SUSTAINABLE PRODUCTION OF BIOLOGICAL MATERIALS FOR FOOD AND AGRICULTURAL APPLICATIONS

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BY

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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

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ABSTRACT

SUSTAINABLE PRODUCTION OF BIOLOGICAL MATERIALS FOR FOOD AND AGRICULTURAL APPLICATIONS

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M.S. in Materials Science and Nanotechnology

Supervisor: Assist. Prof. Dr. Turgay TEKİNAY

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Microalgae are planet's primary biological CO_2/O_2 converters. Today, microalgae are used in a wide range of areas; such as waste-water treatment, production of protein-rich food and feed additives, high value added compounds, carotenoids and biofuels. Nowadays, there is an increasing need for renewable energy sources, specifically biofuels due to the depletion of limited fossil fuels. For this purpose, microalgae have emerged as a promising third-generation biofuel source and present possible solution to energy problems. In the first part of this study, the aim was to determine and compare the effects of sulfur (S) and nitrogen (N) starvation on triacylglycerol (TAG) accumulation, which is used as a biodiesel feedstock, and related parameters in wild type *Chlamydomonas reinhardtii* CC-124 mt(-) and CC-125 mt(+) strains to improve the biodiesel production capacity. Cell division was interrupted, protein and chlorophyll levels rapidly declined while cell volume, total neutral lipid, carotenoid and carbohydrate content increased in response to nutrient deprivation. Microalgae under nutrient starvation were monitored by three-dimensional confocal laser imaging of live cells and by transmission electron microscopy (TEM). FTIR measurement results showed that relative TAG,

oligosaccharide and polysaccharide levels increased rapidly in response to nutrient starvation, especially in S starvation. Neutral lipid, TAG and carbohydrate levels reached their peak values following four days of N or S starvation. However considering that four days of S deprivation leads to an increased total biovolume and stimulates more lipid and carbohydrate accumulation, S starvation seems to be a better way of stimulating biodiesel feedstock production of wild type *C. reinhardtii* compared to N starvation.

Carotenoids are lipid soluble compounds that play important role in acting provitamin-A, color materials and antioxidants that protect cells and tissues from free radicals and singlet oxygen. In nature, approximately 700 carotenoids have been isolated and characterized. However, there are some disadvantages of natural carotenoids such as being unsustainable and non-economic. Microalgae could serve sustainable solution to the production of natural carotenoids. The aim of the second part of this study was to identify new sources of natural, sustainable and inexpensive carotenoids and antioxidants from 12 isolated microalgae by determining their total carotenoid contents and antioxidant activity. These 12 microalgae were isolated from different water sources in Turkey. Results of this study demonstrated that among 12 microalgae strains, STA2, STA3 and STA9 contained substantial amounts of carotenoids in their metabolism and these carotenoids extracts showed strong antioxidant activity. With the ease of cultivation and high growth rate, these three microalgae strains have potential to use as natural and sustainable carotenoids for food, dietary supplement, pharmaceutical, cosmetic, feed and other related applications.

Keywords: biodiesel, *Chlamydomonas reinhardtii*, microalgae, nitrogen starvation, sulfur starvation, triacylglycerol, carotenoids, antioxidant capacity.

ÖZET

GIDA VE TARIM UYGULAMALARI İÇİN BİYOLOJİK MALZEMELERİN SÜRDÜRÜLEBİLİR ÜRETİMİ

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Mikroalgler gezegende bulunan başlıca biyolojik CO_2/O_2 dönüştürücüleridir. Günümüzde kirli su işleme, proteince zengin gıda ve yem katkıları üretimi, yüksek katkılı bileşimler, karotenoidler ve biyoyakıtlar gibi geniş çaplı kullanım alanları bulunmaktadır. Bu günlerde, kısıtlı olan fosil türevli yakıtların tükenmesinden dolayı özellikle biyoyakıt gibi yenilenebilir enerji kaynaklarına giderek artan ihtiyaç bulunmaktadır. Bu amaç doğrultusunda, mikroalgler gelecek vadeden üçüncü nesil biyoyakıt kaynağı olarak ortaya çıkıp günümüzün enerji problemlerine çözüm sunmaktadır. Bu çalışmanın ilk bölümündeki amaç, biyodizel üretim kapasitesini artırmak için vahşi tip *Chlamydomonas reinhardtii* CC-124 mt(-) ve CC-125 mt(+) suşlarına uygulanan azot (N) ve kükürt (S) açlığının, biyodizel hammaddesi olarak kullanılan triaçilgliserollerin (TAG) birikimi ve diğer ilgili parametreler üzerindeki etkilerini belirleyip karşılaştırmaktır. Besin yoksunluğuna cevap olarak hücre hacmi, toplam nötr lipid, karotenoid ve karbonhidrat içeriği artarken hücre bölünmesi kesilip, protein ve klorofil düzeyleri hızla gerilemiştir. Besin açlığı altındaki mikroalgler, canlı hücrelerin üç boyutlu konfokal lazer görüntülüme mikroskobu

ve transmisyon elektron mikroskobu kullanılarak takip edilmiştir. FTIR ölçüm sonuçları, besin açlığına cevap olarak; TAG, oligosakkarit ve polisakkarit düzeylerinin hızla arttığını göstermiştir. Azot (N) ve kükürt (S) açlığını takip eden dört günde, nötr lipid, TAG ve karbonhidrat seviyeleri en üst noktaya çıkmıştır. Ancak dört günlük kükürt (S) açlığının toplam biyohacim artışı, daha fazla lipid ve karbonhidrat birikimine yol açtığı düşünüldüğü zaman, kükürt (S) açlığı azot (N) açlığına karşı *C.reinhadrtii* tipi mikroalglerin biyodizel hammaddesi üretimini teşvik etmede daha iyi bir yol olduğu görülmüştür.

Karotenoidler; A vitamini, renk malzemeleri, hücre ve dokuları serbest radikallerden ve tekli oksijen moleküllerinden koruyan antioksidan gibi davranmakta önemli rol oynayan, yağda çözünen bileşiklerdir. Doğada yaklaşık 700 karotenoid izole edilip karakterize edilmiştir. Ancak, doğal karotenoidlerin ekonomik ve yenilenebilir olmama gibi dezavantajları vardır. Mikroalgler doğal karotenoid üretimine yenilenebilir çözüm sunarlar. Bu çalışmanın ikinci bölümündeki amaç, izole edilmiş 12 mikroalg kültürünün toplam karotenoid içeriği ve antioksidan aktivitesi belirlenerek; doğal, yenilenebilir ve pahalı olmayan yeni karotenoid kaynaklarının tespit edilmesidir. Bu 12 mikroalg kültürleri Türkiye'de bulunan değişik su kaynaklarından izole edilmiştir. Bu çalışmanın sonuçları, 12 mikroalg arasından STA2, STA3 ve STA9'un kendi metabolizmasında önemli miktarda karotenoid içerdiği ve bu karotenoid özlerinin güçlü antioksidan etkinliği gösterdiğini ortaya koymuştur. Kolay yetiştirme ve yüksek büyüme oranı ile bu üç mikroalg suşları; gıda, besin takviyesi, ilaç, kozmetik, yem ve diğer ilgili uygulamalarda doğal ve sürdürülebilir karotenoid olarak kullanılmak için potansiyele sahiptir.

Anahtar kelimeler: biyodizel, *Chlamydomonas reinhardtii*, mikroalg, azot açlığı, sülfür açlığı, triaçilgliserol, karotenoidler, antioksidan kapasitesi.

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1.1. Algal Basic

Algae are sunlight-driven cells that convert carbon-dioxide to potential biofuels, foods, feeds and high value products [1, 2]. They are prokaryotic or eukaryotic photosynthetic organisms that range from small, unicellular or simple multi-cellular organisms (microalgae) to multi-cellular organisms (macroalgae) [3]. They commonly occur in water (fresh, marine or brackish) in which they may be suspended (planktonic) or live at the bottom (benthic). Few algae live at the water - atmosphere interface and are defined as neustonic. Some of them grow on moist rocks, wood, trees and on the surface of moist soils [5].

Algae vary greatly in shape and size range from few micrometers to over 60 m. Some examples are given in Figure 1. *Ostreococcus tauri* (Prasinophyceae) is the smallest eukaryotic algae that have a cell diameter of less than 1 μm [4]. On the contrary, the brown algae, *Macrocystis pyrifera* (Phaeophyceae), they are the dominant organism in kelp forests and grow up to 60 m [5].

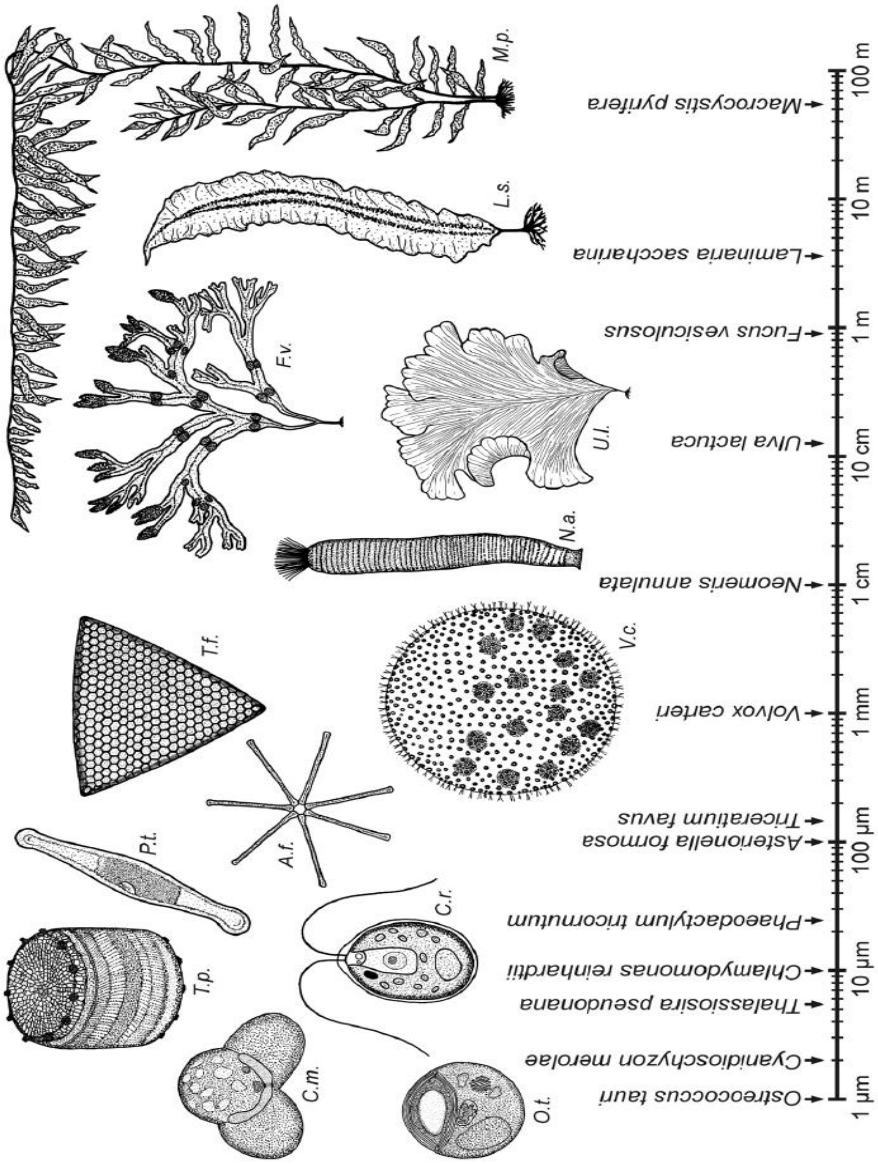


Figure 1. The pictures show phenotypes and sizes of algal species. The approximate sizes are indicated on a logarithmic scale [5].

1.2. Macroalgae vs. Microalgae

Algae are classified into two groups: macroalgae and microalgae. Macroalgae are the larger and multi-cellular photosynthetic organisms. The largest multi-cellular algae are called as seaweed that grow in salt or fresh water. They are classified into three broad groups based on their pigmentation such that brown seaweed (Phaeophyceae), red seaweed (Rhodophyceae) and green seaweed (Chlorophyceae). The second group, microalgae, is highly specialized group of unicellular photosynthetic microorganism that lives in freshwater, marine and salt water. They are very small, plant like organisms which range from 1 – 50 μm and can be seen only using a microscope. Microalgae are classified in multiple major groupings based on their pigmentation, life cycle and basic cellular structure. The four most important classes are diatoms (Bacillariophyceae), green algae (Chlorophyceae), golden algae (Chrysophyceae), cynobacteria (blue-green algae) (cyanophyceae) [6, 7]. Microalgae cells can double every few hours during their exponential growth period [8]. For example, during the peak growth phase, some of them can double every 3.5 h [9].

1.3. Composition of Microalgal Biomass

Microalgal biomass contains three main components: proteins, carbohydrates and lipids in alternating proportions. The percentages of these depend on the type of algae [10]. The chemical compositions of various microalgae are shown in Table 1.

In microalgae, carbohydrates can be found in the form of starch, glucose, sugars and other polysaccharides. Digestibility of them is also very high therefore there is no limitation about using of them in food and feed industry [11, 12].

Lipids and fatty acids are found as membrane components, storage products, metabolites and energy sources. The average lipid content of microalgae differs between 1% and 70% however under certain conditions lipid content could reach 90% of dry weight [8].

Algae consist of saturated or unsaturated fatty acids that have 12 to 22 carbon atoms. In microalgae, changes in nutritional and environmental conditions affect the total or relative amount of fatty acids [1, 13, 14].

Table 1. Chemical composition of algae on a dry matter basis (%) [12].

Species of Sample	Proteins	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Scenedesmus quadricauda	47	--	1.9	--
Scenedesmus dimorphus	8-18	21-52	16-40	--
Chlamydomonas reinhardtii	48	17	21	--
Chlorella vulgaris	51-58	12-17	14-22	4-5
Chlorella pyrenoidosa	57	26	2	--
Spirogyra sp.	6-20	33-64	11-21	--
Dunaliella bioculata	49	4	8	--
Dunaliella salina	57	32	6	--
Euglena gracilis	39-61	14-18	14-20	--
Prymnesium parvum	28-45	25-33	22-38	1-2
Tetraselmis maculate	52	15	3	--
Porphydridium cruentum	28-39	40-57	9-14	--
Spirulina platensis	46-63	8-14	4-9	2-5
Spirulina maxima	60-71	13-16	6-7	3-4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrical	43-56	25-30	4-7	--

Microalgae also contains essential vitamins such as A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid that increase the nutritional value of algal biomass [11]. However, the amount of vitamins changes with environmental factors and harvesting treatment [15].

In addition to these, microalgae contains high amount of pigments such as chlorophyll (0,5% to 1% of dry weight), carotenoids (0,1% to 0,2% of dry weight) and phycobiliproteins that have wide range of commercial applications in human and animal nutrition [12].

1.4. Current Usage of Microalgae

The biotechnology of microalgae has gained considerable importance in recent decades. High lipid, protein, carbohydrate and other composition of microalgae gives important qualities which can be applied on several industries. Applications range from simple biomass production for food and feed to valuable products for ecological applications.

Commercial use of microalgae as a source of specific chemicals began with *D. salina* for the production of β-carotene in the 1970s [16]. Nowadays use of dried microalgal biomass that are sold as powders, tablets, capsules and pastilles for health food supplement is one of the biotechnological applications of microalgae (Table 2) [17]. Algal biomass is also used for animal feed additives especially in poultry production and aquaculture [18]. Also, several microalgal species are used in agriculture as biofertilizer and soil conditioners [17].

In addition to use of dried biomass, microalgae can be used to produce high value added products such as amino acids, essential fatty acids (especially PUFAs such as DHA, EPA, GLA and AA, etc), polysaccharides, vitamins, pigments, carotenoids and antioxidants that are used in food, feed, cosmetic and pharmaceutical industry [18, 19].

Furthermore, microalgae could be used for environmental and agricultural applications such as bioremoval of contaminants from waste water or aqueous solutions [19], biofuel production and CO_2 fixation [3]. Table 2 shows the list of some commercial companies that produce algal products [5].

Table 2. Commercial companies producing and selling algae and algal product [5].

Company	Products
Acroyali Holdings Qingdao Co. Ltd.,	Agar
Agar del Pacifico S.A., Chile	Agar
Algas Vallenar S.A., Chile	Biomass (gracilaria, brown macroalgea)
Algatech, Israel	astaxanthin, microalgae-derived products
Bluebio Bio-pharmaceutical Co. Ltd., China	Biomass (chlorella, Spirulina)
Ceamsa, Spain	Carrageenan
Codif Recherce & Nature, France	Cosmetics
Cognis Nutrition and Health, Australia	β-carotene
Dainippon Ink and Chemicals, Japan	Pigments
Far East Bio-Tec Co. Ltd., Taiwan	Biomass (chlorella, Spirulina), microalgae extracts, health care, cosmetics
FMC Biopolymer, USA	Alginates, carrageenan
Kingland Seaweed Fertilizer Co. Ltd., China	Macroalgae extract fertilizers
Klötze, Germany	Biomass (chlorella)
LVMH group, France	Cosmetics
Lyg Seaweed Ind., China	Alginates, mannitol, iodine
Martek Biosciences Corporation, USA	Fatty acids
MicroGaia, USA	Astaxanthin
Nature Beta Technologies, Israel	β-carotene
Qingdao Richstar Seaweed Industrial Co. Ltd., China	Food additives, pharmaceutical chemicals

1.4.1. Biofuel

Dependence on fossil fuel for energy requirements is one of the major problems that the world is subjected. Studies have shown that almost 85% of the total energy being utilized is provided by the fossil fuels [20]. Use of fossil fuels produces harmful gases like carbon dioxide, nitrogen oxides, sulfur dioxide, volatile organic compounds and heavy metals. Therefore, the increase in the levels of these gases has contributed to environmental impacts such as global warming, acid rain and air quality deterioration [21].

Furthermore, due to high dependence and consumption of fossil fuel for energy and transportation, world's current demand does not give permission to the use of fossil fuels at the same level and for the same price in the future. As a result of these, there is an increasing need for renewable energy sources, specifically biofuels [22].

Biofuels are currently thought as one of the most promising alternative to reducing emission of CO_2, decreasing dependence on fossil fuels, and so improving economies [3, 23].

The most common biofuels are biodiesel and bioethanol that are mainly produced from biomass or renewable energy sources and contribute to lower combustion emissions than fossil fuels per equivalent power output. They can be produced by using existing technologies and be distributed through the available distribution system.

1.4.1.1. Biodiesel

Biodiesel production is currently made from plant and animal oils, but not from microalgae commercially. In the United States, soybean oil is the primary interest as a biodiesel source. Other sources of commercial biodiesel include rapeseed oil, canola oil, animal fat, palm oil, corn oil, cottonseed oil, sunflower, waste cooking oil, and jatropha oil [24, 25]. But, nowadays several companies are attending to commercialize microalgal biodiesel. The production of biodiesel from microalgae is expected to use the typically process for commercial production of biodiesel.

Biodiesel is chemically defined as the mono-alkyl esters of parent oil or fat. The lipid feedstock are composed by 90–98%(weight) of triglycerides and small amounts of mono and diglycerides, free fatty acids (1–5%), and residual amounts of phospholipids, phosphatides, carotenes, tocopherols, sulfur compounds, and traces of water [26]. In biodiesel process, triglycerides that consist of fatty acid molecules are esterified with a molecule of glycerol.

In this process, reaction of triglycerides with methanol is known as transesterification or alcoholysis. After chemical conversion of oils, methyl esters of fatty acids which are used as a biodiesel and glycerol are produced (Figure 2). For this reaction, firstly triglycerides are converted to diglycerides, then to monoglycerides and finally to glycerol [27].

