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Master Thesis

Characterization of a novel strain of Coelastrella terrestris for the biotechnological potential of secondary carotenoids

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Affidavit

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 10.01.2022 Maria KÄFER *(manu propria)*

Preface

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Abstract

The present work aimed at the characterization of rather unexplored green microalga species *Coelastrella terrestris*, isolated from Iceland including morphological analysis, growth behaviour, physiological and pigment composition. For improving biomass and pigment productivity, the microalga was cultivated under mixotrophic conditions, compared with photoautotrophic cultivation. Experiments were conducted in shake flasks and lab-scale stirred photobioreactors. It was observed that *C. terrestris* was capable of synthesizing adequate amounts of a complex mixture of secondary carotenoids, like astaxanthin, adonixanthin, and canthaxanthin. The reddish-orange cells accumulated a maximum yield of 3.125 ± 0.550 mg of secondary carotenoids per g biomass. This was achieved by the addition of 1 $g \cdot L^{-1}$ sodium acetate in Bolt's Basal medium with high light intensity under 21 days of cultivation in photobioreactor. The most important factors influencing secondary carotenoid production were nitrogen starvation and light stress. The average mass composition was 47.45 ± 4.68% astaxanthin, 28.94 ± 2.61% adonixanthin and 23.61 ± 5.13% canthaxanthin. It should also be emphasised that more than 85% of the fatty acids produced were unsaturated. Since approximately 50% of all secondary carotenoids produced were the rare xanthophylls adonixanthin and canthaxanthin and because of the low cultivation temperature, the green microalga has a lot of industrial potential especially for the secondary carotenoid production in cooler areas, like its place of origin. This master thesis represents the first investigation of the accumulation of secondary carotenoids of the species *C. terrestris*.

Kurzfassung

Ziel dieser Arbeit war die Charakterisierung der bisher weitgehend unerforschten grünen Mikroalge *Coelastrella terrestris*. Die Charakterisierung der aus Island stammenden Alge umfasste die morphologische Analyse, das Wachstumsverhalten, die physiologische Zusammensetzung sowie die Pigmentzusammensetzung. Um die Biomasse- und Pigmentproduktivität zu verbessern, wurde die Mikroalge unter mixotrophen Bedingungen kultiviert. Die daraus stammenden Ergebnisse wurden mit Ergebnissen aus photoautotropher Kultivierung verglichen. Die Experimente wurden in Schüttelkolben und Photobioreaktoren im Labormaßstab durchgeführt. Es konnte festgestellt werden, dass *C. terrestris* in der Lage war, eine komplexe Mischung an Sekundär-Carotinoiden wie Astaxanthin, Adonixanthin sowie Canthaxanthin zu akkumulieren. Die rötlich-orangen Algenzellen waren in der Lage einen maximalen Ertrag von 3,125 ± 0,550 mg Sekundär-Carotinoide pro g Biomasse anzureichern. Dies konnte durch Zugabe von 1 $g \cdot L^{-1}$ Natriumacetat in Bolt's Basal Medium in 21 Tagen Kultivierung im Photobioreaktor erreicht werden. Lichtstress und Stickstoffmangel konnten als wichtigste Faktoren, welche die Produktion der Sekundär-Carotinoide beeinflussen, identifiziert werden. Der durchschnittliche Massenanteil an Astaxanthin lag bei 47,45 ± 4,68%, Adonixanthin erreichte 28,94 ± 2,61%, gefolgt von 23,61 ± 5,13% Canthaxanthin. Hervorzuheben ist besonders, dass mehr als 85% der der produzierten Fettsäuren ungesättigt sind. Etwa 50% aller produzierten Sekundäre-Carotinoide von *C. terrestris* nehmen die seltenen Xanthophylle Adonixanthin und Canthaxanthin ein. Diese Tatsache sowie die im Vergleich zu anderen Mikroalgen niedrige Kultivierungstemperatur, machen *C. terrestris* zu einem attraktiven Produzenten von Sekundär-Carotinoiden, insbesondere für die Produktion in kühleren Regionen. Diese Masterarbeit ist der bislang weltweit erste Nachweis über die Akkumulierung von Sekundär-Carotinoiden in der Mikroalge *C. terrestris*.

1 Introduction

1.1 Green Microalgae – Chlorophyta

Microalgae are complex, mostly photosynthetic microorganisms, which are characterized by a high genetic diversity of nuclear, mitochondrial, and chloroplast genomes [1,2]. In fact, their genetic variety makes algae the most diverse organisms on the planet. They are capable of producing a broad range of biochemical molecules like carbohydrates, lipids, proteins, and other essential compounds like vitamins and antioxidants which represent the sustainable and natural options for many chemical-based compounds used in a broad range of industries [2,3]. The concentration of these components, however, does not only depend on the specific strain but is further determined by the physiological response of environmental stress factors like nutrient starvation or light intensity [2].

Apart from various uniforming characteristics, microalgae have developed a significant number of morphological and ecological variations during their evolution, reflecting their extremely diverse habitat [4]. This allows algae to be classified into eleven major phyla: Cyanophyta, Rhodophyta, Chlorarachniophyta, Charophyta, Glaucophyta, Euglenophyta, Cryptophyta, Haptophyta, Heterokontophyta, Dinophyta, and Chlorophyta [2].

The Chlorophyta are capable of producing chlorophyll *a* and *b* and additionally a broad range of carotenoids, including ß-carotene and xanthophylls, which comprise, for instance, astaxanthin and canthaxanthin. The microalgal biomass containing those high-value products is used as supplementation for nutraceutical and functional foods [2].

Those pigments are produced in the chloroplasts, which are embedded by a double layer membrane with thylakoids linked lamellae. Pyrenoids can be found, if present, in the chloroplast encircled by starch, which represents the main type of storage carbohydrates. The cell wall of most green microalgae is firm consisting of cellulose building blocks [4].

Under favorable conditions, microalgae accumulate primarily polysaccharides and lipids to 50-70% of their biomass, which can be used as combustible material in the biofuel industry. Since microalgae can be found everywhere in the environment, they can be seen as very robust with unique adaptation machinery. Metabolism dependent, these unicellular organisms can have a photoautotrophic, photoheterotrophic, or even a mixotrophic or heterotrophic nature [3].

In addition to light, H_2O, and CO_2 as a carbon source, other macronutrients such as nitrogen, phosphorus, and sulfur, as well as micronutrients such as K, Fe, Mg, and Ca are needed to ensure

normal physiological and metabolic activities of the microalgae. Micro- and macronutrient deficiencies can lead to disruptions and changes in these metabolic activities, which in turn can lead to changes in biomass components. [5].

Because of the broad spectrum of natural products produced by microalgae, much attention is drawn to them. Between 1926 and 2016 over 4000 studies on bioactive components of microalgae were conducted. From an industrial point of view, these bioactive products have a significantly higher financial value as compared to whole, dried microalgae biomass, even if the amount, which is produced, is much less [2].

1.1.1 Coelastrella sp.

The green microalga is commonly found growing in soil and belongs to the genus Chlorophyta. It is characterized by the peculiar sculpture of its cell wall [6]. The cells of the strain of *Coelastrella* are characterized by their lack of flagella or other zoospores which make the cells incapable of moving on their own. Other researchers have also investigated the production of secondary carotenoids (SC) under stress conditions in several *Coelastrella* species. The analysis of pigment composition revealed the presence of lutein, neoxanthin, violaxanthin, canthaxanthin, and astaxanthin, while the fatty acid analysis measured mostly C16 and C18 fatty acids (FA), often found in feedstocks that are suitable for biodiesel production [7]. Due to the composition of SC and FA, this strain shows a high potential for application in algae biotechnology [8,9] . Likewise, various studies also have expressed great biotechnological interest in the genus because of its pigment and FA composition [7].

For the past 100 years, the genus *Coelastrella* has been known and only a few species belong to this genus. *Coelastrella* can range in shape from coccoid to elliptical and appear most often in a unicellular form. They multiply by asexual reproduction and the autospores are released by disrupting the parental cell wall. For the identification of this species, structural properties such as cell shape, chloroplast, and pyrenoids' structure are essential [7].

1.1.2 Model organism – *Haematococcus pluvialis*

The microalgae *Haematococcus pluvialis* represents the main source of natural astaxanthin, which offers, despite from a higher production price, more safety for human consumption as compared to synthetic production [10].

Under the influence of certain stress factors, the industrially-used microalga *H. pluvialis* can accumulate the high valuable SC. If the vegetative growing green cells get stressed by e.g. high light illumination, nitrogen, and nutrient starvation, or salinity stress. Under the influence of these factors, the cell division stops, and the viable cells transform to hematocysts or aplanospores. These forms are capable of producing and accumulating astaxanthin and thereby turning from green to red [11].

Haematococcus covers currently only 1% of the market demand. A significant advantage of this alga strain is that it is the only one receiving the United States Food and Drug Administration (FDA) approval for supplementation in human nutrition. This strain represents currently the most promising microorganism for natural astaxanthin production as it can accumulate astaxanthin up to 5 % of its dry cell weight (DCW) [11].

The cultivation of microalgae in total darkness with an organic carbon source as an energy supply is an economically promising option for commercial astaxanthin production. *Haematococcus* can be cultivated heterotrophically, however, the cultivation is not executed due to the low growth rate compared to autotrophic cultivation and the fact that astaxanthin accumulation solely occurs under high light stress [11].

If acetate is supplemented to the medium of *H. pluvialis*, it enhances carotenogenesis. In comparison, the viable cells transform significantly earlier to hematocyst without acetate. The intake rate of acetate under dark conditions was doubled in comparison to light cultivation [11]. *H. pluvials* species tend to be found in more temperate regions and therefore, require a higher cultivation temperature [12].

Several production systems are used for the cultivation of *H. pluvials*, including an indoor closed tank system used in Sweden, resulting in 4% of astaxanthin, and an open pond system in Hawaii yielding 1.5-2.5% of astaxanthin. In addition, there are closed outdoor production systems from AstaREAL ‘‘BioDomeTM’’located in Hawaii, yielding 5-6% of astaxanthin [13].

1.2 Carotenoids

Carotenoids represent one of the most important groups of lipid-soluble pigments with more than 600 types investigated [16,17]. The worldwide carotenoid market is prognosticated to reach 1.53 billion US$ by the end of 2021 and experienced an annual growth of nearly 4% in the last four years [11]. However, they have a minor role in the process of photosynthesis compared to the green pigments chlorophyll *a* and *b* [8]. Carotenoids are synthesized *de novo* from many photosynthetic organisms, such as plants and algae in their chromoplasts or chloroplasts, during a light-harvesting

complex, which is known as photosynthesis [11]. The number of conjugated double bonds of their polyene chain directly correlates to the absorbed photons in the near UV and blue visible regions of light, which determines their color. The pigments can be colored from a range of yellow to orange or red [13]. All of the naturally occurring pigments carry a C40 unsaturated backbone and are biologically derived from a simple isoprene unit. This group of pigments is divided into two classes. One class represents the xanthophylls, which carry one or more oxygen functional groups like hydroxyl (OH^-) or ketone groups. On the contrary, the second class which is represented by the so-called carotenes simply carries one or more hydrocarbons [14].

Carotenoids play a pivotal role in oxygenic photosynthesis as they possess the function of light-harvesting in order to protect the photosynthetic apparatus from excess light by releasing energy. In Addition, carotenoids have an antioxidant effect by quenching singlet oxygens and capturing peroxyl radicals [8].

SC have extra health benefits due to their antioxidant, anti-inflammatory, anti-aging, and anti-angiogenic properties [11]. They may protect against lipid peroxidation, DNA oxidation and can contribute to the prevention of cancer and chronic diseases [17,15]. These health benefits are derived from the conjugated double bonds of their structure[14]. Specifically, the pigments ameliorate oxidative stress, which is connected to the overproduction of reactive oxygen species (ROS). These reactive oxygen species are connected to the aggravation of various diseases [17].

1.2.1 Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is an SC and belongs to the class of xanthophylls since it carries both a ketone and hydroxyl group [14,19]. Due to its 13 double bonds in total, the xanthophylls have the highest antioxidation power of all carotenoids [11]. Therefore, it is viewed to be superior to most other types of carotenoids. According to its lipophilic backbone structure and the hydrophilic groups on the molecular ends, astaxanthin is capable of traveling through the double-layered membrane with no structural effects. This fact enables astaxanthin to release its antioxidant power inside and outside the cell tissue [14]. Furthermore, astaxanthin has been shown to have a high transferability to brain tissue and thus may protect the cerebral vessels from cerebrovascular disorders, such as cerebral ischemia and subarachnoid haemorrhage [18,20]. Due to these quality characteristics, astaxanthin can be regarded as one of the most valuable carotenoids concerning the health of human beings [14]. Apart from medicinal purposes, it can be used as a food colorant, because of its intense red color and therefore used as a feed supplement in aquaculture for shrimp, salmon, and trout [8,19].

Astaxanthin is derived from β-carotene and further oxidized to astaxanthin. However, the biosynthesis of astaxanthin can be different in various microorganisms. The high-value carotenoid has been found in several microalgae including the industrially used strain *H. pluvialis*, *Chlorococcum sp*., and *Chlorella zofingiensis* but has also been found in the marine bacterium *Agrobacterium aurantiacum* and the yeast *Phaffia rhodozyma* [8,19]. Possible intermediates of the astaxanthin pathway are ß-carotene, echinenone, canthaxanthin, zeaxanthin, adonixanthin, and adonirubin. [18]

The oxidative formation of astaxanthin in *H. pluvialis* starts with the precursor ß-carotene to the intermediates echinenone, canthaxanthin, and adonirubin [18]. In *C. zofingiensis* about 70% of astaxanthin is formed via the oxygenation of zeaxanthin [12].

Synthetic astaxanthin, which is gained from petrochemical derivatives, dominates the global astaxanthin market by over 95%. Nevertheless, biologically derived astaxanthin from the main industrial producer, the microalgae *H. pluvialis,* has much better oxidant power because it is esterified with one or two fatty acids which considerably increase its absorption and bioactivity power. Therefore, astaxanthin produced by microalgae is 90 times more potent than astaxanthin derived from petrochemical derivates [11]. Furthermore, carotenoids which are obtained by chemical synthesis produce hazardous by-products, that could harm the environment [15].

1.2.2 Adonixanthin and Adonirubin

Adonixanthin (4-ketozeaxanthin) and adonirubin (phoenicoxanthin) are both biosynthetic intermediates of astaxanthin [19,21]. The precursor of both intermediates is β-carotene [20]. They possess conjugated double bonds and therefore strong antioxidant and anti-inflammatory properties, which lead to nearly the same health benefits as astaxanthin [18,21]. The energy of singlet oxygens excites the carotenoids which convert the singlet oxygens into stable triplets. The excitation energy is converted into thermal energy and released. Additionally, adonixanthin and adonirubin have polar ionone rings at their ends which differ from the ionone rings of astaxanthin. This specific structure difference causes polarity and could possibly result in stronger protective effects than astaxanthin.

Moreover, studies showed that adonixanthin and adonirubin have better cytoprotective effects than astaxanthin. The daily intake of adonixanthin in small amounts may protect the brain from damage induced by cerebral hemorrhage [17]. However, there has no suitable industrial producer of adonirubin and/or adonixanthin been found yet, just a gram-negative aerobic bacterium called *Paracoccus carotinifaciens* which produces 20 to 30% adonixanthin and/or adonirubin of all carotenoids accumulated, however, there is not much attention given to this [20].

1.2.3 Canthaxanthin

Canthaxanthin (β,β-carotene 4,4'-dione) belongs also to the class of xanthophylls and possesses nine conjugated double-bonds. The most extracted form of canthaxanthin is the more stable all-trans canthaxanthin isomer. In nature, there are very few organisms that can produce canthaxanthin as a final product. The precursor of canthaxanthin is ß-carotene, as in adonixanthin and adonirubin. The enzyme catalyzing ß-carotene to the next intermediate echinenone is called the ketolase enzyme [21].

Canthaxanthin is a very valuable product and can be merchandised commercially within a broad range of possible applications. When the ketocarotenoid is consumed by humans it gets incorporated into mixed micelles. In addition, canthaxanthin is capable of releasing its antioxidant power against ROS, like astaxanthin or adonixanthin. Furthermore, in vivo studies proved that canthaxanthin could prevent induced liver damage in rats by its antioxidant effect [21].

Currently, the main application of canthaxanthin is as a skin tanning agent in cosmetics and as a food dye for egg yolk and chicken skin [18,8]. Although canthaxanthin could positively affect the health status of human and animal consumers, there are no studies that aim to investigate the health effect of canthaxanthin in the human body. The canthaxanthin market is an emerging market, expected to reach the size of $85 million in 2024 with an annual growth rate of 2.2%. 40% of the market volume is accounted for animal feed. Canthaxanthin has a license in over 70 countries including Europe and the U.S [21].

The health effect of taking carotenoids dietary turns synthetic production towards natural production. Natural producers can be algae, bacteria, or plants. Therefore, natural producers are sought for cost-effective production systems with high yields, which are, furthermore, good for the environment and safe for consumption [21]. However, the generation of canthaxanthin has been observed in a marginal number of algal species like *C. zofingiensis, Chlorococcum sp., and Tetracystis intermedium*. It was reported that *C. zofingiensis* is capable of producing 8.79 $mg \cdot g^{-1}$ DCW of SC, containing more than 95% canthaxanthin, under conditions of low light and salt stress in 9 days of cultivation [8].

There are many reports aiming to improve the yield of secondary carotenoids in large-scale production. However, only a few papers have the major goal of improving the canthaxanthin yield [21]. Out of all the ketocarotenoids, which were found in higher plants, algae, fungi, or bacteria, astaxanthin and canthaxanthin are the most relevant ones from a biotechnological perspective [8].

1.3 Carotenogenesis – the Production of Secondary Carotenoids

The production of primary carotenoids (PC) in microalgae is essential for photosynthesis whereas SC are produced and accumulated as a natural response to stress factors. The natural process of producing PC and SC in microorganisms is called carotenogenesis. Synthesizing SC is a way of storing energy and increasing the resistance against oxidative stress sources, which increases the probability of the cell's survival. [13].

The building blocks of carotenogenesis are composed of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These are produced by the 2-C-methyl-D-erythritol 4 phosphate cytoplasmic pathway (MEP) and by the mevalonic acid plastid pathway (MVA). Phytoene, which results from the reaction catalyzed by phytoene synthase, becomes lycopene through desaturation and isomerization processes. The lycopene cyclases (β and ε) cause the carotenoid diversity by generating β-carotene and α-carotene. Further reactions take place via hydroxylation and ketolation [21].

