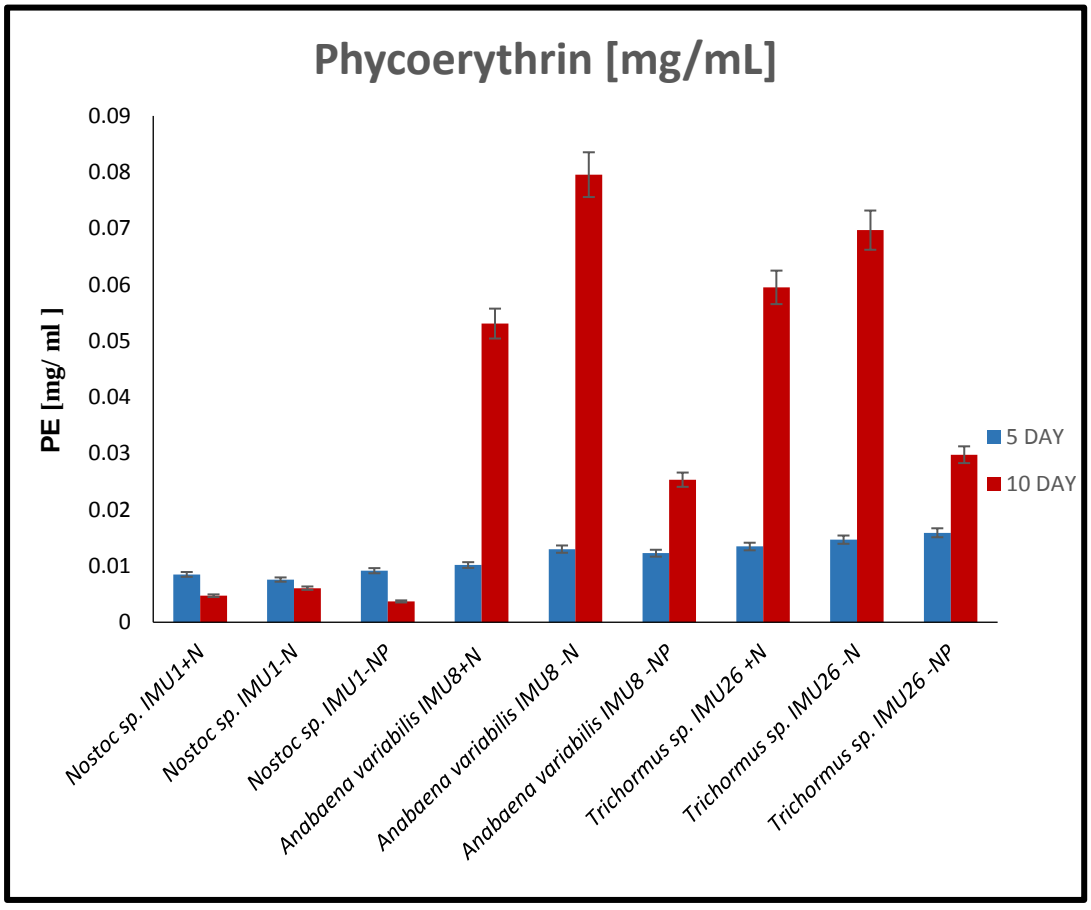
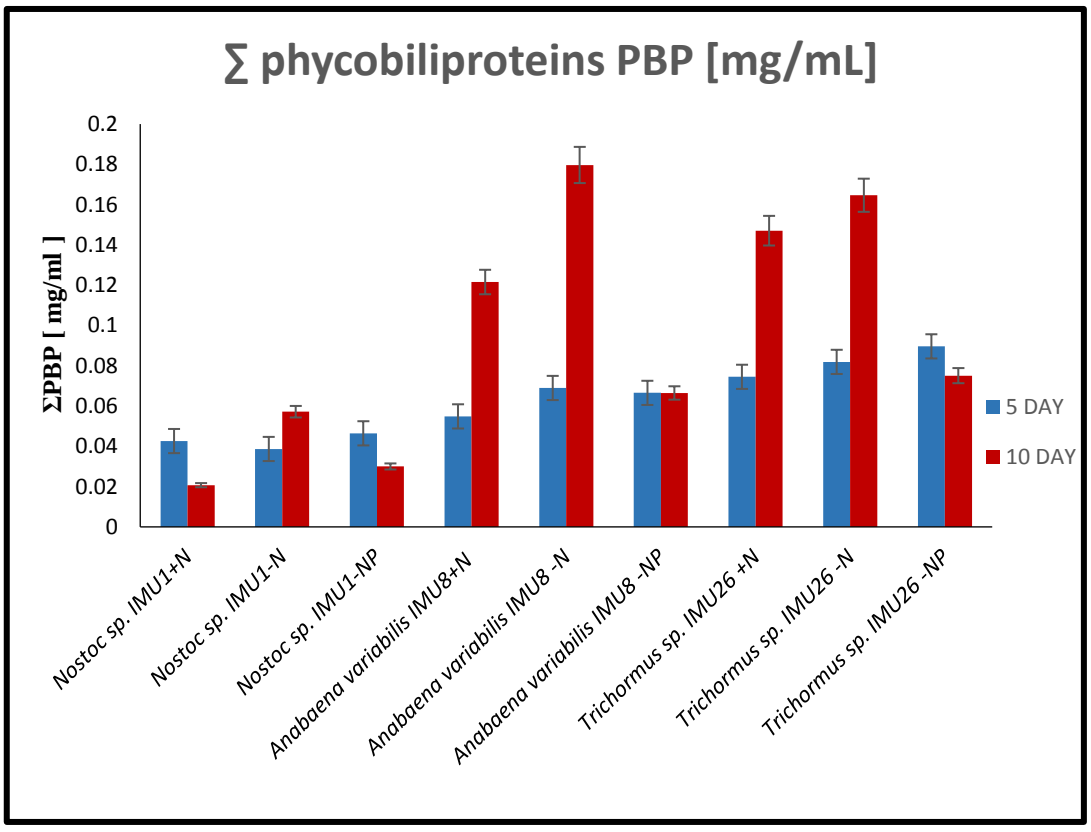


Figure 4.13 Impact of +N, -N and -NP deprivation on phycoerythrin production of three selected cyanobacteria.

4.6.4 Total Phycobiliproteins

Production of total phycobiliproteins from the selected three species of cyanobacteria was shown in (Figure 4.14), -N deprivation was the controlled unit in this experiment. The specific data showed the phycobiliproteins content of *Nostoc sp.* IMU1+N was increased to 10% at the 5th day of growing, while the value of *Nostoc sp.* IMU1+N was decreased to 63% at the 10th day of growing. However, the *Nostoc sp.* IMU1-NP was decreased to 16% at the day 5th day, but it was increased to 90% at the 10th day of growing. The *Anabaena variabilis.* IMU8+N was decreased to 20% in the 5th day, while this value changed to 32 % at the 10th day of growing. However, the *Anabaena variabilis.* IMU8-NP was decreased to 63% in the 10th day of growing. The *Trichormus sp.* IMU26 +N was also 9%, 11% at the 5th day and the 10th day of growing respectively. But The *Trichormus sp.* IMU26 -NP was increased to 9% at the 5th day of growing and was decreased to 54% at the



10th day of growing.

Figure 4.14 Production of total phycobiliproteins from the selected three species of cyanobacteria

4.7 Chlorophyll a and Total Carotenoid Contents

Chlorophyll a is identified as the main dye that is transformed photovoltaic energy to chemical energy. Chlorophyll b as adjunct pigments act indirectly in photosynthesis by transfer. The light is absorbed by A3-chlorophyll. The chlorophyll molecule Mg^{2+} in its center makes it ionic, granular, and non-hydrophobic ring in nature with a carbonic range in the tail makes it polar. It is held in place in the plant cell within a water-soluble chlorophyll (WSCP) protein. Chlorophyll b differs from chlorophyll a only in a single function in which, a porphyrin ring surrounds the group (sense - CHO), and also chlorophyll b is the more soluble type of chlorophyll in polar solvents than chlorophyll a due to carbonyl group no. 18 [41]. Chlorophyll a content of cyanobacteria was analyzed (Fig. 4.15). The strains *Nostoc sp. IMU 1*,

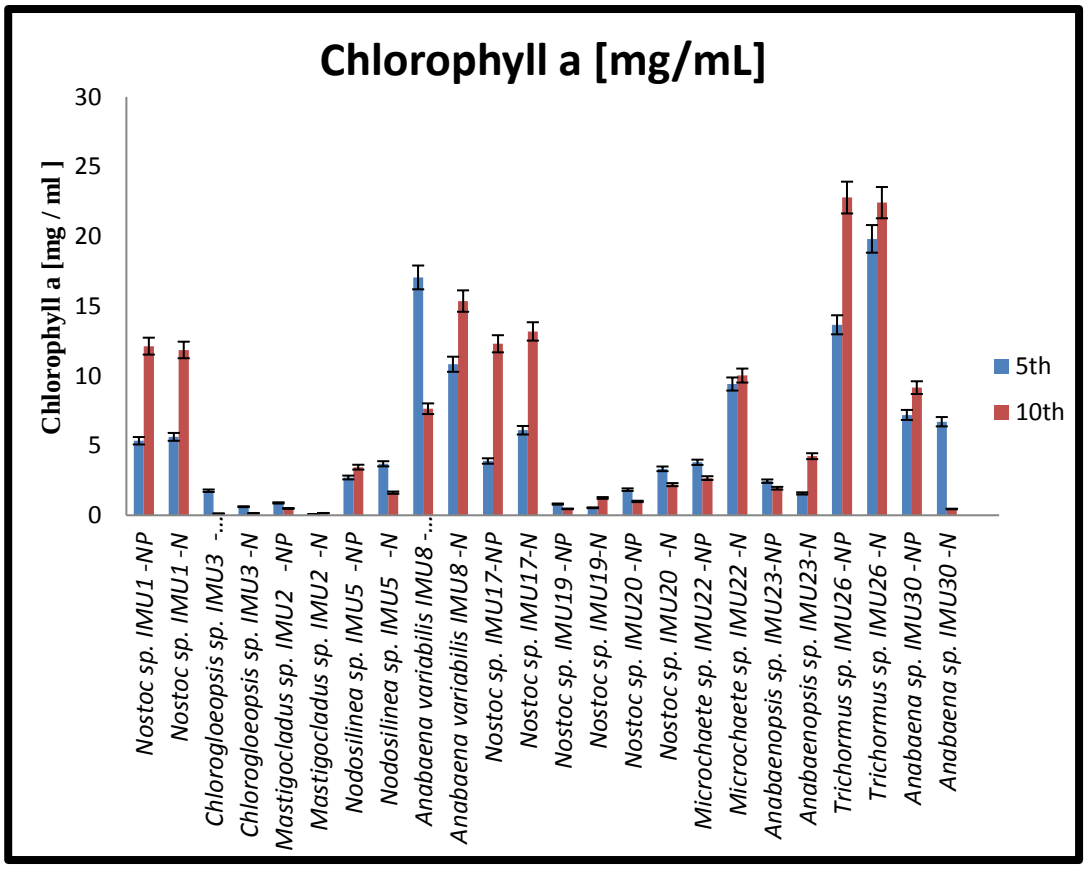


Figure 4.15 Production of chlorophyll a from twelve different types of cyanobacteria species by using BG11-N, BG11-NP media.

A.variabilis L. IMU8 and *Nostoc sp.* IMU1 showed high chlorophyll a content when compared to other strains studies. Phosphorus deprivation induced production of chlorophyll a in *Nostoc sp.* IMU1. On the other hand, *A.variabilis L.* IMU8 showed a rapid increase in chlorophyll a, content at the 5th day of -PN deprivation while it retunes back to the normal levels in the 10th day of growth at -PN deprivation.

The total carotenoid content of cyanobacteria was analyzed (Fig.4.16). The strains *Nostoc sp.* IMU1, *A.variabilis L.* IMU8 showed high total carotenoid content when compared with other strains studies. Phosphorus deprivation induced production of total carotenoid in *Nostoc sp.* IMU1. On the other hand, *A.variabilis L.* IMU8 showed a rapid increase in total carotenoid content on 5th days of -NP deprivation while it returns to the normal levels on the 10th day of -NP deprivation.