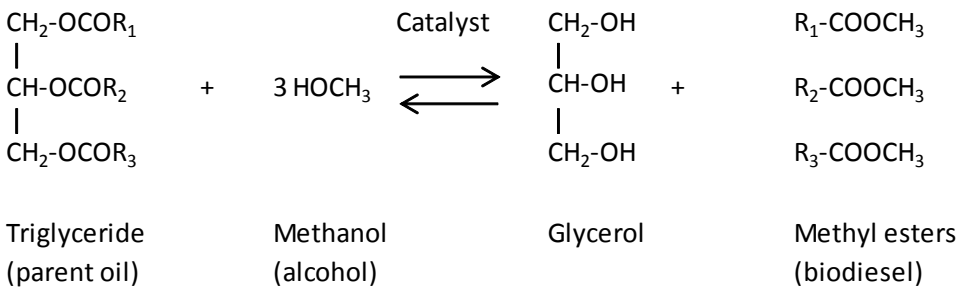


Figure 2. Reaction shows the transesterification of oil to biodiesel. R_{1-3} are hydrocarbon groups [9]**.**

Catalysts that are used for transesterification are acids, alkalis [28] and lipase enzymes [29]. Alkalis such as sodium and potassium hydroxide are commonly used as commercial catalysts. Alkoxides such as sodium methoxide are used increasingly and are better catalysts than sodium hydroxide. Although use of lipases gives some essential advantages, its usage is not applicable presently because of the relatively high cost of the catalyst [28].

In industrial process, although other alcohols can be used, methanol is commonly used catalyst because of being least expensive alcohol. During biodiesel production, the oil and alcohol must be dry and the oil should have a minimum of free fatty acids to prevent yield loss due to saponification reactions [27].

For industrial process, some improvements were suggested such as reactors with improved mixing, microwave assisted reaction [30, 31], cavitation reactors [32, 33] and ultrasonic reactors [34, 35] were used to work in continuous mode with reduced reaction time.

1.4.1.2. Availability of Algae for Biodiesel Production

Microalgae are very efficient solar energy converters and the fastest-growing photosynthetic organisms. In every few days they can conclude a whole growing cycle [36]. Because of these, according to some claims, the yield (per acre) of oil from algae is over 200 times the yield from the best-performing plant or vegetable oils [37]. Various algae species produce different amounts of oil (Table 3) [9]. Some algae species can produce up to 50 % algal oil by dry weight [38]. Based on some theoretical estimates, 47000-308000 L/hectare/year oil supply could be produced by using microalgae species [36].

Table 3. Oil contents of some microalgae [9].

Microalgae	Oil content (wt% of dry basis)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Chlamydomonas reinhardtii	21
Dunaniella primolecta	23
Isochrysis sp.	25-33
Nannochloris sp.	20-35
Nannochloropsisi sp.	31-68
Nepchloris aleoabundans	35-54
Nitzschia sp.	45-47
Schizochytrium sp.	50-77
Tetraselmis sueica	54-23

Microalgae have the ability to survive and multiply over a wide range of environmental conditions. To response to the change in environmental conditions, algae have the ability to modify lipid metabolism [39]. The lipid of algae may contain neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinones and phytylates pyrole derivatives such as the chlorophylls [40].

Microalgae synthesize fatty acids mainly for esterification into glycerol-based membrane lipids that make up about 5–20% of their dry cell weight (DCW) under optimal growth conditions. Fatty acids contain medium-chain (C10–C14), long-chain (C16–18) and very-long-chain (>C20) species and fatty acid derivatives [41]. Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone [40].

The overall fuel properties of biodiesel are determined by the properties of the different individual fatty esters. Microalgae mostly produce polyunsaturates which may constitute a problem by decreasing stability of biodiesel because of higher levels of polyunsaturated fatty acids. However polyunsaturates also have lower melting points than monounsaturates or saturates; therefore algal biodiesel should have better cold weather properties than many other biodiesel [10].

Algae are very promising source of biodiesel theoretically. The lipid and fatty acid contents of microalgae differ in accordance with conditions. In some cases,

lipid content can be enhanced by unfavorable growth conditions or other stress factors [36].

Many algae modify their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW), mainly in the form of triacylglycerol (TAG) under unfavorable environmental or stress conditions for growth. Triacylglycerol (TAG) is an ester of three fatty acids and glycerol. All eukaryotic organisms have the ability to synthesize TAG, which is the main constituent of vegetable oil, algal lipid bodies and animal fats.[13, 42]

TAGs serve mainly as storage of carbon and energy. In microalgae, changes in environmental conditions such as temperature and light intensity or nutrient media characteristics such as iron supplementation and urea, nitrogen or phosphorus limitation are known to enhance lipid accumulation [13, 14, 42, 43].

The pathway of TAG biosynthesis may play a more active role in the stress response in algae. In green algae, the formation and accumulation of lipid bodies are located in the inter-thylakoid space of the chloroplast. After TAGs synthesizing, they are deposited in lipid bodies that are located in the cytoplasm of the algal cell [44]. As can be seen from Figure 3, fatty acids are synthesized in the chloroplast, using either carbon fixed during photosynthesis, or from an external supply of organic carbon. Free fatty acids are taken from the chloroplast and then turned to TAGs in the endoplasmic reticulum (ER), where they are stored into oil bodies in the cytosol [45],[46].

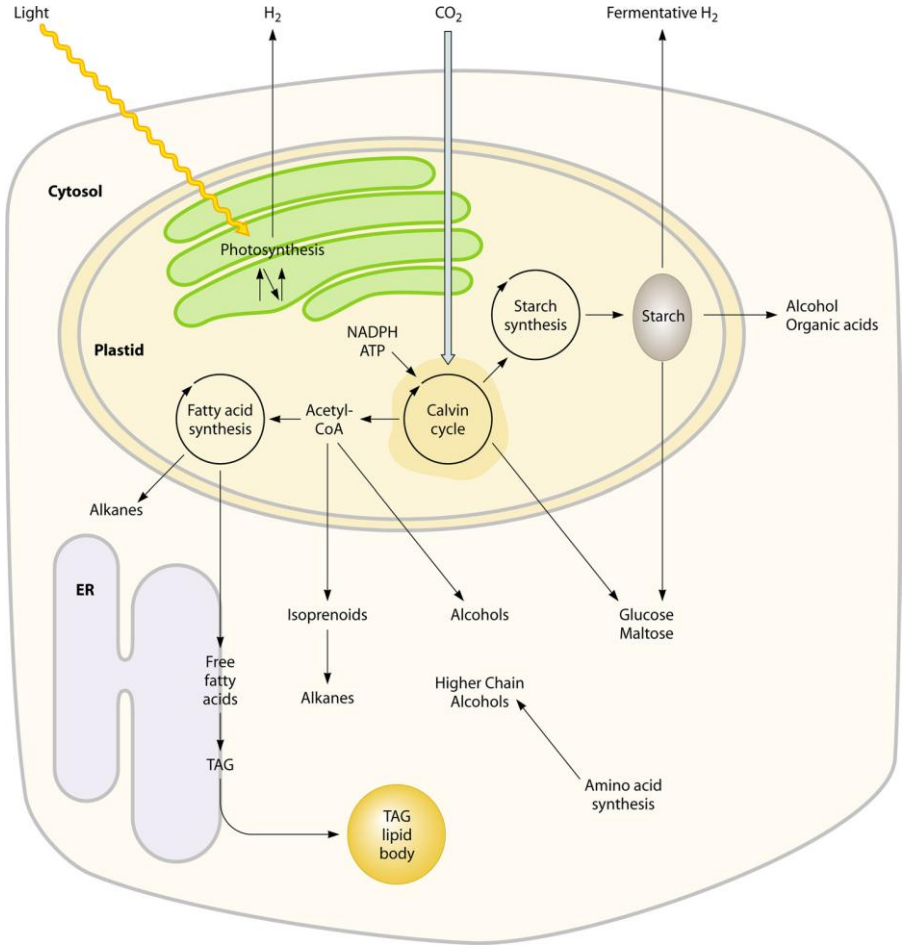


Figure 3. The figure shows the microalgal metabolic pathways that can be leveraged for biofuel production. ER, endoplasmic reticulum [47].

With the abilities to grow both phototrophically and heterotrophically, microalgae respond rapidly to environmental stress with pronounced metabolic changes [4].

Another type of neutral lipid that can be found in algae are hydrocarbons at quantities generally <5% DCW [48]. Under adverse conditions, *Botryococcus braunii*, example from green algae, has been shown to produce large quantities (up to 80% DCW) of very-long-chain (C23–C40) hydrocarbons. This type of

hydrocarbons is similar to those found in petroleum, and therefore microalgae have been searched as a feedstock for biofuels and biomaterials [49].

As a result, algal species have been found to grow rapidly and produce substantial amounts of TAG or oil. Therefore they are referred to as oleaginous algae. They have been assumed that algae could be participated to produce oils and other lipids for biofuels and other biomaterials [50].

1.4.1.3. Advantages and Disadvantages of Using Microalgae for Biodiesel Production

For biofuel production, a number of important properties potentially point out microalgae as an excellent feedstock relative to plants and seed crops. Microalgae can show rapid growth rates such as 1–3 doublings per day, and they can survive in salty waters and water with chemical composition, and also they can tolerate unsuitable lands (e.g. desert, arid- and semi-arid lands) that are not convenient for traditional agriculture.

Large quantities of lipids and oils (20-50 % DCW) are synthesized and accumulated by microalgal cells, and also other harvestable biochemical products that can be sold separately and give additional value are produced by them. Furthermore, the annual productivity of microalgal biomass can be greatly more than that of plants per unit land area (Table 4). Moreover, for keeping microalgal biomass at optimal level harvesting rates can be changed, and also for continuous biodiesel production, the potential of microalgae production assist to avoid from the seasonality of crop plant production [40],[51].

Table 4. Comparison of some sources of biodiesel [9].

Crop	Oil yield (L per hectare)
Corn	172
Soybean	446
Canola	1190
Jatropha	1892
Coconut	2689
Palm	5950
Microalgae[a]	136900
Microalgae[b]	58700

[a] 70% oil (by weight) in biomass

[b] 30% oil (by weight) in biomass

Optimal growth nutrients (e.g. CO_2, N, P, etc.) can be supplied at all times of the year, and their growth can remove other contaminants from wastewater sources, thus they obtain the additional environmental benefits to the wastewater bioremediation [19].

Due to their reduced needs for nutrients, they can be grown in areas that are unsuitable for agricultural purposes therefore their cultivation do not compete for areable land use. In addition, they can produce value-added co-products or by-products (e.g. biopolymers, proteins, polysaccharides, pigments, animal feed, fertilizer and H_2) [51].

Furthermore, in microalgae biodiesel, high levels of polyunsaturates is more suitable than other biodiesel for cold weather climate [36]. Moreover, algae biofuel contains no sulfur, toxic materials and also nitrous oxide release. These toxical materials could be minimized when microalgae are used for biofuel production [52].

The usage of microalgae for biofuels production can also contribute other purposes. These are listed below,

_ By microalgae biofixation, removal of CO_2 from industrial gases [53], while producing biodiesel, reducing the greenhouse gas emissions of a company or process.[3]

_ By removal of NH_4^+, NO_3^-, PO_4^{3-}, treatment of wastewater and growing microalgae by using these water contaminants as nutrients [53].

_ After extraction of oil that are used in biodiesel production, microalgae biomass can be used as organic fertilizer due to its high content of N, P or burned for electricity and heat energy [53];

_ Fats, polyunsaturated fatty acids, oil, natural dyes, sugars, pigments, antioxidants, high-value bioactive compounds, and other fine chemicals and biomass can be obtained from microalgae as a high value added products after biodiesel production [17, 52, 54]. These compounds can be used other industrial and biotechnological areas including cosmetics, pharmaceuticals, nutrition and food additives, aquaculture, and pollution prevention besides of biofuel production [17, 55].

On the other hand, one of the major disadvantages of microalgae for biofuel production is producing unstable biodiesel because of many polyunsaturates [36]. Also other disadvantage of microalgal biodiesel is the low biomass concentration in the microalgal culture and therefore low lipid concentration [52]. The large water content of harvested algal biomass also means its drying would be an energy-consuming process.

However, these problems are expected to be minimized by technological development. As a result, there is obvious that biodiesel from microalgae will eventually become one of the most important alternative energy sources due to the given potentials of microalgae as biofuel producer.

1.4.2. Carotenoids and Antioxidant Activity

Carotenoids are yellow to orange-red pigments that are found in nature. Chemically they are composed of a polyene skeleton which usually consists of 40 carbon atoms and is either acyclic or terminated by one or two cyclic end groups. The collective term xanthophylls refer to substituted derivatives containing hydroxy-, keto-, methoxy-, epoxy- or carboxyl groups. Unsubstituted derivatives are commonly called carotenes [56].

More than 700 structurally defined carotenoids are reported from nature; land plants, algae, bacteria including cynobacteria and photosynthetic bacteria, fungus and animals. Except for animals, these organisms can synthesize many kinds of carotenoids, which are synthesized from diverse carotenogenesis pathways [56].

Carotenoids are a class of widespread fat-soluble pigments. In addition to their role in coloration, carotenoids act as provitamin A and biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Therefore, carotenoids are utilized in pharmaceuticals, health food, dietary supplements, cosmetics, and as a feed additive [1].

Carotenoids are recognized as efficient antioxidants against oxidative damage. They could quench singlet oxygen, resulting in the suppression of lipid peroxidation. Ben-Amotz (1999) indicated that humans could lower incidence of certain cancers, coronary heart disease and other degenerative diseases through eating carotenoid- rich vegetables and fruits [44, 57, 58].

1.4.2.1. Carotenoids from Microalgae

Microalgae contain chlorophylls, the photosynthetic pigments, and a number of other pigments which are mainly used to improve the efficiency of light energy utilization and for protection from damage by the sunlight. From a commercial point of view, the carotenoids seem to be the most important [5].

Many different kinds of carotenoids are found from the algal species. Structures of some important carotenoids in algae are illustrated in Figure 4. Among them, approximately 30 types may have functions in photosynthesis, and others may be intermediates of carotenogenesis or accumulated carotenoids. Some carotenoids are found only in some algal divisions or classes and their distribution in algae is summarized in Table 5 [56].

In many markets, microalgal carotenoids are in competition with the synthetic form of the pigments. Although the synthetic forms are much less expensive than the natural ones, microalgal carotenoids have the advantage of supplying natural isomers in their natural ratio [59] and sustainable solutions. Today it is accepted that the natural isomer of carotenoids is superior to the synthetic all-trans form [19, 60].

Microalgae may serve as a continuous and reliable source of natural products, including antioxidants and carotenoids, because they can be cultivated in bioreactors on a large scale [61]. Furthermore, the qualities of the microalgal cells can be controlled, so that they contain no herbicides and pesticides, or any other toxic substances, by using clean nutrient media for growing the microalgae

[62]. The value of microalgae as a source of natural antioxidants is further enhanced by the relative ease of purification of target compounds [62].

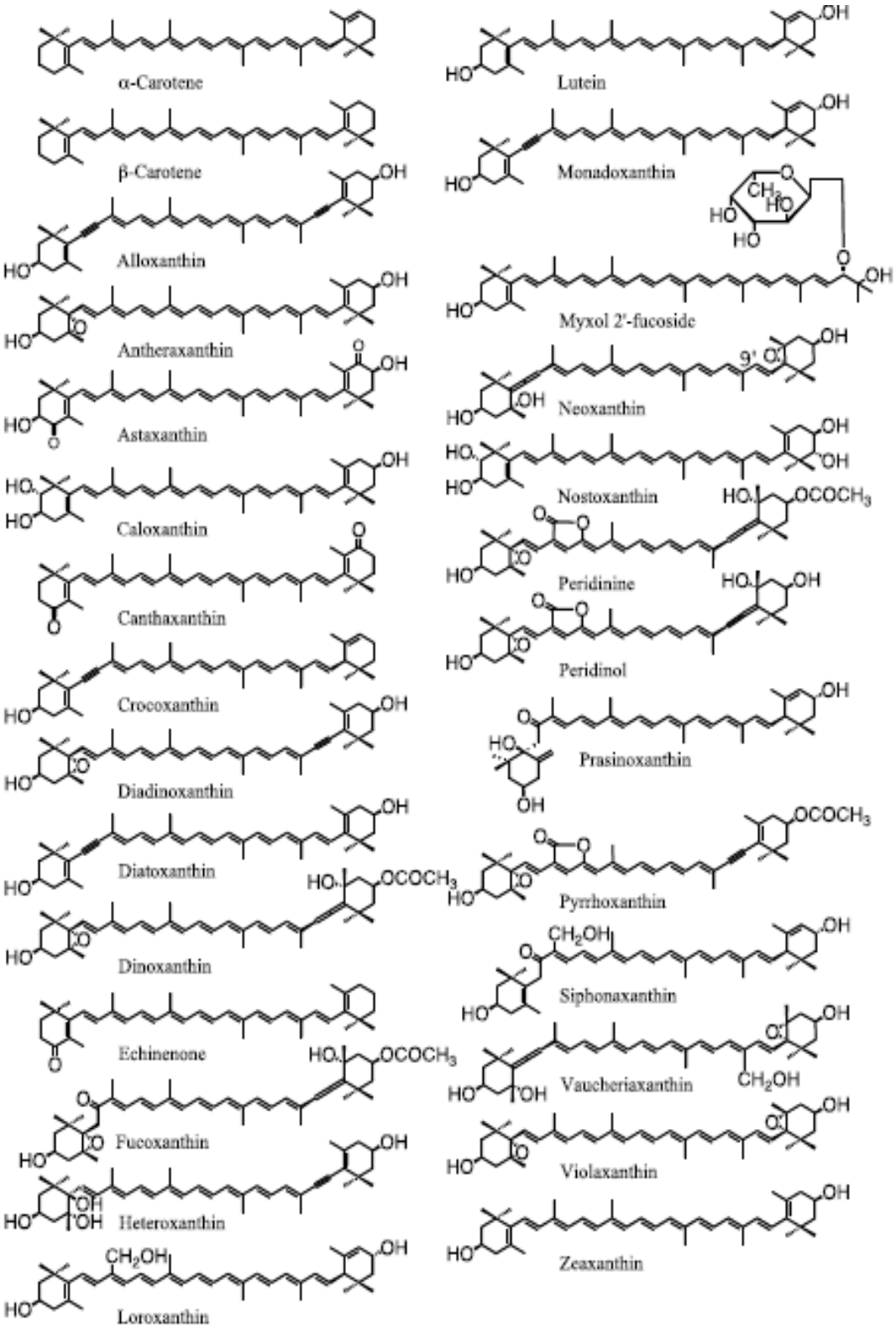


Figure 4. The figures show the structures of some important carotenoids [56].

Table 5. Distribution of carotenoids in algae [56].