The production is first located around the nucleus and later the storage occurs as oil droplets in the cytoplasm or plastids. The synthesis of secondary carotenoids often occurs simultanously with metabolic and morphological changes i.e., the transformation from green macrozoids into red large hematocysts like at the green microalgae *H. pluvialis*. However, SC production is not always accompanied by the transformation of living cells into cysts. Under nutrient starvation conditions, some studies report that the accumulation of astaxanthin was induced in viable cells. For instance, the microalgae *C. zofinogiensis* accumulates the SC astaxanthin and discharges it into lipid bodies outside the chloroplast. Keeping cell division active while producing carotenoids like in *C. zofinogiensis,* could be a great advantage for the biotechnological production of carotenoids. In simple terms, carotenogenesis is a convenient way for cells to store energy and use it after experiencing certain stress conditions to recover and repair all types of damage and to scavenge toxic reactive oxygen species [13].

There are two types of stress sources generated – photochemical and nonphotochemical, which can be quite dangerous for algae without a certain life-saving system for coping with these stress types. Both types of stress produce toxic ROS which occur in form of hydrogen peroxide (H_2O_2), singlet oxygen ($1\ O_2$), hydroxide radical (OH^-) as well as superoxide ion radical (O^{2-}). This generation causes an unbalanced ROS level and results in oxidative stress within the cell, which can damage cell compartments [13].

In detail, these ROS species might interact with DNA, proteins, or lipids. This interaction could affect the function of these biologically important components. This process is perceived as the initial event of many diseases, such as cancer or cardiovascular diseases in humans. Many biological components can be attacked by these free radicals. Whereby, it depends on the components whether they have rapid or slower rates. Whereas amino acids attacked by radicals generate rapid reaction rates, polyunsaturated fatty acids attacked by radicals result in significantly lower rates. The more double bonds a molecule under attack possess, the slower the reaction rate will be [8]. The cell has evolved several defensive mechanisms involving the formation of enzymes as well as the production of antioxidants like carotenoids [13].

1.4 Current Development of SC production

Current production methods, aiming at a high SC yield, differ from each other in terms of metabolic cultivation modes including photo-, mixo-, or heterotrophic cultivation and of cultivation techniques ranging from open ponds to closed PBRs. Another difference is the cultivation environment ranging from indoor to outdoor conditions. Due to different cultivation options, the current production costs of different companies vary significantly [14].

However, there are still some challenges in the biologically derived production of SC. The limited production of these valuable products is probably the biggest issue yet to be solved to keep up with the yield of petrochemical derivatives [22,11]. Therefore, efforts to improve the large-scale production of high-value carotenoids have been ongoing for years. Strain selection and improvement for industrial microbial production might be the most essential stage for process development. The wild-type strains found in nature differ significantly in their phenotypes. The appropriate selection of the best performing strain suitable for production could be crucial for starting a successful process development project. [14]. Another promising approach is metabolic engineering, which contains enhancing carotenoid storage capacity, overexpressing enzymes that are product-limiting, and downregulating of divergent pathways [13]. In addition, the market for natural carotenoids can certainly also benefit from advances in synthetic biology and biotechnological tools for improving the industrial large-scale production of natural carotenoids [21].

1.4.1 Photoautotrophic cultivation

The most preferred large-scale cultivation method for photoautotrophic algae is the open system since natural sunlight can be used as an energy source and atmospheric CO_2 is fixed and utilized as

carbon source. Therefore, the installation and operating costs are rather low compared to controlled closed PBR. For open systems, robust species are needed to grow under harsh conditions like high salinity or extreme pH to lower the risk of contamination. PBR can be designed based on the organism's and the end product's needs to gain high yields and productivity, which go along with high installation and operating costs. However, for producing high-end compounds for pharmaceutical use, controlled closed systems are the preferred cultivation process to guarantee the quality and quantity of the end product and to maintain the cultures and products in their purest conditions [11].

Nevertheless, the cultivation of microalgae with high light demand is one of the major problems since in upscale PBR in dense populations the light cannot be efficiently dispersed [23] and in open systems, the pond depths are also limited due to poor light penetration. Else, carotenoid and biomass yield would suffer [11]. Cost associated, the equipment, and energy consumption in PBR might be too high to compete with open phototrophic cultivation utilizing natural light [14].

1.4.2 Heterotrophic cultivation

A valuable solution for industrial cultivation of microalgae could be heterotrophic cultivation without any intake of light in conventional bioreactors, to overcome the major problem of light supply. Additionally, conventional bioreactors also exist for bacterial fermentations. Heterotrophic microalgae can maintain cell division and metabolize under dark conditions, using solely an organic carbon source as an energy source. Heterotrophic cultivation in conventional industrial fermenters enables controllable process parameters like carbon source supply, pH, and temperature [11].

Under heterotrophic conditions, self-shading of light supply, which limits cell growth, remains off. On the other hand, instead of fixing CO_2 during photosynthesis, heterotrophic cultivation produces CO_2 and negatively affects global CO_2 emissions. The major costs are composed of installation and equipment costs followed by the cost of organic carbon sources [11]. Despite great results in the increase of microalgal biomass, high SC production is still strongly associated with high-intensity illumination [12].

1.4.3 Mixotrophic cultivation

Mixotrophy combines both carbon supplies of photoautotrophic and heterotrophic cultivation. It provides a light source to maintain photosynthesis and to fix an inorganic carbon source (CO_2) and an organic carbon source, like glycerol, glucose, or sodium acetate in the medium. This type of cultivation offers certain advantages, for instance, a faster growth rate due to the fact that the microorganism is

simultaneously provided with organic and inorganic carbon. This results in a saving of reduction of cost of light energy and organic carbon, both compared to autotrophic and heterotrophic cultivation [22].

Considering the normal aquatic habitat of microalgae, which also provides organic carbon sources, mixotrophy is a very common nutritional strategy for microalgae to survive. In addition it is a method to manage stressful conditions e.g., a lack of light or nutrients. A great advantage of mixotrophic cultivation is the overcoming of self-shading problems, like in dense large-scale photoautotrophic cultures. This cultivation method is also widely used in industry to produce biomass and secondary metabolites, however, there is sparse information about the mixotrophic cultivation of microalgae [1].

1.5 Problem statement | State of the Art

Humans are not capable of producing secondary carotenoids *de novo* and have to acquire their need for those essential nutrients like ß-carotene, which is a precursor of vitamin A, by consuming them via dietary sources or through other medicinal products [11,24]. Due to the high demand for antioxidant carotenoids, the industry is trying to improve the biological process of carotenoid production.

The production process of astaxanthin derived from *H. pluvialis* has been under development for more than 35 years. There are more than 10 production plants established worldwide but despite this intensive development, the technology is still in its infancy and requires further research. Due to the complicated biology and physiology of microalgal cells and the biochemistry of astaxanthin production, astaxanthin production from *H. pluvialis* is extremely difficult. Despite more than 20 years of intensive research and operation of the astaxanthin process gained from *H. pluvialis,* companies like Cyanotech Inc., Subitec or BDI-Bioenergy International are still confronted with the challenges of maintaining a consistent and reliable production of astaxanthin [14].

Consequently, many other unicellular algae species have been screened for these health-beneficial pigments for industrial production [22]. Screening of native algae is essential for evaluating the biotechnological potential of each species, specifically in harsh environmental settings, as some of these species might be more favorable than the commercial strains like *H. pluvialis* , that are currently used [7]. In addition, there is no suitable industrial producer found yet for producing industrial reasonable amounts of the high valuable xanthophylls adonixanthin and canthaxanthin [21,16].

1.6 Aim of this Thesis

The primary aim of this master thesis was to characterize the growth of the rather unexplored green microalga *C. terrestris* and to analyse the physiological composition of the fatty acids profile and stored carbohydrates as well as the pigment composition of primary and secondary carotenoids. Afterward, the productivity of *C. terrestris* was evaluated statistically under different influences, such as different levels of light and nitrogen concentration conducted in shake flasks. Finally, experiments in lab-scale PBRs were performed to examine the pigment productivity of *C. terrestris*.

In order to evaluate the carotenoid productivity of *C. terrestris*, a direct comparison was also made with the industrially used microalga *H. pluvialis.*

1.7 Hypothesis

The new algal strain could become an industrial source of secondary carotenoids due to the assumption that requires, (i) no salt stress and (ii) low light for carotenoid formation

thus, facilitating indoor cultivation in photobioreactors. Due to the low cultivation temperature, this strain could be cultivated in colder hemispheres and this advantage could open new markets.

In general, there are no or no suitable microalgae and microorganisms known that are able to produce adonixanthin and canthaxanthin in industrially appropriate amounts as their main SC [8,21,25]. The assumption was made that *C. terrestris* is capable of generating an appropriate amount of adonixanthin and canthaxanthin as one of their main carotenoids due to preliminary indications. This ability could lead to the strain becoming very attractive to the industry as a producer of adonixanthin and/or canthaxanthin.

1.8 Novelties of this work

The green alga *C. terrestris* has never been reported to produce SC and no attempt was made to cultivate this alga in a PBR by adjusting the culture conditions yielding in a high SC content.

The novelty of this work lies in the examination of how different levels of light and nitrogen starvation affect the growth and the respective SC productivity of the new strain *C. terrestris* regarding different phototrophic and mixotrophic cultivation strategies.

Experiments in PBR to determine the SC content that can be achieved, constitute the second novelty of this work.

The third novelty lies in the head-to-head comparison of the growth and SC content of *C. terrestris* with the industrial workhorse *H. pluvialis*.

1.9 Approach

The experiments performed to test the hypotheses of this work were statistical DoE done in erlenmeyer flasks and cultivation in PBRs.

Every experiment was performed under mixotrophic conditions since mixotrophy offers significant advantages, for instance, a faster growth rate by consuming both organic (carbohydrate in medium) and inorganic (CO_2 fixed via maintaining photosynthesis) carbon simultaneously [22]. For every

experiment, the mixotrophic approaches are compared with photoautotrophic reference to gain further knowledge about the growth behavior of the strain.

For direct comparison and to test the reactor setup and cultivation conditions, the SC productivity of *C. terrestris* and an industrially utilized microalga called *H. pluvials* will be observed by culturing both algae with a *Haematocuccus* based strategy used in industry.

1.10 Roadmap

1. Shake flask experiment to find nitrogen concentration and carbohydrate source for high biomass and carotenoid production
2. DoE: with different nitrogen concentrations and different photosynthetic photon flux densities (PPFD) performed in shake flasks to observe the light and nitrogen condition and to investigate the relation between both variables to generate high biomass and carotenoid yield
3. PBR experiments: gained knowledge from shake flask experiments and DoE is used to cultivate *C. terrestris* in PBRs for maximal possible secondary carotenoid yield
4. Comparing productivity of *C. terrestris* and industrially used *H. pluvialis* with *H. pluvialis* based process

An overview of every experiment can be found in the materials and methods section.

2 Materials and methods

2.1 Working Strain - *Coelastrella terrestris*

Coelastrella terrestris, the microorganism to be investigated, is a green microalgae strain that was discovered and provided by Dr.rer.nat. Remias Daniel from the University of Applied Science FH Upper Austria. The strain was found in Iceland and isolated in Vienna. The data for the strain identification of the 18S sequencing is not shown here.

2.2 Reference Strain - *Haematococcus pluvialis*

The reference organism for an experiment to compare secondary carotenoid productivity was provided by Dr.rer.nat. Remias Daniel from the University of Applied Science FH Upper Austria. The strain was found on the rooftop of the University of Applied Science FH in Upper Austria and isolated from DI Doppler Philipp.

2.3 Light microscopy

The different cell stages of *C. terrestris* and the reference strain *H. pluvials* were analyzed by a Zeiss Axio Imager M1m (Carl Zeiss, Oberkochen, Germany) light microscope equipped with a Zeiss x1000/0.7 objective. For imaging, a Canon EOS 250D (Tokyo, Japan) was used.

2.4 Media composition

The composition of Bolt's Basal Medium (BBM) is shown in Table 1. For all cultivation experiments, BBM medium was used [26].

Table 1: BBM composition

Ingredients	**Concentrations**
$NaNO_3$	250 $mg \cdot L^{-1}$
$MgSO_4 \cdot 7H_2O$,	75 $mg \cdot L^{-1}$
NaCl	25 $mg \cdot L^{-1}$
K_2HPO_4	75 $mg \cdot L^{-1}$
KH_2PO_4	175 $mg \cdot L^{-1}$
$CaCl_2 \cdot 2H_2O$	25 $mg \cdot L^{-1}$
trace metal mix	1 $mL \cdot L^{-1}$
vitamin mix	1 $mL \cdot L^{-1}$

The original nitrogen content of BBM results in a final $NaNO_3$ concentration of 250 $mg \cdot L^{-1}$. Correspondingly, 0.6N-BBM has a $NaNO_3$ content of 150 mg L^{-1} and 0.2 N-BBM a content of 50 $mg \cdot L^{-1}$.

The 1000 times concentrated stock solution of the trace metal mix was prepared according to [26]:

Table 2: 1000x Trace Metal Mix Stock Solution

Ingredients	**1000x Trace Metal Mix**
$ZnSO_4 \cdot 7H_2O$	8.82 $g \cdot L^{-1}$
$MnCl_2 \cdot 4H_2O$	1.44 $g \cdot L^{-1}$
MoO_3	0.71 $g \cdot L^{-1}$
$CuSO_4 \cdot 5H_2O$	1.57 $g \cdot L^{-1}$
$Co(NO_3)_2 \cdot 6H_2O$	0.49 $g \cdot L^{-1}$
H_3BO_3	11.4 $g \cdot L^{-1}$
$Na_2EDTA \cdot 2H_2O$	50.0 $g \cdot L^{-1}$
$FeSO_4 \cdot 7H_2O$	4.98 $g \cdot L^{-1}$

For all shake flasks experiments (C-source screening and DoE) 5 mM of HEPES has been added to the medium, and the pH value was set to 7.0 by 5 M NaOH before autoclaving. For all experiments carried out in PBR's, no HEPES has been added to the medium and the pH was not set, but a lower limit of 6.3 was regulated with 1M Na_2CO_3 after autoclaving.

After autoclaving, the vitamin stock solution mix has been added under aseptic conditions by a syringe with a 0.22 µm filter [26]. The composition of the vitamin stock solution was mixed according to [26].

Table 3: Stock solution vitamin mix

Vitamin Stock Solution Mix	
Vitamin H (Biotin)	0.025 mg · 100 mL^{-1}
Vitamin B12 (Cyanocobalamin)	0.015 mg · 100 mL^{-1}
Vitamin B1 (Thiamin HCl)	0.1 g · 100 mL^{-1}

2.5 C-source Screening Experiment

Shake flask experiments for characterization of growth conditions with different carbohydrate sources were carried out in 100 mL Erlenmeyer flasks. For the evaluation of appropriate carbon sources for the microalga, the flasks were filled with a volume of 50 mL either BBM, 0.6N-BBM, or 0.2N-BBM supplemented with the addition of 1 $g \cdot L^{-1}$ of different carbohydrates. The supplemented carbohydrates were: glucose, glycerol, sodium acetate, xylose, ribose, and fructose. 1 mL of pre-culture added in every flask, started the experiment. For cultivation, the flasks were positioned in a Minitron incubation shaker (Infors, Basel, Switzerland) with continuous shaking of 150 rpm. The light-darkness cycle was set to 14 h light/ 10 h darkness with a photosynthetic photon flux density (PPFD) of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The experiment was conducted for approximately 50 days for investigating the growth characterization and secondary carotenoid (SC) production.

The growth behaviour was monitored with OD measurements along the whole cultivation experiment. At the end of the cultivation, 10 mL of fermentation broth of each shake flask were harvested. The suspension was centrifuged in falcon tubes that were previously weighted, followed by 48 h of lyophilization of the biomass. After that, the biomass was stored at -20 °C and the dry cell weight of the samples was gravimetrically determined. Those biomass samples were used for the determination of the carotenoid content. All approaches were executed in biological duplicates. The results obtained in this experiment served as the basis for further performed experiments.

2.6 Design of Experiment

In order to get the optimal cultivation conditions for *C. terrestris* and therefore gain a high SC yield, a Design of Experiment (DoE) in shake flasks was performed. Shake flask experiments for

characterization of growth conditions with different light photon flux densities (PPFD) and different $NaNO_3$ concentrations were carried out in 100 mL shake flasks. The $NaNO_3$ concentrations selected were 50 (0.2N), 150 (0.6N) and 250 (1N) $mg \cdot L^{-1}$ and the photosynthetic photon flux densities (PPFD) were chosen to be 30, 90 and 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. A volume of 50 mL BBM medium was used for each shake flask. pH and temperature set-points were selected to pH 7.0 and 20 °C. The experiment started with 1 mL of preculture grown in BBM medium. For starting the experiment, the inoculated shake flasks were put into a Minitron incubation shaker (Infors, Basel, Switzerland) with 14 h light/ 10 darkness cycle with 150 rpm. The experiment lasted for 29 days and at the end of the experiment, the biomass was harvested for dry cell weight (DCW) determination and pigment extraction. Samples were taken approximately every 10 days to check the optical density (OD_{600}) and to take samples to measure the concentration of nitrate, phosphate, and glucose or sodium acetate. The responses of the DoE are the SC content [$mg \cdot g^{-1}$] and the biomass concentration [$g \cdot L^{-1}$]. All approaches were executed in duplicates. The evaluation of the DoE was performed using MODDE 10 (Umetrics, Sweden). The following table was established as an experimental design for this experiment (*Table 4*).