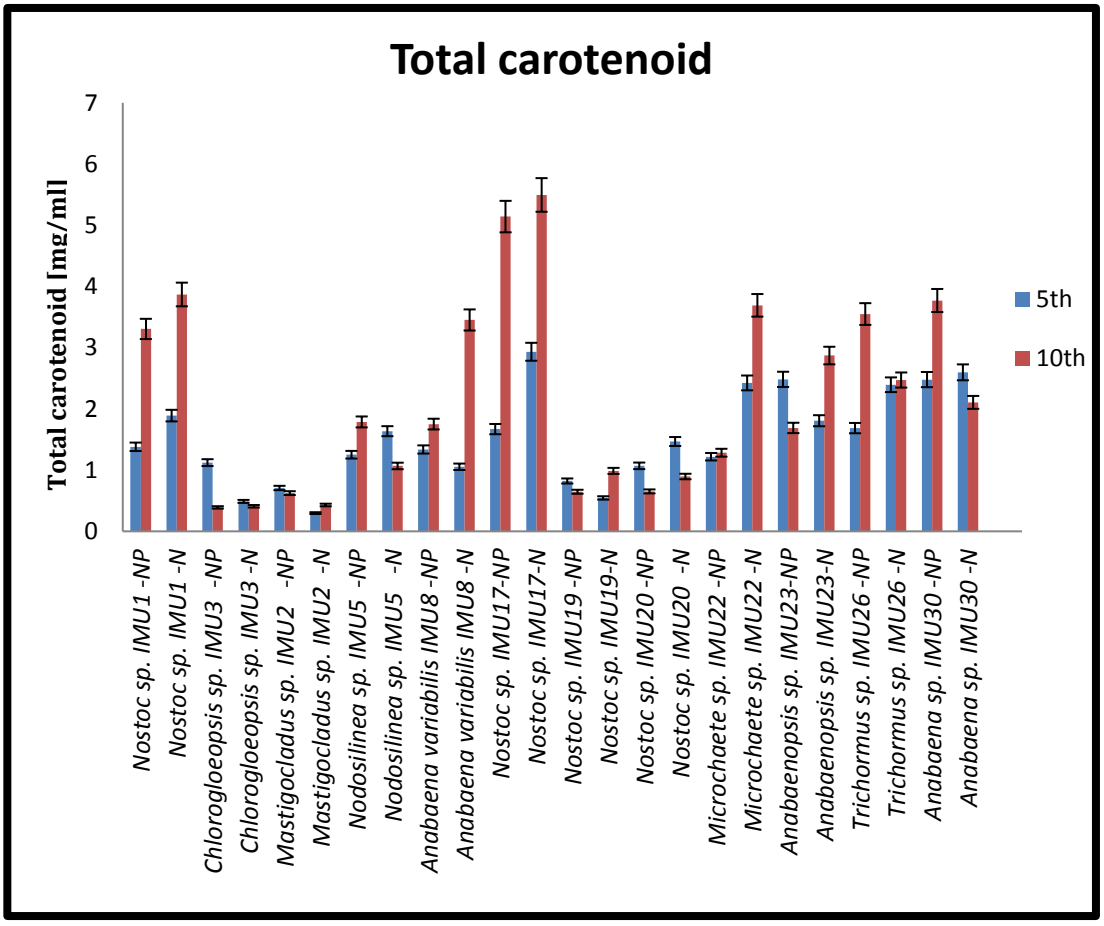
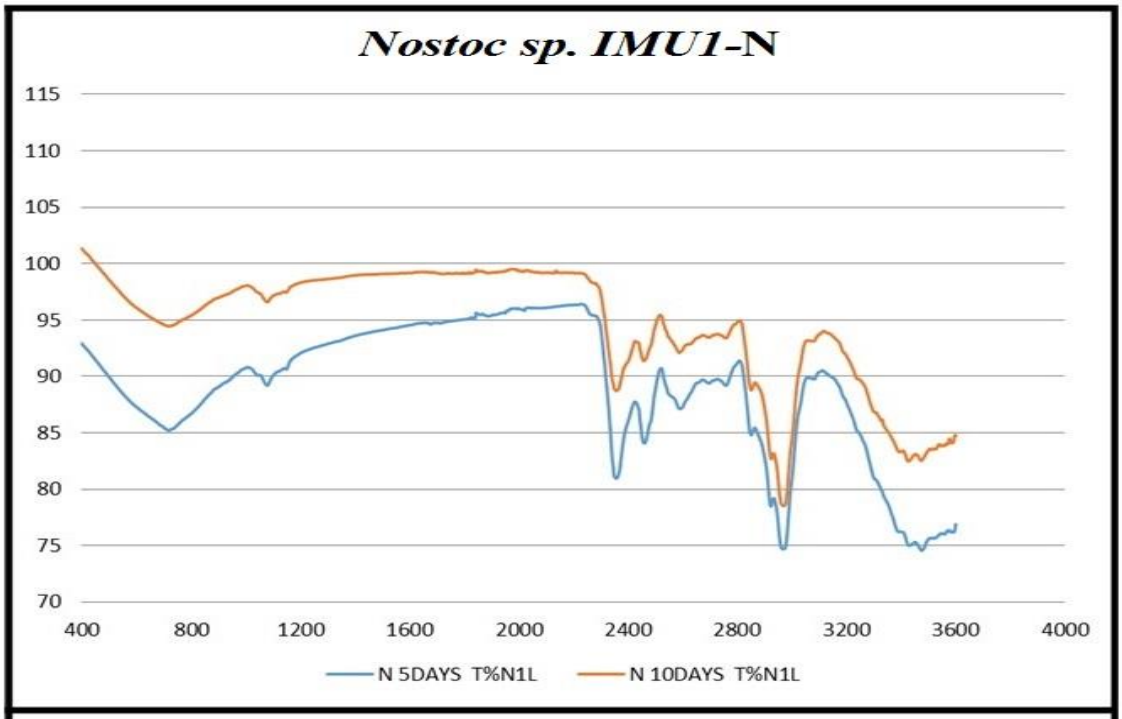


Figure 4.16 Measurment of total carotenoid from twelve different species of cyanobacteria.

4.8 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The results obtained by the FT-IR data were validated using a different analytical approach. A single point resonance FT-IR was performed through the three different profiles of the cyanobacterial layer from different positions (Fig.4.17, 4.18 and 4.19).

The results were obtained by the FT-IR of +N, -N and -NP deprivation on the production of were compared to every species of cyanobacteria alone. All analyses were obtained from identical cell aggregates at a specific depth. The 886 nm excitation shows no enhancement of the red-shifted band closer to the surface position but instead possesses a more enhanced red-shifted band at lower positions. The uppermost two spectra in possess a weaker resonance FT-IR of carotenoids superimposed on a stronger fluorescence background; FT-IR dominates the spectral record when using 886 nm excitation, as shown in baseline-corrected spectra. Figures (Fig.4.17, 4.18 and 4.19) mentioned about FTIR analysis of three different types of cyanobacteria. This figures explained the difference between FTIR measurements of the selected three species of cyanobacteria *Nostoc sp.* IMU1, *A.variabilis L.* IMU8*, Trichormus sp.* IMU26.


Nostoc sp. IMU1-N
115
110
105
100
95
90
85
80
75
70
400
800
1200
1600
2000
2400
2800
3200
3600
4000
N 5DAYS T%N1L
N 10DAYS T%N1L

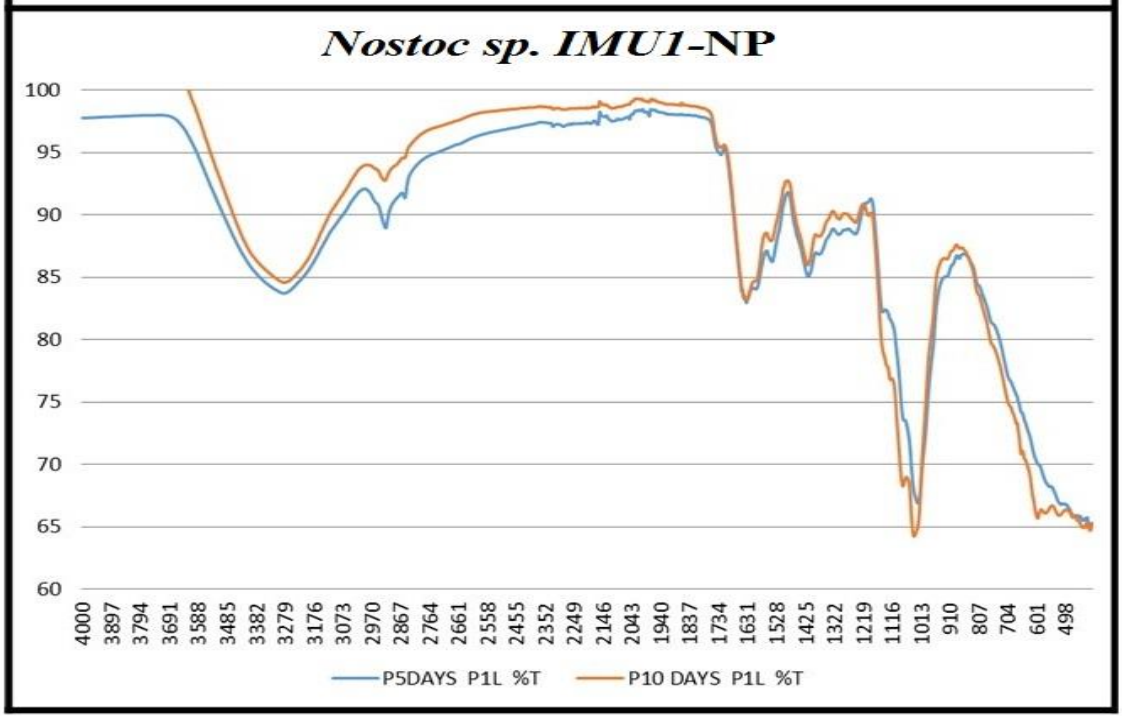

Nostoc sp. IMU1-NP
100
95
90
85
80
75
70
65
60
4000
3897
3794
3691
3588
3485
3382
3279
3176
3073
2970
2867
2764
2661
2558
2455
2352
2249
2146
2043
1940
1837
1734
1631
1528
1425
1322
1219
1116
1013
910
807
704
601
498
P5DAYS P1L %T
P10 DAYS P1L %T

Figure 4.17 FTIR content of *Nostoc sp.* IMU1 in BG11-N as controlled media, BG11+N, and BG11-NP media in the 5th day and the 10th day.

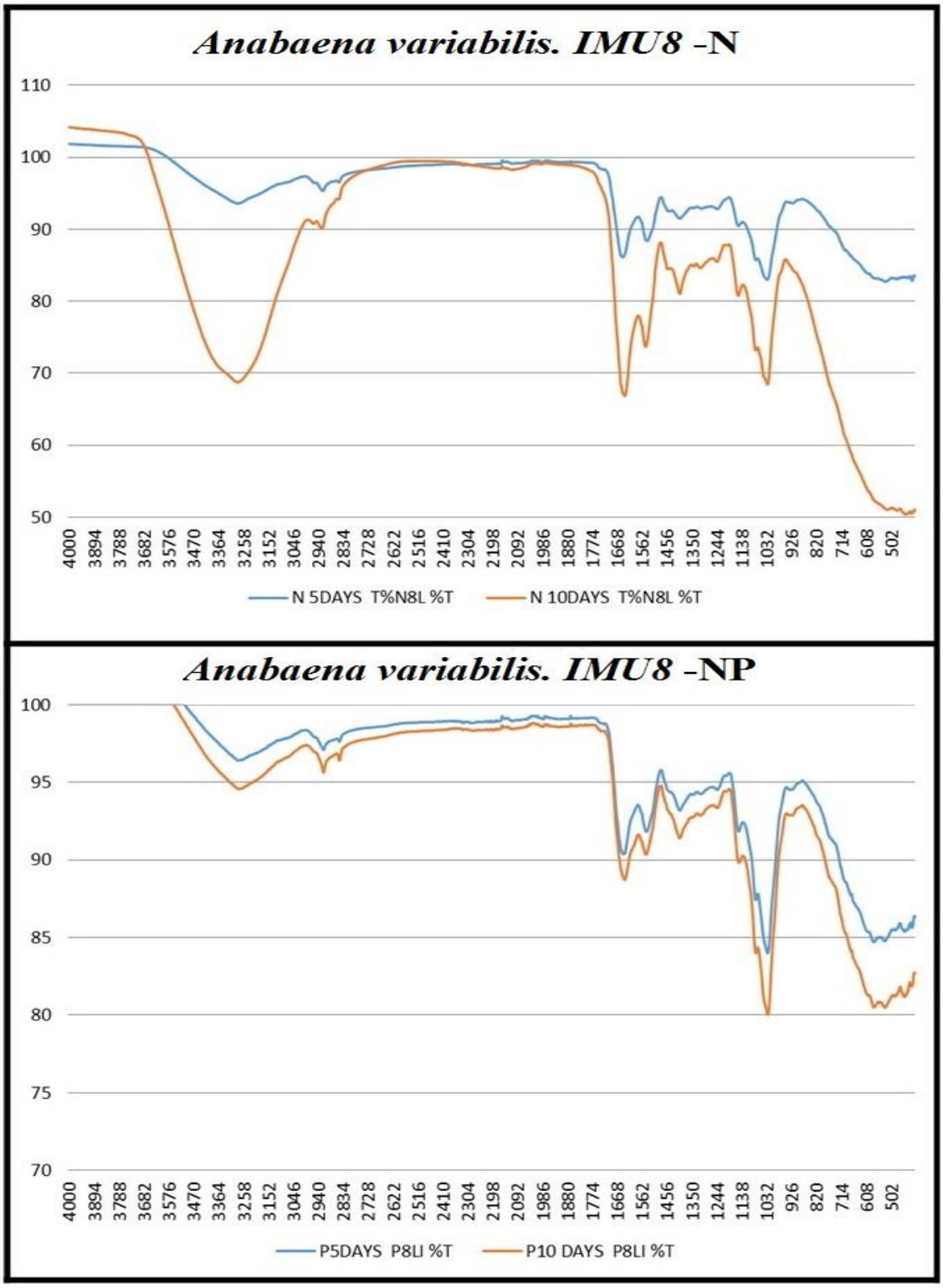


Figure 4.18 FTIR content of *Anabaena variabilis*. IMU8 in BG11-N as controlled media, BG11+N, and BG11-NP media in the 5th day and the 10th day.

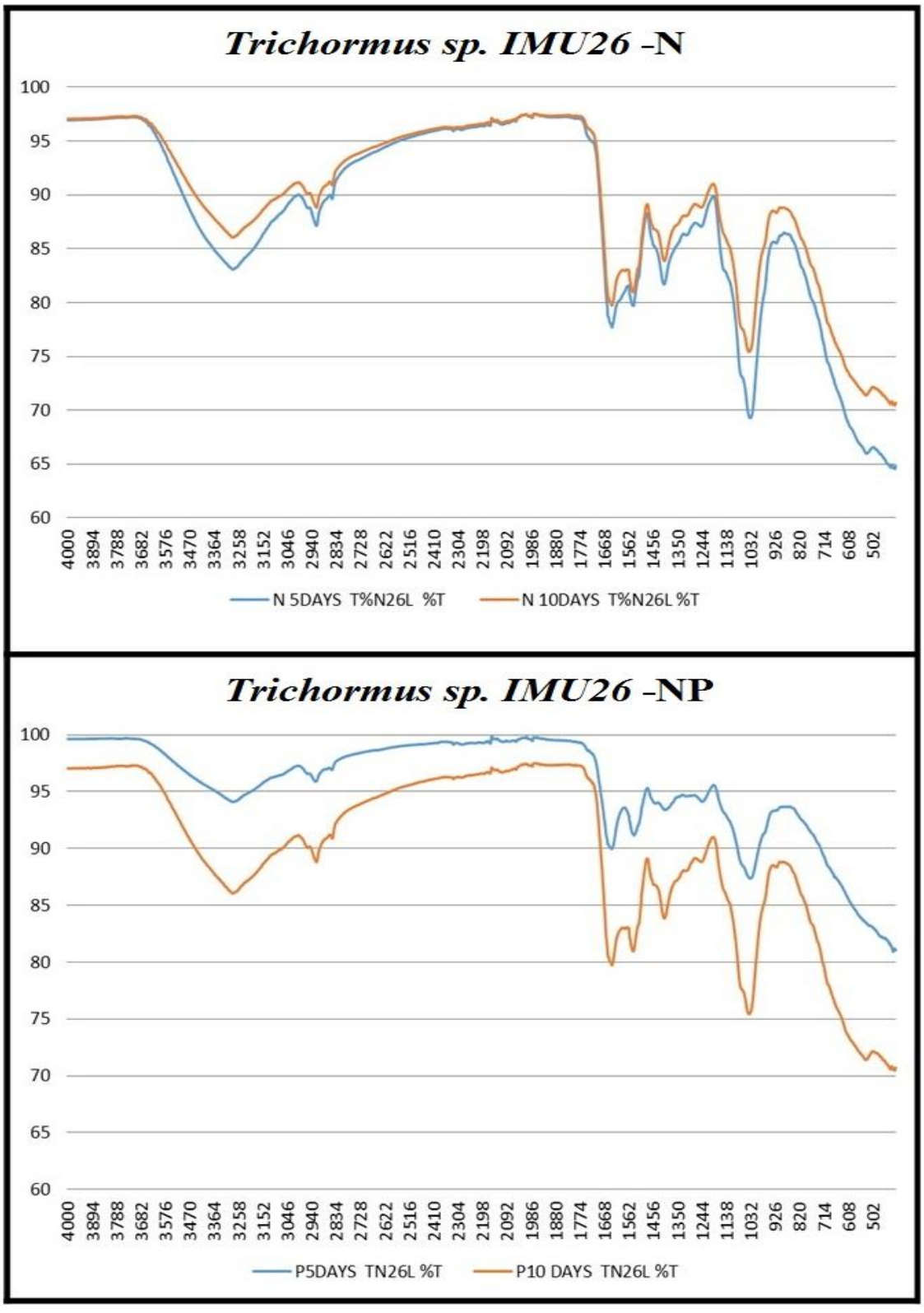


Figure 4.19 FTIR content of *Trichormus sp.* IMU26 in BG11-N as controlled media, BG11+N, and BG11-NP media in the 5th day and the 10th day.