Division	Carotene		Xanthophyll									Chlorophyll		
Class	β	α	Ze	Vi	Ne	Da	Dd	Fx	Va	Lu	Lo	Sx	Other xanthophyll(s)	*a*	*b*	*c*
Cyanophyta	H	L	H										No, L; Ec, H; My, H	H	L	
Glaucophyta	H		H											H		
Rhodophyta																
Unicellular type	H		H											H		
Macrophytic type	L	L	H	L				L		H				H		
Cryptophyta		H	L										Al, L; Cr, L; Mo, L	H		H
Heterokontophyta																
Chrysophyceae	H		L			L	L	H	L					H		H
Raphidophyceae	H		H	L		L	L	L						H		H
Bacillariophyceae	H		L			L	L	H						H		H
Phaeophyceae	H		H	H		L	L	H						H		H
Xanthophyceae	H		L			H	H						Va-FA, L	H		H
Eustigmatophyceae	H			H					L					H		
Haptophyta	H		L			L	H	H					Fx-FA, L	H		H
Dinophyta	L		L			L	H	L					Pe, H	H		H
Euglenophyta	H		L		L	L	H				L	L		H	H	
Chlorarachniophyta	H		L	L	L					L	L		Lo-FA, L	H	H	
Chlorophyta																
Prasinophyceae	H	L	L	H	H					L	L	H	Pr, L; Lo-FA, L; Sx-FA, H	H	H	
Chlorophyceae	H	H	L	H	H					H	L	L	Sx-FA, L	H	H	
Ulvophyceae	H	L	L	H	H					L	L	L	Sx-FA, H	H	H	
Trebouxiophyceae	H		L	H	H					H				H	H	
Charophyceae	H		L	H	H					H				H	H	
Land Plants	H	L	L	H	H					H				H	H	

*Abbreviations H, Major carotenoid in most species of the class; L, Low content in most species or major carotenoid in some species. α, α-carotene; β, β-carotene; Al, alloxanthin; Cr, crocoxanthin; Da, diatoxanthin; Dd, diadinoxanthin; Ec, echinenone; -FA, fatty acid ester; Fx, fucoxanthin; Lo,

loroxanthin; Lu, lutein; Mo, monadoxanthin; My, myxol glycosides and oscillol glycosides; Ne, neoxanthin; No, nostoxanthin; Pe, peridinin; Pr, prasinoxanthin; Sx, siphonaxanthin; Va, vaucheriaxanthin; Vi, violaxanthin; Ze, zeaxanthin. Red, α-carotene and its derivatives.

CHAPTER 2- Effects of Nutrient Limitation on Biodiesel Feedstock Production of *Chlamydomonas reinhardtii*

Part of this study was published as "Differential effects of Nitrogen and Sulfur deprivation on growth and biodiesel feedstock production of *Chlamydomonas reinhardtii* " Cakmak T., Angun P., Demiray Y.E., Ozkan A.D., Elibol Z., Tekinay T. Biotechnology and Bioengineering. 109 (8):1947-57 and "Nitrogen and sulfur deprivation differentiate lipid accumulation targets of *Chlamydomonas reinhardtii"* Cakmak T., Angun P., Ozkan A.D., Cakmak Z., Olmez T.T., Tekinay T. Bioengineered. 3(6):1-4

2.1. OBJECTIVE

In microalgae, *Chlamydomonas reinhardtii* is an attractive model for investigation of a wide range of biological functions such as starch metabolism [63], lipid metabolism [64], flagella formation [65] photosynthesis [66], synthesis of bioenergy carriers [67] or nutrient stress [68]. Although a large volume of literature is present on starch biosynthesis; TAG metabolism is relatively less documented in microalgae, including *C. reinhardtii*. As TAG production is important to the use of microalgae as biofuel, investigation of its synthesis mechanism is of considerable interest.

Nutrient deficiency is known to induce a wide variety of cellular response mechanisms in living organisms. Increase in lipid accumulation in different *C. reinhardtii* mutants during nitrogen (N) limitation and increased anaerobic H_2 production under sulfur (S) deprivation were previously reported [69, 70]. However, comparison of the effects of N and S starvation on

TAG accumulation and related parameters in microalgae has not been studied. In the first part of this thesis, the aim was to determine and compare the effects of N and S starvation on biodiesel feedstock production levels and evaluate the importance of mating type on the nutrient starvation response of *C. reinhardtii*.

2.2. MATERIALS AND METHODS

2.2.1. Algae, Culture Conditions and Experimental Outline

The CC-124 wild type mt (-) 137c and CC-125 wild type mt (+) 137c strains were obtained from the *Chlamydomonas* Resource Center (www.chlamy.org). The incubation temperature was at 23 °C under continuous light (150 μmoles photons m^2 s^{-1}) in liquid culture on a rotary shaker (120 rpm). The cells were grown in Standard Tris-Acetate-Phosphate (TAP) medium, which includes acetate (17.4 mM) as carbon source and tris-base (20 mM) as buffering [71]. (Table 6)

Table 6. Standard Tris-Acetate-Phosphate (TAP) medium components

Stock Solution (SL)	Volume	Components	Concentration in SL	Conc. In Final Medium
Tris base	2,42 g	$H_2NC(CH_2OH)_3$ Tris(hyrroxymethyl)-aminomethan		$2.00 . 10^{-2}$ M
TAP-salts (Beijerinck salts)	25 ml	NH_4Cl	15 $g.L^{-1}$	$7.00 . 10^{-3}$ M
		$MgSO_4.7H_2O$	4 $g.L^{-1}$	$8.30. 10^{-4}$ M
		$CaCl_2.2H_2O$	2 $g.L^{-1}$	$4.50 . 10^{-4}$ M
Phosphate solutions	1 ml	K_2HPO_4	28.8 g. 100 ml^{-1}	$1.65 . 10^{-3}$ M
		KH_2PO_4	14.4 g. 100 ml^{-1}	$1.05 . 10^{-3}$ M
Trace Elements Solutions(Hutners trace Elements)	1 ml	$Na_2EDTA.2H_2O$	5.00 g.100 ml^{-1}	$1.34 . 10^{-4}$ M
		$ZnSO_4.7H_2O$	2.20 g.100 ml^{-1}	$1.36 . 10^{-4}$ M
		H_3BO_3	1.14 g.100 ml^{-1}	$1.84 . 10^{-4}$ M
		$MnCl_2.4H_2O$	0.50 g.100 ml^{-1}	$4.00. 10^{-5}$ M
		$FeSO_4.7H_2O$	0.50 g.100 ml^{-1}	$3.29 . 10^{-4}$ M
		$CoCl_2.6H_2O$	0.16 g.100 ml^{-1}	$1.23 . 10^{-4}$ M
		$CuSO_4.5H_2O$	0.16 g.100 ml^{-1}	$1.00 . 10^{-4}$ M
		$(NH_4)_6MoO_3$	0.11 g.100 ml^{-1}	$4.44. 10^{-6}$ M
Acetic acid, conc.	1 ml	CH_3COOH		

Table 7. Standard TAP, TAP-N (TAP medium without N) and TAP-S (TAP medium without S) medium components for 1 ml.

Stock Solution (SL)		**TAP**	**TAP-N**	**TAP-S**
	Components			
Tris base	$H_2NC(CH_2OH)_3$ Tris(hyrroxymethyl)-aminomethan	2420 mg/L	2420 mg/ml	2420 mg/ml
TAP-salts (Beijerinck salts)	NH_4Cl	400 mg/L	-	400 mg/L
	$MgSO_4.7H_2O$	100 mg/L	100 mg/L	$MgCl_2$/ 39.3 mg/ml
	$CaCl_2.2H_2O$	50 mg/L	50 mg/L	50 mg/L
Phosphate solutions	K_2HPO_4	108 mg/L	108 mg/L	108 mg/L
	KH_2PO_4	54 mg/L	54 mg/L	54 mg/L
Trace Elements Solutions(Hutners trace Elements)	$Na_2EDTA.2H_2O$ $ZnSO_4.7H_2O$ H_3BO_3 $MnCl_2.4H_2O$ $FeSO_4.7H_2O$ $CoCl_2.6H_2O$ $CuSO_4.5H_2O$ $(NH_4)_6MoO_3$	1 ml	N-Hutners 1 ml	1 ml
Acetic acid, conc.	CH_3COOH	1 ml	1 ml	1 ml

The starting cell density, approximately $2.86x10^4$ cells ml^{-1}, was inoculated in all groups. For N starvation studies, cells were centrifuged at 2000 g for 3 min at room temperature; cell pellets were kept and washed twice in TAP medium without N (TAP-N medium) studies (Table 7). For S deprivation, instead of using N free TAP medium, TAP medium without S (TAP-S medium) was used (Table 7). Each treatment consisted of triplicate flasks. In all media, initial pH values were set to 7 before algal cell inoculation and pH value of the media was checked every 24 hours during 7 days of incubation period. During this period, initial pH values did not deviate more than 5%.

By counting cells by using hemocytometer, lugol solution (Sigma) and Image-J, a java-based image processing program developed at the National Institutes of Health [72], cell growth and size were monitored. Total cell biovolume was calculated using the equation "B=CV", in which B is the total biovolume, C is cell count, and V is cell volume.

For relative dry weight measurement, approximately $1x10^9$ cells were centrifuged at 3000 rpm for 5 minutes, pellet was dried for 5 minutes at room condition, weighed and incubated at 80 °C for 48 hours, thereafter cells were re-weighed. Cells from all experiment groups (CC-124 and CC-125 strains grown in TAP, TAP-N and TAP-S media) were harvested every 24 hours for 7 days following N and S starvation.

2.2.2. Quantification of Chlorophyll and Carotenoid Content

For quantification of chlorophyll a, b, c and carotenoid, protocol described by Jeffery and Humphrey et. al. [73] was used with some modifications. Approximately $1x10^7$ frozen cells were resuspended in 500 µL of 90% acetone, incubated by mixing for 15 minutes and centrifuged at 15000 rpm for 5 minutes. After that, the supernatant was loaded in a 96-well-plate. The absorbance of the supernatant at 470, 630, 647, 664 and 750 nm wavelengths were measured and chlorophyll a, b, c and carotenoid contents were calculated the formulae given in literature [73, 74]. Total chlorophyll results were presented as a sum of chlorophyll a, b, and c.

2.2.3. Quantification of Protein Content

Protein extraction was performed according to Weiss et. al. [75] with some modifications. Frozen cell pellets were resuspended in lysis buffer consist of 50 mM Tris–HCl pH 8.0, 2% SDS, 10 mM EDTA, protease inhibitor mix. This suspension was subjected to sonication (3510E-DTH, Branson) for 1 min at 60% power (~7 watts/pin), frozen in liquid nitrogen for 1 minute, thawed and centrifuged at 13000 rpm for 20 minutes at 4 ºC. The supernatant was then used for protein determination with Bradford method [76].

2.2.4. Quantification of Neutral Lipids and Starch Content

Quantification of neutral lipid by using Nile Red was performed as described previously [77]. Approximately $29.3x10^4$ cells ml^{-1} were stained with 22 μL of 7.8×10^{-5} M Nile Red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) (Invitrogen) dissolved in acetone (final concentration 0.26 μM). Solution was left to incubate on a shaker for 15 minutes under dark conditions and washed twice with TAP-N or TAP-S media. Relative fluorescence intensity of Nile Red staining was quantified on a fluorescence spectrometer (SpectraMax M5, MDS Analytical Technologies) using 490 nm excitation and 585 nm emission values.

Total neutral lipid levels were also determined gravimetrically with Bligh and Dyer method described previously [78]. For this experiment, approximately $1x10^9$ cells were used .The lipids were extracted and separated with a final solvent ratio of chloroform:methanol:water of 1:1:0.5. In order to collect neutral lipids, the chloroform layer was transferred into a pre-weighed vial. After that, it is evaporated in a water bath (30 °C) using a rotary evaporator, dried in a vacuum oven (Vacucell, Model No: 2255); then, vials were reweighed.

For quantification of starch, cells were stained with 0.02% Safranin O (Sigma) and relative fluorescence intensity of Safranin O staining was measured using 435 nm excitation and 480 nm emission wavelengths [79].

2.2.5. Confocal Imaging of Live Cells for Observing Lipids and Chlorophyll

Nile Red (5 μg/ml final concentration; Invitrogen) was used for cell staining as described by Wang et al. [80]. Images were acquired using an LSM 510 confocal microscope (Carl Zeiss) and a Plan Apo 63 oil immersion objective lens with a numerical aperture of 1.40 - 0.60. The Nile Red signal was captured using a laser excitation line at 488 nm, and the emission was collected between 560 and 600 nm; chlorophyll fluorescence was captured using a laser excitation line at 633 nm, and the emission was collected at 650 nm. Images were merged and pseudo-colored using ZEN 2008 CLSM user interface software. For three-dimensional imaging, *Z* stacks through an entire cell were acquired at 0.2 to 0.4 μm intervals, and each set was computationally projected using ZEN 2008.

2.2.6. Fourier Transform Infrared Spectroscopy (FTIR)

A 1.3 ml sample was aliquoted from each replicate flask for each experimental group. The samples were centrifuged and the supernatant was removed. After that, the cells were resuspended in 70 μl of distilled water. 30 μl of them was then deposited on a 96 well silicon microplate, and oven-dried at 42 ºC for 45 minutes [69]. The plate was placed in a micro well plate accessory and FTIR spectra were collected using a Nicolet 6700 Research FT-IR Spectrometer (Thermo Scientific).

FTIR spectra were collected over the wave number range 4000–600 cm^{-1}.

Each sample was analyzed in triplicate. Bu using the automatic baseline correction, spectra was corrected and they were scaled to the amide I peak. In order to observe the change of bands independent from the cell number, the obtained information was analyzed on a per cell level.

2.2.7. Transmission Electron Microscope (TEM) Imaging of Algal Cells for Observing Cellular Structure

The preparation method was adapted from Santhana et. al. with slight modifications [81]. The samples taken from algae were centrifuged and the supernatant was removed. After that, the pellets (20 µl) are incubated with 1 ml of 1% Paraformaldehyde, 1% Glutaraldehyde in a 1-0.5 M Sodium Phosphate buffer (pH=7) for overnight. The cells were washed with sodium buffer of 0.1 M for three times. After centrifuging at 1000 g for 10-15 min, the cells were exposed to 1% of Osmium Tetroxide solution dissolved in 0.5 M Sodium buffer and they were incubated for 4 h at 4 ºC. After rinsing the cells with distilled H_2O, they were dehydrated with 70%, 96% and 100% ethanol, respectively, for two times, incubated at 10-15 min and centrifuged at 1000 g. Polymerization was done with pure resin in embedding oven at 65°C for 24-48 hours after the microalgae infiltrated by mixture of ethanol and resin (1:1) for 1 hour. The blocks were trimmed and cut to 100 nm ultra thin sections by using ultramicrotome and placed them on carbon grids, suitable for TEM. The specimens then were stained with Uranyl Acetate and Lead Acetate.

2.2.8. Statistical Analysis

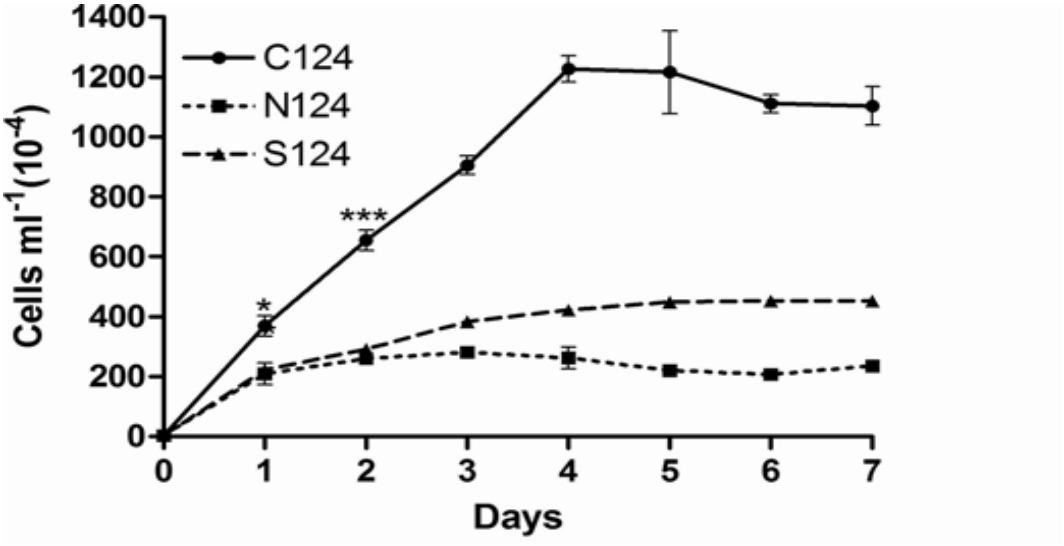
In this part, shown data are the mean values of at least three separate samples that are collected at two different times (n=6). The fluctuation ranges of each point on the figures were not indicated to avoid complication of the figures. Statistical analysis was accomplished by means of average values, standard errors and t- test (two tails, pair type) with the significance criterion of 0.05, 0.01 or 0.001.

2.3. RESULTS AND DISCUSSION

2.3.1. Effects of Nitrogen and Sulfur Starvation on Growth of Algae

In order to observe the effects of S and N starvation on algae growth, cell count and volume measurements were performed during seven days of nutrient deprivation. Starting cell density which was approximately $2.86x10^4$ cells/ml was incubated in 50 ml of culture media. While N starved CC-124 cells entered stationary phase on the first day with $209x10^4$ cells, control and S starved cells entered stationary phase with a maximum of $1227x10^4$ and $422x10^4$ cells on day 4 (Figure 5). CC-125 control and S starved cells entered stationary phase with a maximum of $1050x10^4$ and $534x10^4$ cells on day 6 while N starved cell density reached their highest level with a maximum of $364x10^4$ cells on day 4 (Figure 5).

A



B

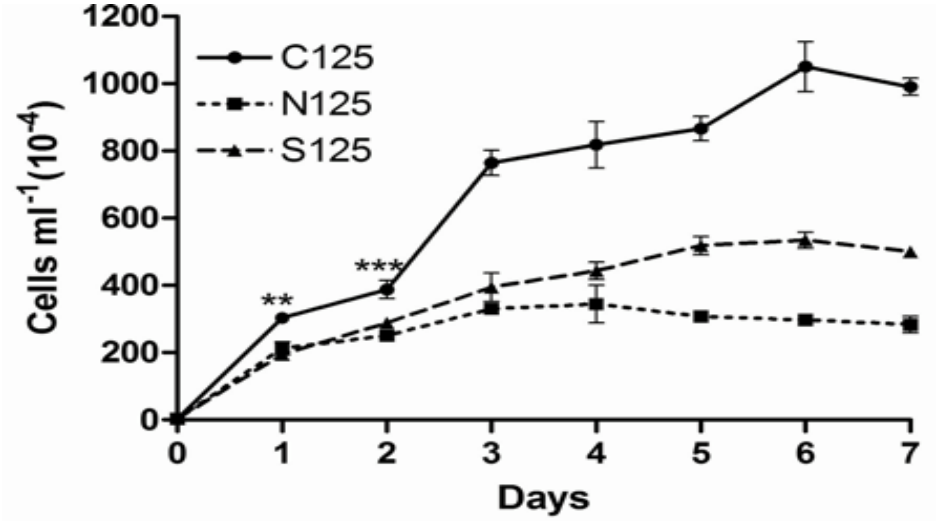


Figure 5. Graphs represent the changes in cell counts of CC124 (A) and CC125 (B) strains in response to N and S deprivation. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively. *, **, *** symbols represent significance evaluated across all experiments (P<0.05*, 0.01* or 0.001***).

Initial cell biovolumes in CC-124 and CC-125 were 87.8±19.1 and 103.9±13.4 μm^3 per cell, respectively. Increase of cell biovolume in response to S deprivation was much higher than the increase under N deprivation in both strains during seven days of experiment. Maximum increase in cell volume in N or S starved CC-124 and CC-125 strains was observed four days after nutrient starvation with 2.9-6.1 and 1.7-5.8 folds respectively. During seven days of nutrient starvation, this increase was followed by a subsequent decline resulting in a final increase of 1.8-4.4 and 1.6-4.1 fold respectively, (Figure 6).