Table 4: Experimental design for shake flask experiment, created with MODDE 12.1

Experiment No.	**Illumiation [$\mu mol \cdot m^{-2} \cdot s^{-1}$]**	**NaNO3 [$mg \cdot L^{-1}$]**	**C-source [$1g \cdot L^{-1}$]**
N1	30	50	-
N2	90	50	-
N3	150	50	-
N4	30	150	-
N5	90	150	-
N6	150	150	-
N7	30	250	-
N8	90	250	-
N9	150	250	-
N10	30	50	glucose
N11	90	50	glucose
N12	150	50	glucose
N13	30	150	glucose
N14	90	150	glucose
N15	150	150	glucose
N16	30	250	glucose
N17	90	250	glucose
N18	150	250	glucose

N19	30	50	NaOAc
N20	90	50	NaOAc
N21	150	50	NaOAc
N22	30	150	NaOAc
N23	90	150	NaOAc
N24	150	150	NaOAc
N25	30	250	NaOAc
N26	90	250	NaOAc
N27	150	250	NaOAc
N28	90	150	-
N29	90	150	-
N30	90	150	-

2.7 Cultivation in Stirred Photobioreactor

A Ralf multi-bioreactor system (Bioengineering, Wald, Switzerland) with 2.0 L total volume per reactor was featured with an EasyFerm Plus pH probe (Hamilton, Bonaduz, Switzerland), and a Pt100 temperature sensor each. The four (1.25 L working volume) reactors were installed with two 55 mm turbine impellers for mixing. The glass vessel was equipped with a glass double jacket for temperature regulation. Illumination was provided externally via LED light stripes which were wrapped around the glass jacket. Further, the reactors were equipped with ports for base addition and sampling. The inner diameter of the reactor was 95 mm.

A volume of 1.25 L of BBM medium was put in the vessel, added either with glucose orNaOAc as C-source or nothing as phototrophic reference. After autoclaving the medium-filled vessel, the vitamin mix was supplemented under aseptic conditions by a syringe with a 0.22 µm filter through a septum. The pH 6.3± 0.1 of BBM medium was a result of the 3.0% CO_2-enriched air supply. The temperature was monitored to 20 °C and the stirrer speed was adjusted to 300 rpm. The light-darkness cycle was set to 14 h light/ 10 h darkness. 15 mL of preculture, which was grown on BBM medium, was inoculated into the bioreactor via a septum. To defeat the difference of preculture conditions, the sampling and the experiment was started at an OD600 of 0.10 ± 0.01. For the first three days of cultivation, the culture was exposed to low light. The illumination in the center of the bioreactor was measured, reaching a PPFD of 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$, at the edge of the bioreactor the illumination level was 183.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After the first three days, the light source was replaced with a more powerful

one. The illumination level in the center was 850 µmol·m^{-2} ·s^{-1}, at the edge the illumination level was860 µmol·m^{-2} ·s^{-1}. The illumination was measured in water by an ULM-500 with an US-SQS/L sensor (Walz, Effeltrich; Germany)

4 mL of fermentation broth were manually taken, put into Eppendorf tubes, and centrifuged at 4°C at 14 000 rpm. 1 mL of supernatant was stored in Eppendorfer tubes and two biomass pellets were stored at -20°C for further analysis. The supernatant was used for nitrate determination (IC) and acetate and glucose determination (HPLC).

Samples were taken every day for the first seven days of the experiment, after that three samples per week were carried out, every two days. After 15 days, a longer sample break of 6 days was taken. After 21 days, the fermentation was finished with a final sample collection. For sampling 4 mL of fermentation broth were taken and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant as well as the pellets were separately stored in Eppendorfer tubes at -20°C. The pellet was further used for carbohydrate quantification analysis (IC) and the supernatant was used for sodium acetate and glucose determination (HPLC) and for NO_3- determination (IC). To determine the DCW in grams per litre, additionally every second sampling time 20 mL samples were taken after the seventh day of the experiment. The 20 mL samples were lyophilized to determine the DCW [g·L^{-1}] gravimetrically. The gained biomass was also used to determine the pigment composition. For cell growth monitoring, OD600 measurements were performed in 1 mL cuvettes on Nanodrop One photometer (Thermo Fisher Scientific, Waltham, MA, USA). The deviations for *C. terrestris* were calculated using RGP function, which represents the standard errors of the coefficients.

2.8 Determination of Nitrate Concentration

For the determination of NO_3^- concentration, 1 mL of the sample was centrifuged, and the supernatant was used without any further preparation for measurement on an ICS-6000 ion chromatography (IC) system (Thermo Fisher Scientific, Waltham, MA, USA). The flow of a Dionex IonPac AS11 with a guard column (Thermo Fisher Scientific, Waltham, MA, USA) was adjusted to 2.0 mL·$min^{-1,}$ and the temperature was set to 30°C. Mobile phase A consisted of 100 % H_2O and phase B consisted of 100 mmol L^{-1} of NaOH. The gradient from 0 to 1 min consisted of 0.2% phase B. After that, the mobile phase B was increased to 5% from minute 1 to minute 6, phase B was increased further to 24 % from minute 6 to minute 13, and then was further increased to 38% between minute 13 to 13.5. The concentration stagnated to minute 14.5 and then decreased to the initial concentration of 0.2% after minute 15 and was held to minute 20 for equilibration. The detection was executed by a conductivity detector, which was combined with an AERS suppressor(Thermo Fisher Scientific, Waltham, MA, USA).

The nitrate quantification of the samples was performed by nitrate standards (Sigma Aldrich, St. Lewis, MO, USA). The resulting data was analyzed by Chromeleon 7.2.8 (Thermo Fisher Scientific, Waltham, MA, USA)

2.9 Determination of Carbon Concentration (HPLC)

For the determination of carbon concentration (glucose, fructose, glycerol, sodium acetate, xylose, ribose) 1mL supernatant of a centrifuged sample was used without any further preparation for measurement on high-performance liquid chromatography (HPLC). The measurement was performed on a Vanquish Core HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The column for the stationary phase was the Aminex H87 (Thermo Fisher Scientific, Waltham, MA, USA) with a flow rate of 0.6 $mL \cdot min^{-1}$ and the temperature was set to 60°C. The mobile was composed of 4 mmol L^{-1} of H_2SO_4. The carbon concentration was performed by carbon standards (Sigma Aldrich, St. Lewis, MO, USA). The resulting data was analyzed by Chromeleon 7.2.8 (Thermo Fisher Scientific, Waltham, MA, USA)

2.10Carbohydrate Extraction, Identification, and Quantification

The preparation of samples for the carbohydrate quantification was performed according to [27]. 1 mL of the sample has been centrifuged and the gained pellet was dried at 75°C for 24 h. 1 mL of 7.5% (v/v) H_2SO_4 in H_2O was added to the dried pellet. After vortexing, the solution was put on a heating block for two hours at 900 rpm and 95°C. Next, the solution was centrifuged again at 14,000 rpm for 10 min at 4°C and 100 µL of the supernatant was diluted with 400 µL of H_2O to get a solution of 1:5.

The carbohydrate quantification of the samples was performed via glycogen (Sigma Aldrich, St. Lewis, MO, USA) standards, which were prepared identically. The prepared standards and samples were measures on an ICS-6000 IC system (Thermo Fisher Scientific, Waltham, MA, USA).). The flow of a Dionex CarboPac PA10 with an amino trap column (Thermo Fisher Scientific, Waltham, MA, USA) was adjusted to 1.0 $mL \cdot min^{-1}$ and the temperature was set to 30°C. Mobile phase A consisted of 100 % H_2O and phase B consisted of 100 mmol L^{-1} of NaOH. From 0 to 15 min, the mobile phase consisted of 18 mmol L^{-1} NaOH. After that, the mobile phase was increased to 100 mmol L^{-1} NaOH from 16 to 26 minutes. The concentration then decreased to the initial concentration of 18 mmol L^{-1} NaOH after minute 27 and was held for 15 minutes for equilibration, before the next injection.

The detection was executed by an electrochemical (EC) detector. The carbohydrate quantification of the samples was performed by carbohydrate standards (Sigma Aldrich, St. Lewis, MO, USA). The resulting data was analyzed by Chromeleon 7.2.8 (Thermo Fisher Scientific, Waltham, MA, USA)

2.11 Pigment Extraction, Identification, and Quantification

2.11.1.1 Pigment Extraction

For the extraction of pigments 30 mg of lyophilized biomass was put into screw-capped reaction tubes together with 0.3 g of 1 mm and one 5 mm glass beads. The reaction tubes were put into liquid nitrogen, followed by the disruption of the biomass with the aid of a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). The device was set to 4.0 $m \cdot s^{-1}$ for 60 s. The first two homogenization runs were performed without the addition of acetone. For the third and last run, 1 mL acetone was added before starting the run. Between runs, samples were frozen with liquid nitrogen. After the homogenization, the samples were centrifuged for 10 min at 14 000 rpm. The supernatant was transferred into a 5 mL volumetric flask and 1 mL of acetone was pipetted into the tubes and were mixed by vortexing. This procedure was replicated at least four times until all pigments were extracted from the cell pellet. To protect the pigments from decomposing, this procedure was performed under dimmed light. The acetone-pigment mixture was filtered with a 0.22 µm filter before being measured by high-performance liquid chromatography (HPLC).

2.11.1.2 Pigment Identification (HPLC-MS)

The Identification of pigments was performed by high-performance liquid chromatography linked to mass spectrometry (HPLC-MS). The acetone-pigment mixture was measured by a 1290 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA) featured with a diode array detector (DAD) at a wavelength of 450 nm. The stationary phase consisted of the column Acclaim C30, 3 µm, 2.1 × 100 mm^2 (Thermo Fisher Scientific, Waltham, MA, USA) with a temperature setting of 20°C with a total flow of 0.3 $mL \cdot min^{-1}$. Mobile phase A consisted of 91% methanol (MeOH), 5% methyl tert-butyl ether (MTBE), 3.9% H_2O, and 0.1% formic acid, and phase B consisted of 46% MeOH, 50% MTBE, 3.9% H_2O, and 0.1% formic acid. One measurement procedure lasted 36 min. The gradient from 0 to 6 min consisted of 1% phase B. After that, the mobile phase B was increased to 100 % from minute 6 to minute 24. The concentration stagnated to minute 28.5 and decreased again from 100% to 1% B in

0.5 min. For equilibration, the concentration of 0.5% was kept for another 7 minutes before the next measuring started.

The mass detection was executed by a 6545 LC/Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), which was linked by a multimode ion source operated in atmospheric pressure chemical ionization (APCI). It was set to positive mode. Hence, the mass range of 100 to 1700 m/z was detected. The identification software was Mass Hunter Qualitative Analysis 10.0 (Agilent Technologies, Santa Clara, CA, USA). According to [28], detected masses with equal retention times were compared with the theoretical mass and thus identified. For the characterization of the monoesters (ME) and diesters (DE) of astaxanthin and adonixanthin, the absorption spectra of the esterified forms were compared with the unesterified forms and thus identified according to [29].

2.11.1.3 Pigment Quantification (HPLC)

The primary and secondary carotenoid quantification was performed on a Vanquish Flex HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The flow rate, the column, the eluents, and gradients were set the same as for HPLC-MS measurements. The detection was performed by a diode array detector (DAD). The external standards were prepared with pure astaxanthin (Sigma Aldrich, St. Lewis, MO, USA) which was dissolved in acetone. All carotenoids were then quantified in terms of their absorbance relative to the astaxanthin amount at a wavelength of 450 nm and expressed in mg carotenoids per g lyophilized dry cell weight (DCW). The resulting data was analyzed by Chromeleon 7.2.8 (Thermo Fisher Scientific, Waltham, MA, USA)

2.11.1.4 Chlorophyll Quantification

The quantification of the pigments chlorophyll *a* and chlorophyll *b* was performed according to Hess et al (2016) [30]. A volume of 0.25 mL sodium dihydrogen phosphate buffer (NaH_2PO_4) at pH 7.8 was added to 1 mL of acetone to get an aqueous sample solution with 80% of acetone. The absorbance of 647, 664, and 750 nm was detected with a Nanodrop One photometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations of the green pigments were calculated in the following way according to [30]:

Equation 1: Calculation of *chlorophyll a [nmol·mL^{-1}]*

$$Chl_a = 13.71A^{663.6} - 2.85A^{646.6}$$

Equation 2: Calculation of chlorophyll *b [nmol·mL^{-1}]*

$$Chl_b = 22.39A^{646.6} - 5.42A^{663.6}$$

2.12 Lipid Extraction and Analysis of Fatty Acids (GC-MS)

The lipid extraction for fatty acid analysis (FAA) was performed according to Folch et al. (1957) [31]. For the extraction of lipids 50 mg of lyophilized biomass was weighted into screw-capped reaction tubes with 0.3 g of 1 mm and one 5 mm glass beads. The tubes were dipped into liquid nitrogen, followed by biomass disrupting by FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). The device was set to 4.0 m·s $^{-1}$ for 60 s. Between runs, samples were frozen with liquid nitrogen. For the third and last run, 1 mL of 2:1 (v/v) chloroform:methanol was added. The mixture was diluted to 5 mL and put into a 15 mL test tube. A washing step for the interfacial area was performed with 1 mL of 0.9% of NaCl solution with H_2O. The washing solution was added to the chloroform-methanol extract and mixed afterward. When the two phases were separated again, the washing solution was removed by pipetting. 10 mL of the pure solvents upper phase mixture of chloroform, methanol, and water by a ratio of 8: 4: 3 by volume was added to the 5 mL chloroform:methanol extract. By that, a biphasic system was created. The upper phase consisted of 3: 48: 47 of chloroform, methanol, and water by volume, and the lower phase respectively of 86: 14: 1. The upper phase was removed via pipetting. Another washing step for the interfacial area was performed. A volume of 200 µL of pure solvent upper phase mixture was added and removed and repeated twice. The lower phase was put into a drying cabinet at 75°C for approximately 5 hours until the pellet was dry. The dried lipid-pellet was dissolved in 0.2 mL toluene, 1.5 mL methanol, and 0.3 mL 8% hydrochloric acid (HCl) (v/v). According to Ichihara et al (2010) [32], the derivatization was carried out by HCl-catalyzed transesterification in methanol to produce fatty acid methyl esters (FAMEs). The derivatization reaction was carried out in an incubation shaker at 100°C for 1 hour at 180 rpm. Afterward, 1 mL of hexane and 1 mL of H_2O were added and the upper hexane layer was used for gas chromatography linked to a mass spectrometer analysis (GC-MS).

The GC-MS system 7890A/5975C (Agilent Technologies, Santa Clara, CA, USA) was coupled with a CTC Combi PAL autosampler. The helium carrier gas with a flow rate of 1.04 mL·min^{-1} was pressed through a capillary column Permabond FFAP (Macherey Nagel, Düren, German) with a size of 30 m length, 0.25 µm film thickness, and 250 µm inner diameter. The gradient composition was transferred from Liu et

al (2010) [33]. For the first 2 min, the oven-heating program was set to 50°C. After that, an increase of 10°C per minute was performed until 200°C was reached. This temperature was held for 10 min, followed by another increase of 10°C per minute to 220°C. This temperature was held for a timespan of 15 min. The transfer line temperature was set to 280°C. For simultaneous measurement, the flow was separated to 1:5 to quantify the fatty acid methyl esters by a flame ionization detector (FID) and to detect the mass values by a mass spectrometry detector. The source temperature was set to 230°C, the scanning range to 35 to 750 m/z, and the electron energy to 70.3 eV for the MS detector. The resulting data was analyzed and identified by a reference library search with Mass Hunter Qualitative Analysis (Agilent Technologies, Santa Clara, CA, USA).

3 Results

3.1 Morphological characterization of *C. terrestris*

To obtain a morphological characterization of *C. terrestris* and to investigate morphology changes between normal and stressed condition, samples were taken on different growth stages and evaluated by light microscopy (*Figure 1*).

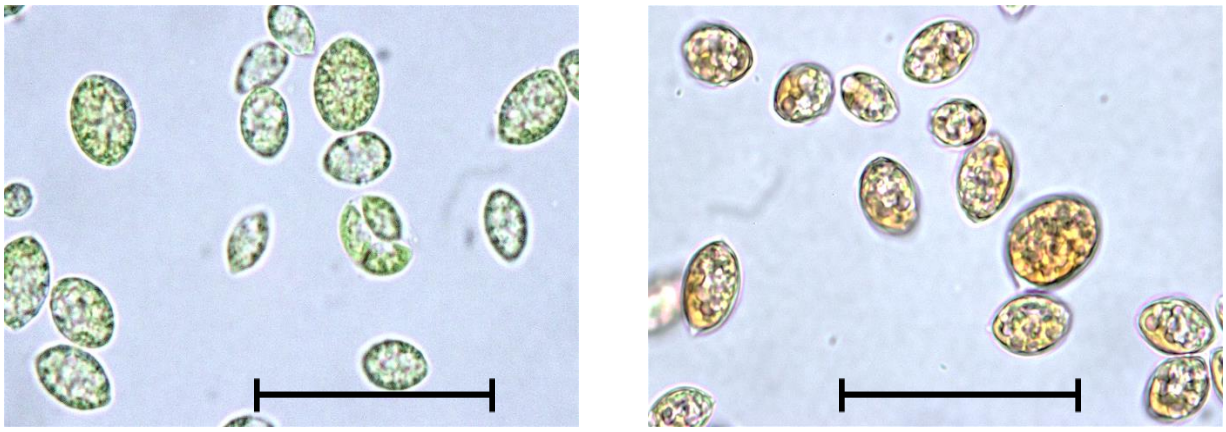

Figure 1: Light microscopy images of Coelastrella terrestris at different cell stages and levels of stress. Image (a) was taken in the green viable stage. (b) was taken on the stressed stage – carotenoid producing stage of cell cultivation. The scale bar is 50 µm.

The isolated strain was analyzed as unicellular green microalga. The Chlorophyta grew in variable shapes from spheroidal, elliptical to citric form to occasional almost round shapes (*Figure 1*a). C. *terrestris* lacks in the flagellum and therefore lacks motility. The cell size was also variable but was most often between 9 and 13 µm long and between 5 to 10 µm wide (*Figure 1*a). Asexual reproduction was observed via autosporulation and occurs by 2 to 3 autospores (*Figure 1*a,b). Furthermore, several vacuoles, lipid/carbohydrate droplets were investigated in all stages of cultivation (*Figure 1*a,b)

The microscope embarked a difference between viable green cells (*Figure 1*a) and stressed cells (*Figure 1*b). The color changed from green to orange, which was also visible to the naked eye. Furthermore, it could also be observed that the poles of the stressed cells become flattened, and in general, the cells appeared more circular (*Figure 1*b).

3.2 Identification of Pigments

For pigment identification of *C. terrestris*, orange biomass of the strain was harvested at the end of fermentation in a photobioreactor at high light and nitrogen starvation and used for pigment identification by High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS). The results are illustrated in *Figure 2*.