A strong correlation of the (C=C) carotenoid band position with depth was observed in all profiles, thus confirming the results described above. A strong signal due to phycobiliproteins in the 1560–1660 cm−1 range was detected at 785 nm excitation, arising from stretching vibrations of C=C in methane bridges and pyrrole rings, with some contribution from C=N stretchings. Medium to weak bands of phycobiliproteins were detected at 1333, 1287, 1242, 1241, 1149, 1113, 1064, 975, and 619 cm−1. Depth-dependent variations of the relative signal intensity of phycobiliproteins were registered by point FT-IR measurements and confirmed by FT-IR imaging (unpublished data). The FT-IR of the phycobiliproteins is enhanced at deeper positions from the rock surface. The red excitation also provides corroborative chlorophyll features at 1556, 1517, 1329, 1189, 1, 918, 886 cm−1, as well as other bands of weaker intensity. The chlorophyll signal intensity relative to overall carotenoid signal remains at comparable levels at different depths.

4.9 Growth Content

Filamentous of cyanobacteria, except for Oscillatoria sp., regularly would produce heterocysts in the absence of combined nitrogen (NO_2^-, NO_3^- and NH_4^+) in the BG11-N medium [37]. Chlorophyll a was used to calculation the measurement constants of the growth rate of the three species of cyanobacteria bacteria that selected.

Figure 4.20 explains all these variables. The growth measurements content of *Nostoc sp.* IMU1 species containing BG11-N (as controlled media), the BG11+N and BG11-NP were measured in the 2-16 day of growing. The simply growth rate was significantly increased in BG11-N by the 14^{th} day while the specific growth rate was significantly decreased in the as controlled media and Bg11-NP media.

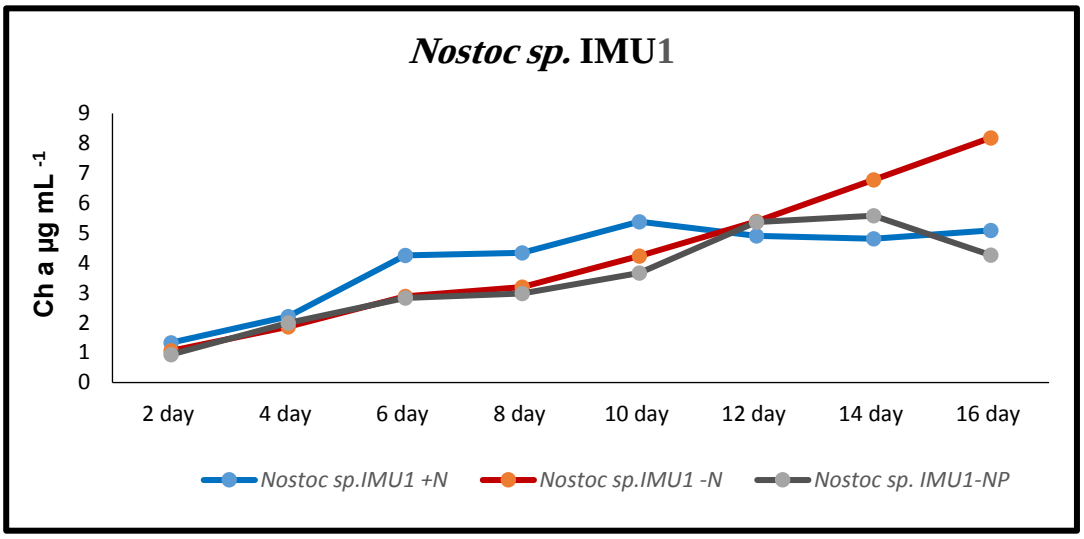


Figure 4.20 Growth content of *Nostoc sp.* IMU1

Figure 4.21 explains the growth of *A.variabilis.* IMU8. Increase the maximum absorption of pigment with its concentration in the environment may affect light transferred in water, which ultimately affects the photosynthesis and growth of aquatic blue bacteria. According to the growth of *A.variabilis L.* IMU8, both chlorophyll and protein synthesis were prevented from increasing dye concentration. Some anionic dyes inhibit the growth of blue bacteria because these pigments interact with physiologically important ions i.e., Ni^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} [85].

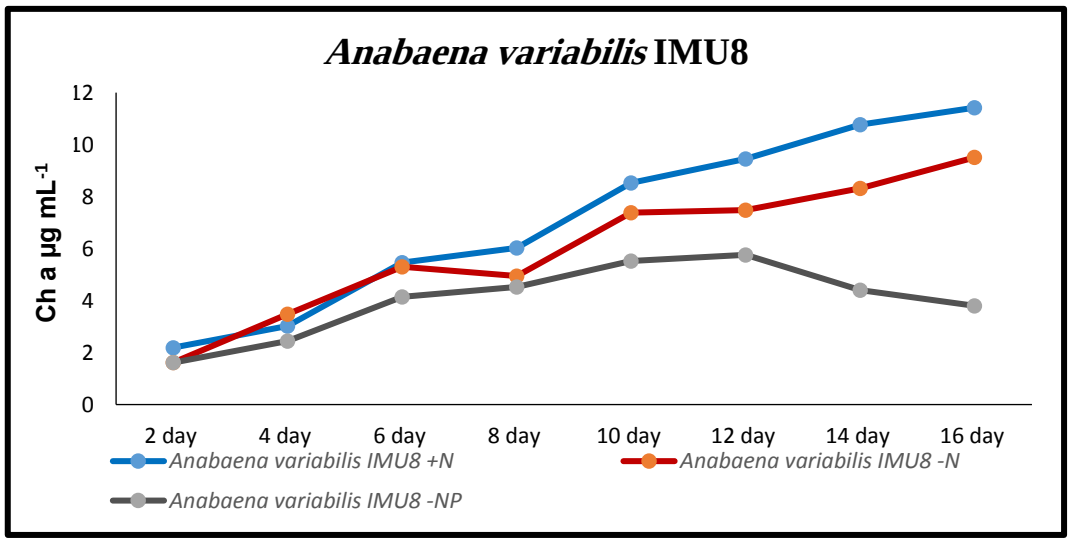


Figure 4.21 Growth content of *Anabaena variabilis.* IMU8

The growth measurements content of *A.variabilis.* IMU8 species containing BG11-N (as controlled media), the BG11+N and BG11-NP were measured at the 16th day of growing. The growth increased in the controlled media and BG11-N by the 12th day while it was significantly decreased in the Bg11-NP media (Fig 4.21).

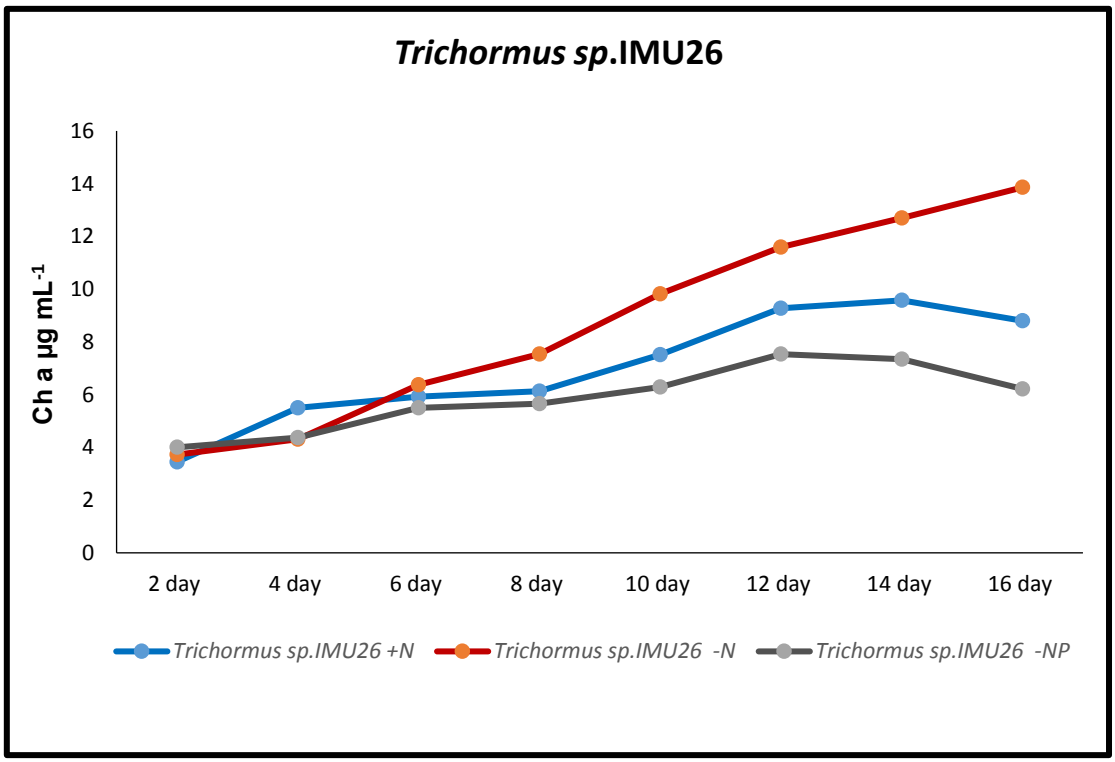
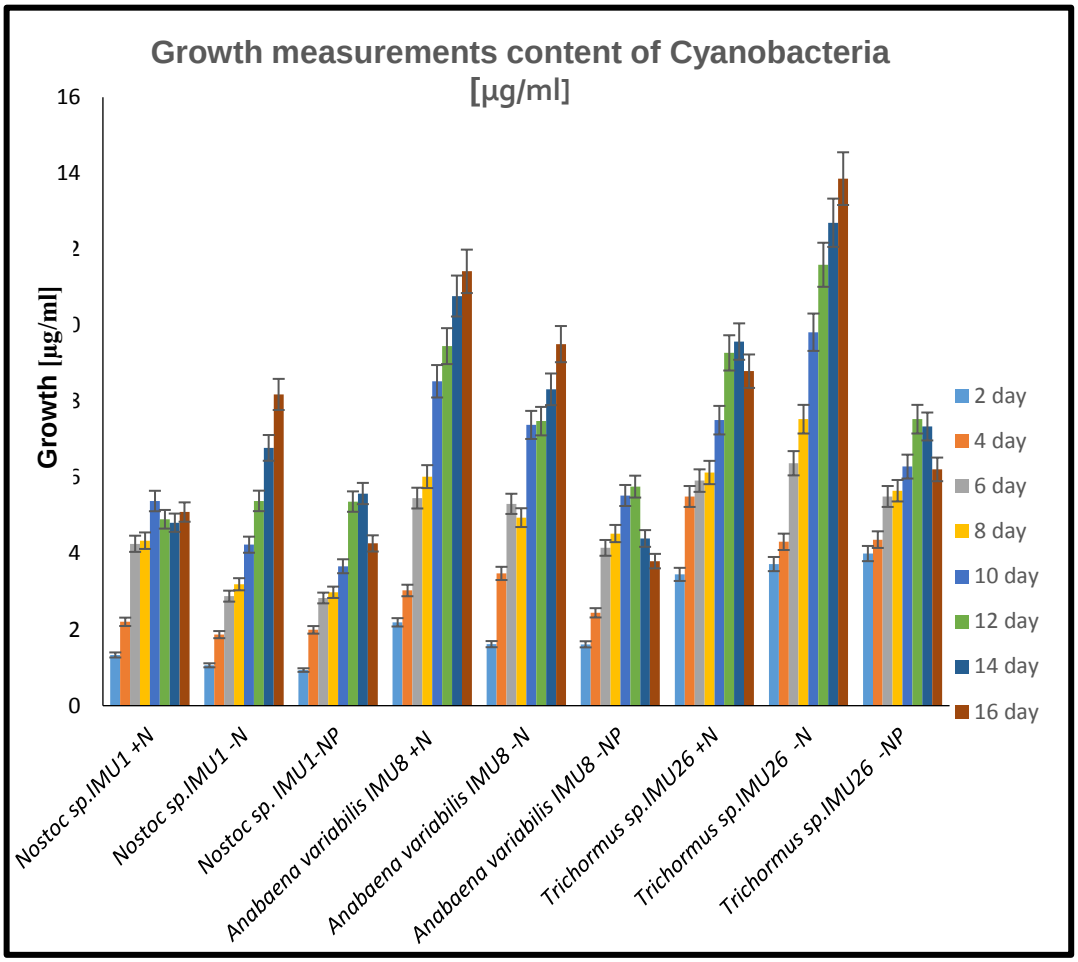


Figure 4.22 Growth content of *Trichormus sp*. IMU26

Figure 4.22 explains growth measurements content of *Trichormus sp.* IMU26*.* The growth measurements content of *Trichormus sp.* IMU26 species containing BG11-N (as controlled media), the BG11+N and BG11-NP were measured at the 2-16 day of growing. The growth significantly increased in the controlled media and BG11-N until 8th day while it was significantly decreased in the BG11-NP media (Fig 4.22).

Figure 4.23 explains the growth measurements of cyanobacteria content. The specific data showed the growth measurements of *Nostoc sp.* IMU1+N was increased (47%) at the 6th day of growing, but from the other side, the value of *Nostoc sp. IMU*1_NP was decreased (9%) at the 12th day of growing. The *Anabaena variabilis. IMU8* +N was decreased (17%) at the day 6th, while this value was changed to 0.3%

at the 12th day of growing. However, the *Anabaena variabilis.* IMU8 -NP was increased at the 6th day around 3% and 26% at the 12th day of growing. The *Trichormus sp.* IMU26 +N was decreased to 7% at the 6th and 20% at the 12th day of growing. While the *Trichormus sp.* IMU26 -NP was decreased to 13% and 35% at



the 6th and the 12th day of growing respectively (Fig 4.23).

Figure 4.23 Growth content of three cyanobacteria species by using BG11, BG11-N, and BG11-NP media.

4.10 Total Protein Content

Figure 4.24 explains total protein measurements content of cyanobacteria species. The nitrogen supply increased the total protein production in *Nostoc sp.*IMU1. There was approximately 30% increase in total protein content as measured at the end of 12 days of incubation. On the other hand, -NP deprivation resulted in significant decreases in total protein content of *Nostoc sp.* IMU1 when compared to -N deprived ones. -NP deprivation decreased total protein content, approximately 20% and 60% at the 6th and the 12th day, respectively.