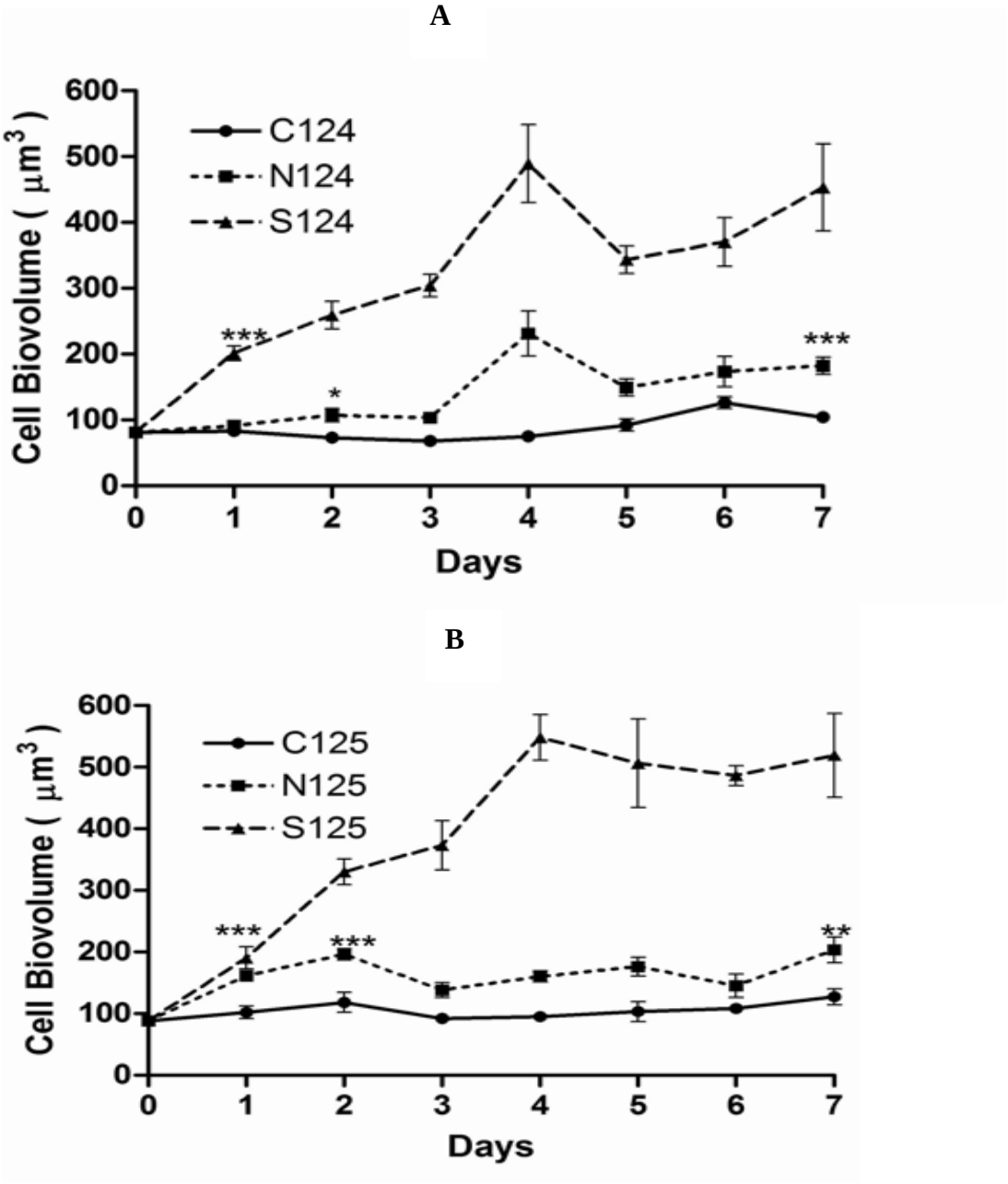


Figure 6. Graphs represent the changes in cellular volume of CC124 (A) and CC125 (B) strains in response to N and S deprivation. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively. Symbols (*, **, ***) denote significance evaluated across all experiments (P<0.05*, 0.01* or 0.001***).

Maximum increase in cell biovolume was observed four days after N and S starvation therefore it was wanted to check possible changes in relative dry weight of cells (Table 1). Relative dry weight measurement showed that 4 days of N and S starvation lead to approximately 31.8% and 27.4 %, and 23.3% and 20 % decrease in CC-124 and CC-125 strains when compared to those of respective controls (Table 8).

Table 8. Relative dry weight and total neutral lipid levels (on a dry weight basis) of 4-day control, N starved and S starved CC-124 and CC-125 strains.

	124C	**124N**	**124S**
Dry weight (%)	12.1±1.1	8.3±0.7	9.3±0.6
Total neutral lipid (%)	16.8±2.2	39.8±5.5	37.6±4.1
	125 C	**125N**	**125S**
Dry weight (%)	11.4±0.9	8.2±1.1	9.1±0.7
Total neutral lipid (%)	14.2±1.2	41.4±3.7	39.7±5.1

After seven days starvation, total biovolume was gradually decreased by 62.6% and 54.6% in N-starved CC-124 and CC-125 cells, respectively. However, it increased by 2.2 and 3.1 fold after 4 day incubation; and this increase was followed by a subsequent decline in S-starved CC-124 and CC-125 cells (Figure 7).

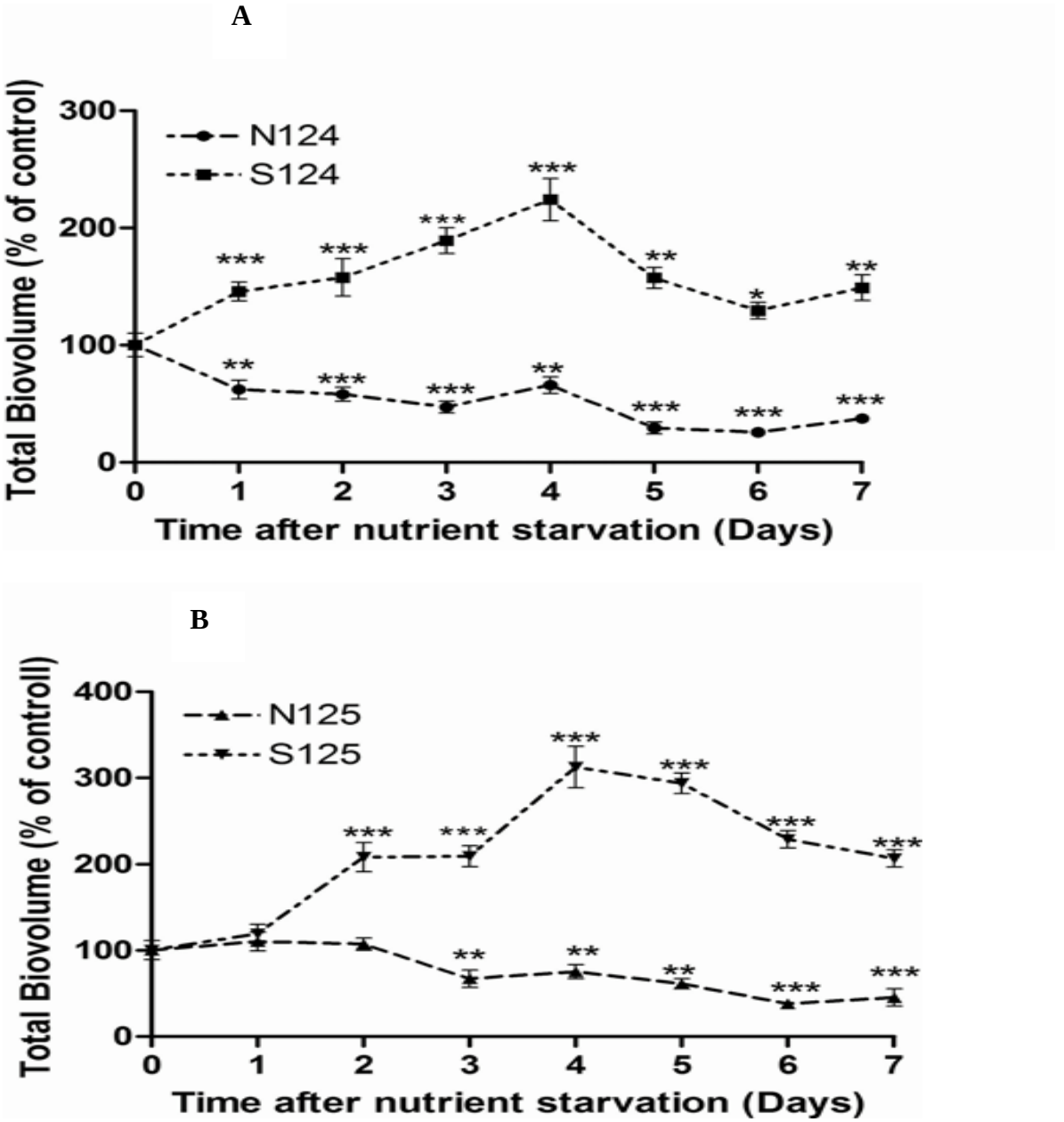


Figure 7. Graphs represent the changes in total biovolume of CC124 (A) and CC125 (B) strains in response to N and S deprivation. N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.*, **, *** symbols denote significance evaluated across all experiments (P<0.05*, 0.01* or 0.001***).

Nutrient starvation induces a wide range of changes through different mechanisms in microalgae. Some of these changes may result in increased lipid production and therefore increased biodiesel production. As a result of N and S starvation, decrease in growth rate of microalgae and increase in cell volume have been previously reported [82, 83]. On the other hand, some studies reported that algal growth rate and cell volume may both decrease upon N starvation [84].

In this study, it was shown that microalgal cell division was inhibited, relative dry biomass decreased, and total biovolume was reduced in despite of the increases in cellular biovolume under N deprivation. On the other hand, S starvation resulted in decrease of cell growth. The relative dry biomass is lower and cell volume increase is higher than N starvation resulting with the increased total biovolume. This result would also be an important factor for selection of an approach for biodiesel production strategies as cytoplasmic lipid accumulation is thought to be related to the volume of a microalgae [80].

2.3.2. Effects of N and S starvation on Chlorophyll and Carotenoid Content of Microalgae

Microalgae need to balance chlorophyll and carotenoid levels in order to use their carbon source efficiently and meet their energy demands. Under normal conditions, chlorophyll: carotenoid ratios of the CC-124 and CC-125 strains were 4.88 and 5.48 on average (Figure 8). However, in response to nutrient starvation there was a rapid and gradual decrease in chlorophyll: carotenoid ratio (Figure 8).

Decrease in chlorophyll content was higher in N starved cells than those of S starved ones. The decrease in total chlorophyll content was approximately 64-60% and 76-63% on first day and lasted with 78-65% and 89-46% during seven days of N-S starvation in CC-124 and CC-125 cells respectively (Figure 9).

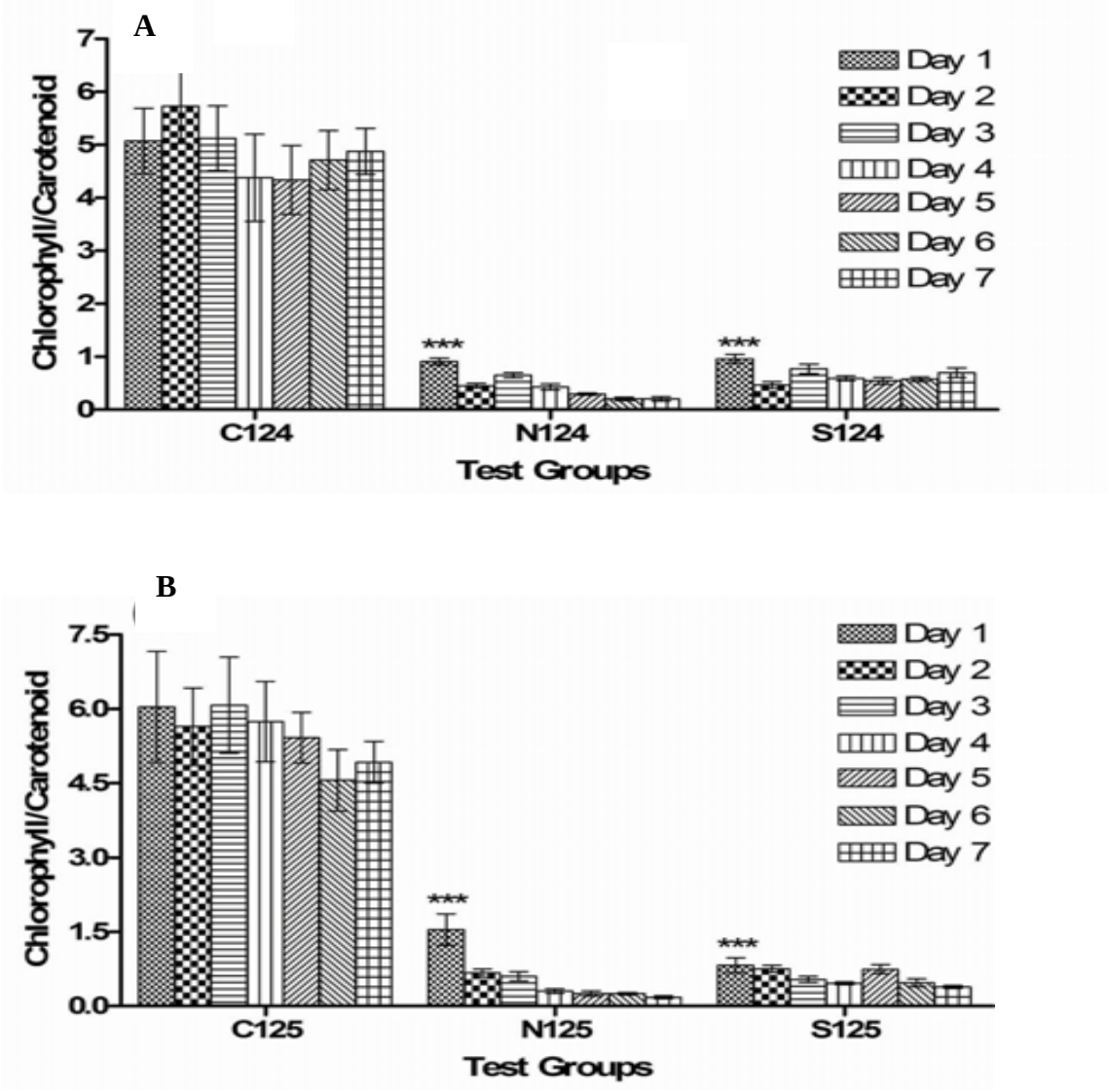


Figure 8. Graphs represent the changes in chlorophyll/carotenoid content of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point chlorophyll and carotenoid content were quantified from $1x10^7$ cells per sample. Total chlorophyll content was presented as a sum of Chlorophyll a, b, and c. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

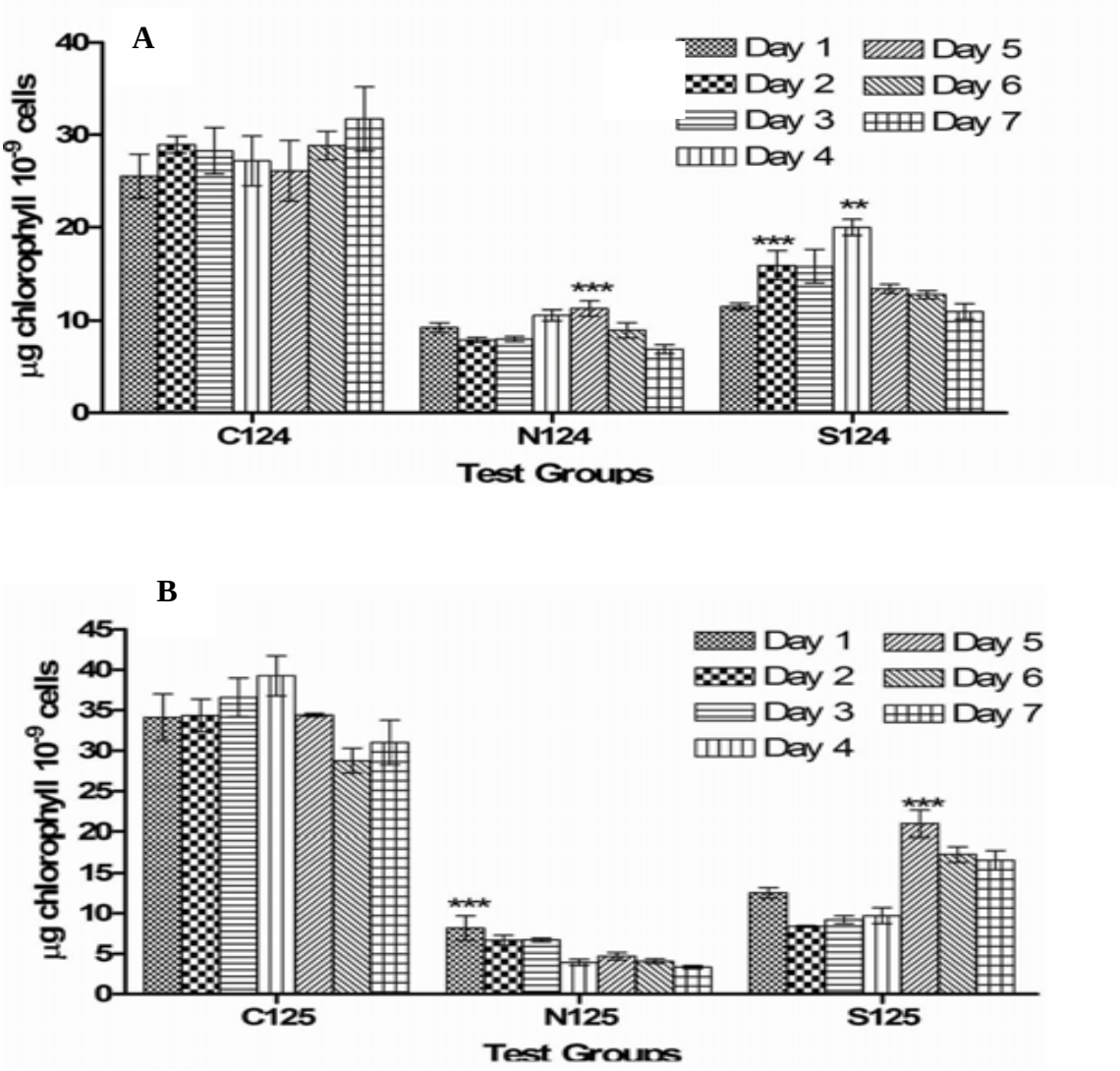


Figure 9. Graphs show the changes in chlorophyll content of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point chlorophyll content were quantified from $1x10^7$ cells per sample. Total chlorophyll content was presented as a sum of Chlorophyll a, b, and c. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

Response to N starvation, increase in carotenoid content was higher in CC-124 but lower in CC-125 strain than that of S starvation. Carotenoid content increased up to 634% or 427% after 5 days N or S starvation. This increase was followed by a subsequent decline and resulting with 514% and 183% increase after seven days of N or S starvation in CC-124. However, after seven days of N and S starvation, carotenoid levels were gradually increased from an initial value of 135-228% to a final value of 260-442% in CC-125 (Figure 10).