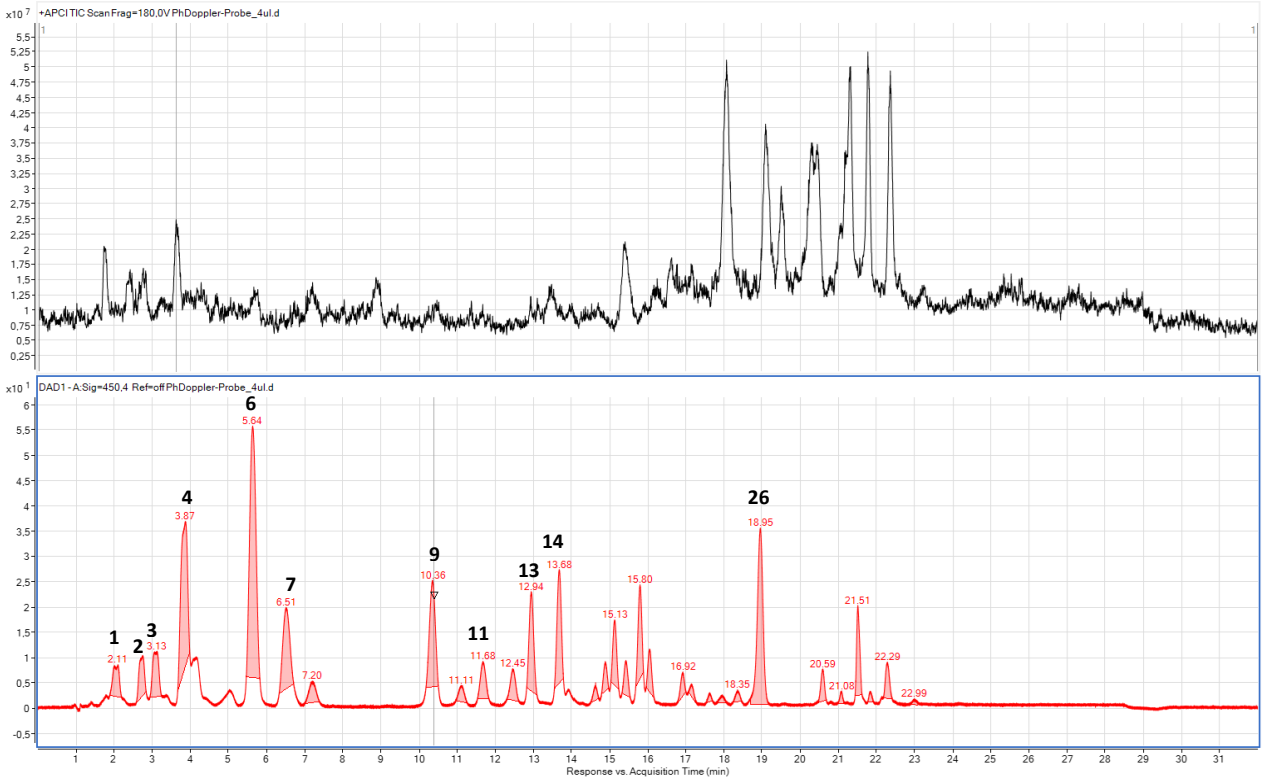

Figure 2: HPLC-MS Chromatogram of all primary and secondary carotenoids of C. terrestris

The following pigments were separated by HPLC and then identified based on the mass to charge ration (m/z) by MS (*Table 5*).

Table 5: Peak Identification of primary and secondary carotenoids with the associated retention time

Peak number	Retention time [min]	Carotenoid
1	2.11	neoxanthin & violaxanthin co-elution + water loss
2	2.75	astaxanthin
3	3.13	adonixanthin+ water loss
4	3.87	lutein + zeaxanthin co-elution
6	5.64	canthaxanthin + water loss
7	6.51	Chl *b*
9	10.36	Chl *a*

11	11.68	echinenone
13	12.94	astaxanthin - monoester
14	13.68	adonixanthin-Monoester
26	18.95	ß-carotene

The following pigments were identified:

- Primary carotenoids: violaxanthin, neoxanthin, lutein, zeaxanthin, echinenone, and β-carotene
- Secondary carotenoids: astaxanthin, adonixanthin, and canthaxanthin
- Chlorophyll *a* and chlorophyll *b*

Free astaxanthin and adonixanthin and their esterified forms as well as free canthaxanthin were the fundamental SC in *C. terrestris*. Lutein represents the major PC in *C. terrestris*.

3.3 C-source screening for mixotrophic cultivation

C. terrestris was grown in shake flasks filled with a volume of 50 mL of 1N, 0.6N, and 0.2N-BBM medium supplemented with 1g L^{-1} of different C-source to gather basic knowledge about carbohydrate uptake, the development of the carotenoid composition, the intracellular carbohydrate rate as well as the corresponding biomass. All this data is used for upscaling experiments in the photobioreactor. Mixotrophic culturing with light and C-source as energy sources was used as culturing method for accelerated growth. A culture with the three different nitrogen levels but without carbohydrates for photoautotrophic growth was used as reference. 1 mL of *C. terrestris* culture was inoculated and positioned in an incubation shaker with a 3% CO_2 supply. Samples of the different flasks were taken irregularly for 53 days for the determination of optical density at a wavelength of 600 nm and the carbon and NO_3^- uptake and to evaluate the carbohydrate storage. Furthermore, at the end of the experiment, 20 mL of culture broth was harvested for the dry cell weight (DCW) and carotenoid quantification.

The biomass was harvested and the results of the DCW are shown in the following tables (*Table 6*,*Table 7*,*Table 8*)

Table 6: Mixotrophic cultivation: DCW of BBM (250 mg L^{-1} $NaNO_3$) with different carbon sources

BBM	**DCW [g·L^{-1}]**
glucose	4.67 ± 0.10
glycerol	3.47 ± 0.085
sodium acetate	3.20 ± 0.39
fructose	4.24 ± 0.16
ribose	4.03 ± 0.21
xylose	0.59 ± 0.080
reference (photoautotrophic)	4.54 ± 0.18

Table 7: Mixotrophic cultivation: DCW of 0.6N-BBM (150 mg L^{-1} $NaNO_3$) with different carbon sources

0.6N-BBM	**DCW [g·L^{-1}]**
glucose	3.63 ± 0.037
glycerol	3.00 ± 0.26
sodium acetate	3.03 ± 0.043
reference (photoautotrophic)	3.32 ± 0.040

Table 8: Mixotrophic cultivation: DCW of 0.2N-BBM (50 mg L^{-1} $NaNO_3$) with different carbon sources

0.2N-BBM	**DCW [g·L^{-1}]**
glucose	1.93 ± 0.030
glycerol	1.50 ± 0.030
sodium acetate	1.44 ± 0.028
reference (photoautotrophic)	1.47 ± 0.025

The shake flasks with 1 g·L^{-1} supplemented glucose resulted in the highest DCW in every $NaNO_3$ level from 50 to 250 mg·L^{-1} reaching 4.67 ± 0.10 g·L^{-1} at BBM, 3.63 ± 0.037 g·L^{-1} at 0.6N-BBM, and 1.93 ± 0.030 g·L^{-1} at 0.2N-BBM. In contrast, the shake flasks with 1 g·L^{-1} supplemented sodium acetate had among the lowest DCW values in each N-BBM media category resulting in 3.20 ± 0.39 g·L^{-1} at BBM, 3.03 ± 0.043 g·L^{-1} at 0.6N-BBM, and 1.44 ± 0.028 g·L^{-1} at 0.2N-BBM. The phototrophic cultures had the second-best DCW values reaching 4.54 ± 0.18 g·L^{-1} at BBM medium and 3.32 ± 0.040 g·L^{-1} at 0.6N-BBM. The addition of glycerol in the category of 0.2N-BBM media resulted in the second most DCW with 1.50 g·L^{-1}.

According to the results illustrated in *Figure 3* highest chlorophyll content was reached of the shake flasks with BBM medium.

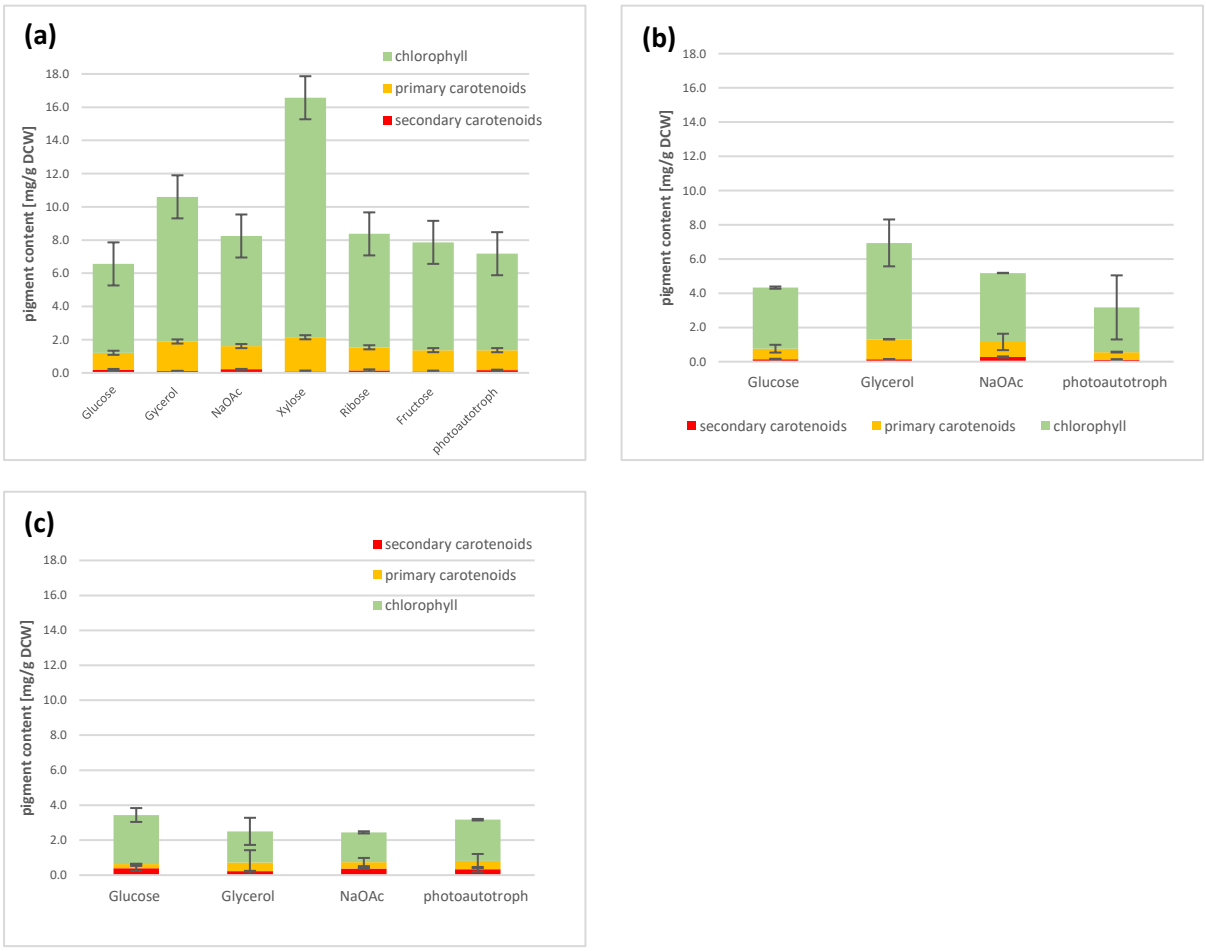

Figure 3: Primary, secondary carotenoid content and chlorophyll content of C. terrestris cultured with different nitrogen concentrations and different c-sources, a) 1N BBM (250 mg $NaNO_3$), b) 0.6 N BBM (150 mg $NaNO_3$) c) 0.2 N BBM (50 mg $NaNO_3$). The primary carotenoids include the pigments lutein, zeaxanthin, neoxanthin, violaxanthin, ß-carotene and echinenone. The secondary carotenoids include the pigments canthaxanthin, adonixanthin, adonixanthin monoester, astaxanthin, astaxanthin monoester. The amount of chlorophyll contains chlorophyll a and chlorophyll b

The highest chlorophyll *a* and *b* content comprised 14.42 ± 3.27 $mg \cdot g^{-1}$ with BBM supplemented with 1 $g \cdot L^{-1}$ xylose as a C-source, followed by the 1 $g \cdot L^{-1}$ glycerol supplemented culture reaching 8.71 ± 0.98 $mg \cdot g^{-1}$. The lowest chlorophyll values were obtained in the shake flasks with the 0.2N-BBM medium. For the shake flask cultures with 1 $g \cdot L^{-1}$ supplemented glucose with 0.2N-BBM, the measured chlorophyll *a* + *b* content was 2.80 ± 0.40 $mg \cdot g^{-1}$, for the 1 $g \cdot L^{-1}$ glycerol culture 1.79 ± 0.78 $mg \cdot g^{-1}$, for 1 $g \cdot L^{-1}$ supplemented sodium acetate culture 1.70 ± 0.06 $mg \cdot g^{-1}$ and for the reference with no C-source addition 2.36 ± 0.04 $mg \cdot g^{-1}$ The highest primary carotenoid content was also reached in the shake flask cultures with BBM. Repeatedly, the highest primary carotenoid content was reached also by the 1 $g \cdot L^{-}$

[1] supplemented xylose culture yielding in 2.07 ± 0.08 mg·g^{-1}, followed by the 1 g·L^{-1} supplemented glycerol shake flask culture measuring 1.79 ± 0.18 mg·g^{-1}. The lowest primary carotenoid content followed the same pattern as the chlorophyll content. The lowest values of PC were found in the 0.2N-BBM cultures. The primary carotenoid content for the 1 g·L^{-1} supplemented glucose culture was 0.25 ± 0.01 mg·g^{-1}, for 1 g·L^{-1} supplemented glycerol culture 0.49 ± 0.71 mg·g^{-1}, for 1 g·L^{-1} supplemented sodium acetate culture 0.37 ± 0.23 mg·g^{-1} and for the reference culture (photoautotrophic) 0.48 ± 0.40 mg·g^{-1} DCW. In contrast, the highest levels of SC were found at 0.2N-BBM cultures, and the lowest levels of SC were found at BBM cultures. The amount of SC for the 0.2N-BBM cultures for 1 g·L^{-1} supplemented glucose culture was 0.38 ± 0.14 mg·g^{-1}, followed by 1 g·L^{-1} supplemented sodium acetate culture reaching 0.38 ± 0.01 mg·g^{-1} and 1 g·L^{-1} supplemented glycerol culture yielding 0.22 ± 0.02 mg·g^{-1} compared to 0.33 ± 0.14 mg·g^{-1} which was measured in the reference culture (*Figure 3*a,b,c). For a more detailed insight into the distribution of SC, *Figure 4*a,b,c was created.

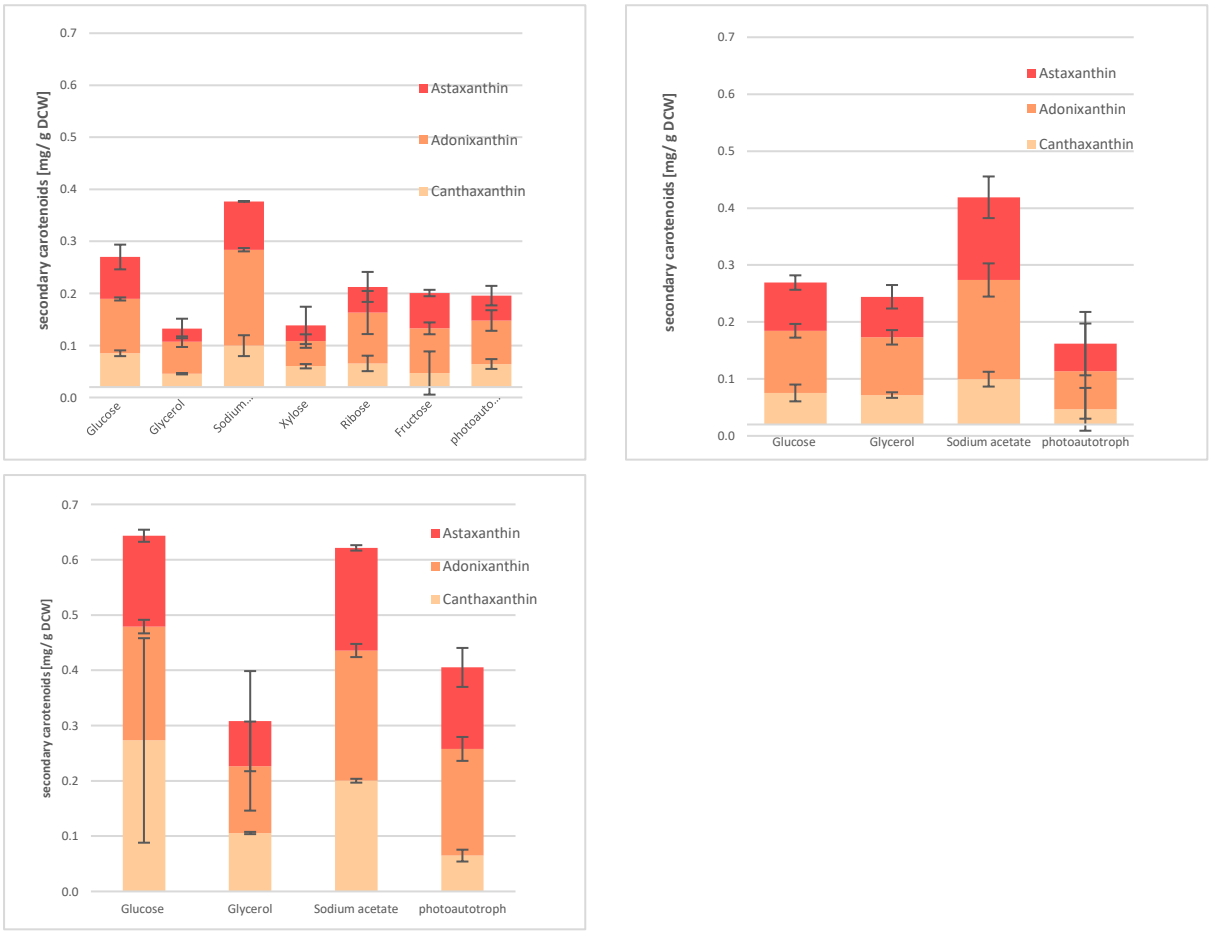

Figure 4: *Detailed composition of secondary carotenoids including canthaxanthin, adonixanthin, astaxanthin and the esterified derivates of adonixanthin and astaxanthin. shake flask experiment cultured with different carbohydrate sources and different nitrogen concentrations, a) BBM (250 mg $NaNO_3$), b) 0.6N-BBM (150 mg $NaNO_3$), c) 0.2N-BBM (50 mg $NaNO_3$).*

The highest amount of canthaxanthin was detected in the 0.2N-BBM 1 $g \cdot L^{-1}$ supplemented glucose culture reaching 0.273 ± 0.185 $mg \cdot g^{-1}$, although the canthaxanthin content of the two duplicates has a very large error rate, followed by 0.200 ± 0.003 $mg \cdot g^{-1}$ of the 1 $g \cdot L^{-1}$ supplemented sodium acetate culture. The smallest amount of canthaxanthin is represented by the BBM 1 $g \cdot L^{-1}$ supplemented glycerol culture yielding 0.026 ± 0.001 $mg \cdot g^{-1}$. The highest content of adonixanthin was found at the 0.2N-BBM 1 $g \cdot L^{-1}$ supplemented sodium acetate culture reaching 0.235 ± 0.012 $mg \cdot g^{-1}$, followed by the 0.2N-BBM 1 $g \cdot L^{-1}$ supplemented glucose culture resulting in 0.206 ± 0.012 $mg \cdot g^{-1}$. All cultures with the three different $NaNO_3$ concentrations supplemented with 1 $g \cdot L^{-1}$ sodium acetate reached relatively high levels of adonixanthin. The highest astaxanthin amounts were also found in the 0.2N-BBM cultures. The 0.2N-BBM 1 $g \cdot L^{-1}$ supplemented sodium acetate culture also represented the culture with the highest astaxanthin content reaching 0.186 ± 0.005 $mg \cdot g^{-1}$, followed by the 0.2N-BBM 1 $g \cdot L^{-1}$ supplemented glucose culture resulting in 0.164 ± 0.011 $mg \cdot g^{-1}$. The smallest astaxanthin amount was found in the BBM 1 $g \cdot L^{-1}$ supplemented glycerol culture yielding 0.025 ± 0.019 $mg \cdot g^{-1}$.