On the other side, the nitrogen supply reduced in the total protein production in *Anabaena variabilis.* IMU8. to 6.7% at the day 6th day, while this value was hanged to 11% at the 12th day of growing. However, the *Anabaena variabilis.* IMU8 -NP was decreased at the 6th day around 14.3% and 34.9% at the 12th day of growing, respectively.

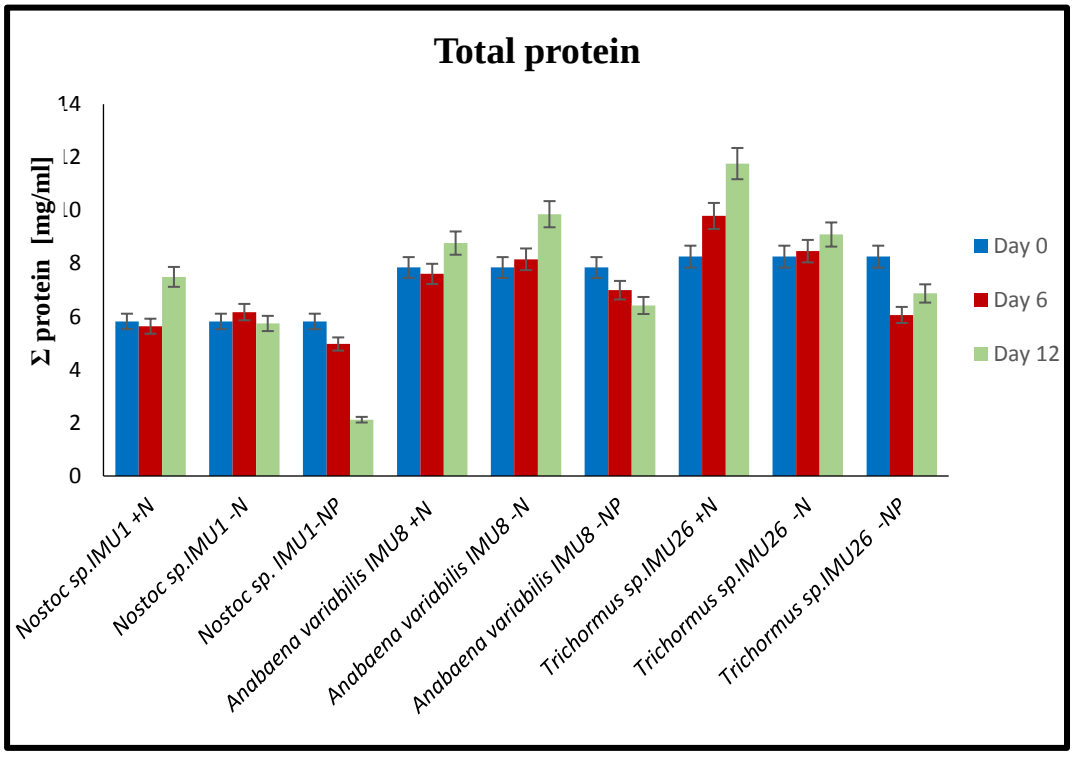


Figure 4.24 Total protein content of three cyanobacteria species.

However, the nitrogen supply increased total protein production in *Trichormus sp.* IMU26. There was approximately 30% increase in total protein content as measured

at the end of the 12th day of incubation. On the other hand, -NP deprivation resulted in significant decreases in total protein content of *Trichormus sp.* IMU26 when compared to -N deprived ones. -NP deprivation decreased the total protein content, approximately 28% and 24% at the 6th and the 12th day respectively.

4.11 Total Saccharide Content

Figure 4.25 shows total saccharide content of cyanobacteria species. The specific data showed the total saccharide content of *Nostoc sp.* IMU1*+N* was increased from 10% to 60% on day 6 and 12 of growing, respectively. While the value of *Nostoc sp.* IMU1-*NP* was decreased to 25% at the 12th day of growing. The *Anabaena variabilis.* IMU8 *+N* was increased to 19.8 % in day 6, while this value was changed to 26.2% at the 12th day of growing. However, the *Anabaena variabilis.* IMU8 *-NP* total saccharide level was increased by 42.7% at the 6th day and reached around 118% at the day 12th of growing. The *Trichormus sp.* IMU26 *+N* was also increased (108.3%) at the day 6th day and reached 168.3% at the 12th day of growing, while the *Trichormus sp.* IMU26 *-NP* was decreased from 50% to 38% at the 6th day and the 12th day of growing respectively (Fig 25).

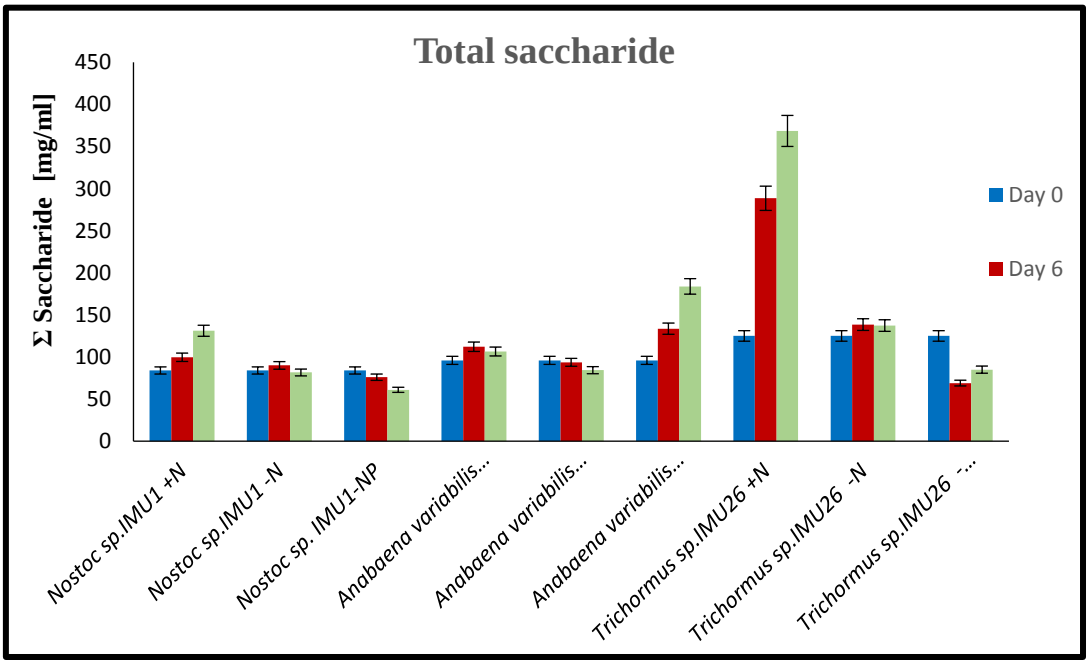


Figure 4.25 Total saccharide content of cyanobacteria species.

4.12 High-Performance Liquid Chromatography– (HPLC)

In the present study, different types of carotenoids were selected (lutein, zeaxanthin, canthaxanthin, chlorophyll a, echınenone, alpha carotene, and beta carotene) to study in the *Trichormus sp.* IMU26. After that, the vitamin B2 was analyzed in the *Trichormus sp.* IMU26. However, the vitamin B_2 also was analyzed in the *Trichormus sp. IMU26* species.

4.12.1 Carotenoid HPLC Analysis

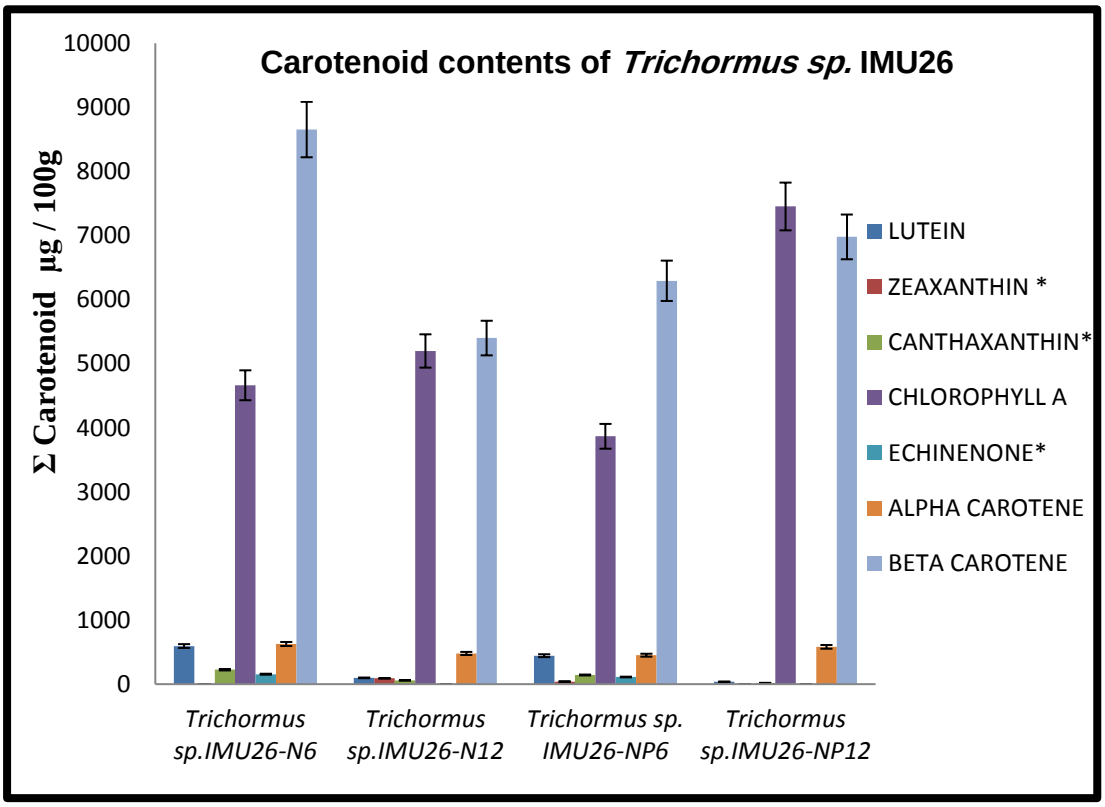


Figure 4.26 Carotenoid contents of *Trichormus sp.* IMU26.

Figure 4.26 shows carotenoid contents of *Trichormus sp.* IMU26. Different changes in β-carotene, canthaxanthin, echinenone, and zeaxanthin levels were measured in *Trichormus sp.* IMU26 and incubated in -N or -NP lacking media for 6 and 12 days of incubation. The β-carotene and alpha carotene contents increased after 6 and 12 days of the incubation in the -NP deprivation.

4.12.2 Vitamin B_2 HPLC Analysis

Figure 4.27 shows vitamin B_2 contents of *Trichormus sp.* IMU26. The changes in vitamin B_2 levels were measured in *Trichormus sp.* IMU26 incubated in -N or -NP lacking media for 6 and 12 days of incubation. The vitamin B_2 content increased of 6 and 12 days of the incubation in the -NP deprivation when compared to –N deprived *Trichormus sp.* IMU26.

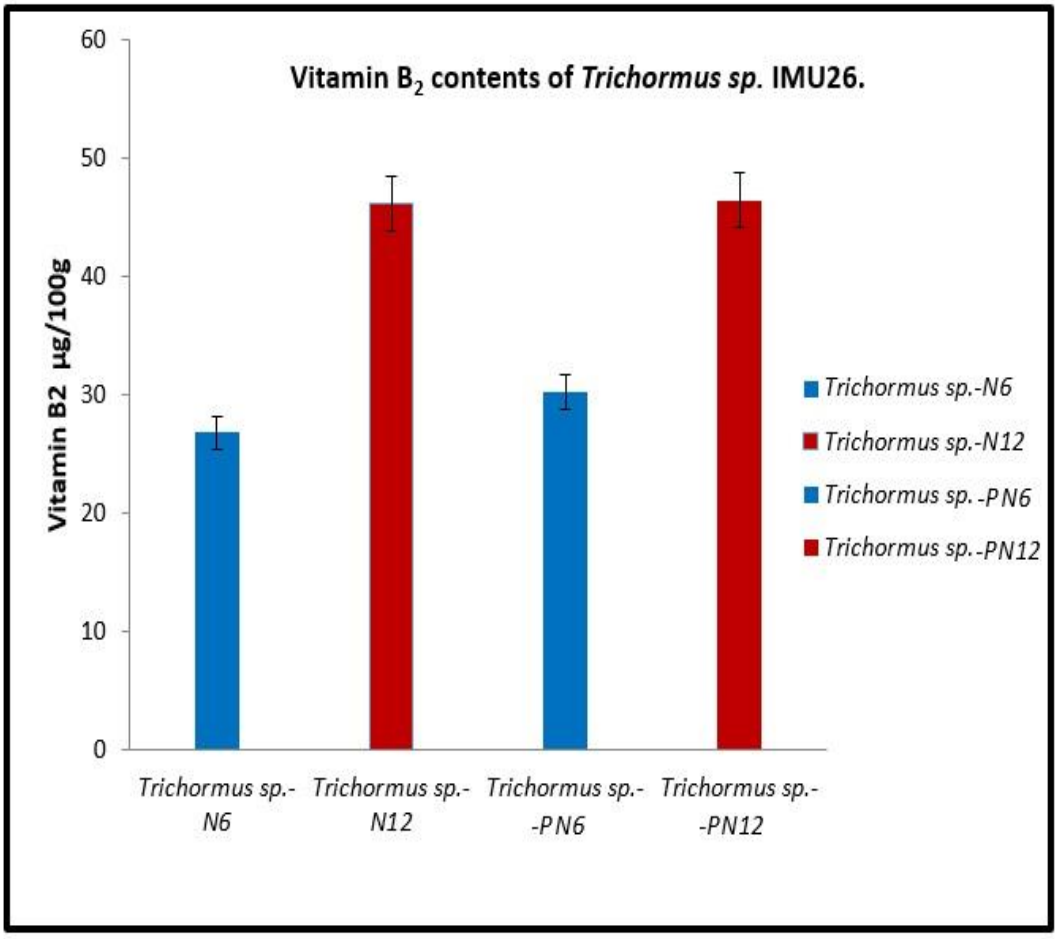
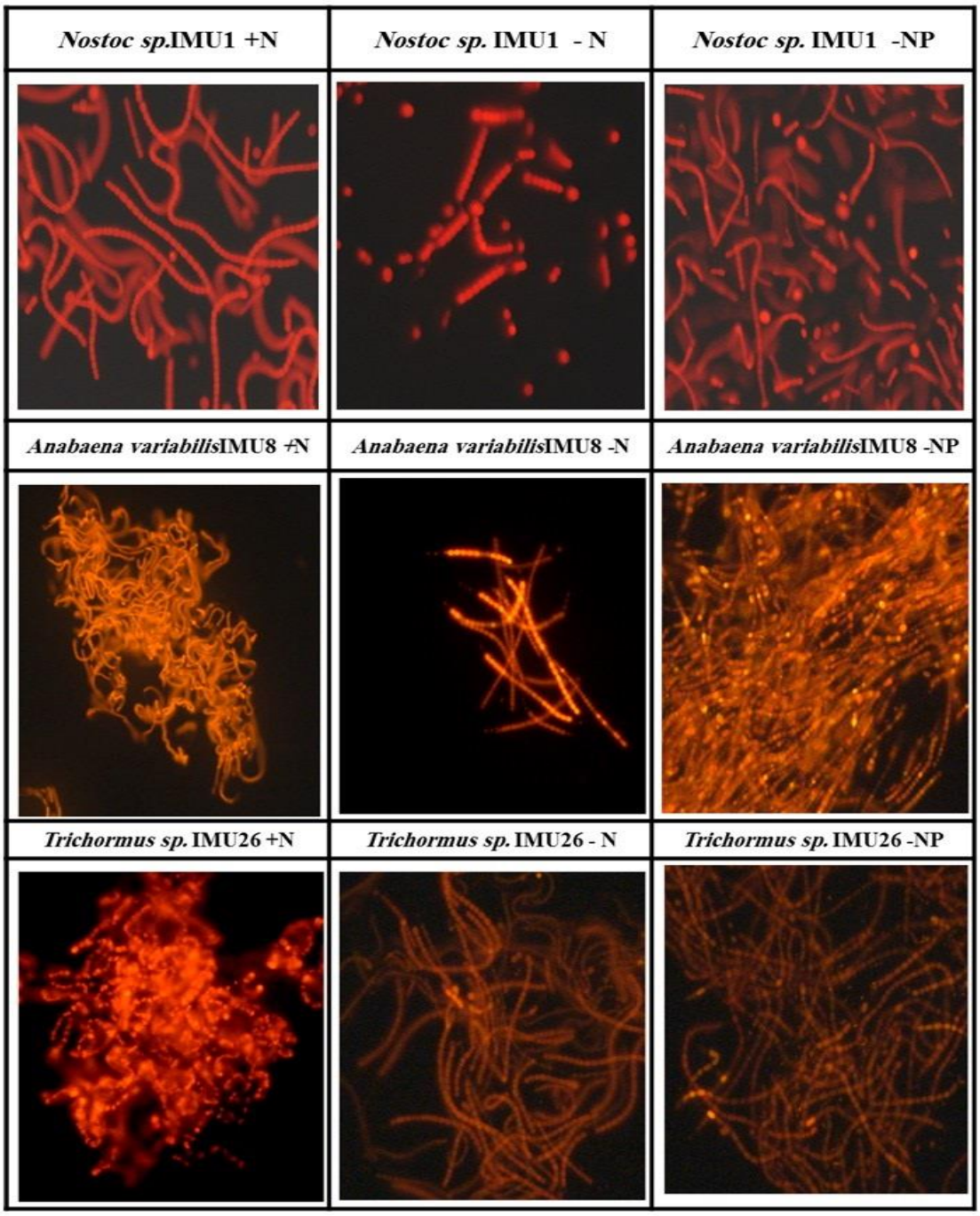