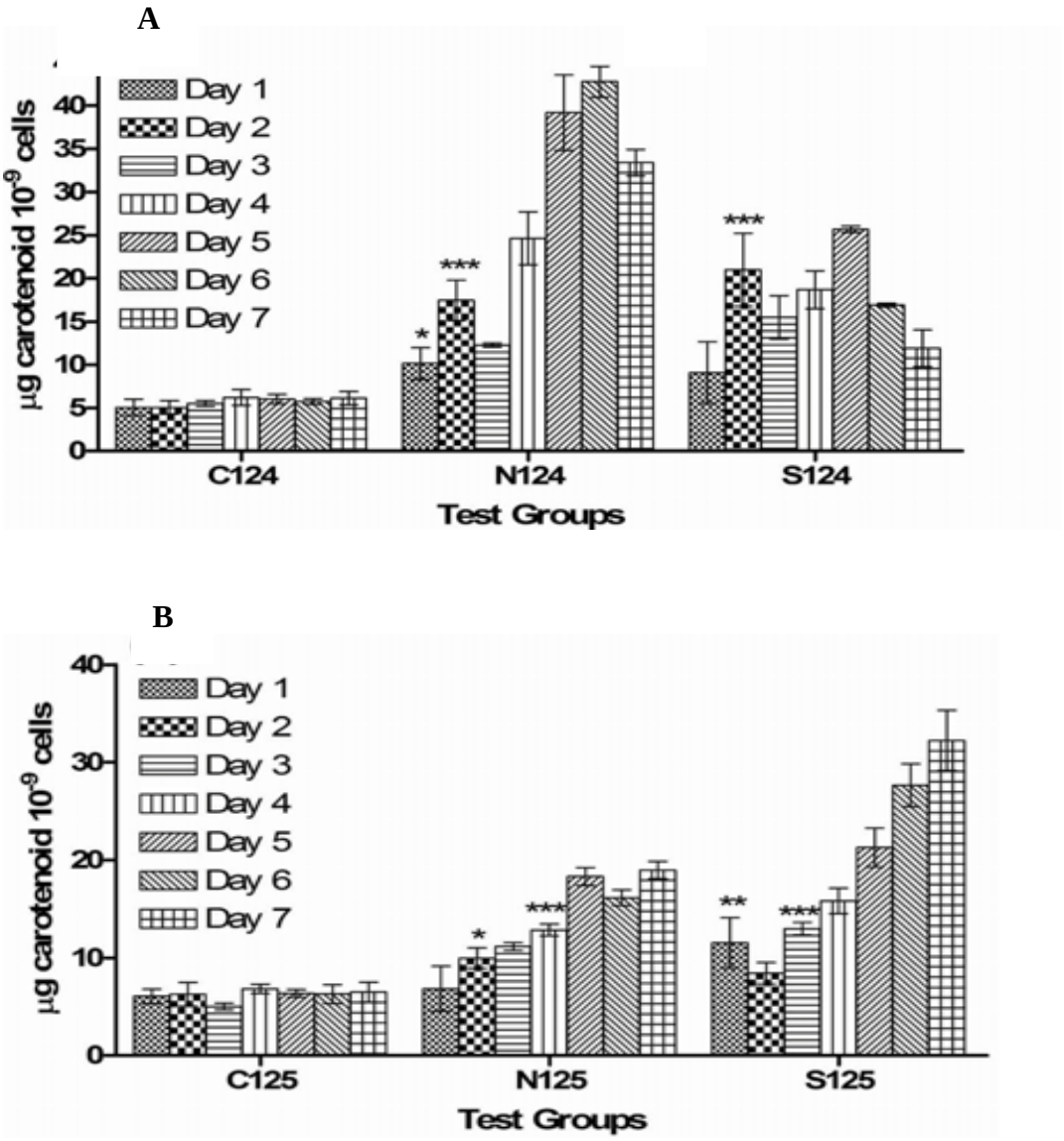


Figure 10. Graphs represent the changes in carotenoid content of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point carotenoid content were quantified from $1x10^7$ cells per sample. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

It has been previously reported that decrease in chlorophyll content in response to N and S starvation causes restricted ability to maintain the photosynthetic functions [83, 85]. According to some researches, some algae accumulate huge amounts of carbon in the form of carotenoids, starch and lipids [86]. Under stress condition, accumulation of carotenoids means the protection of algal cells from light damage [87].

In this study, results represent that N and S deprivation causes a decrease in chlorophyll levels and increase of carotenoid content. However CC-124 and CC-125 strains gave different mechanism responses to N and S starvation. Especially, increase in carotenoid content in S starvation was lower in CC-124, but higher in CC-125 than that of N starvation. This difference may be related to the mating type of CC-124 (mt-) and CC-125 (mt+).

2.3.3. Effects of N an S starvation on Protein, Total Neutral Lipid and Starch Levels in Microalgae

In response to nutrient starvation, concentration of total soluble protein in the algal samples decreased drastically. It was observed that an 82% decrease in protein content was occurred after 1 day of nutrient deprivation and a decrease of over 92% was observed by day 7 (Figure 11).

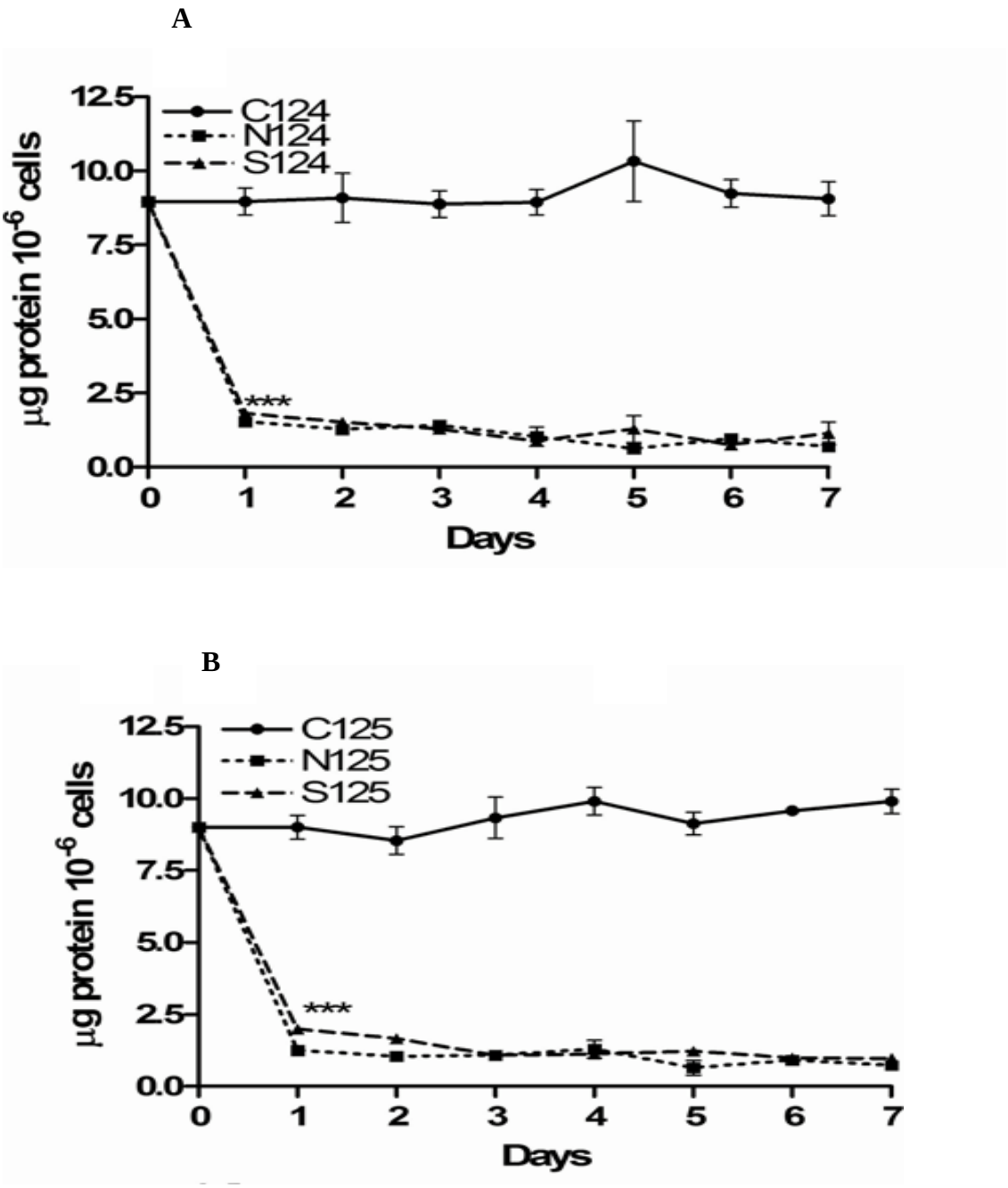


Figure 11. Graphs represent the changes in total soluble protein of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point the protein absorbance values were quantified from $1x10^6$ cells per sample. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

On the other hand, decrease in protein content inversely related to increase in total neutral lipid and starch levels. After 4 days of nutrient starvation, it is reached to maximum increase in total neutral lipid content for both strains. Increase in lipid content was 240% and 258% in CC-124 and 165% and 302% in CC-125 in response to N and S starvation respectively (Figure 12). In addition to Nile Red Fluorescence determination, analytical measurements also supported the increase in neutral lipid content of 4 days N and S starved cells. After 4 days of nutrient starvation, Increase in neutral lipid content for CC-124 N and S starved cells was approximately 136% and 123%, for CC-125 cells it was 190% and 172% when compared to their respective controls.

After 3 days of N and S starvation, starch levels increased rapidly to the maximum level approximately 706% and 713% in CC-124, 490% and 571% in CC-125 respectively (Figure 13).

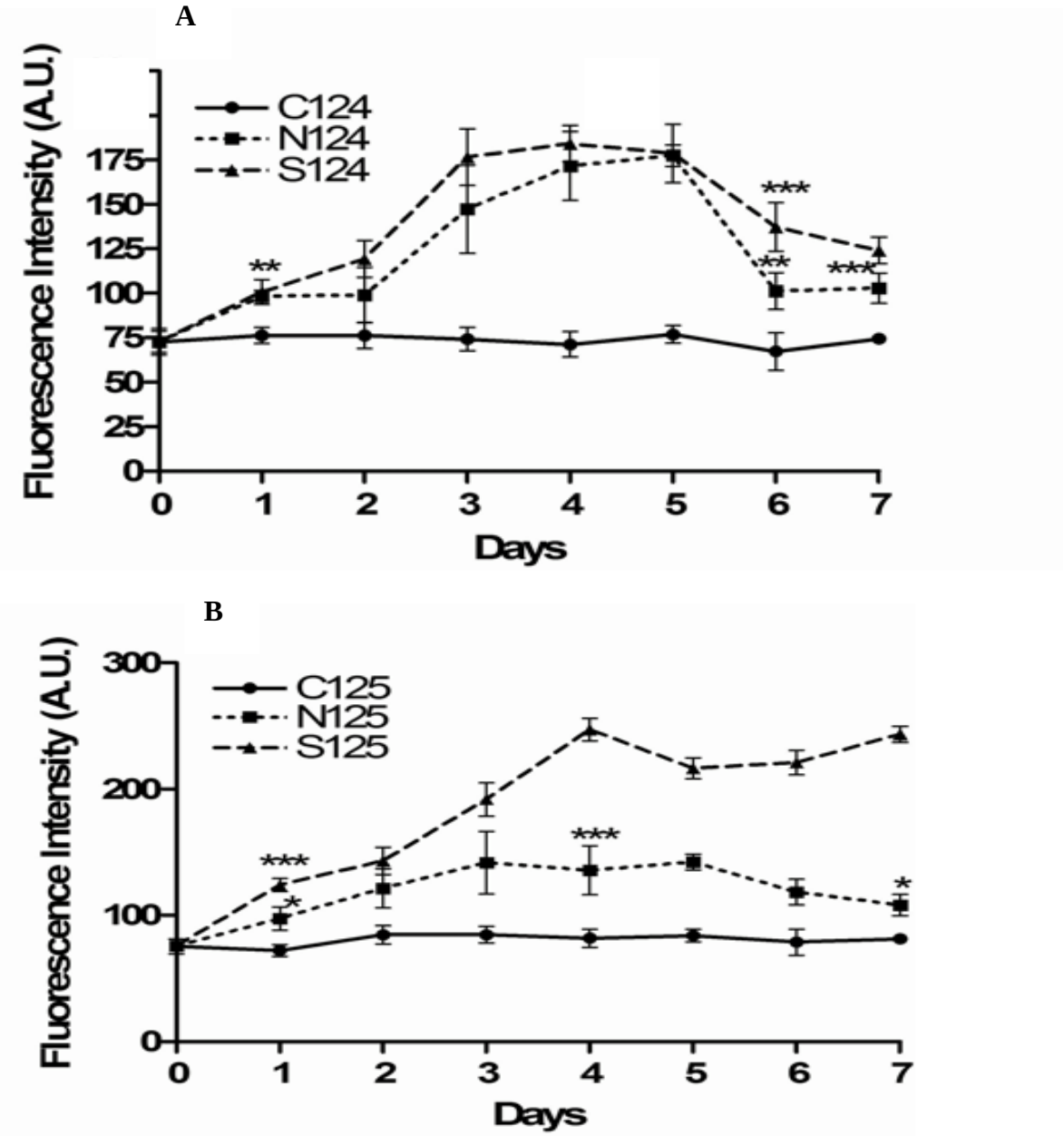


Figure 12. Graphs represent the total lipid content of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point the fluorescence emission of Nile Red (lipid) absorbance values were quantified from $1x10^6$ cells per sample. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

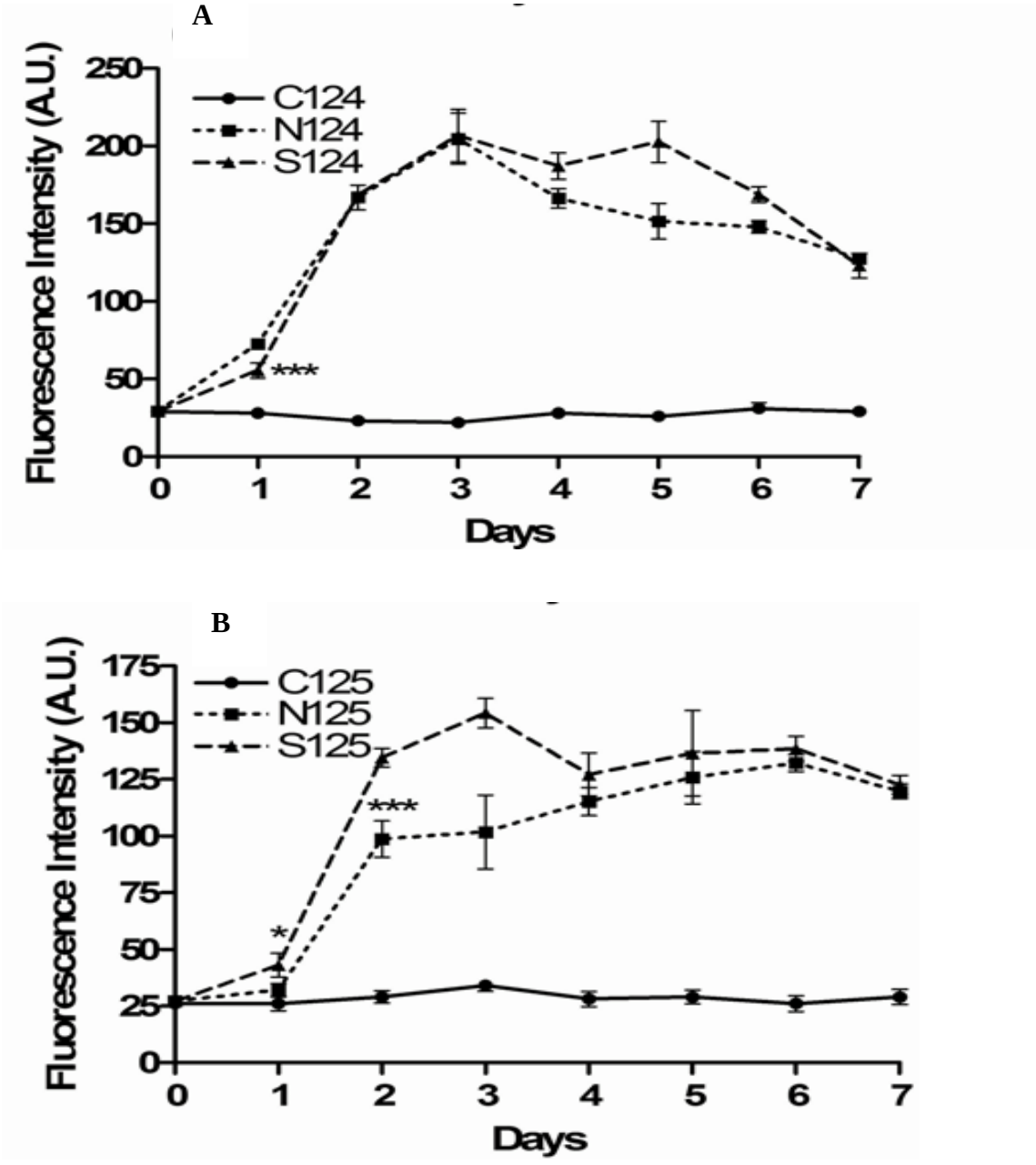


Figure 13. Graphs represent the starch content of CC-124 (A) and CC-125 (B) strains in response to N or S deprivation. For each treatment and time point the Safranin O (starch) absorbance values were quantified from $1x10^6$ cells per sample. Each data point is the mean (+-SE) of at least six samples. C124 and C125 indicate control cells, N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

In most cases, decrease in protein content was followed by a simultaneous increase in lipid and starch content in both strains upon exposure to nutrient starvation. Particularly, S deprivation caused higher lipid and starch accumulation than N deprivation. Moreover, in response to nutrient deprivation, increase in starch content was faster and higher than increase in lipid content.

When selecting biofuel production strategies, there is an important factor which is the potential competition between synthesis of lipid derivatives and carbohydrates. If starch metabolism interferes with lipid production in N or S-starved algae, then disabling starch synthesis may be a simple and effective method to increase net lipid production. Increased lipid synthesis on a dry weight basis has been reported in *C. reinhardtii* mutants with defective starch synthesis machinery [88], though lipid content was not investigated on a per cell basis. Another starchless *C. reinhardtii* mutant was determined to show 1.5 to 2.0-fold increase in lipid content per cell, suggesting that lipid and starch syntheses do antagonize each other [80]. However, high production of both starch and oils has been recently reported from *C. reinhardtii* and this result is inconsistent with the competition hypothesis [89]. These studies show that increase in both starch and lipid content was greatly affected in response to nutrient starvation. However, maximum increase in starch content was 3 fold higher than increase in lipid content.

These results may point out that firstly *C. reinhardtii* wild type strains accumulate starch and then lipid synthesis is stimulated together with carbohydrate accumulation under nutrient starvation.

2.3.4. Analysis of Confocal Microscopy to Determine the Effects of N an S Starvation

In the initial stages of N and S starvation, Ellipsoid-shaped cells which correspond to the normal morphology of *C. reinhardtii* cells were replaced with larger and spherical cell shapes gradually (Figure 14). After longer nutrient starvation, this situation was followed by cell mass reductions.

In this study, it is observed that triacylglycerols which may account for the increase in cell volume are stored in cytosolic lipid bodies in microalgae. Essentially, three dimensional pictures taken from day 4 of N or S starved cells show that the amount and volume of cellular cytosolic lipid bodies are lower in N starved cells than that of S starved ones (Figure 14). This suggests that the density and volume increase of lipid bodies is directly related to increase in biovolume of the *C. reinhardtii*.