3.4 Influence of different $NaNO_3$/Illumination levels

C. terrestris was cultivated in shake flasks filled with 50 mL of 1N, 0.6N, and 0.2N-BBM medium supplemented with 1 $g \cdot L^{-1}$ of glucose or sodium acetate according to the experimental design *Table 4*. The shake flasks were irradiated with a photosynthetic photon flux density (PPFD) of either 30, 90, or 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 29 days of cultivation. This experiment was executed to gain basic knowledge about the correlation of light, nitrogen, and carbon source influencing the yield of secondary carotenoids and biomass production. The experiments were performed in duplicates according to *Table 4*.

In the DoE study, light intensity (30 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and $NaNO_3$ content (50-250 $mg \cdot L^{-1}$) were set as quantitative factors, the two different types of carbohydrates plus cultures only cultivated photoautotrophically were set to qualitative factors. This required a total of 27 experiments plus three repeated experiments for the center points to result in a full factorial design with every factor varied at three levels to gain a resolution. An excellent model was obtained using multiple linear regression (MLR) for the prediction of the yield of biomass and SC content.

The replicate plots illustrated in *Figure 5* and *Figure 6* show the variation of the results of all 30 experiments.

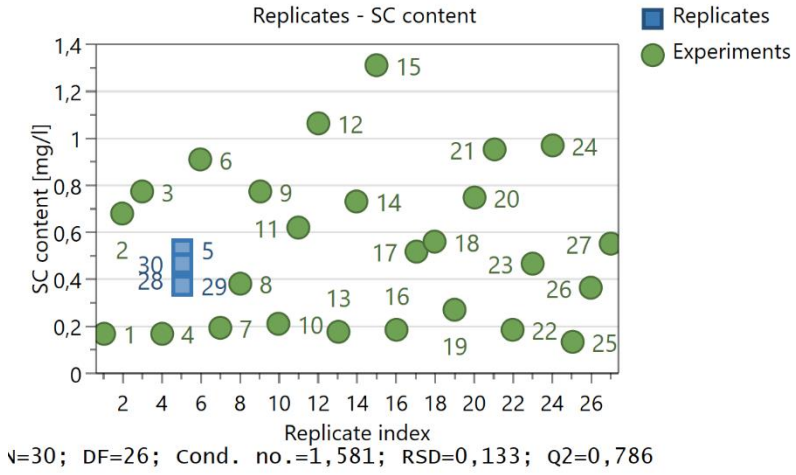

Figure 5: Replicate plot: variation of SC content across the 30 experiments in the DoE. Repeated experiments carried out once are illustrated as green dots, the repeated experiments are connected by a line and are illustrated in a different color (blue rectangle)

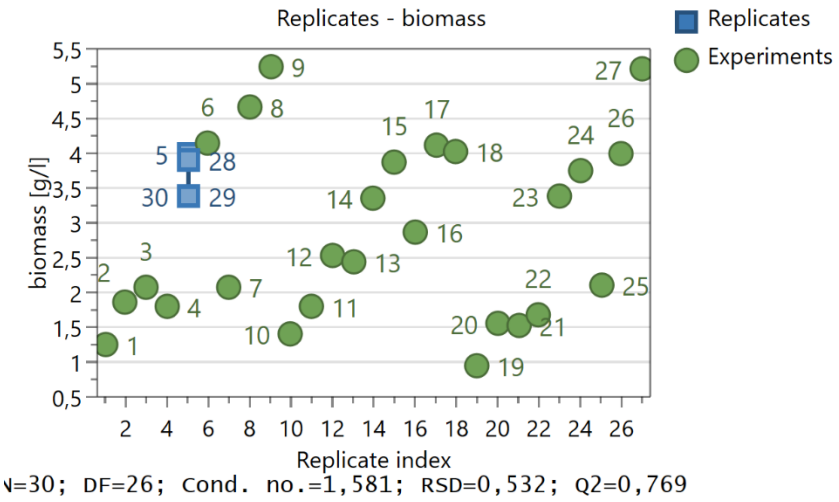

Figure 6: Replicate plot: variation of biomass content across the 30 experiments in the DoE. Repeated experiments carried out once are illustrated as green dots, the repeated experiments are connected by a line and are illustrated in a different color (blue rectangle)

Experiments that have been carried out once are shown as green dots (average of the duplicates). The repeated experiments are connected by a line and shown as blue rectangles. The variability of both responses (biomass and SC content) of the replicates could be seen as relatively small. The three center points (blue rectangles) for both responses resulted in consistent biomass and SC content, as can be seen in *Figure 5* and *Figure 6*, which clarify that the overall variability of all independent experiments (green) was much higher than the variability of the repeated experiments. This result showed that the experiment can be seen as a useful model.

The following coefficient plots (*Figure 7*, *Figure 8*) of the coefficient plot provided information on which factor is affecting the yield of the microalgal biomass.

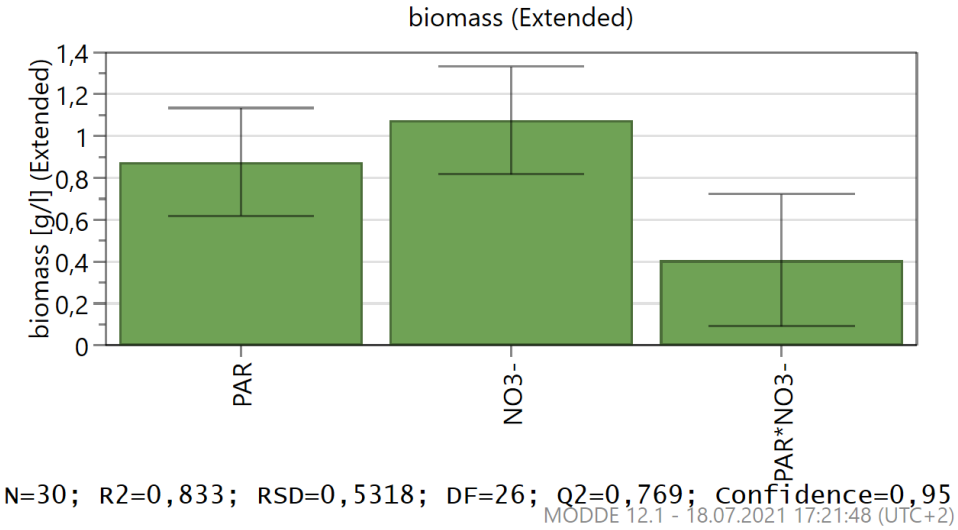

Figure 7: Coefficient plot generated from MODDE 12.1 experimental design showing all factors influencing the productivity of biomass. PAR -Illumination intensity; NO_3^- : nitrate concentration

Every factor significantly influencing the biomass is represented by a green bar. The green bars illustrate shown in *Figure 7*, showing the average effect of the yield of the biomass. The most significant factor influencing biomass productivity is the $NaNO_3$ concentration in the medium. The nitrate concentration positively affected the biomass productivity of *C. terrestris* giving on average a 1.075 $g \cdot L^{-1}$ increase of the biomass. The photosynthetic photon flux density (PPFD) (PAR) had also a significant impact on an average increase of 0.876 $g \cdot L^{-1}$ on the production of biomass. With a higher concentration of $NaNO_3$ and a higher light level, an improvement in biomass could be expected. The interaction between the two factors was also shown in *Figure 7*, but is not fully resolved. Therefore, care must be taken in the interpretation. Among the non-significant factors was the selection of carbohydrates. The addition of 1 $g \cdot L^{-1}$ glucose, NaOAc, and photoautotrophic culture did not significantly affect biomass productivity.

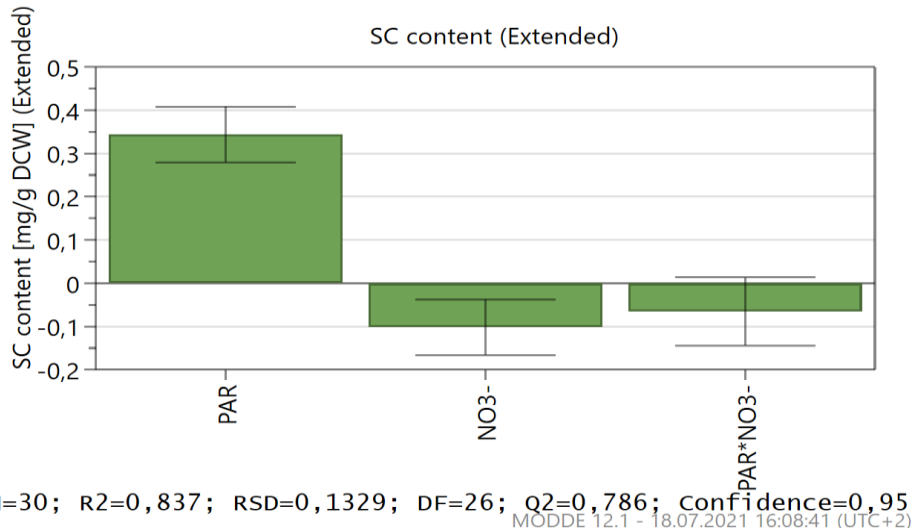

Figure 8: Coefficient plot generated from MODDE 12.1 experimental design showing all factors influencing the SC yield. PAR -Illumination intensity; NO3- : nitrate concentration

The increasing light level positively affected the SC content giving on average a 0.344 $mg \cdot g^{-1}$ increase of SC content. On the other hand, the increase of $NaNO_3$ in the medium negatively affected the SC content by an average of -0.102 $mg \cdot g^{-1}$. In other words, an increase of $NaNO_3$ in the medium was resulting in a decrease in SC content. The interaction between the two factors was also illustrated (*Figure 8*), but the factors were not significantly connected. Also, in terms of SC content, no significant correlation could be found regarding carbohydrates in the medium. The addition of 1.0 $g \cdot L^{-1}$ glucose, sodium acetate, or photoautotrophic culture did not significantly affect biomass productivity.

It suggests that the biomass can be increased by increasing the light level with a low level of $NaNO_3$ (50 $mg \cdot L^{-1}$) from approximately 1.40 $g \cdot L^{-1}$ biomass at 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD to approximately 2.34 $g \cdot L^{-1}$ at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD according to the interaction plot (*Figure 9*).

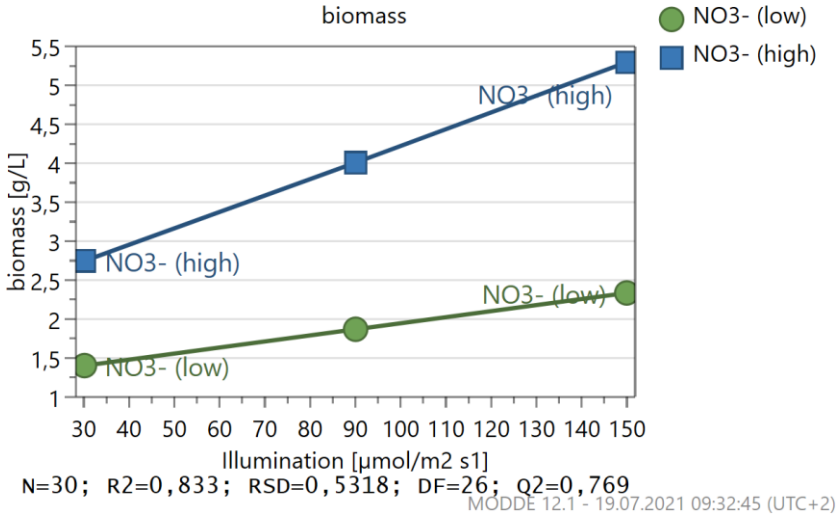

Figure 9: Interaction plot showing the interaction between the two most impactful components: illumination level and the $NaNO_3$ concentrations on the biomass yield

This resulted in an increase of biomass resulting in 0.94 $g \cdot L^{-1}$ by increasing PPFD from 30 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The increase of biomass productivity by increasing the PPFD was even higher at a high level of $NaNO_3$ in the medium (250 $mg \cdot L^{-1}$). It was observed that the biomass increases from 2.74 $g \cdot L^{-1}$ at 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD to approximately 5.30 $g \cdot L^{-1}$ at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD. The increase in light from 30-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD resulted in an increase of biomass reaching 2.56 $g \cdot L^{-1}$. This suggested that the optimal conditions for a high microalgal biomass yield are a high $NaNO_3$ concentration (250 $mg \cdot L^{-1}$) in the medium combined with a high level of PPFD (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

The interaction plot (*Figure 10*) illustrates the interactions between $NaNO_3$ concentration in the medium and illumination level on microalgal SC yield.

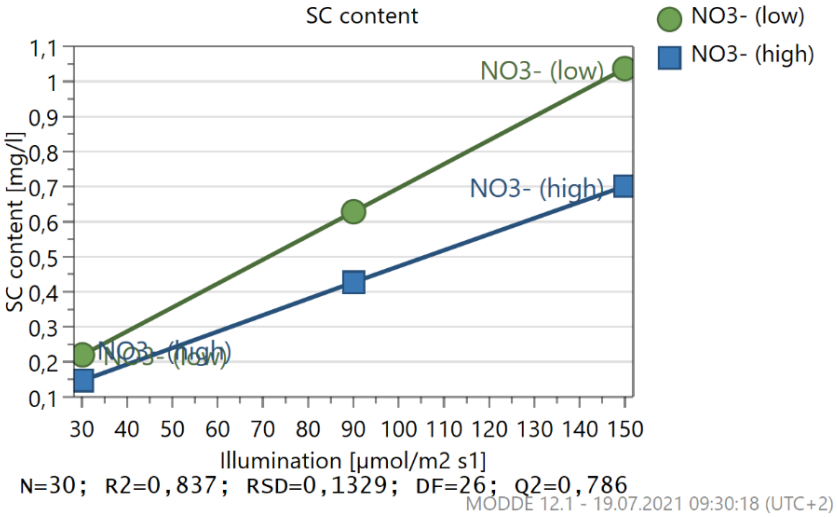

Figure 10: Interaction plot showing the interaction between the two most impactful components: illumination level and the NaNO3 concentrations on the SC yield

It can be generally suggested that the SC content increases with a rise of light (30 to 150 µmol·m^{-2}·s^{-1}). On average, at low nitrogen levels (50 mg·L^{-1}) the SC content increases from approximately 0.220 mg·g^{-1} DCW at 30 µmol·m^{-2}·s^{-1} PPFD to approximately 1.038 mg·g^{-1} at 150 µmol·m^{-2}·s^{-1} PPFD. The increase in light from 30-150 µmol·m^{-2}·s^{-1} PPFD resulted in an increase of biomass yielding 0.818 mg·g^{-1}.

On high $NaNO_3$ levels (250 mg·L^{-1}) the SC content gained is generally much lower than the SC content of the low levels of $NaNO_3$ (50 mg·L^{-1}). It was observed that the SC content increases from 0.147 mg·g^{-1} at 30 µmol·m^{-2}·s^{-1} PPFD to approximately 0.704 mg·g^{-1}at 150 µmol·m^{-2}·s^{-1} PPFD. The increase in light from 30-150 µmol·m^{-2}·s^{-1} PPFD resulted in an increase of SC content yielding 0.557 mg·g^{-1}. From that, it can be assumed that the optimal conditions for a high SC yield contradict the optimal conditions for high biomass yield. It suggested that the optimal cultivation conditions for a high SC yield are a high level of PPFD (150 µmol·m^{-2}·s^{-1}) combined with low $NaNO_3$ concentrations (50 mg·L^{-1}).

As shown by the contour plots in *Figure 11* a and b, it could be clearly seen that the factors for increasing biomass and SC are contradictory.