Figure 4.27 Vitamin B2 contents of *Trichormus sp.* IMU26.

4.13 Fluorescence Imaging of Cyanobacterial Lipids

In the present study, the effects of various nitrogen and phosphorus sources were investigated on total lipid contents. Cyanobacteria species were grown in BG-11+N, BG11-N, and BG11-NP for 16 days with a source of nitrogen and phosphorus



parallelly running [80].

Figure 4.28 Fluorescence imaging of cyanobacterial lipids

Figure 4.28 shows the fluorescence imaging of cyanobacterial lipids. The cyanobacteria analyses were obtained from cell aggregates at a specific depth; the special light in the nitrogen-fixing heterocyst was so bright and shining. This means that the lipid content was very high in the cyanobacteria species. But the N*ostoc sp.* IMU1 did not affect the nitrogen or nitrogen phosphorus deprivation in terms of lipids. However, the *Anabaena variabilis.* IMU8-*NP* increases the production of the lipid in terms of or nitrogen phosphorus deprivation so much, if compared with the nitrogen content in *Anabaena variabilis* IMU8*+N*. But on the other hand, the *Trichormus sp* IMU26 *+N* increased lipids production in terms of nitrogen more than the *Trichormus sp* IMU26 -*NP* content.

5
Discussion of the Results

5.1 Types of Media Used in The Study

The results which were obtained through the study of different types of blue bacteria and identified as the best strains that can be utilized in the fields of biotechnology, advanced technology and knowledge of the best agricultural media that can grow these types of bacteria and determine the optimum conditions of heat, energy sources and quantities. These factors are almost different from one place to another (BG11, FW, Z8, and Allen media) as in Fig 4.2.

It was found that cyanobacterial species under study that grow in hot spring water resources located in Ankara could be cultured well by growing them in BG11, BG11-N and BG11-NP media and the best medium was BG11. The quantity and the quality of growth in BG11-N soled were better than in Z8-N, Z8, and the other media [69,81].

In other studies which were conducted in hot springs water, they were used different types of cyanobacterial strains that isolated from Gunung Pancar (Leptolyngbya HS-16 and Leptolyngbya HS-36) were incubated at 20 °C, 35 °C, and 50 °C in BG-11 liquid medium culture at pH of 7.4. BG-11 medium contains macronutrients and micronutrients [86, 87].

Different environmental and operational factors, which change the biology, environments of these organisms and their biomass productivity as well as biomass, composition must be regarded into account.

The most significant factors are nutrients, pH, and alkalinity, source of light, cultural cell density, temperature, and contamination by another m.o. [88]. The cyanobacteria can also be used both CO_2 and HCO_3^- as a source of carbon while

cyanobacteria and chlorophyceae can start growth in 18% dissolved CO_2 in the culture medium, but cyanobacteria use CO_2 at a higher rate than chlorophyceae [89, 90].

5.2 Isolation, Purification, and Cultivation of Cyanobacteria

The choice of suitable algal strains was an important factor in the overall success of biofuel production from microscopic algae [82]. As the algal species grew in a medium of 50 ml in 100 mL flasks 50 mL medium in 100 mL flasks under the continuous light intensity of 100 μE/(m^2/s) at temperature-controlled orbital shaker with a 120 rpm speed under 25 °C temperature. BG11-N0 medium, which was used as control and $NaNO_3$, was not included in the growth medium for nitrogen deprivation (Figure 4.3). In this study cyanobacterial strains were not depended on the nitrogen and phosphorus as a source of nutrients but in other research they used them [91, 92].

Phosphorus is also a necessary macronutrient for microalgae growth, although cyanobacterial biomass does not take large amounts of phosphorus, as it receives less than 1% of it, phosphorus is an essential growth-limiting factor, especially in natural environments where phosphorus is limited [93,94]. The low phosphorus absorption is related to low cell frequencies [95]. Moreover, cyanobacteria can collect excess amount of phosphorus as polyphosphate reserves, which can be satisfactory for extended culture in phosphorus-deficient media [94]. The utilization of phosphate is energy-dependent, and its uptake rate is slower in the dark than in source light [96, 98]. However, the uptake of phosphate was determined by pH; uptake rates reduce in acid and almost alkaline environments [97]. Additionally, the carriage of ions also influences the uptake of phosphate; namely, lack of ions such as K^+, Na^+, and Mg^{2+} modify the phosphate uptake rate [95, 98].

However, In the other study the temperature was the necessary physical factor, which significantly influences the oxygen unfolding activity of the photosystem II (PSII), has several effects on the cyanobacterial membranes, and affects nutrient availability and its uptake [99,100].

There is a relationship between temperature, light, and photoinhibition at a low degree of temperatures. Cyanobacteria were photoinhibition by the high- light effects, and thus, the temperature can be deemed as the most critical limiting factor in easy cultivation during the winter. The photoinhibition can be considerably

decreased by an increase in temperature [99]. Temperature is a part that also affects cyanobacterial biomass composition. Moreover, that protein content reduces by the increased in temperature [101], but the related time, carbohydrate content was increased [102].

5.3 Genomic Information and Genomic DNA Sequence of the Cyanobacteria Species

In this study, the species were identified based on their microscopical and genomic information. Genomic DNA was selected from algal species following the phenol-chloroform method on a pellet collected by centrifugation of 10 mL of algal culture at the late-log phase. DNA amplification from genomic DNA carrying a partial 16S ribosomal RNA region was performed by PCR using the following primers: Forward (27F): (5’ AGAGTTTGATCMTGGCTCAG 3') and Reverse (809R): (5'GCTTCGGCACGGCTCGGGTCGATA3'). The same primers were utilized for Sanger sequencing. Sequence identification of the 16S rRNA genes was performed using the NCBI databases with BLAS search (National Center for Biotechnology Information) and BioEdit-graphical biological sequence editor v7.0.9. The total DNAs were extracted from all isolates in this study and were used as a template for PCR assays. The purities and concentrations of genomic DNAs were measured using Biodrop spectrophotometer as shown in Table 4.4 and Figure 4.4, but also the analysis of 16S ribosomal RNA region by using polymerase chain reaction denaturing gradient gel electrophoresis gives as 400 Bp in Figure 4.5, The samples including PCR prodacts were sent for sequencing to Sentromer DNA Technologies in istanbul Turkey (https://www.sentromer.com/) , after that the results were compared with the data from the gene bank program.

However, other researchers found that the PCR - based quality control (QC) as shown on the extracted DNA samples to determine the sufficiency of at least 30,000 reads. Sample with unfinished reads was re-extracted from the supplementary sample. Sequencing were performed by the Australian Genome Research Facility in this recerch, Queensland.

The V1- V3 hypervariable and flanking ranges of the 16S rRNA gene was amplified using primers 27F: (5' AGAGTTTGATCMTGGCTCAG 3') and 519R: (5' GWATTACCGCGGCKGCTG 3') [103,104].

Other researchers also found that 16S rRNA-based NGS library development primers were designed for the V4 region in the microbial 16S rRNA gene, and the primers utilized for PCR introduced Illumina Bridge PCR-compatible primers, barcode primers, and the V4 universal primers 515F: (5′GTGCCAGCMGCCGCGGTAA-3′) and 806R: (5′GGACTACHVGGGTWTCTAAT-3′).

PCR products were cleared with magnetic beads, and the sizes were verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). This was followed by quantification with Qubit 3.0 (Thermo Fisher Scientific, Inc.), qPCR, and sequencing with the Illumina HiSeq 4000 platform (Illumina, Inc.)], [104,105].

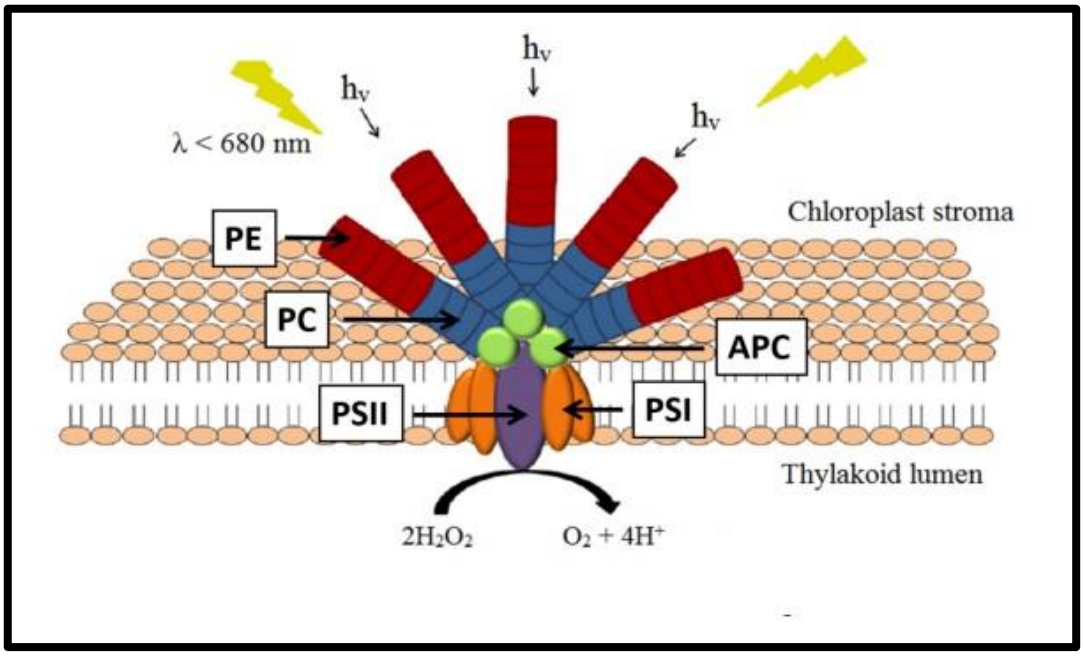
The homology of the three selected species, *Nostoc sp*. IUM1 around 98% similar to *Nostoc sp*.PCC7017, *Anabaena sp*. IMU8 gives 97% similar to *Anabaena variabilis* .CIBNOR23 and the *Trichormus sp*. IMU26 gives 95% similar to *Trichormus sp*.MACC643.

5.4 Extraction and Estimation of Phycobiliproteins (PBPs)

Phycobiliproteins (PBPs) in Figure: 5.1 are the principal component of light-harvesting systems in cyanobacteria and red algae. As well as their real, genuine, actual, essential role in photosynthesis, PBPs have many reasonable applications in medical analysis, treatment of diseases, foods, and makeups, nevertheless, fundamental studies and technological discoveries were mostly needed for searching those potentials, such function, and structure, their biosynthesis as well

as and purification of the downstream. For medical use and application, mechanisms underlying their therapeutic effects [106].

However, other researchers found that the extracted of phycobiliprotein can be operated as a fluorescent marker in biological research. After pigment extraction, continuing biomass can be utilized for carbohydrate and protein-rich feedstock for either food supplement or as a carbon source in fermentation media for other biofuel (e.g., ethanol and butanol) productions. Rodjaroen et al., studied eleven cyanobacterial cultures in BG-11 medium at 60 µmole photon m-2 s -1 light intensity maintaining 12:12 day-night ratio and 28°C temperature and reported that *Oscillatoria sp*. produces 19.32 % w/w on dry biomass basis) carbohydrate



[107].

Figure 5.1 General exemplification of a phycobilisome structure phycobiliproteins (phycoerythrins PC or phycoerythrocyanin PE, allophycocyanins APC) [110].

The accumulation of carbohydrates and depletion of phycobiliprotein in the same time period has also been reported by other investigators in a different strain of cyanobacterial [108]. Three cyanobacterial strains were isolated and cultured in BG-11 medium, incubated at 27 °C with a ratio of 12:12 days at night and a light density at 41 μmol m-2 s -1 by Parekh et al., [109]. In this research, the authors note that the extracellular polysaccharide composition. *Opsillatoria* sp. was 34.4 mg protein / gm EPS and Nostoc sp. produced 40.1 mg protein / gm EPS [82].