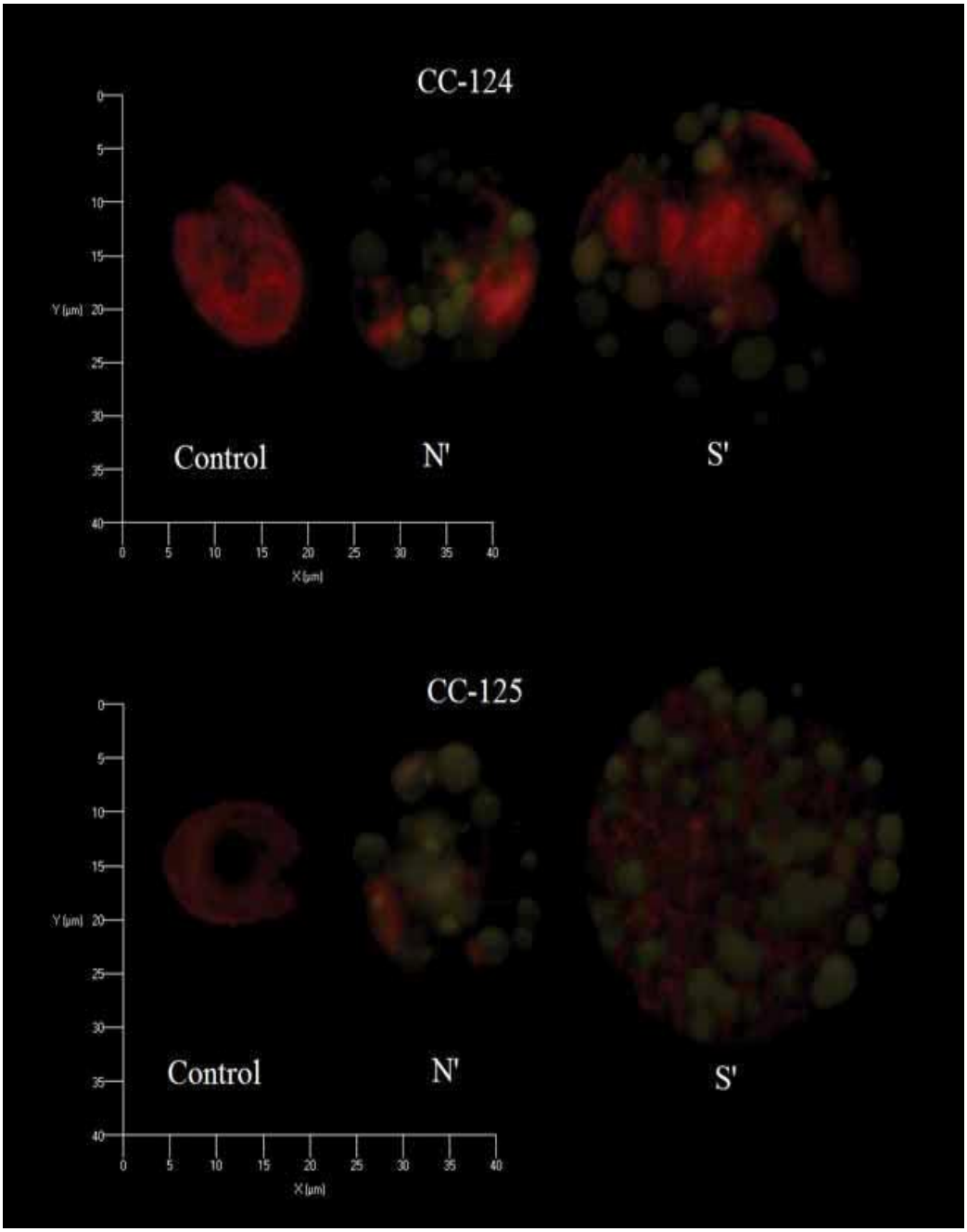


Figure 14. Graphs show the representative three-dimensional confocal fluorescence microscopy images of four days N or S starved CC-124 (top) and CC-125 (bottom) strains. Red and green droplets mean chlorophyll autofluorescence and Nile Red fluorescence, respectively. C, N' and S' means control cells nitrogen starved cells and sulfur starved cells, respectively.

2.3.5. Quantification of Relative Triacylglycerol, Oligosaccharide and Polysaccharide by Using FTIR

In this study, FTIR measurement for detection of polysaccharide, oligosaccharide and triacylglycerol levels was performed after spectrophotometric detection of neutral lipid and starch levels, by using FTIR, quantification of biomolecules is a relatively new approach.

Using transmission mode, infrared spectra were recorded with 128 scans in the range 4000-600 cm^{-1}. The bands were designated to specific molecular groups on the basis of biochemical standards previously described [90]. Bands were attributed to asymmetric stretching vibration of CH_2 of acyl chains (2922 cm^{-1}); Amide I absorption (1652 cm^{-1}); C-N stretching and CHN bending vibrations of amides from proteins (Amide II, 1544 cm^{-1}); asymmetric CH_3 bending of the methyl groups of proteins (1449 cm^{-1}); δCH_3 stretching of C- O groups (1380 cm^{-1}); PO_2^- asymmetric bonds associated with phosphorus compounds (1260 cm^{-1}) and PO_2^- symmetric stretching of phosphodiesters (1075 cm^{-1}).

There are three bands that were of particular interest. These are attributed to ester group (C=O) vibration of triglycerides (1744 cm^{-1}), membrane-bound oligosaccharide C-OH bond (1145 cm^{-1}), and C-O stretching frequencies coupled with C-O bending frequencies of the C-OH groups of polysaccharide (1045 cm^{-1}).

In response to nutrient starvation, protein concentration decreased by up to 93% according to result of total soluble protein determination experiment. However, FTIR spectra levels of amide I band obtained from control cells did not at any

time deviate more than 32%, therefore we chose the amide I band for normalization of the FTIR spectra and ratio determination. Quantification of relative TAG, polysaccharide and oligosaccharide contents were performed by calculating the ratio of TAG (1744 cm^{-1}), polysaccharide (1045 cm^{-1}), and oligosaccharide bands (1145 cm^{-1}) to the amide I band (1652 cm^{-1}). For each data set, we calculated fold-change values compared to their respective controls. For each time point, fold changes and standard errors were determined by fitting a linear model and empirical Bayes smoothing was applied to the standard errors for all samples studied. For control samples, ratios of TAG, polysaccharide and oligosaccharides to Amide were determined to a value of 1, independently for each time point. Increases in TAG, polysaccharide and oligosaccharide contents of nutrient starved samples were defined as values displaying "fold increase" in ratios of interested bands to Amide I band (Figure 16, Figure 17 and Figure 18).

On the other hand, in the sample preparations procedure, there are some variations that cause nonlinear deviations in results such as the thickness of dried sample layers on microtiter plates. The distribution of dried cells on the microtiter plates is usually inhomogeneous. However, as a result of the drying procedure during sample preparation, all cells collapse forming a thin film of biomolecules on the silica plate sample holder (Figure 15). Thus, the thickness of this layer is crucial for the absorbance through the sample, rather than the optical property of the microalgae cells itself.

In this study, we minimized deviations because of drying procedure by limiting cell numbers and forming a sample film that give a maximum amide I absorption value of 0.2–0.3.

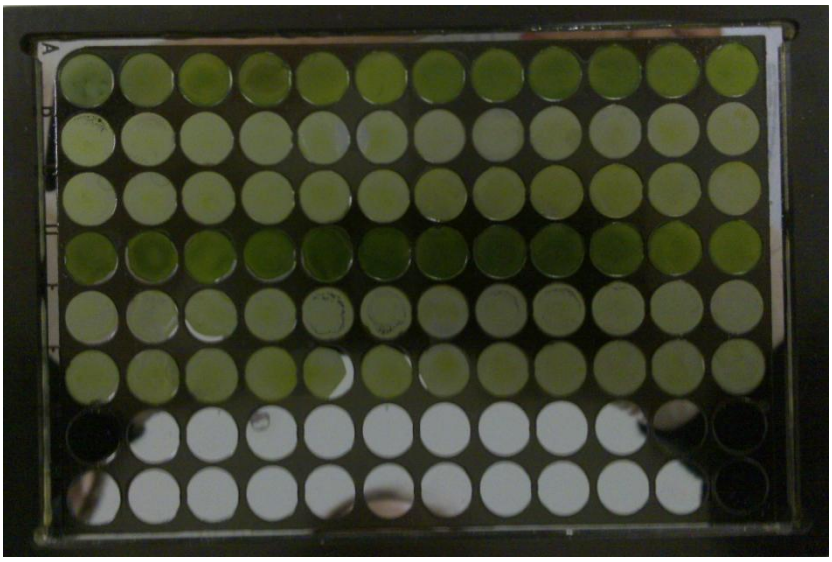


Figure 15. The picture shows the appearance of slica plate sample holder used in FTIR measurements. First three lines consist of CC-124 cells; second three lines consist of CC-125 cells. From above to bottom, control, N deficient and S deficient cells are aligned in one group.

In response to nutrient deprivation, infrared spectroscopy results showed that there are rapid increments in relative TAG, oligosaccharide and polysaccharide content. After 4 days of N or S starvation, increase in relative TAG content started with an initial increment of about 5 or 3.1 fold in CC-124 and 2.3 or 2.1 fold in CC-125, reached its maximum level with 6.9 or 15.3 fold in CC-124 and 29.1 or 16.5 fold in CC-125, respectively (Figure 16).

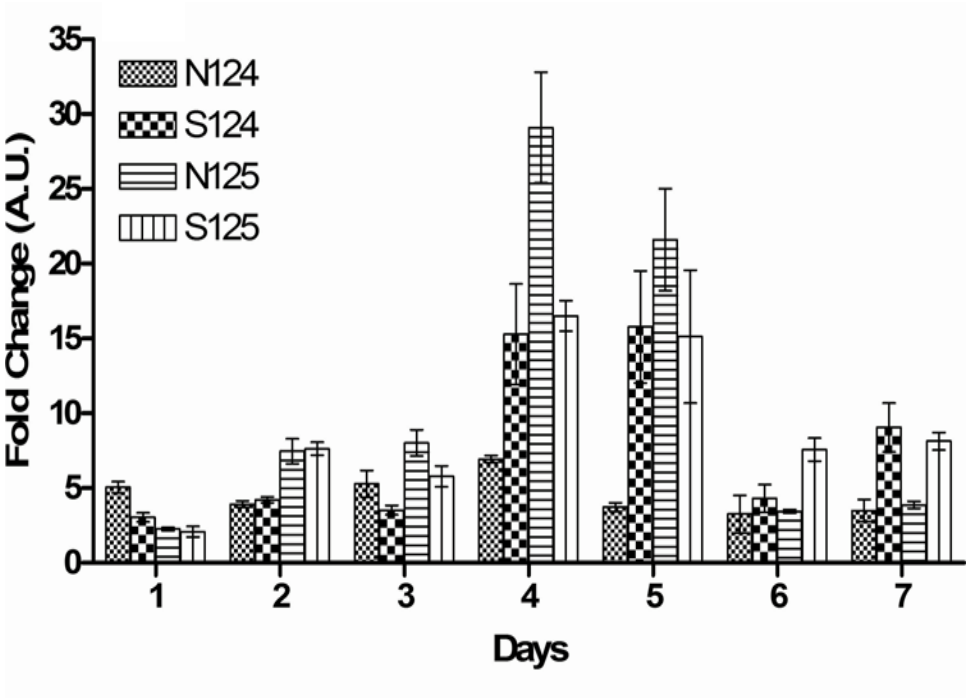


Figure 16. Graph shows the changes in TAG: amide I ratio within CC-124 and CC-125 strains in response to N or S deprivation. For all data sets, each point is the mean (±) of at least three FTIR spectra. N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

Obviously it is seen that S starvation caused higher TAG accumulation in both strains at the end of seven days of nutrient starvation. Generally, algae adjust to the environmental conditions by changing their lipid metabolism and composition [86].

Particularly under N-deprivation, it is reported that some algal species synthesize TAG as an efficient carbon source [39]. Also, it has been shown that nitrogen starvation increases the C/N ratio due to reduced level of amino acids synthesis and proteins which results to storage of an increased level of carbon in polysaccharides and/or lipids [69, 91]. Although S starvation has been analyzed as a potential tool for increasing hydrogen production in microalgae, there is no study for using S starvation or comparison of S and N starvation on increasing biodiesel feedstock production in literature. In this study, it is obtained that S starvation is a better way of increasing biodiesel feedstock production than N starvation.

In order to determine content of carbohydrates in microalgae, both oligosaccharide and polysaccharide peaks were analyzed. Increase in oligosaccharide content was higher in S starved cells than those of N starved ones. During seven days of nutrient starvation, the ratio of oligosaccharide to amide I displayed a gradual increase from an initial value of 1.2-0.9 to a final value of 6.3-9.8 in N and S starved CC-125 strains (Figure 17). However, in both N and S starved CC-124 cells, the ratio increased up to 7.9-12.9 on day 5 and ended with values of 6.8-9.1 after nutrient starvation period (Figure 17).

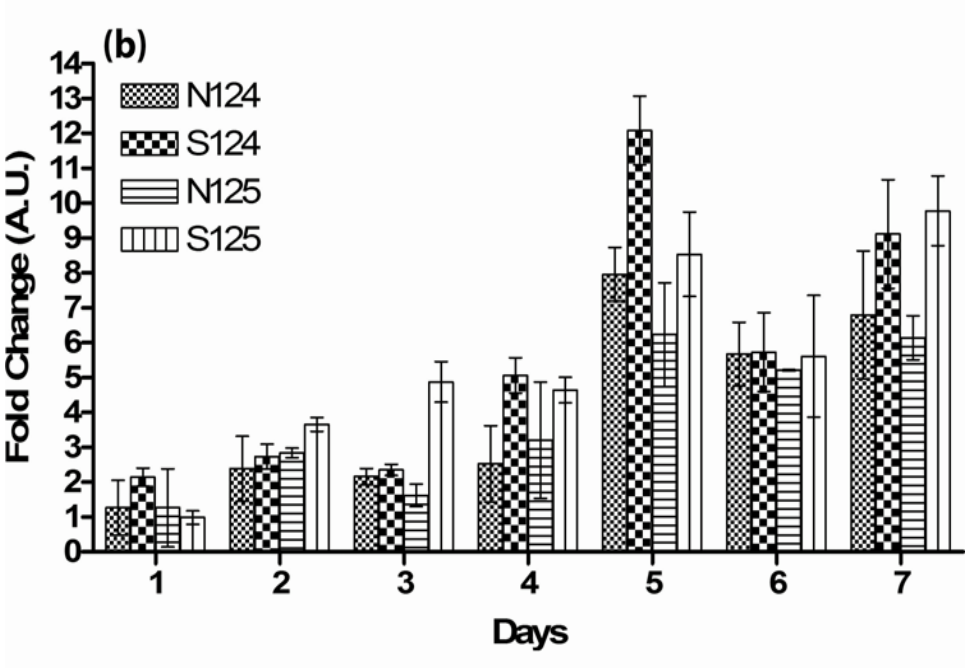


Figure 17. Graph shows the changes in oligosaccharide: amide I ratio within CC-124 and CC-125 strains in response to N or S deprivation. N124 and N125 indicate N starved cells; S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

Algae and plants generate some molecules in response to abiotic stress such as nutrient deprivation or biotic stress such as attack by pathogens. Systematic or localized resistance is induced by proteins, lipids, or oligosaccharides. Oligosaccharides were described as regulatory molecules which were biologically active at very low concentrations (nM) and were called elicitors [92]. During the past two decades, oligosaccharides were studied for their action on growth regulation, development, elicitation of defense systems against biotic or abiotic stress (such as nutrient limitation) in plants and algae [93]. In this study our results represent that oligosaccharide content may play significant role on storage of carbon molecules as polysaccharide or lipid structures. While increase in oligosaccharide content is so small at the first day of starvation, this increase gradually accumulated in response to nutrient starvation.

Increase in relative polysaccharide content in response to S starvation was higher in CC-124 compared to increase in N starvation. During seven days of nutrient deprivation, relative polysaccharide content gradually increased from an initial value of 3.8-2.7 to a final value of 9.8-17.6 in N and S starved CC-124 strains (Figure 18). However, in CC-125 cells, after four day of N and S starvation increase in relative polysaccharide content reached its maximum level with a value of 13.1-8.6 and this increase was followed by a subsequent decline, reaching a value of 5.7-5.6 on day 7(Figure 18).

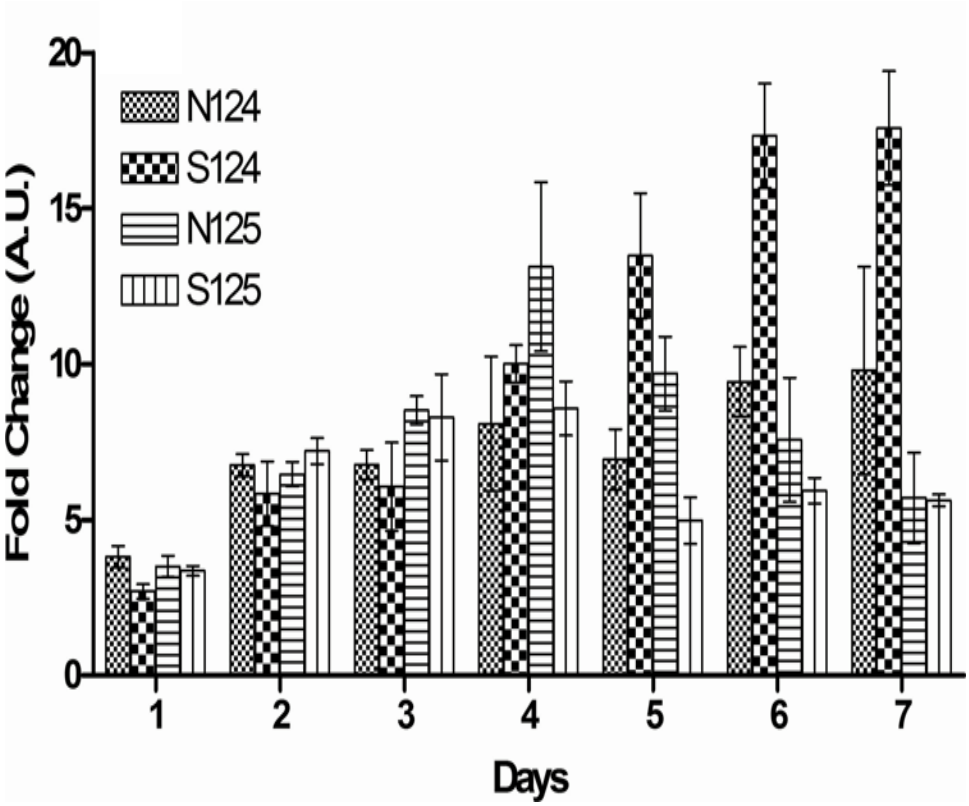


Figure 18. Graph shows the change in carbohydrate: amide I ratio within CC-124 and CC-125 strains in response to N or S deprivation. N124 and N125 indicate N starved cells, S124 and S125 indicate S starved CC-124 and CC-125 cells respectively.

In many plant and algae , carbohydrate and lipids are two major carbon and energy storage compounds especially under stress conditions. Although, lipid and carbohydrate synthesis pathways are well studied, during nutrient starvation possible interactions between the two pathways are less understood. In our results, fluorescence and infrared spectroscopy experiments show that the increase in carbohydrate level was fast in response to nutrient starvation. In addition to these, fluorescence spectroscopy measurement revealed that maximum increase in starch content was 3-fold higher than the increase in lipid content.

Finally, because of continuous increase in oligosaccharide content starting from first day of nutrient starvation, it is supposed that changes in oligosaccharide levels may have an important role in regulating carbon structure storage as lipid or carbohydrate compounds.

2.3.6. Analysis of TEM Microscopy to Determine the Effects of Nutrient Starvation on Microalgae Anatomy

According to researches, N is a significant macroelement for microalgal metabolism and the limitation of this element causes radical changes in several metabolic pathways. During the process of N deficiency, it is reported that microalgae degrade ribosomes and decrease enzyme activities involved in photosynthesis, glyoxylate cycle, gluconeogenesis and photosynthetic carbon fixation cycle while simultaneously stimulating carotenoid production to protect against oxidative stress, increasing the expression levels of TAG synthesis related genes and differentiating into gametes, which is considered a potential survival strategy since zygotes can resist adverse conditions [69, 94].

Nitrogen starvation causes dramatic anatomical changes in *C. reinhardtii* (Figure 19, Figure 20). Especially chloroplast of microalgae is degraded into smaller sphere-like sub-compartments and also cytoplasmic lipid droplets are formed (Figure 19). In addition to these, during S starvation chloroplast degradation is not as fast as N deprivation (Figure 20).