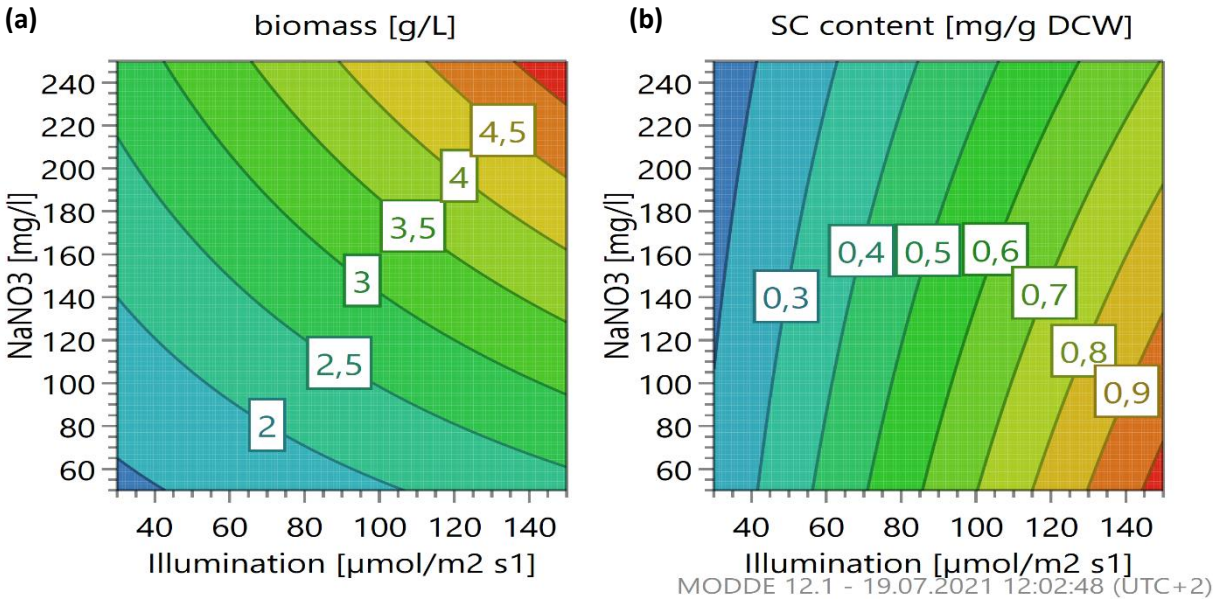

Figure 11: Response contour plot for biomass and SC yield depending on $NaNO_3$ concentration and Illumination (PPFD).

The response to an increase of $NaNO_3$ concentration in the medium was opposite for both factors, which can also be seen in the interaction plot in *Figure 5* and *Figure 6*. The highest biomass yield could only be achieved with a high concentration of $NaNO_3$. Conversely, the highest SC yield could only be achieved at lower $NaNO_3$ concentrations. These factors conflicted with each other. However, both responses of biomass and SC yield reacted in the same way to the increase in PPFD. The yield of both increased with the increase in light.

3.5 Cultivation in Stirred Photobioreactor

To get as close as possible to the industrial production of secondary carotenoids, upscaling experiments in 1.25 L photobioreactors have been performed. Therefore, the cultivation data from the shake flasks experiment were used. As a carbon source sodium acetate and glucose were used, as they achieved the best results in terms of carotenoid production and biomass growth. In addition, photoautotrophic cultivation was used as a reference. Since photobioreactors have a better CO_2 supply compared to the shake flasks experiments, higher growth rates can be expected, different nitrogen concentrations were chosen. *C. terrestris* was grown in PBR filled with 1.25 L BBM and 2N-BBM medium supplemented with 1 $g \cdot L^{-1}$ glucose, 1.36 $g \cdot L^{-1}$ sodium acetate, (C-equimolar) and without any carbon source to find the most suitable setup for a high secondary carotenoid content and an appropriate amount of biomass. Samples were taken to determine the optical density (OD_{600}), the NO_3^- and organic carbon uptake, the carotenoid quantification, the dry cell weight, and the intracellular carbohydrate storage. The experiments lasted for 21 days. The deviations were calculated using RGP function, which represents the standard errors of the coefficients.

OD measurements were taken to observe the process. The OD measurements are shown in *Figure 12*. It can be seen that the fermentations supplemented with 2N-BBM medium had in general higher absorption at 600 nm than the fermentations with BBM medium.

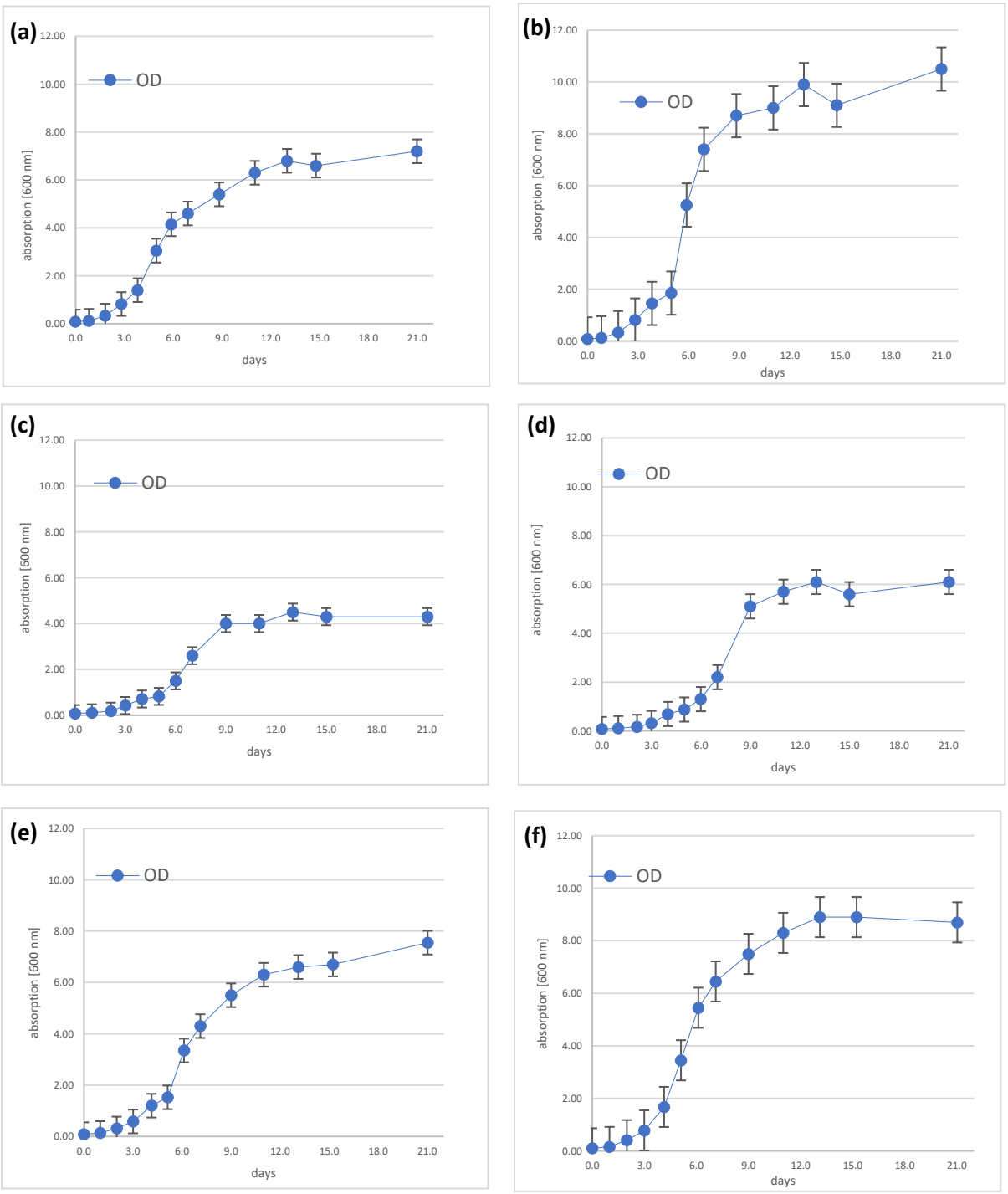

Figure 12: Optical density measurements at 600 nm to evaluate the growth of C. terrestris cultured in PBR a) BBM with 1 g·L^{-1} glucose b) 2N-BBM with 1 g·L^{-1} c)BBM with 1.36 g·L^{-1} sodium acetate d) 2N-BBM with 1.36 g·L^{-1} sodium acetate e) BBM photoautotrophic (reference culture) f) 2N-BBM photoautotrophic (reference culture).

The highest absorption has been measured at the 2N-BBM-glucose fermentation at the end of cultivation with an OD of 10.50 ± 0.8. The lowest OD value measured at the end of cultivation was at the BBM-NaOAc fermentation yielded in 4.30 ± 0.37. In general, it can be said, that the OD value does not change much from cultivation day 15 to cultivation day 21. In fact, in the 2N-BBM-autotroph cultivation, the OD value decreased from 0.2 and in the BBM-NaOAc fermentation, the value neither increased nor decreased. The highest OD increase was observed in all fermentations from day 3 to day 9 with exponential growth. After day 9 it could be assumed based on *Figure 12* that the stagnated growth phase has started.

The amount of harvested microalgal biomass is shown in *Table 9*.

Table 9: DCW harvested on day 21 of cultivation. After the 21. day cultivation was stopped.

	Glucose	**NaOAc**	**Photoautotrophic**
1N-BBM	2.87 ± 0.47 $g \cdot L^{-1}$	2.40 ± 0.41 $g \cdot L^{-1}$	3.79 ± 0.36 $g \cdot L^{-1}$
2N-BBM	3.82 ± 0.39 $g \cdot L^{-1}$	3.50 ± 0.54 $g \cdot L^{-1}$	4.00 ± 0.57 $g \cdot L^{-1}$

The largest amount of DCW in the BBM medium category at the end of the fermentation was detected in BBM-autotrophic fermentation reaching 3.79 ± 0.36 $g \cdot L^{-1}$, followed by the BBM-glucose fermentation resulting in 2.87 $g \cdot L^{-1}$ and the BBM-NaOAc fermentation yielding approximately 0.50 $g \cdot L^{-1}$ less than the BBM-glucose fermentation. The highest DCW content in the 2N-BBM category was also detected at 2N-BBM-autotrophic fermentation reaching 4.0 ± 0.57 $g \cdot L^{-1}$.

It can be seen that the uptake of nitrate in both BBM and 2N-BBM 1 $g \cdot L^{-1}$ supplemented glucose fermentation (*Figure 13*a,b) was faster than in the two supplemented sodium acetate fermentation(*Figure 13*c,d).

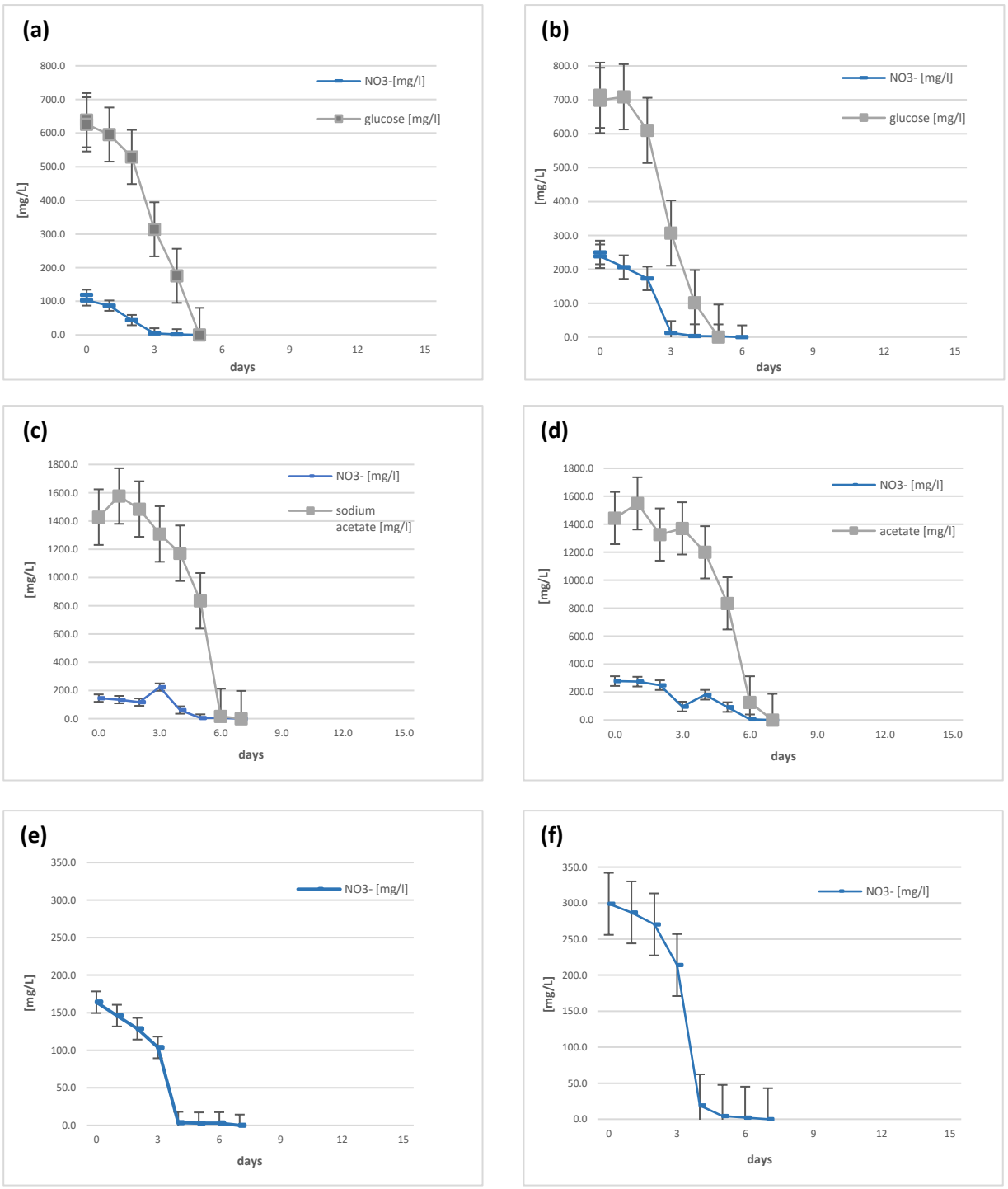

Figure 13: Quantitative uptake rate of NO_3^- (nitrate), glucose and sodium acetate of C. terrestris in PBR over 21 days. a) BBM medium with 1 g·L^{-1} glucose supplementation b) 2N-BBM medium with 1 g·L^{-}glucose supplementation c) BBM medium with 1.36 g·L^{-1} sodium acetate supplementation d) 2N-BBM medium with 1.36 1 g·L^{-1} sodium acetate supplementation e) BBM medium - photoautotrophic growth f) 2N-BBM medium - photoautotrophic

The total nitrate content of BBM-glucose fermentation was already 4.53 mg·L^{-1} on day 4, whereas in direct comparison the nitrate content of BBM-NaOAc fermentation was still more than ten times higher (61.53 mg·L^{-1}) on the same day of fermentation. The nitrate uptake of N-BBM-autotrophic fermentation performs similarly to the uptake of BBM-glucose fermentation. The nitrate content on day four was 3.61 mg·L^{-1} (*Figure 13*d). The nitrate uptake behaves similarly in all 2N-BBM fermentations. On day 7, no more nitrate was measured in the 2N-BBM cultures (*Figure 13*b,d,f).

The chlorophyll content decreased from 5.13 to 3.28 mg·g^{-1} in the BBM-glucose fermentation (*Figure 14*a), from 4.81 to 2.26 mg·g^{-1} in the BBM-NaOAc fermentation (*Figure 14*c), and from 6.17 to 2.44 mg·g^{-1} in the BBM-autotrophic fermentation (*Figure 14*e).

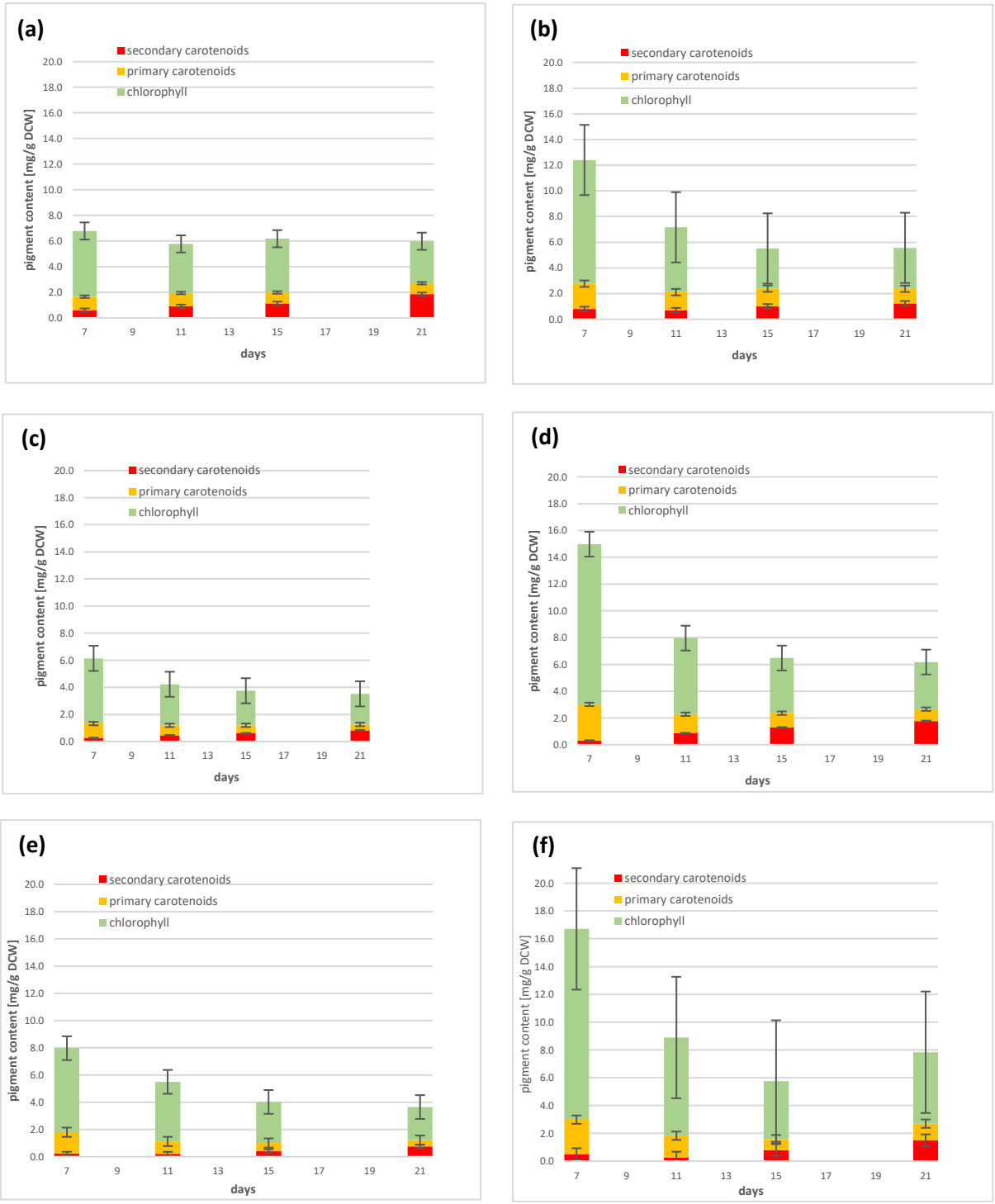

Figure 14: Primary, secondary carotenoid content and chlorophyll content of C. terrestris cultured in PBR a) BBM with 1 g·L^{-1} glucose b) 2N-BBM with 1 g·L^{-1} c) BBM with 1.36 g·L^{-1} sodium acetate d) 2N-BBM with 1.36 g·L^{-1} sodium acetate e) BBM photoautotrophic (reference culture) f) 2N-BBM photoautotrophic (reference culture). The primary carotenoids include the pigments lutein, zeaxanthin, neoxanthin, violaxanthin, ß-carotene and echinenone. The secondary carotenoids include the pigments canthaxanthin, adonixanthin, adonixanthin monoester, astaxanthin, astaxanthin monoester. The amount of chlorophyll contains chlorophyll a and chlorophyll b

In the 2N-BBM fermentations, the chlorophyll decreases from 9.63 to 3.19 $mg \cdot g^{-1}$ in the glucose fermentation (*Figure 14*b), from 11.96 to 3.52 $mg \cdot g^{-1}$ in the NaOAc fermentation (*Figure 14*d), and from 13.73 to 5.14 $mg \cdot g^{-1}$ in the autotrophic fermentation (*Figure 14*f). The primary carotenoid content in the BBM fermentations also decreased from 1.06 to 0.86 $mg \cdot g^{-1}$ in the glucose fermentation (*Figure 14*a), from 1.07 to 0.431 $mg \cdot g^{-1}$ for the NaOAc fermentation (*Figure 14*c), and for the reference culture the PC content decreased from 1.58 to 0.46 $mg \cdot g^{-1}$(*Figure 14*e). The reduction of the PC content for the 2N-BBM fermentations was from 1.97 to 1.13 $mg \cdot g^{-1}$ for the glucose fermentation (*Figure 14*b), from 2.71 to 0.888 $mg \cdot g^{-1}$ for the NaOAc fermentation (*Figure 14*d), and 2.49 to 1.20 $mg \cdot g^{-1}$ for the reference culture (*Figure 14*f).