Moreover, Subramanian et al. isolated *Phormidium* sp.from aqueous source found 23% (w / w based on dry biomass) protein accumulation [109]. In their investigation, it was observed that two isolated *Phormidium* sp. (NITAAP005 and NITAAP012) produced approximately 65% (w / w based on dry biomass) protein in 15 days. Furthermore, *Oscillatoria* sp. and *Microcoleus* sp. they were also a potential source of protein as these strains contain about 50% (weight / weight based on dry biomass) in 15 days.

Another research [110] mentioned the strong light intensity usually reduces the accumulated amount of pigments (chlorophyll and PBP) due to a mechanism to protect against photosynthesis damage promoted by free radical production. The optical image occurs through a two-step process:

1- Destruction of the manganese group in the oxygen evolution complex

2- The direct effect of light disruption of the photochemical reaction center of the second photodiode system (PSII) by light containing it absorbed by chlorophyll.

Moreover, the repair of photosynthetic PSII is prevented by reactive oxygen species, which mainly suppress the synthesis of de novo proteins [111]. However, high light intensity promotes the growth of microalgae, which represent the opposite condition required for the production of phycocyanin [112]. Phycocyanin content in cyanobacteria increases when it grows in low light intensity (0–120 20l 2 in 1) [113, 114].

Other researchers were indicated that the results of phycobiliprotein in tested strains of cyanobacteria revealed to the maximum amount of phycoerythrin (PE) in fragile *Phormidium* (32.28±0.34µg mL-1) and the minimum in *Phormidium species* (1.57±0.67 µg mL^{-1}). On the other hand, the *Phormidium sp.,* which contain a much higher amount, than phycocyanin (PC) (14.33±0.09 µg ml^{-1}) and the minimum *Synechococcus elongatus* (2.75±0.01 µg ml-1 respectively). Thus, these two strains act as rich sources of dyes and can be technically exploited. Similarly, the content of Phycocyanin (PC) was maximum in *Lyngbya diguetii* (17.5 µg ml^{-1}) and minimal in

Carost Nostoc (12.1 µg ml^{-1}). The content of Phycoerythrin (PE) was at the maximum in *Oscillators* (48.7 µg ml^{-1}) and the minimum at *Muscola sylindrosperm*,

(15.34 µg ml^{-1}). Allophycocyanin (APC) had a maximum in *Lyngbya diguetii* (25-µg ml^{-1}) and a minimum in *Moscula sylindrospermum* (15.34 µg ml^{-1}).

The total content of phycobiliprotein was maximum in *Oscillatory oscillations* (81.4 µg ml^{-1}), and minimum in *Moscula sylindrospermum* (45.98 µg ml^{-1}) 119. However ,when comparing dyes, the best results was found in fragile *Phormidium* (322.8 ± 0.034 µg ml-1 dry weight) were more significant than *Oscillatoria subbrevis* (48.7 µg ml^{-1}) for phycoerythrin and *Phormidium* sp. (143.3 ± 0.09 µg ml^{-1} dry weight) larger than *Lyngbya* diguetii (17.5 µg ml^{-1}) for phycocyanin. The total current market value of phycobiliproteins products (including fluorescent agents) is estimated at more than US $ 60 million. [115].

Unlike most other photosynthetic organisms, cyanobacteria are able to make photosynthesis efficiently in the low chlorophyll absorption spectrum over the wavelength range 450 to 655 nm by means of PBPs [116]. Phycoerythrin (PE, λA max = 540-570 nm; λF max = 575-590 nm), phycocyanin (PC, λA max= 610-620 nm; λF max: 645-653 nm) and allophycocyanin (APC, λA max = 650-655 nm; λF max = 657-660 nm) are main PBPs in cyanobacteria [117].

The overall content and proportions of PBPs differentiate depending on the type of cyanobacterium and fluctuations in the environmental conditions in (Fig 4.8, 4.10,

4.12). In this study, *Nostoc sp.* IMU1, *Anabaena sp*. IMU8, and *Trichormus sp*. IMU26 in (Fig 4.9, 4.11, 4.13) respectivly were isolated from the same spring water resource at the same time and the stock cultures were maintained in N-lacking BG-11 medium for approximately 20 months before the experimentation. Thus, the strains were considered as N-deprivation acclimated cyanobacteria. Analysis of the PBP content of exponentially growing cells showed that *Trichormus sp.* IMU26 produce approximately 4.8, and 2.6 folds higher PBP equivalent than *Anabaenopsis* sp., and *Nostoc* sp. respectively: Figure 4.14. and the cyanobacterium *Trichormus sp.* IMU26 was selected for further analysis [118].

In cyanobacteria, the PBP content and composition is dynamically regulated in response to environmental changes such as light intensity, temperature, pH, and

nutrient availability [136]. Amongst nutrients, N and P are of special importance as mainly N availability affects C and N allocation to phycobiliproteins, and P availability affects spore formation in filamentous cyanobacteria [118]. Phosphorus and N are the main principal nutrients limiting microalgal growth in most water habitats [119].

It was reported that the proportion of cyanobacteria increased with distance downstream in a manner related to the large decreases in N and P concentrations that occur in stream water [120]. Thus, in order to evaluate further physiological differences, *Trichormus sp.* IMU26 was incubated in N-sufficient, N-lacking, and N- and P-lacking BG-11 growth medium. When compared to N-deprived ones, N supply improved initial growth and N-P-deprivation did not cause a significant change in first 6 days whereas a substantial decrease of growth in both groups was observed after the 8^{th} day of incubation. Both N-sufficient and N-P-deprived cells entered the stationary phase after the 10^{th} day of incubation while N-deprived cells were still growing exponentially with a higher cell density by the end of 16 days of incubation. Phosphorus is essential for cell growth, as it is a vital macromolecular constituent of phospholipids, proteins, polysaccharides, and cofactors [121]. However, an initial increase in cell density followed by a lower saturation level of population density in N-sufficient cells was somewhat interesting. This may stem

from the fact that the cyanobacterium is acclimated to N-deprivation over 20 months of maintaining.

Recently, [122] reported that N-free growth of diazotrophic *Nostoc sp.* resulted in higher biomass and PC production. Likewise et al. Reported that growing *Anabaena sp.* in N-free medium is superior to nitrate, ammonium or urea supply in terms of PBP production [123]. Supportively, our results show that 12 days of nitrate supply lower the level of PBP when compared to N-deprived ones. However, the level of PBP equivalents remained unaffected by the increased population density in N-deprived ones. Decreased level of PBP production accompanied by saturation of population density may reflect the overall PBP production in the existence of nitrogen source in diazotrophic cyanobacterium *Trichormus sp.* IMU26. Thereby, our results refer that long thermal diazotrophic growth of N_2-fixing cyanobacteria

may increase the PBP yield and the saturation level of the population density. Short thermal N- and P-deprivation stimulated higher PBP production but a rapid degradation of PBP equivalents was observed on a longer time period of 12 days of incubation. In fact, the PBPs do not contain phosphorus. The uptake and intracellular accumulation of phosphate are common in filamentous cyanobacteria [124]. The phosphate stock is used under P deficiency to support growth and cellular metabolism [125]. Thus, increased PBP levels in the first 6 days may reflect growth related photosynthetic adaptation strategies to overcome the absence of P and the diminished PBP levels observed in 12 days of P-deprivation might be the consequence of decreased PBP biosynthesis relative to the rate of cell division.

Thus, increased PBP levels in the first 5 days may reflect growth-related photosynthetic adaptation strategies to overcome the absence of P and the diminished PBP levels observed in 10 days of P-deprivation might be the consequence of decreased PBP biosynthesis relative to the rate of cell division, The biomass characteristics in cyanobacteria tend to change as a response to environmental fluctuations. Thus, a potential change in biomass characteristics of

the three types of strains that selected and grown in N-replete, N-lacking, and N-P-lack.

5.5 Chlorophyll a and Total Carotenoid Contents

The results of chlorophyll content of cyanobacteria were analyzed in (Figure 4.15). Strains of *Nostoc sp.* IMU 1, *A.variabilis L.* IMU8 and *Trichormus sp.* IMU26 showed a high percentage of chlorophyll when compared to studies of other strains. Deprivation of phosphorus from chlorophyll production in *Nostoc sp.* IMU1. on the other hand, *A.variabilis L.* IMU8 showed a rapid increase in chlorophyll content a on the fifth day of deprivation -PN while returning to normal levels on the tenth day of growth in -PN deprivation.

The total carotene content of cyanobacteria was analyzed (Fig. 4-16). The strains of *Nostoc sp.* IMU1 and *A.variabilis L.* IMU8 showed a high percentage of total carotene compared with studies of other strains. Deprivation of phosphorus from the

production of total carotenoids in *Nostoc sp.* IMU1. On the other hand, *A.variabilis* L. IMU8 showed a rapid increase in total carotenoids content in the fifth days of denial of -NP while returning to normal levels on the tenth day of denial of -NP.

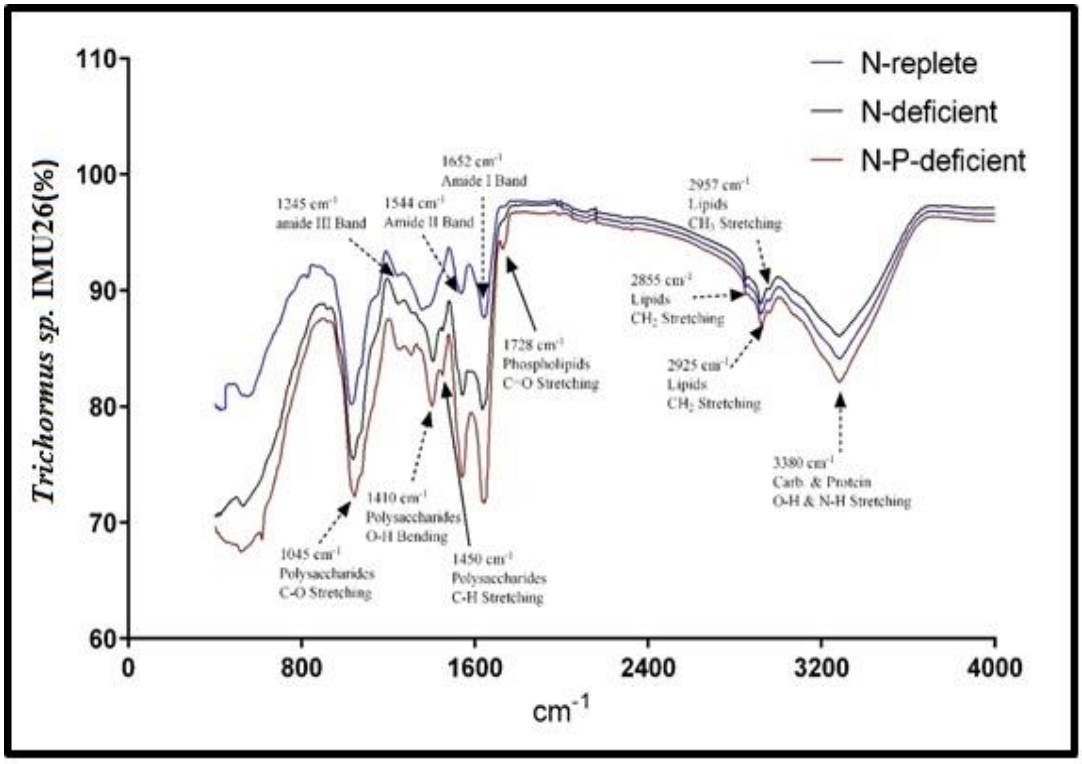
In another study, it was observed that two isolated *Phormidium sp.* (NITAAP005 and NITAAP012) had high carotenoids content (around 23 mg/gm dry biomass). Therefore, these two *Phormidium sp*. can undergo continuous photosynthetic growth under high light intensity condition. With the growing demand of natural pigments in the market, there is a need for enhancing production of pigments from biological sources. Hence, proper selection of strain or finding a new strain with a faster growth rate and high content of desired pigment product is huge beneficial for industry [126]. Moreover, the isolated, *Phormidium sp.* (NITAAP005) having a rapid growth rate may be considered as valuable resources for industrial-scale cultivation and eventually larger quantity production of carotenoids, [127].

Other researchers were initiated to characterize the biochemical contents in five selected species of marine cyanobacteria comprised of two unicellular and three filamentous isolates, in terms of chlorophyll a phycobilins pigments, total carotenoid, total protein as xanthan, and polyphenolic contents were analyzed. In *Synechococcus elongatus*, *Synechococcus aeruginosus, Oscillatoria subrevrevis, Phormidium, and Phormidium*, chlorophyll a and protein were recorded at an interval of two days until day 15. The maximum amount of chlorophyll a and protein was recorded on the seventh day and rarely on a ninth day. The amount of total carotenoid in *Synechococcus elongatus* (7.49±0.21 µg g-1) was the maximum amount of yield accompanied by *Synechococcus aeruginosus* (2.84±0.10 µg g-1 respectively). The last level of the carotenoid was near in *Phormidium fragile* (0.93±0.25 µg g-1). Similar issues were observed in *Calothrix marchica* (5.26 µg ml–1) was significantly (P<0 05) efficient in phases of carotenoid production and in the remaining four species carotenoid content changed from (2.31 µg ml–1 4.82 µg ml–1) [128,129].

Paliwal [130] segregated fifty seven isolates based on their carotenoid production using principal component analysis (PCA) and hierarchical clustering and reported that *Oscillatoria sp., Phormidium sp.* and *Microcoleus sp.* were synthesized 1.71 mg g^{-1}, 2.07 mg g–1, and 1.96 mg g–1 carotenoid respectively while grown in BG-11 culture medium maintaining 25±2°C under a light concentration of 60 µmolm2 s –1 and 12:12 h light-dark period [121]. It is well known now that cyanobacterial stresses producing high carotenoid content also have more extensive immunity for high light concentrations [130].

5.6 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

In our study, all analyses were obtained from identical cell aggregates at a specific depth. The 886 nm excitation shows no enhancement of the red-shifted band closer to the surface position but instead possesses a more enhanced red-shifted



band at lower positions. The uppermost two spectra in possess a weaker resonance FT-IR of carotenoids superimposed on a stronger fluorescence background; FT-IR dominates the spectral record when using 886 nm excitation, as shown in baseline-corrected spectra.

Figure 5.2 FTIR spectrum of N-replete, N-deficient, and N-P-deficient *Trichormus sp.* IMU26. Dashed arrows show major bands detected in all groups and continuous arrows address specific peaks emerged in N-deprived and N-P-deprived cells.