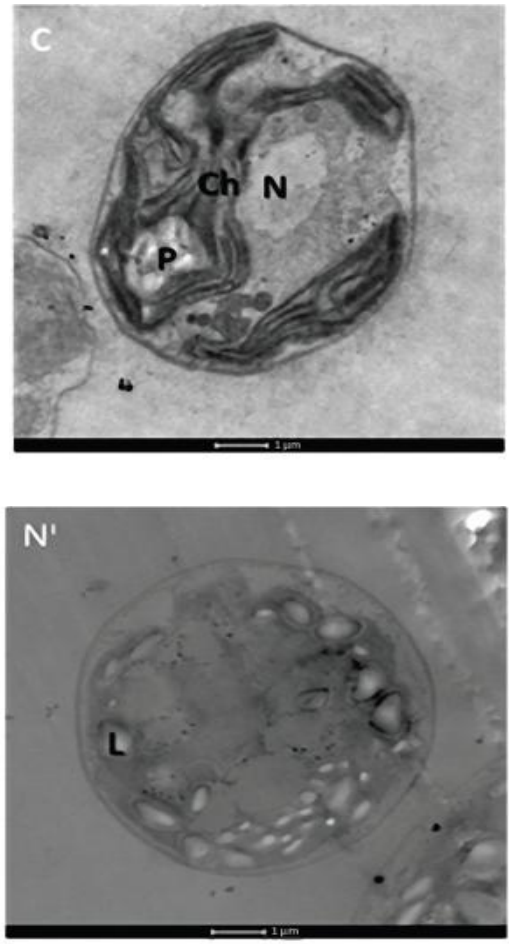


Figure 19. The pictures show transmission electron microscopy images of control, N-starved *C. reinhardtii* cells sampled on fifth day of incubation. Abbreviations C, N', Ch, P, N and L mean control cells, N-deprived cells, chloroplast, pyrenoid, nucleus, lipid bodies.

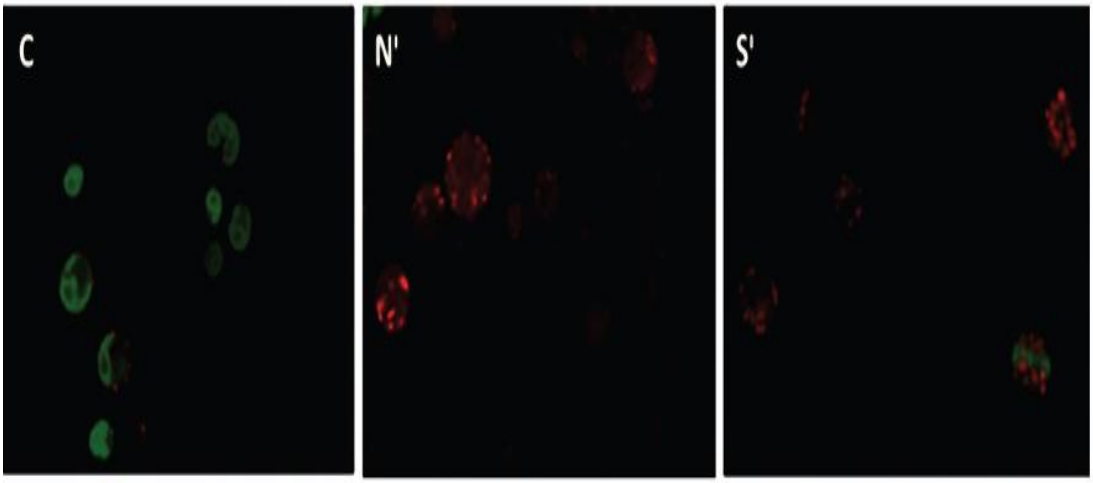


Figure 20. The pictures show confocal fluorescence microscopy images of control, N-starved and S-starved *C. Reinhardtii* cells sampled on fifth day of incubation. Green represents chlorophyll autofluorescence and red represents Nile red fluorescence. Abbreviations C, N' and S' mean control cells, N-deprived cells and S-deprived cells.

2.3.7. Comparison of N and S Starvation Effects on CC-124 and CC-125 Samples

In this study, under N starvation conditions, chlorophyll content of *C. reinhardtii* mt(+) (CC125) and mt(-) (CC124) strains showed a rapid decrease, while their S-deprived cells exhibit increased growth rates, cell volumes, neutral lipid and TAG accumulation compared to N-deprived cells (Table 9).

Moreover, in response to nutrient deprivation, the metabolic change was occurred in a time-dependent manner. Particularly, they reached a maximum level on the fourth and fifth days of deprivation and decreased or remained stable afterwards. This tendency may show that vegetative cells of *C. reinhardtii* can reduce the effects of N and S starvation for four to five days before the

stress associated with long-term nutrient deprivation leads to autophagy to recycle part of the cytoplasm including organelles.

In literature, it was reported that N deficiency induces autophagy, a self-degrading process common in eukaryote. Autophagy provides energy and raw materials that is needed for cellular repair, in many organisms [95] including *C. reinhardtii* [96]. Longer starvation period would lead cell death because the autophagy response is insufficient in longer period of nutrient deficiency. Dead cells may then be removed by other cells for their N or S content, therefore this situation allows limited growth and a stable cell count.

Table 9. Changes in growth and biochemical parameters in wild type *C. reinhardtii* CC-124 and CC-125 strains after four days of N or S deprivation.

Parameters tested	***C. reinhardtii* CC-124 (mt -)**		***C. reinhardtii* CC-125 (mt +)**	
	N deprivation	**S deprivation**	**N deprivation**	**S deprivation**
Cell Growth	83% decrease	65% decrease	66% decrease	49% decrease
Total biovolume	62.6% decrease	220% increase	54.6% decrease	310% increase
Relative dry weight	32% decrease	27% decrease	23% decrease	20% decrease
Protein level	88% decrease	89% decrease	87% decrease	89% decrease
Chlorophyll content	61% decrease	26% decrease	89% decrease	74% decrease
Carotenoid content	3.6 fold increase	2.8 fold increase	1.9 fold increase	2.3 fold increase
Cell biovolume	2.9 fold increase	6.1 fold increase	1.7 fold increase	5.8 fold increase
Starch level	2.3 fold increase	3.4 fold increase	4.3 fold increase	4.7 fold increase
Relative polisaccharide level	8.1 fold increase	9.9 fold increase	13.1 fold increase	8.6 fold increase
Total neutral lipid level	2.4 fold increase	2.6 fold increase	1.7 fold increase	3 fold increase
Relative TAG level	6.9 fold increase	15.3 fold increase	29.1 fold increase	16.5 fold increase

In this part of thesis, it was shown that N starvation generally exhibited similar effects as S starvation, but there were some negative impacts on cell count, total protein and chlorophyll levels that were more severe than S starvation (Table 9). This result is because of the relative importance and abundance of N compared to S. In detail, while S can be salvaged from dead cells or obtained from intracellular stores, N must be supplied constantly for adequate growth [80]. Also, N molecules in the biomass of dry *C. reinhardtii* is known to be over ten-fold greater than the S content [80]. It means that greater N content should be necessary for growth of *C. reinhardtii* cells and in order to facilitate growth of *C. reinhardtii* cells, comparatively lesser amount of S is needed. In addition, N-starved *C. reinhardtii* cells showed a lower enlargement in comparison with S-deprived cells. This was also correlated with the greater metabolic stress on N-starved samples (Table 9).

It was observed that effects of N starvation on cellular functions of algae are occurred more rapidly. Due to the necessary presence of N in proteins and metabolites that cannot be compensated by autophagy or other recycling pathways, N starvation causes sudden responses. However, S starvation leads less sudden but again severe responses in overall metabolism and cellular functions (Table 9). This delay probably promotes cellular recycling by autophagy and better accumulation of stress marker molecules (carotenoid, TAG etc.). (Table 9) (Figure 21)

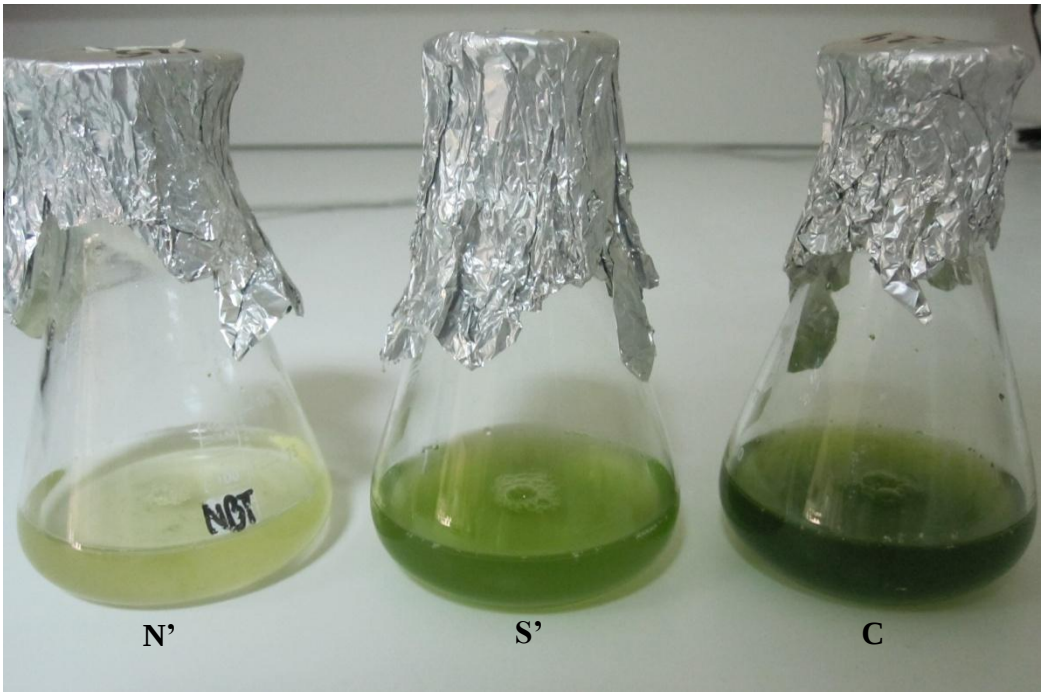


Figure 21. The picture shows the appearance of *C. reinhardtii* cells in different growth medium after seven days of incubation. Abbreviations C, N' and S' means control, N-deprived and S-deprived cells grow in control, N' and S' media.

2.3.8. Detailed Description of Relation between Nutrient Deprivation and TAG Production in Microalgae

It was observed that chlorophyll content decreased rapidly after S and N starvation period, while a corresponding increase in carotenoid content was also observed. It is known that *C. reinhardtii* restructure its photosynthetic mechanism during S deprivation period, resulting in a decrease in the expression of many proteins that constitute the photo system complexes I and II within 24 h [97]. Such adjustments occur to reduce oxidative stress, as reactive oxygen species (ROS) are generated during photosynthesis and the shutdown of the latter may afford a measure of control over their levels [98]. Also, the decrease in chlorophyll synthesis is interpreted to be a part of the alteration, deactivation and disassembly of photosynthetic complexes as a response to oxidative stress resulting from S starvation.

Similarly, N deprivation is closely related with the degradation of ribulose-1,5-bisphosphate carboxylase oxygenase to recycle the latter's N content [99] and the depletion of this protein may require alterations in the mechanism of photosynthesis, leading to the decrease in chlorophyll content observed in N-deficient *C. reinhardtii* [100, 101].

Carotenoid content increase is a response to the stress conditions brought by nutrient deficiency. Carotenoids are known to protect microalgae against oxidative stress, which can result from both starvation types tested [102]. As such, their accumulation may be a stress response intended to prevent oxidative damage.

In this part of thesis, it is showed that both starch and neutral lipids greatly accumulate in S-deprived *C. reinhardtii* and that those increases correspond to the rapid decrease in protein levels observed during the first day of starvation. Production of starch took priority over lipid synthesis, suggesting that the two metabolites may compete and that the disruption of starch metabolism may increase lipid production capacity, as has been suggested previously [80, 88]. While both metabolites were found to increase greatly upon starvation with no apparent antagonistic effects, this is likely the result of cell enlargement caused by S deprivation instead of a true lack of competition between lipid and starch syntheses. According to our experiment result, disappearances in energetic functions are observed. Flagella are lost, chlorophyll levels drop meaning anabolic reactions are severely reduced. Here in this study one of our conclusions is that upon reduction of energy consumption, the trend of metabolism favors storage of energetic, denser molecules. Lipids are highly energetic molecules having higher yield per gram than sugars and shown to increase up to great levels under nutrient deficiency while starch is known to be the densest form of sugars that is usually used for storage because of its biochemical structure.

Records detailing the use of N-starvation to increase lipid production for biodiesel production exist in literature [80], our study offers that S-starvation is the preferable approach due to the lack of adequate cell growth and biovolume attainment upon N-exposure.

2.4. CONCLUSION AND FUTURE PROSPECTS

In this part of thesis, the aim was to determine and compare the effects of N and S starvation on biodiesel feedstock production levels and evaluate the importance of mating type on the nutrient starvation response of *C. reinhardtii*.

Recently, the applicability of FTIR spectroscopy for the quantification of cellular amounts of macromolecules or elemental ratios has been studied [103]. Our study shows that the use of FTIR is a reliable method for quantitative measurement of biological macromolecules. The FTIR results were supported by fluorescence spectrometric measurements in this study.

Our results suggest that mating type is an important factor determining physiological response to mineral deprivation. Wild type CC-124 and CC-125 strains exhibit different levels of regulation responses under mineral deficiency.

Lastly, there are a large number of studies reporting increased hydrogen production using S deprivation [104] and increasing lipid production via N deprivation [105]. However, to our knowledge, there is no study so far reported on use of S starvation and the comparison of the effects of S and N deprivation in biodiesel feedstock production. Our results showed that four days of N or S starvation is a promising way of increasing algal biodiesel feedstock production. However considering that four days of S deprivation leads to an increased total biovolume and stimulates more lipid and carbohydrate accumulation, S starvation seems to be a better way of stimulating biodiesel feedstock production of wild type *C. reinhardtii* compared to N starvation.

In addition, as a further study, growth conditions (temperature, pH, carbon source, nitrogen source and some salts percentages) of S-starved microalgae may be optimized in a large scale to increase the efficiency of biodiesel production. Also, extraction of TAG as a biodiesel feedstock might be optimized as a continuation of the project.

After the extraction of lipid molecules used in biodiesel production, remained parts (biomass, carbohydrates, proteins, carotenoids, antioxidant molecules and etc) can be examined to use as a natural and sustainable high value added products in food, feed, cosmetic, agriculture and other industries.

CHAPTER 3 - Evaluation of Total Carotenoid Content and Antioxidant Capacity of Isolated Microalgae

3.1. OBJECTIVE

Dietary carotenoids are related to their possible actions as preventive agents in disease associated with oxidative stress. These electron-rich compounds can act as antioxidant and their possible role of protection from reactive oxygen has received much attention [106]. Also, some synthetic antioxidants, such as BHT and BHA, as they were found to be toxic and carcinogenic in animal models therefore synthetic types are needed to be replaced with natural antioxidant [107]. Thus, it is important to identify new sources of safe and inexpensive antioxidants of natural origin.

Some algae are considered as rich sources of natural carotenoids and antioxidants [108]. Although macroalgae have received much attention as potential natural antioxidants [109], there has been very limited information on antioxidant activity of microalgae [107].

Microalgae represent a resource of natural antioxidants, due to their enormous biodiversity that was much more diverse than higher plants. However, not all groups of microalgae can be used as natural sources of antioxidants, due to their widely varied contents of target products, growth rate or yields, ease of cultivation, and/ or other factors. Reports on the antioxidant activity of microalgae are limited [110, 111]. Therefore, it was desirable to identify some rich sources of carotenoid and antioxidants from a large group of microalgae.

The aims of this study were to identify new sources of natural and inexpensive antioxidants from 12 microalgae by determining their total carotenoid contents and investigating the relationship between antioxidant capacity and carotenoid content.

3.2. MATERIAL AND METHODS

3.2.1. Algae and Culture Conditions

In this study, used microalgae (STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11, and STA12) are isolated from different water resources. For growth of them, the incubation temperature was 23°C under continuous light (150 μmoles photons m^2 s^{-1}) in liquid culture on a rotary shaker (120 rpm). The cells were grown in Standard Tris-Acetate-Phosphate (TAP) medium, which includes acetate (17.4 mM) as carbon source and tris-base (20 mM) as buffering [71, 112] (Table 6).

3.2.2. Absorbance Measurement

Algal concentration estimation can be done by direct cell counts, chlorophyll content measurement, and absorbance or turbidity numerical correlations [113]. In this study, spectrophotometrical absorbance is the chosen method; optical densities were measured at 700 nm. For optical density measurements of algae, the wavelength of 700 nm is usually recommended [114]. The starting cell density, approximately $2x10^4$ cells ml^{-1}, was inoculated in all flasks. Absorbance was measured at 700 nm using Microplate reader (SpectraMax M5). In a 10-day period, 250 μl of samples taken at each day were put into wells of 96 well plate triplicates and measured by Microplate reader duplicates.

3.2.3. Dry Weight Measurement

For dry weight measurement, approximately $1x10^9$ cells were centrifuged at 3000 rpm for 5 minutes, pellet was dried for 5 minutes at room condition, weighed and incubated at 80 °C for 48 hours, thereafter cells were re-weighed. Cells from all experiment groups were harvested after 10 days of growth.

3.2.4. Carotenoid and Chlorophyll Content Measurement and Selection of Extraction Solvent

For quantification of chlorophyll a, b, c and carotenoid, protocol described by Jeffrey and Humphrey [73] was used with some modifications. Approximately equal cell numbers from all groups are centrifuged at 5000 rpm for 5 min, supernatants were discarded, and the pellets were washed with distilled H_2O. After discarding supernatant again, 5 ml of different extraction solvents (100% acetone, 100% ethanol, 100% methanol) were added to cell debris and vortexed for 90 seconds. The falcons were incubated at 25 ºC, 125 rpm for 1 h. After incubation period, they were centrifuged at 6000 rpm for 10 min. This extraction procedure was repeated until all pellets were observed as white. After that, the collected supernatant was loaded in a 96-well-plate. The absorbance of the supernatant at 470, 630, 647, 664 and 750 nm wavelengths were measured and chlorophyll a, b, c and carotenoid contents were calculated the formulae given by Jeffrey and Humphrey et al. and Lichtenthaler et al. [73, 74]. Total chlorophyll results were presented as a sum of chlorophyll a ,b and c.

3.2.5. Antioxidant Capacity Assay of Carotenoid Extract by Measuring DPPH Radical Scavenging Activity

The method was performed as described in literature with slight modification [115, 116]. An aliquot of each sample (30 μL) in Acetone/MeOH (1/1, v/v) was mixed with 200 μL of 100 μM 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) that was prepared with methanol. The mixture was shaken vigorously and then left to stand at room temperature for 60 min in the dark. The absorbance was measured spectrophotometrically at 520 nm against an acetone/MeOH (1/1, v/v) blank. Commercial β-carotene (Sigma) was used as a control in this experiment. The lower absorbance indicated the stronger scavenging activity. EC50 value (mg sample/mL) means the effective concentration at which 50% of the DPPH radicals were scavenged. The scavenging activity was calculated based on the percentage of DPPH radical scavenged.

3.2.6. Statistical Analysis

In this part, shown data are the mean values of at least three separate samples that are collected at two different times (n=6). Statistical analysis was accomplished by means of average values and standard errors.

3.3. RESULTS AND DISCUSSION

3.3.1. Isolation of Microalgae

For this study, in order to identify new sources of natural and sustainable carotenoids and antioxidants, 12 isolated microalgae were examined to determine their carotenoid content and antioxidant activity. These algae were isolated from different water resources in Turkey. The water samples was inoculated on TAP agar and 12 single colonies of microalgae were isolated and grown separately (Figure 22). These strains of microalgae were called as STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11 and STA12. STA1, STA2, STA3, STA6, STA7, STA9, STA10, STA11 and STA12 are green colonies while STA4 and STA8 are orange colony and STA5 is red colony (Figure 23).