On the other hand, the highest SC content was obtained in the BBM- glucose fermentation resulting in 1.85 $mg \cdot g^{-1}$ (*Figure 14*a) followed by the 2N-BBM-NaOAc culture yielding 1.77 $mg \cdot g^{-1}$ (*Figure 14*d). The lowest SC level reaching 0.75 $mg \cdot g^{-1}$ was measured at the BBM-autotrophic culture (*Figure 14*e). Due to the results of *Figure 14*, it could be generally said that the chlorophyll content decreased during the fermentation as well as the PC content. However, the SC content had increased from day 7 to day 21 significantly. The highest progress was observed from day 15 to day 21 by all fermentations except the 2N-BBM-glucose fermentation (*Figure 14*b). For the investigation of accumulated and produced SC content in the fermentation in detail, *Figure 15* was created.

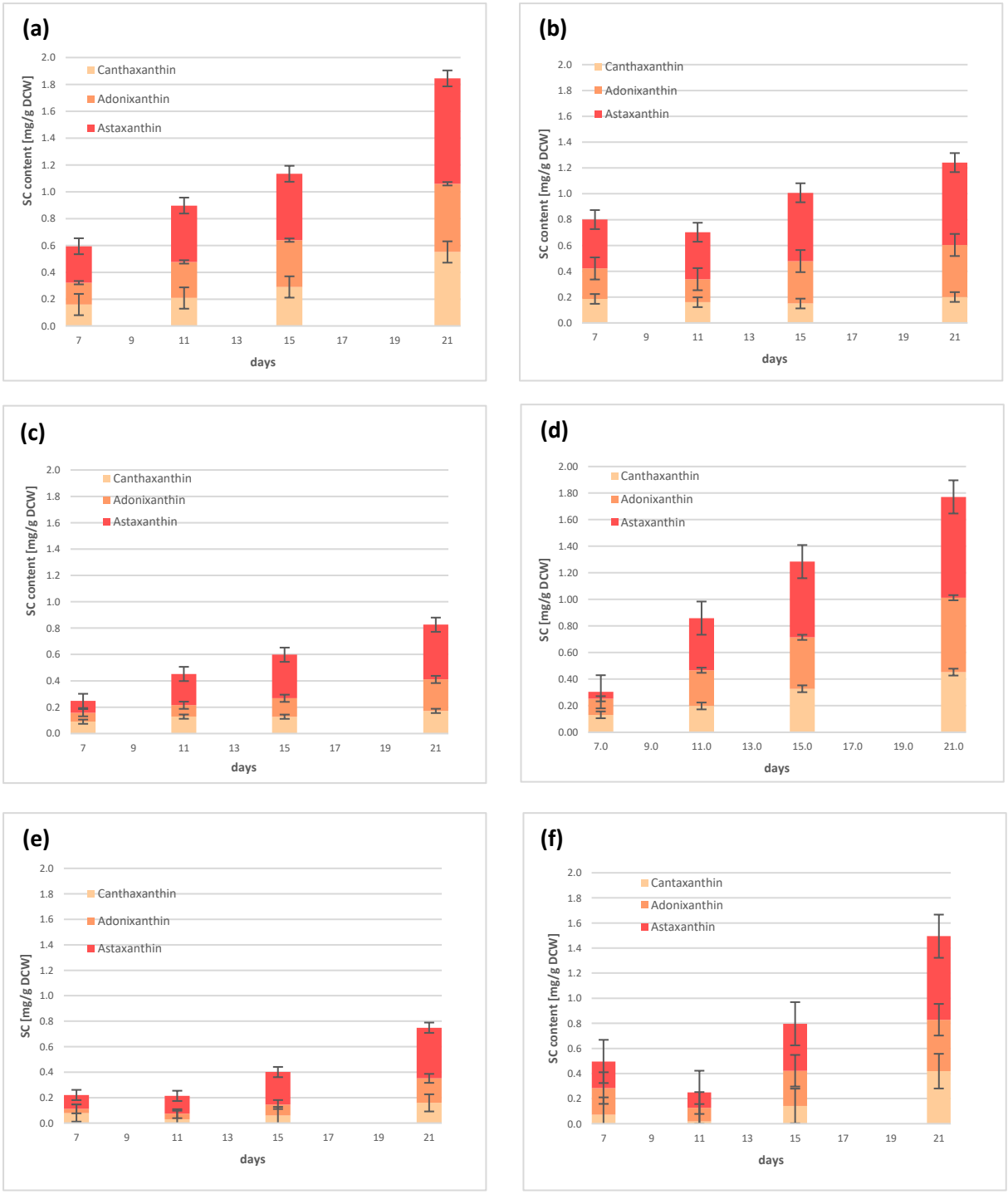

Figure 15: Detailed composition of secondary carotenoids including canthaxanthin, adonixanthin, astaxanthin and esterified derivates of C. terrestris. batch experiment cultured in PBR a) BBM with 1 g·L^{-1} glucose b) 2N-BBM with 1 g·L^{-1} glucose c) 1N-BBM with 1.36 g·L^{-1} NaOAc d) 2N-BBM with 1.36 g·L^{-1} NaOAc e) BBM photoautotrophic (reference culture) f) 2N-BBM photoautotrophic (reference culture).

The astaxanthin content from BBM PBR experiments increased from day 7 to day 21 from 0.271 to 0.785 mg·g^{-1} in the glucose fermentation. The starting astaxanthin value on day 7 was 0.0896 mg·g^{-1}

and increased to 0.415 mg·g^{-1} by day 21 NaOAc fermentation compared to the reference culture the astaxanthin content increased from 0.109 mg·g^{-1} on day 7 to 0.398 mg·g^{-1}. The highest canthaxanthin content was obtained at the BBM-glucose fermentation yielding 0.551 mg·g^{-1}. The highest astaxanthin levels were also detected in the BBM-glucose fermentation, resulting in 0.785 mg·g^{-1} astaxanthin. However, the highest adonixanthin level was investigated in the 2N-BBM-NaOAc fermentation reaching 0.560 mg·g^{-1}. On the other hand, the lowest adonixanthin and canthaxanthin values in the BBM group were detected, reaching 0.192 mg·g^{-1} and 0.159 mg·g^{-1}, both measured in the autotrophic fermentation. The lowest astaxanthin, adonixanthin, and canthaxanthin levels in the 2N-BBM media group were all measured in the glucose fermentation. To ensure better traceability of the results, *Table 10* and *Table 11* were created.

Table 10: Produced secondary carotenoid content of C. terrestris including canthaxanthin, adonixanthin, and astaxanthin including esterified derivatives measured at the end of the PBR experiment (after 21 days) BBM medium fermentation

	glucose	**sodium acetate**	**reference**	**unit**
astaxanthin	0.785	0.415	0.398	mg·g^{-1}
adonixanthin	0.509	0.239	0.192	mg·g^{-1}
canthaxanthin	0.551	0.172	0.159	mg·g^{-1}
total SC content	1.85	0.826	0.749	mg·g^{-1}

Table 11: Produced secondary carotenoid content of C. terrestris including canthaxanthin, adonixanthin, and astaxanthin including esterified derivatives measured at the end of the PBR experiment (after 21 days) 2-N BBM medium fermentation.

	glucose	**sodium acetate**	**reference**	**unit**
astaxanthin	0.638	0.759	0.666	mg·g^{-1}
adonixanthin	0.403	0.560	0.410	mg·g^{-1}
canthaxanthin	0.201	0.453	0.419	mg·g^{-1}
total SC content	1.24	1.77	1.49	mg·g^{-1}

The highest canthaxanthin content was observed in the 2N-BMM-autotrophic fermentation yielding 1.68 mg·L^{-1} followed by the BBM-glucose fermentation and the 2N-BBM-NaOAc fermentation resulting both in 1.58 mg canthaxanthin per liter gained fermentation broth, which can be observed in *Table 12*.

Table 12: Produced canthaxanthin, adonixanthin, and astaxanthin and total SC in mg per L fermentation broth for all PBR experiments after 21 days

PBR-experiment	DCW [g·L^{-1}]	canthaxanthin [mg·L^{-1}]	adonixanthin [mg·L^{-1}]	astaxanthin [mg·L^{-1}]	total SC conc. [mg·L^{-1}]
BBM-glucose	2.86	1.58	1.46	2.25	5.28
BBM-NaOAc	2.40	0.41	0.57	1.00	1.98
BBM-autotroph	3.86	0.60	0.73	1.51	2.83
2-BBM-glucose	3.82	0.77	1.54	2.44	4.74
2-BBM-NaOAc	3.50	1.58	1.96	2.65	6.19
2-BBM-autotroph	4.00	1.68	1.64	2.66	5.98

1.96 mg adonixanthin per liter gained fermentation broth was the highest adonixanthin amount detected in the 2N-BBM fermentation supplemented with NaOAc. The highest astaxanthin amount was found in the 2N-BBM-autotrophic fermentation reaching 2.66 mg·L^{-1}, closely followed by 2.65 mg·L^{-1} astaxanthin in the 2N-BBM fermentation supplemented NaOAc. A total of 6.19 mg of SC per liter could be obtained in the 2N-BBM fermentation supplemented with NaOAc, which was the highest amount that could be extracted.

The largest proportion of secondary carotenoids could be attributed to astaxanthin in all fermentations, which is illustrated in *Figure 16*.

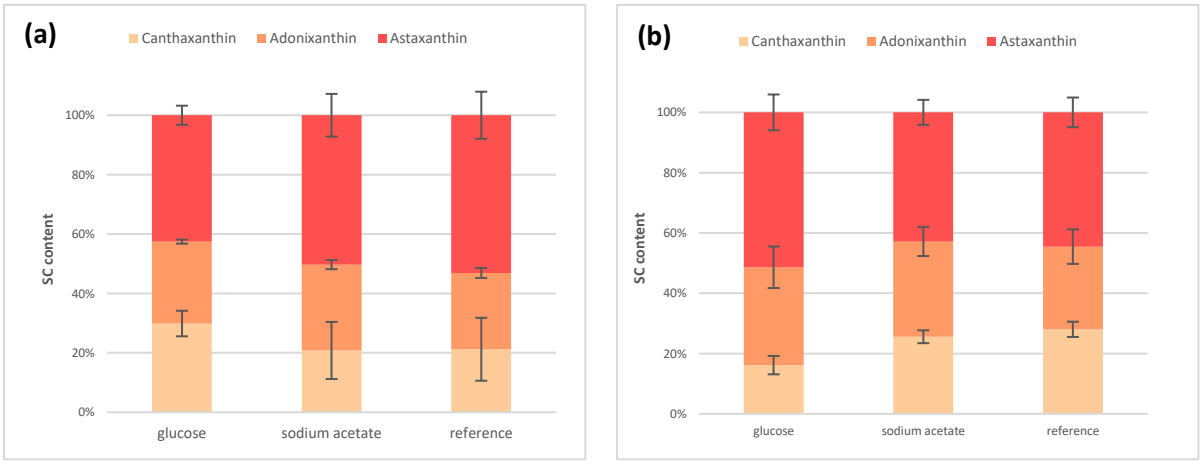

Figure 16: Mass composition of secondary carotenoids of C. terrestris including canthaxanthin, adonixanthin and astaxanthin including the esterified forms a) BBM with 1 g·L^{-1} glucose, 1.36 g·L^{-1} NaOAc and reference culture (photoautotrophic) b) 2N-BBM with 1 g·L^{-1} glucose, 1.36 g·L^{-1} NaOAc and reference culture (photoautotrophic)

The mass composition of astaxanthin ranged from 42.6% to 53.1%. Adonixanthin represented the second-highest proportion of secondary carotenoids in all but the BBM-glucose and 2N-BBM-autotrophic fermentation. In those two experiments, adonixanthin ranked just behind canthaxanthin with 29.9% and 28.0%, respectively. The mass composition of adonixanthin was between 25.7% and 32.5%. The mass composition of canthaxanthin ranged from 16.17% to 29.9%. The average mass composition of astaxanthin was 47.45 ± 4.68%, the average mass composition of adonixanthin reached 28.94 ± 2.61%, followed by 23.61 ± 5.13% average mass composition of canthaxanthin.

From *Table 13*, it can be suggested that the BBM-glucose fermentation produced the most carbohydrate content of all fermentations yielding 26.8 ± 2.94%.

Table 13: Produced carbohydrate content at the end of all fermentations (after 21 days)

Cultivations	**Carbohydrate content [g·g^{-1}]**	**Carbohydrate ratio of DCW [%]**
BBM-glucose	0.268 ± 0.0294	26.8 ± 2.94
BBM-NaOAc	0.209 ± 0.0292	20.9 ± 2.92
BBM-autotroph	0.230 ± 0.0415	23.0 ± 4.15
2-BBM-glucose	0.136 ± 0.0151	13.6 ± 1.51
2-BBM-NaOAc	0.233 ± 0.0685	23.3 ± 6.85
2-BBM-autotroph	0.224 ± 0.0351	22.4 ± 3.51

Also, a large amount of carbohydrate storage was produced in the 2N-BBM-NaOAc fermentation, reaching 23.3 ± 4.15% of DCW, followed by 23.0 ± 4.15% carbohydrate content of the BBM-autotrophic fermentation. It was very noticeable that the carbohydrate content of the 2N-BBM-glucose fermentation is around 10% less than that of all the others, reaching only 13.6 ± 1.51%.

3.6 Fatty Acid Analysis

For the fatty acid analysis, the extraction of lipids was performed with orange-colored *C. terrestris* biomass, which was harvested at the end of a previous stress-induced fermentation to gain secondary carotenoids. The fatty acids profile is shown in *Table 14*.

Table 14: Relative content in percent of all fatty acids, which were found in C. terrestris biomass

Fatty Acids	**Relative Content**
C16:0	12.6% ± 0.15%
C16:1(7Z or 9Z)	0.8% ± 2.42%
C16:2 (7Z, 10Z)	1.3% ± 1.25%
C16:3 (7Z, 10Z, 13Z)	12.5% ± 0.52%
C18:0	1.1% ± 2.98%
C18:1 (7Z,9Z)	30.5% ± 0.14%
C18:2 (9Z, 12Z)	15.6% ± 0.52%
C18:3 (9Z)	25.5% ± 0.89%
Total saturated	**13.7% ± 3.13%**
Total monounsaturated	**31.4% ± 3.35%**
Total polyunsaturated	**54.9% ± 3.2%**

All fatty acids between 14 to 22 C-atoms were measured. The most predominant fatty acid was monosaturated oleic acid (C18:1) resulting in 30.5 ± 0.47%. The most predominant polyunsaturated fatty acid was α-linolenic acid (C18:3) reaching 25.5 ± 0.89% and the most predominant saturated fatty acid was palmitic acid (C16:0) yielding in 12.6 ± 0.15%. Polyunsaturated fatty acids represented the majority of all detected fatty acids reaching 54.9%, followed by monounsaturated fatty acids yielding in 31.4%. This implies that more than 85% of the fatty acids produced are unsaturated. The smallest amount of all detected fatty acids represented the saturated fatty acids.

3.7 Comparison of the productivity of *C. terrestris* and *H. pluvialis*

To compare the SC productivity of the microalgae strain *C. terrestris*, the industrial workhorse for astaxantin production, *H. pluvialis*, was used to find out if *C. terrestris* could be a potential alternative for industrial SC production. Furthermore, this experiment aims to find out if the reactor setup allows a high accumulation of SC at all. This can be observed by comparing the SC yield gained with our set up of the *H. pluvialis* fermentations with the SC yield find in the literature. The *H. pluvialis* fermentation was performed in duplicates in 1.25 L photobioreactors. Due to a lack of time the *C. terrestris* fermentation was performed once. As a carbon source, 1. $g \cdot L^{-1}$ sodium acetate was used. The PBRs were filled with BBM. The starting inoculation volume was 15 mL for both microalgal cultures. Samples were taken to determine the optical density, NO_3^- uptake, carotenoid quantification, DCW, and intracellular carbohydrate storage. The experiments lasted for 21 days. The only difference between the fermentation was the cultivation temperature. *C. terrestris* was cultivated at 20°C and *H. pluvialis* at 24°C.