The biomass characteristics in cyanobacteria tend to change as a response to environmental fluctuations. Thus, shown in (Figure 5.2), a potential change in biomass characteristics of *Trichormus sp.* IMU26 grown in N-replete, N-lacking, N-P-lacking conditions for 6 days of incubation were evaluated by means of FTIR analysis. The peak emerged at 1450cm^{-1} attributed to C-H stretching of polysaccharides was visible in N-, and N-P-deficient cell biomass; however, it was lost in when *Trichormus sp* IMU26 was grown in N-replete medium. Besides, a new peak attributed to C=O stretching of Phospholipids (1728 cm-1) emerged when the cyanobacterium was N-P-deprived for 6 days of incubation. Phospholipids are the main component of cell membranes and they maintain a gradient of chemical and electrical processes to ensure cell survival [131].

Thereby, it seems that there was a reorganization of surface and composition of diazotrophic *Trichormus sp.* IMU26 biomass as a response to N- and P-deprivation. Because there was a clear induction of total protein and saccharide production in N-replete *Trichormus sp.* IMU26, while total protein and saccharide levels showed dramatically decreases when the cyanobacterium was grown in N-P-lacking medium. Supportively, it was reported an extensive change in biomass composition and membrane structure of *S.platensis* as a response to changes in element composition and salinity of the growth medium [132]. Other researchers indicated that the natural strain of the heterogeneous cyanobacterium *Trichormus* variabilis VRUC 168 was slowly grown in a low-cost bioreactor of the combined

product of polyunsaturated fatty acids (PUFA) and non-toxic substances (EPS) of the same blue biomass.

From the other hand FTIR analysis was performed to see overall changes in biomass characteristics in response to N- and P- availability (Figure 5.2). Infrared spectra was recorded in transmission mode with 64 scans in the range 4,000–600 cm^{-1}. The bands were assigned to specific molecular groups on the basis of biochemical standards and published studies as previously described [133]. The major bands observed in all groups were attributed to C-O stretching frequencies of the C-OH groups of polysaccharides (1045cm^{-1}), Amide I absorption (1652cm^{-1}), Amide II absorption (1544 cm^{-1}), asymmetric stretching vibration of acyl chains (2925cm^{-1}), O-H stretching of carbohydrates and N-H stretching of proteins (3380 cm^{-1}).

On the other hand, as shown in the (Figure 5.3), some bands were clearly visible in N-deprived and N-P-deprived cells while they were lost in N-replete cells. The bands attributed to PO_2^- asymmetric stretching of phosphodiesters (1076cm^{-1}), membrane-bound oligosaccharide C-OH bond (1145cm^{-1}), and symmetric CH_3 bending modes of the methyl groups of proteins (1401 cm^{-1}) were visible in N-deprived and N-P-deprived *A.variabilis sp.* IMU8 while they were lost in N-replete cells. These bands might be related to systemic resistance. Membrane-bound oligosaccharides were described as biologically active elicitors at low concentrations [134].

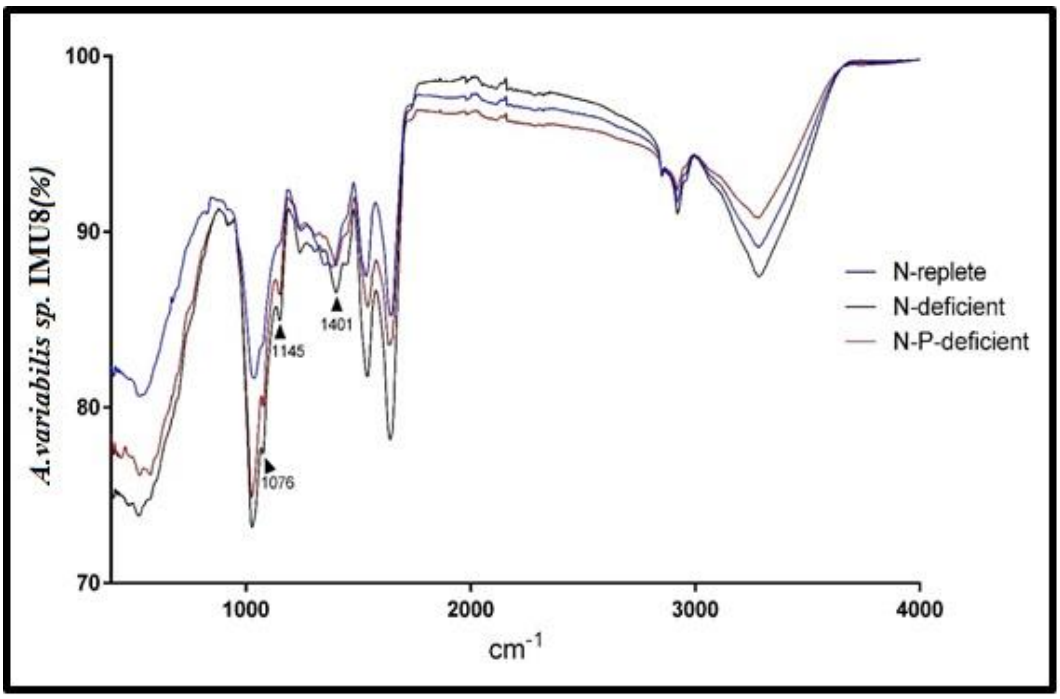


Figure 5.3 FTIR spectrum of old N-replete, N-deficient, and N-P-deficient *A.variabilis sp. IMU8.*

In plants and algae, oligosaccharides were reported to undertake regulatory roles on growth, development, and defense against stressors including nutrient limitation [135]. Our results refer that N-availability itself might be responsible for the induction of membrane-bound oligosaccharide production in diazotrophic cyanobacterium *A.variabilis sp.* IMU8*.*

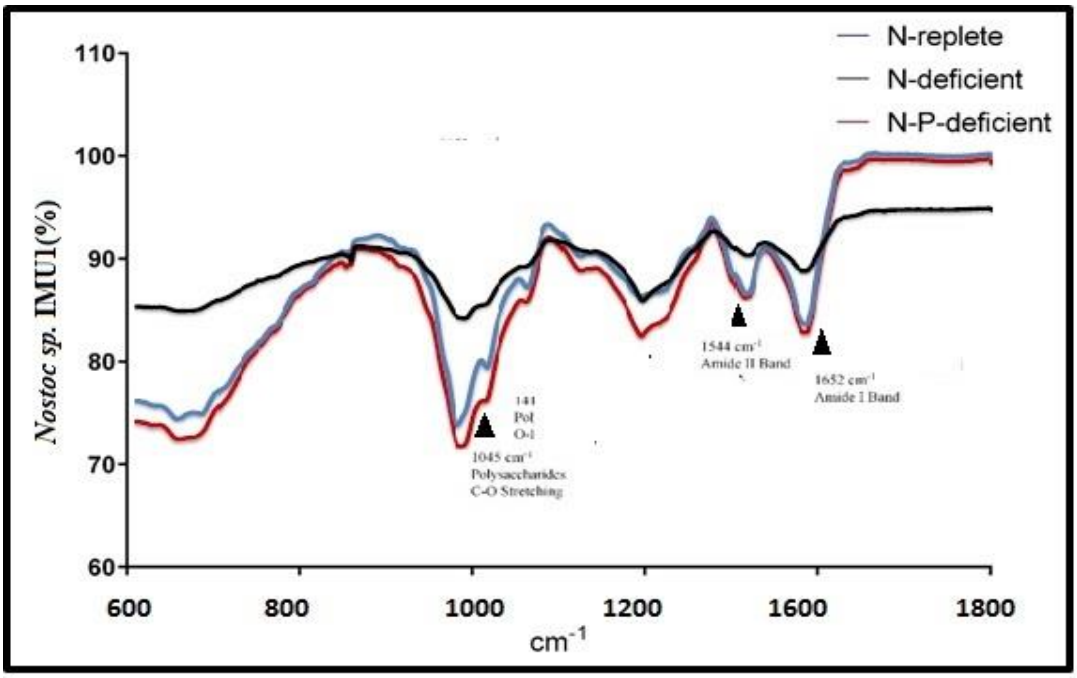


Figure 5.4 FTIR spectrum of old N-replete, N-deficient, and N-P-deficient *Nostoc sp.* IMU1.

On the other hand, as shown in the (Figure 5.4), some bands were clearly visible in N-deprived and N-P-deprived cells while they were lost in N-replete cells. The bands attributed to membrane-bound oligosaccharide C-OH bond (1145cm-1), and Amide groups bending (1544 cm-1) were visible in N-deprived and N-P-deprived deficient Nostoc sp. IMU1. While they were lost in N-replete cells. These bands

might be related to systemic resistance. Membrane-bound oligosaccharides were described as biologically active elicitors at low concentrations [134].

5.7 Growth Measurements Content

In our study the growth measurements content of *A.variabilis* L. IMU8 species containing BG11-N (as controlled media), the BG11+N and BG11-NP were measured in the 16th day of growing. The growth increased in the controlled media and BG11-N by the 12th day while it was significantly decreased in the Bg11-NP media (Fig 4.23). However, in the Figure 4.23 explains growth measurements content of

Trichormus sp. IMU26. The growth significantly increased in the controlled media and BG11-N until 8th day while it was significantly decreased in the BG11-NP media (Fig 4.23). But the figure 4.23 explains the growth measurements of cyanobacteria content. The specific data showed the growth measurements of *Nostoc sp.* IMU1+N was increased (47%) in the 6th day of growing, but from the other side, the value of *Nostoc sp.* IMU1-NP was decreased (9%) in the 12th day of growing . The *Anabaena variabilis* IMU8 +N was decreased (17%) in the day 6th, while this value was changed to 0.3% on the 12th day of growing. However, the *Anabaena variabilis* IMU8 -NP was increased in the 6th day around 3% and 26% in the 12th day of growing. The *Trichormus sp.* IMU26 +N was decreased to 7% on the 6th and 20% on the 12th day of growing. While the *Trichormus sp.* IMU26 -NP was decreased to 13% and 35% in the 6th and the 12th day of growing respectively (Fig 4.23).

The other researchers find in both cultures, biomass increased during the period of light and decreased during darkness. Low biomass during the dark period is a known biological response because cells use the energy accumulated during the lighting period to meet maintenance requirements. No nitrogen was absorbed in the dark, and therefore chlorophyll was not manufactured during the dark period, [136,137].

From another side other researchers found, the cyanobacteria had a fast growth rate, high photosynthesis efficiency, and require less space. In this study, three strains of cyanobacteria were studied to produce phycobiliprotein and the effect of potassium nitrate, sodium nitrate, and ammonium chloride on the growth and formation of phycobiliprotein strains. In the batch culture period of 12 days, *Phormidium sp*. and *Pseudoscillatoria sp.,* they were able to utilize all the tested nitrogen sources; however, ammonium chloride was the best source of nitrogen for both strains to achieve maximum growth rate μ = 0.284 ± 0.03 and μ = 0.274 ± 0.13 day - 1, chlorophyll 16.2 ± 0.5 and 12.2 ± 0.2 mg – 1, [138].

Also other researchers improved that the growth of small freshwater algae *Chlamydopodium fusiform* and to verify the process of cell adaptation to different amounts of nitrogen, the researchers developed cells in Bristol and BG11, under

constant conditions from 10 hours to 14 hours in the light / dark cycle. Cultures were grown in BG11 showed an increase in biomass accumulation, with cell weight reaching 1.94 ± 0.1 g L-1, while in Bristol reaching 1.58 Bristol 0.1 g L-1. In cells grown in BG11, the chlorophyll content increased continuously to 79.6 mg L-1 (ninth day), while in Bristol, it was significantly lower (29.5 mg L-1).

In our study observed that one of the results obtained from in-depth working to the three different types of cyanobacteria and compared to research and study that have already been worked that explain most models of cyanobacteria were able to adapt themselves to continue to grow and reproduce with the change of the food source, especially after the absence and presence of the nitrogen and phosphorous elements. This is evidence that these discovered strains are Strains of good scientific benefit and can benefit from their production of compounds of technological advantage.

5.8 Total Protein Content

In our study, at total protein in cyanobacteria species (Fig 4.24), the nitrogen supply increased the total protein production in *Nostoc sp*. IMU1. There was approximately 30% increase in total protein content as measured at the end of 12

days of incubation. On the other hand, -NP deprivation resulted in significant decreases in total protein content of *Nostoc sp.* IMU1 when compared to -N deprived ones. -NP deprivation decreased total protein content, approximately 20% and 60% at the 6th and the 12th day, respectively. On the other side, the nitrogen supply reduced in the total protein production in *Anabaena variabilis* IMU8 to 6.7% in the day 6th day, while this value was hanged to 11% at the 12th day of growing. However, the *Anabaena variabilis* IMU8 -NP was decreased at the 6th day around 14.3% and 34.9% at the 12th day of growing, respectively.

But in another research, they found that comparative evaluation of soluble full protein contents of five isolated bacterial strains was investigated for 15 and 30 days of transplantation that total soluble protein strains of bacteria were higher for 15 days compared to 30 days. Moreover, a maximum amount (64% of dry biomass)

of total dissolved protein was synthesized from the strain *Phormidium sp*. (NITAAP012) at a minimum of 15 days (28% of dry biomass). The full soluble protein was produced from the *Microcoleus sp.* strain (NITAAP023) in 30 days. The ratio of dissolved protein content was observed in 15 days to 30 days maximum (2.1 fold) for *Microcoleus sp*. (NITAAP023) while the minimum (1.2 fold) of one *Phormidium sp.* (NITAAP004) isolated from a water source. The results suggested that 15 days was better cultivation for more than 30 days to achieve high protein production [127].

Other researchers find three types of bacterial strains were isolated and transplanted into BG-11 medium, incubated at 27 °C with a 12:12 day-night light intensity ratio at 41 µmol m-2 s-1 by Parikh et al. Extracellular polysaccharide (*Opsillatoria sp.)* had 34.4 mg protein/gm EPS and *Nostoc sp.* produced 40.1 mg protein / gm EPS [126]. Moreover, Subramanian et al. isolated *Phormidium sp.* from a water source, he found 23% (W / w based on dry biomass) protein accumulation [109]. In another investigation, it was observed that two isolated *Phormidium sp*. (NITAAP005 and NITAAP012) produced approximately 65% (w / w based on dry biomass) protein in 15 days. Furthermore, *Oscillatoria sp.* and

Microcoleus sp. were also a potential source of protein as these strains contain about 50% (weight/weight based on dry biomass) in 15 days.

The Comparing values of our study with other studies indicate that protein ratios and amounts increase in different proportions of cyanobacteria types with more important ratios for growth periods between 1-15 days more than bacteria types whose growth ranges between 1 to 30 days. This study and previous studies also showed that the lack of nitrogen and phosphorous for periods worked to increase the rates of protein production, but in proportions that differed according to the type of bacteria.