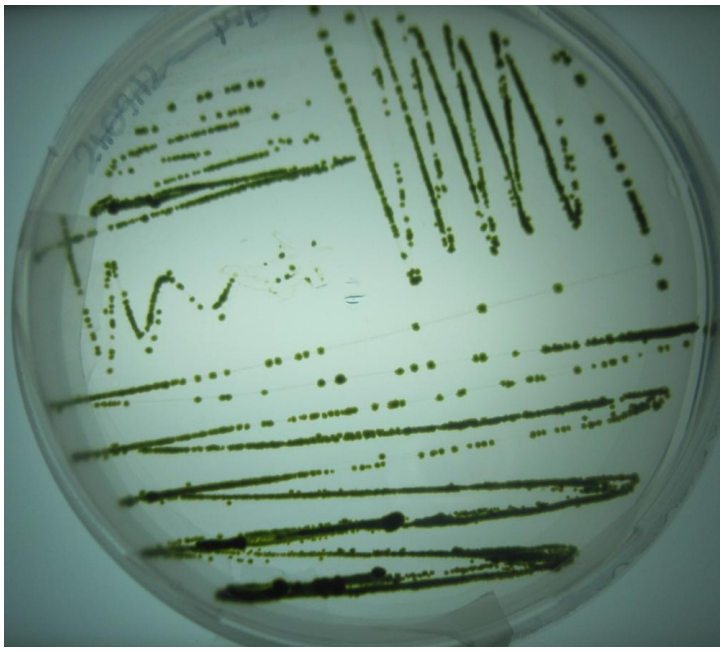


Figure 22. The picture shows the appearance of microalgae that are grown on TAP agar separately.

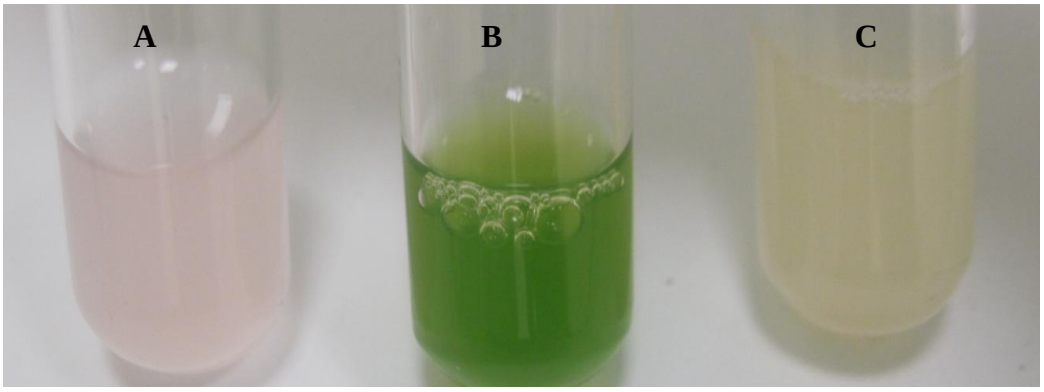


Figure 23. The picture shows the appearance of red (A), green (B) and orange (C) microalgae in TAP medium.

3.3.2. Selection of Solvent for Carotenoid Extraction

During this processes, algal carotenoid was extracted by using 3 types of solvents; Acetone, Ethanol and Methanol; at room temperature and the result was shown in Figure 24. Randomly selected microalgae which have different color ; STA2, STA5, STA7 and STA8, were grown in a TAP medium for 7 days and their carotenoids were extracted after equalizing their absorbance at 700 nm. After 3 extraction steps that provide obtaining white cell pellets, acetone were proved to be better than the other solvents for all algae type for extracting carotenoids (Figure 24).

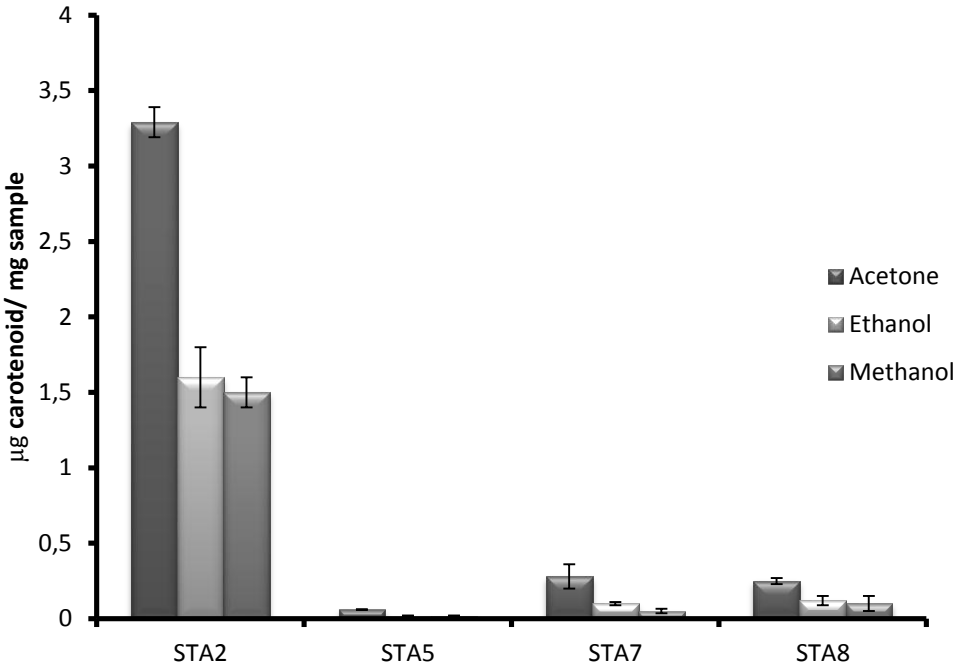


Figure 24. The graph shows the effect of different solvents (Acetone, Ethanol and Methanol) for extracting carotenoids of microalgae (STA2, STA5, STA7, STA8). Each data point is the mean of at least three samples.

Solvent extraction of algal constituents is widely used to extract metabolites like astaxanthin, β-carotene and essential fatty acids [117]. Among the different solvents used for extraction of carotenoids from microalgae, n-hexane, isopropyl alcohol, ethyl acetate, acetone could extract maximum carotenoids and xanthophylls. Methanol extract contained chlorophyll contaminants [117]. In this study, acetone, ethanol and methanol were chosen as a solvent for extracting carotenoids of microalgae. Because of reaching maximum extracted amount of total carotenoid with acetone, it was used for determining total carotene in all type of microalgae used in this study.

3.3.3. Determining Total Carotenoid Content of Microalgae

After determining efficient extracting solvent for microalgae, total carotenoid contents of STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11 and STA12 were determined. Carotenoids were extracted from 8 day grown algae in a TAP medium. Growth time was assigned after observing growth rate in a TAP medium. 8 days is required to reach stationary phase for microalgae used in this study. Figure 25 shows the results of total carotenoid content. STA3 contained 3.7 μg carotenoid/mg dry microalgae that was the highest amount of carotenoid content in all microalgae used in this study. However, STA5, red microalgae, contained 0.07 μg carotenoid/mg dry microalgae that was the lowest amount. Although carotenoid contents of other microalgae were between these values, STA2 and STA9 were also prominent to contain high amount of carotenoids.

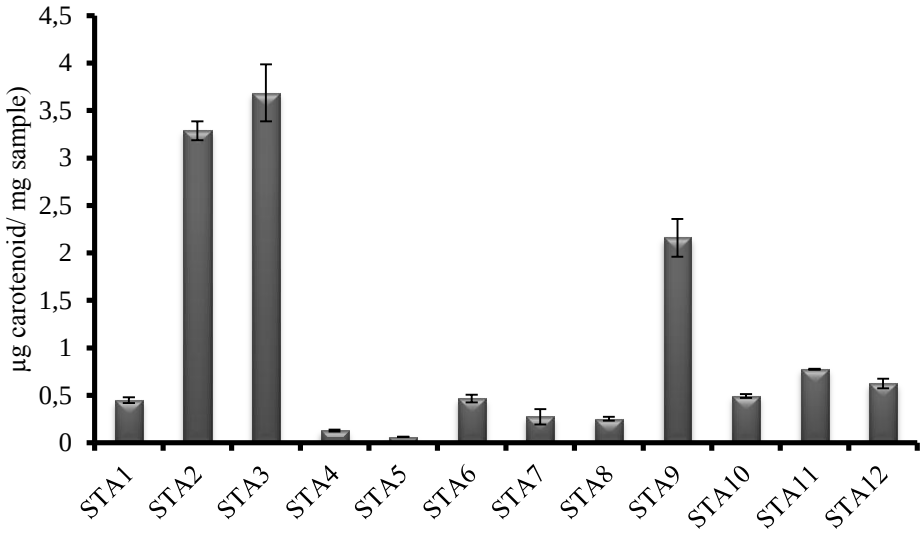


Figure 25. The graph shows the total carotenoid contents (µg carotenoid/mg sample) of microalgae (STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11 and STA12). Each data point is the mean of at least three samples.

3.3.4. Determining Antioxidant Capacity of Carotenoid Extract by Measuring DPPH Radical Scavenging Activity

The antioxidant activity of algal carotenoid extract was determined by measuring DPPH radical scavenging activity. EC50 means the effective concentration of sample that can decrease DPPH concentration by 50% [116]. Figure 26 presents the EC50 values of scavenging DPPH radicals of the all microalgal extract. Lowest effective concentration means the highest antioxidant activity of algal extract [107, 116]. Therefore with the 0.4 mg sample/ ml EC50 value, STA3 demonstrated maximum antioxidant activity while carotenoid extract of STA1 showed minimum antioxidant activity with the maximum EC50 value which was 3.8 mg sample/ ml (Figure 26). Besides to STA3; STA2, STA8 and STA9 had much higher antioxidant activity than other algal extracts (Figure 26).

According to the literature, carotenoids are potential biological antioxidants that are able to absorb the excitation energy of singlet oxygen radicals (SOR) into their complex ringed chain. The radical scavenging lessens tissue decay by reducing the damage. Furthermore, the processes brought about by SOR have also been linked to the ageing process and to the pathogenesis of several diseases, and several neurological disorders [59, 118]. For instance, lutein has been recommended for the prevention of cancer and diseases related to retinal degeneration [119, 120].

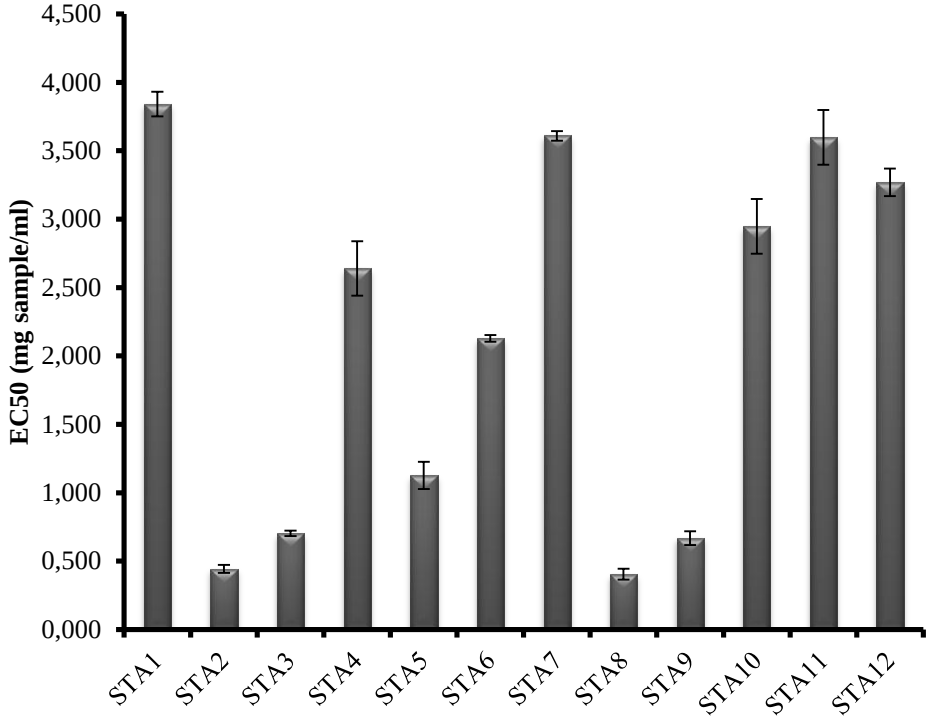


Figure 26. The graphs show EC50 values* of DPPH radical quenching activity of the carotenoid extract of microalgae (STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11 and STA12). Each data point is the mean of at least three samples.

*EC50 value means the effective concentration of sample that can decrease DPPH concentration by 50%.

By using Chlorella sp. containing carotenoids such as β-carotene and lutein as s feed for animals, Nakashima et al. found that improvement in cognitive impairment could be prevented to a significant extent [121]. Generally, microalgae are rich in carotenoids, some of which have been recognized to exhibit a strong antioxidant capacity.

In this study, we observed similar results to the previous researches. STA2, STA3 and STA9 that have high total carotenoid content among others (Figure 25) showed also higher antioxidant activity according to the results of DPPH radical scavenging activity test (Figure 26).

In order to use these microalgae as a source of natural and sustainable carotenoid and antioxidants, their growth rate should be substantially high to extract these compounds efficiently. Figure 27 shows growth of STA2, STA3 and STA9 in a TAP medium. Calculated biomass as a dry weight after growing all microalgae in a TAP medium for 10 days are observed in Table 10. These results displayed that adequate amount of STA2, STA3 and STA9 biomass could be achieved to obtain high concentration of carotenoid and antioxidant (Table 10).

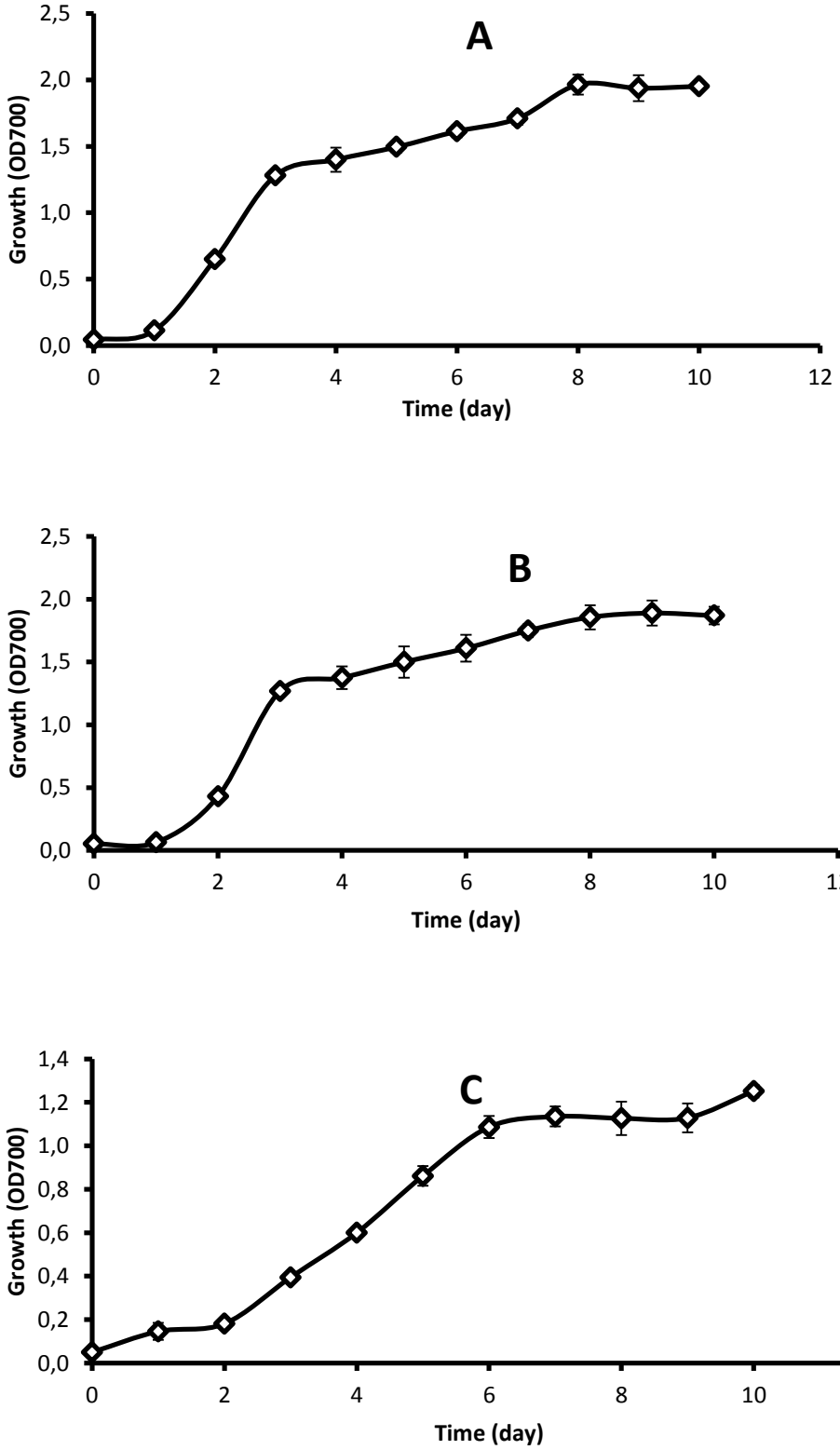


Figure 27. The graphs show the growth curves of STA2 (A), STA3 (B) and STA9 (C) respectively. Each data point is the mean of at least three samples.

Table 10. Relative dry weight, total carotenoid content and EC50 values of microalgae (STA1, STA2, STA3, STA4, STA5, STA6, STA7, STA8, STA9, STA10, STA11 and STA12).

Microalgae	Dry weight (mg/ml)	Carotenoid Content (µg Carotenoid/mg sample)	EC50 (mg sample/ml)
STA1	0,65±0,035	0,45±0,03	3,84±0,09
STA2	0,84±0,023	3,29±0,1	0,44±0,03
STA3	0,75±0,02	3,67±0,3	0,70±0,02
STA4	0,15±0,01	0,13±0,01	2,64±0,2
STA5	0,24±0,04	0,063±0,001	1,13±0,1
STA6	0,50±0,005	0,47±0,04	2,13±0,025
STA7	0,43±0,013	0,27±0,08	3,60±0,035
STA8	0,29±0,002	0,25±0,02	0,40±0,04
STA9	0,97±0,04	2,16±0,2	0,67±0,05
STA10	0,61±0,02	0,49±0,02	2,95±0,3
STA11	0,49±0,005	0,77±0,006	3,60±0,2
STA12	0,55±0,008	0,62±0,05	3,27±0,1

3.4. CONCLUSION AND FUTURE PROSPECTS

There is a considerable interest, demand and search for novel and sustainable source of carotenoids in general. This is mainly due to its functionality of precursor for Vitamin A, antioxidant potentials and also natural color. With current consumer awareness regarding the benefits of naturally derived carotenes over synthetic ones, extensive research is going on utilizing of biotechnology as a tool for such compounds. Even though carotenoids produced by synthetic route are economical, they have potential to be cancerous on long term usage instead of anti-cancerous agent [122] and they are also less efficient as provitamin-A due to monoisomeric form compared to naturally derived ones. Microalgae can serve some advantages for producing carotenoids such as being natural, sustainable and safe.

The objective of present study was to identify new sources of natural, sustainable and inexpensive carotenoids and antioxidants from 12 microalgae isolated from different water resources by determining their total carotenoid contents and investigating the relationship between antioxidant capacity and carotenoid content. Our results demonstrated that among 12 microalgae, STA2, STA3 and STA9 contained substantial amounts of carotenoids in their metabolism and these carotenoids extracts showed strong antioxidant activity. With the ease of cultivation and high growth rate, these three microalgae might have a potent to use as a natural and sustainable carotenes in food, nutraceutical and some other related applications.

As s further study, culture conditions such as nutrient requirements, illumination conditions, temperature and pH of the media could be optimized for improving algal cell growth and high carotenoids production. Enhancement of carotenoids production was carried out adopting various stress conditions like changing salt concentration, nutrient deficiency, light and temperature. Therefore these parameters would be studied in order to improve carotenoid production and antioxidant activity efficiency to meet the demand of economical, sustainable and natural carotenoids.

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