5.9 Total Saccharide Content

In our study, the total saccharide content of cyanobacteria species (Fig 4.25), The specific data showed that the total saccharide content of *Nostoc sp.* IMU1+N was increased from 10% to 60% on day 6 and 12 of growing, respectively. While the value of *Nostoc sp.* IMU1-NP was decreased to 25% in the 12th day of growing. The *Anabaena variabilis* IMU8+N was increased to 19.8 % in day 6, while this value was changed to 26.2% on the 12th day of growing. However, the *Anabaena variabilis* IMU8 -NP total saccharide level was increased by 42.7% in the 6th day and reached around 118% in the day 12th of growing. The *Trichormus sp.* IMU26 +N was also increased (108.3%) on the day 6th day and reached 168.3% on the 12th day of growing, while the *Trichormus sp*. IMU26 -NP was decreased from 50% to 38% on the 6th day and the 12th day of growing respectively .

Other investigation, Barrick et al., found 70% (w / w based on dry biomass) carbohydrates in *Oscillatoria sp.* among the three isolates grown in the center of BG-11 at a temperature of 27°C temperature, 41 μmol m-2 s -1 light intensity and a ratio of 12:12 on the night [126]. At another study isolated *oscillator SP.* (NITAAP003), total carbohydrates produce an average of 45% dry biomass in 30

days of planting. In another investigation, under continuous white fluorescent tubes at a photon 80 μmol photon m-2 s -1 along with a temperature of 28 ± 1 °C, *Phormidium sp*. cultivated in BG-11 medium and 36% (w / w based on dry biomass), carbohydrate accumulation has been reported [127]. In this connection, it should be noted that their separate *Phormidium species* (NITAAP004, NITAAP005, and NITAAP012) had accumulated by about 41%, carbohydrates on the other hand. Their isolated *Microcoleus sp.* (NITAAP023) also contains the highest amount (62% dry biomass) of carbohydrates in 30 days of growth. Therefore, this particular strain has the potential of carbohydrates and protein-rich supplements. It was also noted from their results that in all isolated strains, the accumulation of carbohydrates became higher in 30 days of growth compared to 15 days, while the synthesis of fatty protein and protein became less in the same period time of growth [127].

It should be noted that the three strains studied have accumulated a quantity of total saccharide content in different proportions during 12 days of growth. However, these three strains from the first six days, there was an apparent effect of increasing the formation of this type of total saccharide. It should be noted that the three strains studied have accumulated a quantity of saccharide content in different species during 12 days of growth, unlike other studies that showed a clear effect during 30 days of growth, where The accumulation of total saccharide content is higher during 30 days of growth compared to 15 days, as the absence of nitrogen and phosphorus had a significant effect on the proportions and amount of total saccharide content in the strains studied in different proportions.

5.10 High-Performance Liquid Chromatography (HPLC)

In the present study, different types of carotenoids were selected (lutein, zeaxanthin, canthaxanthin, chlorophyll a, echinenone, α-carotene, and β-carotene) to study in the *Trichormus sp.* IMU26 species. After that, the vitamin B_2 was analyzed in the *Trichormus sp.* IMU26. However, the vitamin B_3 also was analyzed in the *Trichormus sp.* IMU26 *species.*

On the other hand, another study of the same selected bacteria *Trichormus variabilis* the relative standard deviations of chromatographic responses in HPLC ranged from 4.61% to 5.33% and in HPLC from 0 to 6.12% in one day and from 4.20% to 12.59% after several days. The lowest standard concentration (1 ng) is subject to the most significant errors. Concerning the speed of the analysis, HPLC has a significant advantage compared to HPLC [139].

Erika Bellini and others also worked on the same strain, where it was obtained that a low content of peptide or protein was also RP-HPLC and spectral data of the REPS solution, indicating the lack of both water-priced compounds and chromophore and high amount from the saccharide [140].

5.10.1 Carotenoid HPLC Analysis

Carotenoids in cyanobacteria serve as light-harvesting pigments in photosynthesis, modulators of membrane microviscosity, and they work against photooxidative damage [141]. The strain-specific composition of the carotenoids fluctuates in response to environmental changes such as cell density, light regime, and nutrient composition of the growth medium [142]. Major carotenoids in cyanobacteria are β-carotene and xanthophylls such as canthaxanthin, echinenone, zeaxanthin, astaxanthin, and myxoxanthoplhylls [143]. Recently, it was reported that carotenoids are required for proper functioning and structural maintenance of PBPs [144]. In this study changes in β-carotene, canthaxanthin, echinenone, and zeaxanthin levels were measured in Trichormus sp. IMU26 incubated in N- or NP-lacking media for 6 days of incubation when increased PBPs production was observed in NP-deprived cyanobacteria (Fig 5.5). There was a remarkable induction of β-carotene and zeaxanthin production accompanied by a decreased level of canthaxanthin and echinenone in NP-deficient Trichormus sp. IMU26. Zeaxanthin is a hydroxyl derivative of β-carotene while echinenone and canthaxanthin are its keto derivatives (Fig 5.5).

Increased β-carotene and zeaxanthin production in NP-deprived cells might refer to the protection of pigment systems from photoinhibition and oxidative stress. Recently, it was shown that zeaxanthin and echinenone protect the repair part of the PSII recovery cycle from photoinhibition via reactive oxygen scavenging activities [145]. Our results showed that there was approximately 209% increase in zeaxanthin production while only 16.2% decrease in echinenone production in NP-deprived cells. On the other hand, it was reported that zeaxanthin, a polar carotenoid, induces membrane rigidity while echinenone may have a neutral or fluidizing effect [146].

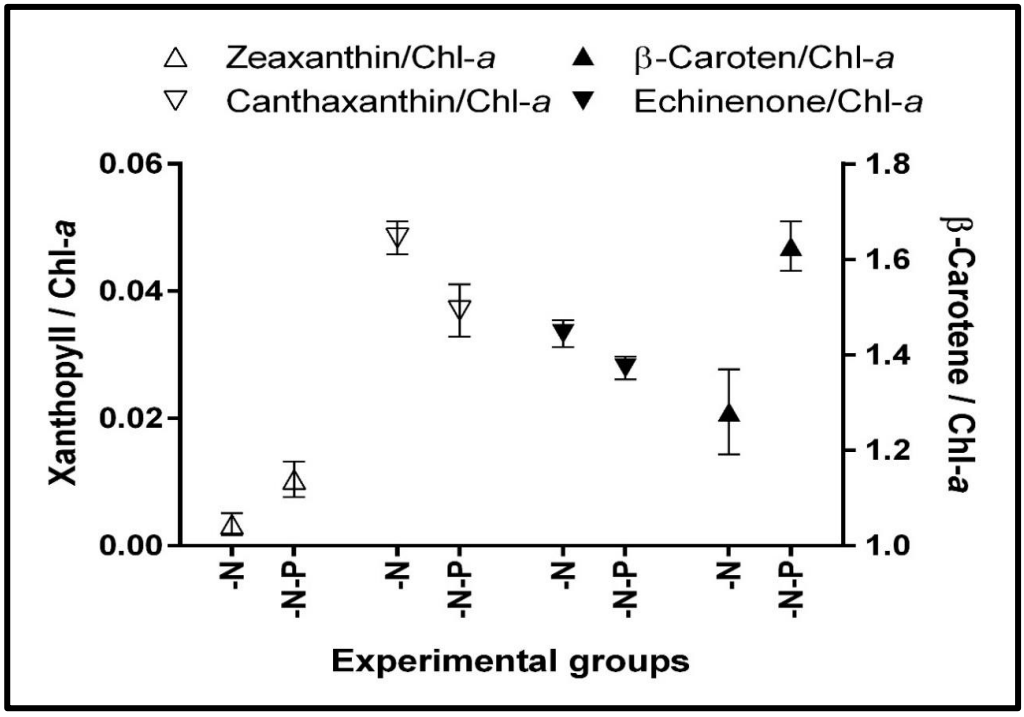


Figure 5.5 Change in β-carotene/Chl-a, zeaxanthin/Chl-a, echinenone/Chl-a, and canthaxanthin/Chl-a levels in *Trichormus* sp. IMU26 in response to N-deprivation, or N-P-deprivation.

While the canthaxanthin and echinenone levels decreased at the same time of incubation in the -NP deprivation when compared to –N deprived *Trichormus sp.* IMU26 increased to around 27% in β-Carotene/Chl-a. The level was accompanied by a dramatic increase of 20. % in zeaxanthin/Chl-a production. On the other hand,

canthaxanthin/Chl-a and echinenone/Chl-a levels decreased to 23.5% and 16.2% respectively.

Thereby, a new peak attributed to C=O stretching of phospholipids (1728 cm-1) observed in FT-IR analysis of NP-deprived *Trichormus sp.* IMU26 may refer to the membrane modulation activity of carotenoids in filamentous cyanobacteria. Lastly, blue-green light absorbing Orange Carotenoid Protein (OCP) in cyanobacteria is known to act as a stress sensor and energy quencher which is able to bind phycobilisomes when it is activated [147]. Activation or deactivation of OCP necessitates a dynamic adjustment in carotenoid-chlorophyll and carotenoid-bilin interactions [148].

Thus, elevated phycobiliprotein levels in response to 6-day NP-deprivation of Trichormus sp. IMU26 seems to stem from induced β-carotene and zeaxanthin production accompanied by a slight decrease in echinenone and canthaxanthin levels, from these results, if compared to the effects of other research, it may be a little different. Still, on the other hand, they may be positive by discovering new types and strains that grow in hot spring water, and this type from strains. It can produce different types of carotenoids in different proportions, knowing that these strains managed to live in -N deficiency.

5.10.2 Vitamin B_2 HPLC Analysis

The coloredin cyanobacteria species (blue green algae) had been extensively tested. This species is particularly rich in the number of low molecular mass compounds including carotenoids and vitamins as well as a battery of chemicals that act as antioxidants [149]. Therefore, many medical applications involving cyanobacteria like diffrent types of vitamin B have been described [150]. Spirulina is relatively easy to grow and harvest on an industrial scale, and could be the source of biomass mass for growing global demand for food, biofuels and chemical production. However, the changes in vitamin B2 levels were measured in Trichormus sp. IMU26 incubated in N- or NP-lacking media for 6 and 12 days of incubation. The vitamin B2 content increased after 6 and 12 days of the incubation

in the NP-deprivation when compared to N-deprived Trichormus sp. IMU26 in [151].

In another reserch study can used B12 that preduced from The Chlorella sp, and the high amounts of vitamin B12 commercial microalgae and has demonstrated the importance of the extraction and quantification methods in the analysis of B vitamins. [154].

From these results obtained and compared to previous studies, we found that some types of bacteria extracted from different kinds of algae can produce various kinds of vitamins and in different quantities. This is what makes them unique. These vitamins can be used in the pharmaceutical industries and also act as antioxidants and in the production of other materials that are valuable and useful.

5.11 Fluorescence Imaging of Cyanobacterial Lipids

In the present study, the effects of various nitrogen and phosphorus sources were investigated on total lipid contents. Cyanobacteria species were grown in BG-11+N, BG11-N, and BG11-NP for 16 days with a source of nitrogen and phosphorus parallel running [80]. In order to analysis of the fluorescence imaging of cyanobacterial lipids, the cyanobacteria analyses were obtained from cell aggregates at a specific depth; the special light in the nitrogen-fixing heterocyst was so bright and shining. This means that the lipid content was very high in the cyanobacteria species. But the Nostoc sp. IMU1 did not affect the nitrogen or nitrogen phosphorus deprivation in terms of lipids. However, the Anabaena variabilis IMU8-NP increases the production of the lipid in terms of or nitrogen phosphorus deprivation so much, if compared with the nitrogen content in Anabaena variabilis IMU8+N. But on the other hand, the Trichormus sp. IMU26 +N increased lipids production in terms of nitrogen more than the Trichormus sp. IMU26 -NP conten. But in the other hand the another researchers found in the past decade, the development of bacterial strains with the potential to accumulate high concentrations of fats has gained increasing attention, as these fats can be used in different industries. Here we describe two ways to assess fat accumulation in cyanobacteria, following our thinking about the issues surrounding these methods.

First, we introduce the Bligh and Dyer protocol as a conventional extraction method using organic solvents to quantify fat and the next Nile River, and selective fluorescent stain, which has been used as a rapid approach to both quantitative and quantitative lipid measurement [152,153].

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Publications from the thesis

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Scientific Publications

1. M. F. Hadad, "No TitleBiochemical Analysis of Urolithiasis in Nenavah Governorate," Tikrit J. Pure Sci., vol. 14, no. 2, pp. 15–119, 2009.

2. M. F. Haddad, S. M. Dawood, "No Title Determination of Sodium & Potassium for Patients Thalassemia by Using Different Techniq," Biol. Sci., Res. Zool., 2009

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4. B. A .Abdulla, E. A. Al-layla, M. F. Haddad, “No Title Serum Screening About Cytomegalovirus (CMV) Infection in High Risk Women in Mosul,” Biol. Sci., Res. Zool., 2009.

5. H. A. Al-Wahab, and M. F. Hadad, “No Title 5. Determination of Some Biochemical Variables in Human Blood Serum Using Some Electrochemical methods & it s Comparison with Spectrophotometric Methods,” Biol. Sci., Res. Zool., 2003.

6. N. S. Mohamed, M. F. H. Haddad, H. K. Al-Rubai, “No Title Detection of Norovirus and Rotavirus in Children under Five Years During Winter 2012-2013 in Mosul Province,” Al-Nahrain Journal of Science., vol. 19, no. 3, pp. 101–107, 2016.

Papers

1. M. F. Haddad, T. Dayioglu, B. Nalbantoğlu, and T. Cakmak, “Long term diazotrophic cultivation induces phycobiliprotein production in Anabaena variabilis IMU8,” Biocell., vol. 43 no. 2017, pp. 327–333, 2019.

2. M. F. Haddad, T. Dayioglu, M. Yaman, B. Nalbantoglu, and T. Cakmak, “Long-term diazotrophic cultivation of Trichormus sp. IMU26 : evaluation of physiological changes related to elevated phycobiliprotein content,” J. Appl. Phycol., pp 1–8, 2020.

Conference Oral Presentations

1. M. Fadhil Haddad*, T. Dayioglu, B. Nalbantoglu, and T. Cakmak, (2019). “Screening Indigenous Cyanobacteria for Phycobilprotein Production”, II. International Green Biotechnology Congress 2019 (09-11 September in Istanbul).

2. T. Dayioglu.*, Z. Elibol Cakmak, M. Haddad, T. Cakmak, "Polyhydroxybutyrate Production Potential of Some Indigenous Cyanobacteria", IPSAT (II. International Plant Science and Technology Congress), 07-10.10.2018, Bodrum, TURKEY.

3. Z. Elibol Cakmak*, T. Dayioglu., M. Haddad, T. Cakmak, "Diazotrophic Cyanobacteria Isolated from Paddy Fields in Edirne Province", IPSAT (II. International Plant Science and Technology Congress), 07-10.10.2018, Bodrum, TURKEY.

4. M. Haddad*, Z. Elibol Cakmak, T. Dayioglu, T. Cakmak, " Phycobiliproteins Producing Cyanobacteria Isolated from Paddy Fields in Edirne Province ", IPSAT (II. International Plant Science and Technology Congress), 07-10.10.2018, Bodrum, TURKEY.

Projects

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