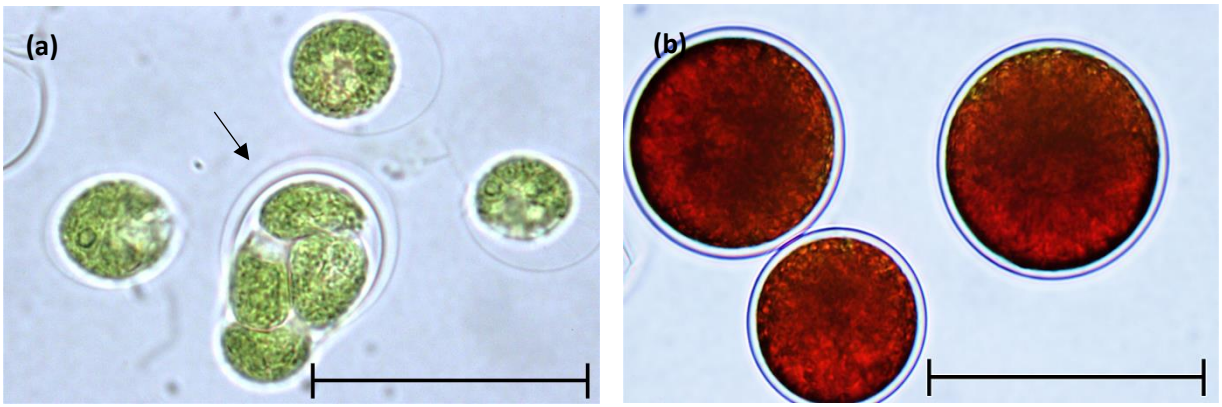

Figure 17: Light microscopy images of H. pluvialis at different cell stages and levels of stress. (a) is showing viable chlorophyll producing cells. (b) is showing astaxanthin accumulating haematocysts. Asexual reproduction can be observed via autosporulation, illustrated in picture (a). The scale bar is 50 µm.

The unicellular *H. pluvialis* is shaped circular in the viable and stressed state (*Figure 17*a,b). The cross-section dimension in the green stage is between 15 and 20 µm (*Figure 17*a). The color changed from green to red under certain levels of stress like nitrogen starvation, which was observed by light microscopy. There was not only a color change but also a change in size. The cells size increased from 15 and 20 µm to 30 to 45µm approximately (*Figure 17*a,b). Asexual reproduction was observed via autosporulation (*Figure 17*a). When viable chlorophyll-producing cells were stressed, they formed cysts and accumulated secondary carotenoids.

In *Figure 18*, the absorption at 600 nm at the end of the *C. terrestris* fermentation yielded an OD value of 3.9±0.6.

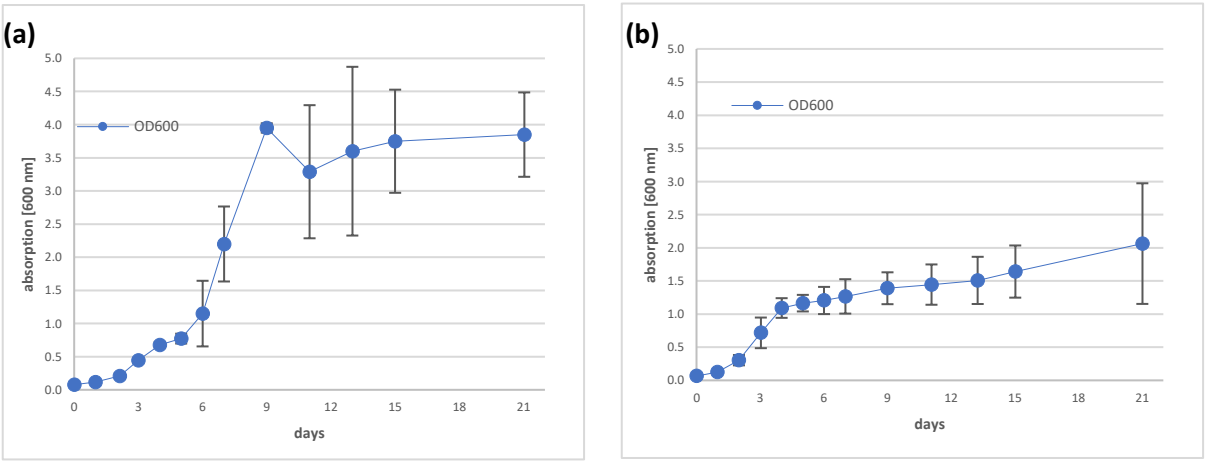

Figure 18: Optical density measurement at 600 nm to evaluate the growth of C. terrestris and H. pluvialis cultured in PBR a) C. terrestris 1N-BBM with 1 g·L^{-1} NaOAc b) H. pluvialis BBM with 1 g·L^{-1} NaOAc

On the other hand, the measured OD value of *H. pluvialis* fermentation at the same time was nearly two times less with 2.1±0.91. In general, it can be said, that the OD value does not change much from cultivation day 11 to cultivation day 21. The highest OD increase was observed in *C. terrestris* from day 5 to day 9 with exponential growth. Whereas the period of the exponential growth phase was much shorter from day 2 to day 4.

The chlorophyll content of the *C. terrestris* fermentation, illustrated in *Figure* 19, decreased from 5.16 to 1.72 ± 1.11 mg·g^{-1}, whereas in the *H. pluvialis* fermentation the amount of chlorophyll declined from 7.51 ± 0.791 to 3.88 ± 0.801 mg·g^{-1}.

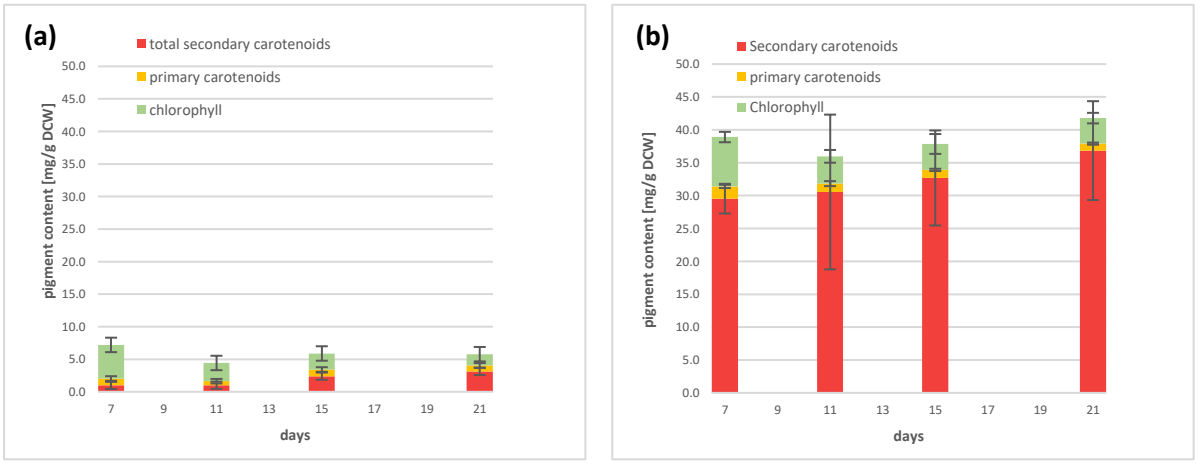

Figure 19: Primary, secondary carotenoid content and chlorophyll content of C. terrestris (a) and H. pluvialis (b) cultured in a PBR. Production developement of secondary carotenoids of fermentation form day 7 to day 21.

This decrease in chlorophyll was quite similar in both fermentations. It dropped of 3.44 mg·g^{-1} (in *C. terrestris*) and 3.63 mg·g^{-1} (*H. pluvialis*) of chlorophyll, respectively. In *H. pluvialis* fermentation, a decrease in primary carotenoids was detected from 1.86 ± 0.243 to 1.07 ± 0.162 mg·g^{-1} during the fermentation, and almost no zeaxanthin was found compared to *C. terrestris* fermentation. Surprisingly, almost no decrease in primary carotenoids was recorded in *C. terrestris* fermentation. The loss was only 0.135 mg·g^{-1}. In comparison, the loss in *H. pluvialis* fermentation was almost 10 times higher. Probably the biggest difference between the two fermentations was the amount of secondary carotenoids produced. In *C. terrestris*, the secondary carotenoids astaxanthin, adonixanthin, and canthaxanthin were found. In comparison, only a high amount of astaxanthin and extremely small amounts of canthaxanthin and adonixanthin were detected in *H. pluvialis*. The amount of secondary carotenoids in *C. terrestris* resulted in 3.125 ± 0.550 mg·g^{-1}, which was the highest amount observed which was ever gained from all the experiments. In comparison, 36.77 ± 7.61 mg·g^{-1} of SC were detected in *H pluvialis* fermentation, which is more than 10 times higher than in *C. terrestris* (*Figure 19*a,b). For a more detailed view of the SC, the following graphs were prepared.

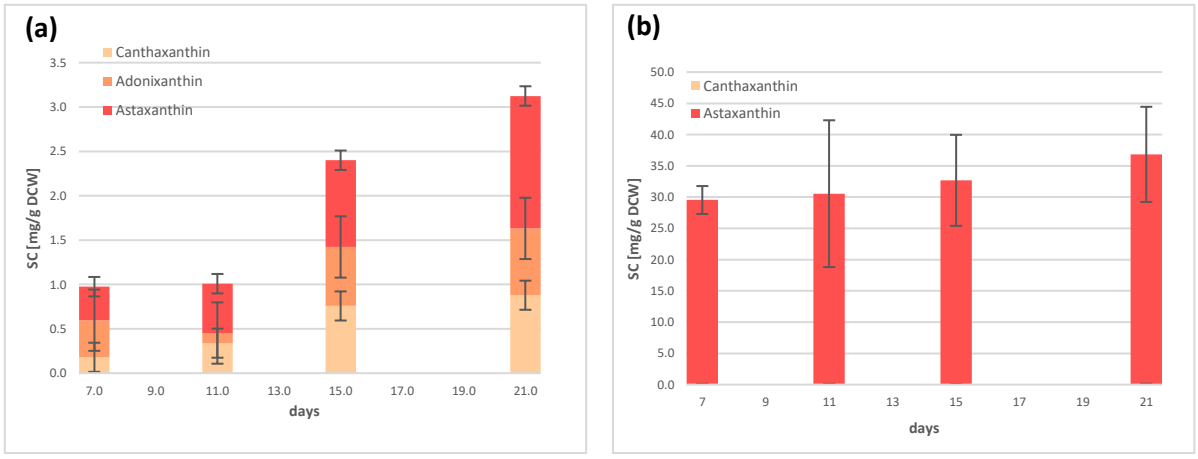

Figure 20: detailed composition of secondary carotenoids including canthaxanthin, adonixanthin, astaxanthin and esterified derivates of C. terrestris (a) and H. pluvialis (b) Production developement of secondary carotenoids of fermentation form day 7 to day 21.

The largest proportion of SC could be attributed to astaxanthin in both fermentations. 36.77 ± 7.61 mg·g^{-1} of astaxanthin was detected at the end of the *H. pluvialis* fermentation, compared to 1.493 ± 0.110 mg·g^{-1} at the end of the *C. terrestris* fermentation. The greatest amount of SC was produced between day 7 and day 21 in the *C. terrestris* fermentation. The increase in secondary carotenoids from day 7 to day 21 was 68.80%, whereas the increase in the *H. pluvialis* fermentation resulted in only 19.80% from day 7 to day 21. It could be assumed that most of the SC were produced before day

7 of the *H. pluvialis* fermentation. The final canthaxanthin content comprised 0.879 ± 0.164 mg·g^{-1}. A final amount of 0.753 ± 0.345 mg·g^{-1} adonixanthin, could be extracted at the end of the *C. terrestris* fermentation. The DCW of *C. terrestris* fermentation increased to an end-DCW of 1.95 ± 0.38 g·L^{-1}, compared to 3.66 ± 0.30 g·L^{-1} of *H. pluvialis* fermentation. To ensure better traceability of the results, *Table 15* and *Table 16* were created.

Table 15: Production development of secondary carotenoids of C. terrestris of fermentation from day 7 to day 21 gained canthaxanthin, adonixanthin, and astaxanthin and total SC in mg per L fermentation broth for all PBR experiments after 21 days

C. terrestris	**DCW [g·L^{-1}]**	**Canthaxanthin [mg·g^{-1}]**	**Adonixanthin [mg·g^{-1}]**	**Astaxanthin [mg·g^{-1}]**	**Total SC conc. [mg·g^{-1}]**
Day 7	0.675 ± 0.383	0.179 ± 0.164	0.418 ± 0.345	0.378 ± 0.110	0.975 ± 0.550
Day 11	1.38 ± 0.383	0.338 ± 0.164	0.114 ± 0.345	0.556 ± 0.110	1.009 ± 0.550
Day 15	1.83 ± 0.383	0.758 ± 0.164	0.665 ± 0.345	0.978 ± 0.110	2.400 ± 0.550
Day 21	1.95 ± 0.383	0.879 ± 0.164	0.753 ± 0.345	1.493 ± 0.110	3.125 ± 0.550

Table 16: Production development of secondary carotenoids of H. pluvialis of fermentation from day 7 to day 21 gained canthaxanthin, adonixanthin, and astaxanthin and total SC in mg per L fermentation broth for all PBR experiments after 21 days

H. pluvialis	**DCW [g·L^{-1}]**	**Canthaxanthin [mg·g^{-1}]**	**Adonixanthin [mg·g^{-1}]**	**Astaxanthin [mg·g^{-1}]**	**Total SC conc. [mg·g^{-1}]**
Day 7	1.91 ± 0.134	0.096 ± 0.025	0.00 ± 0.003	29.44 ± 2.23	29.54 ± 2.26
Day 11	2.33 ± 0.085	0.099 ± 0.028	0.04 ± 0.054	30.45 ± 11.74	30.56 ± 11.82
Day 15	2.84 ± 0.077	0.039 ± 0.055	0.00 ± 0.003	32.65 ± 7.28	32.69 ± 7.23
Day 21	3.66 ± 0.304	0.063 ± 0.089	0.00 ± 0.001	36.77 ± 7.61	36.84 ± 7.52

The *C. terrestris* fermentation in *Table 17* results in 6.093 ± 1.073 mg SC per liter harvested fermentation broth, which is approximately 22 times lower than the SC amount per liter of the *H. pluvialis* fermentation.

Table 17: Produced canthaxanthin, adonixanthin and astaxanthin and total SC in mg per L fermentation broth for C. terrestris and H. pluvialis productivity fermentation

fermentation	DCW [g·L^{-1}]	Canthaxanthin [mg·L^{-1}]	Adonixanthin [mg·L^{-1}]	Astaxanthin [mg·L^{-1}]	Total SC conc. [mg·L^{-1}]
C. terrestris	1.95 ± 0.383	1.713 ± 0.320	1.469 ± 0.674	2.911 ± 0.214	6.093 ± 1.073
H. pluvialis	3.66 ± 0.304	0.230 ± 0.325	-	134.59 ± 27.85	134.8 ± 27.52

However, the majority of SC produced by *H. pluvialis* was represented by astaxanthin. Other SC except of very microscopic sizes of canthaxanthin and adonixanthin were not present in the *H. pluvialis* fermentations. The *C. terrestris* fermentation had a variety of SC, yielding 1.469 ± 0.674 mg·L^{-1} adonixanthin, 1.713 ± 0.320 mg·L^{-1} canthaxanthin and 2.911 ± 0.214 mg·L^{-1} astaxanthin.

4 Discussion

4.1 Morphological characterization of *C. terrestris*

From the light microscopy evaluation of the stressed cells of *C. terrestris* it could be concluded that this strain of microalga does not form cysts when stressed like it is the case with other SC producing algae species such as *H. pluvialis. For H. pluvialis*, the cell division stopped, and the viable green cells were transformed to hematocysts or aplanospores. Those forms accumulate astaxanthin and turn, in this process, from green to red [11]. These cyst cells are no longer capable of reproduction. It can be concluded that *C. terrestris* accumulates SC in viable cells like it is the case in the microalgae *C. zofingiensis.* The strain accumulates SC and discharges them into lipid bodies outside the chloroplast [13]. As stated before, this feature of keeping the cell division and simultaneously SC synthesis active could be a great advantage for the biotechnological production of carotenoids, as this could lead to more biomass and SC amount [13].

4.2 C-source screening for mixotrophic cultivation

The C5 sugars xylose, ribose, and the triol glycerol can be completely excluded as a suitable carbon source for *C. terrestris* as there was no uptake of those three c-sources. Therefore, no further experiments were performed with those substances. In contrast, the alga showed a high affinity to the following C6 sugars: glucose, fructose, and salt NaOAc. The biomass growth was similar with the same levels of supplemented nitrogen. Only the biomass with xylose addition was out of line. It seemed that xylose inhibited algae growth. The shake flasks with the glucose addition resulted in the highest DCW in every nitrogen range of 4.67 $g \cdot L^{-1}$ at BBM, 3.63 $g \cdot L^{-1}$ at 0.6N-BBM, and 1.93 $g \cdot L^{-1}$ at 0.2N-BBM. Due to the results, it could be assumed that glucose accelerates the growth of biomass.

In contrast, the shake flasks with NaOAc addition have among the lowest DCW values in each media category of 3.20 $g \cdot L^{-1}$ at 1N-BBM, 3.03 at 0.6N-BBM, and 1.44. $g \cdot L^{-1}$ at 0.2N-BBM. This is explained by the fact that NaOAc acts as a carbon source for the alga and at the same time as a salt that causes stress which could have a negative impact on the biomass, however it could also amplify the SC content [13].

Pigment content

From the results, it could be concluded that the nitrogen content in the medium is significant for secondary carotenoid production as well as for primary carotenoid production and chlorophyll *a* and *b* content. Whereby the amount of PC, as well as chlorophylls, decreased significantly as the concentration of nitrogen in the medium decreased. In contrast, the amount of SC increased as the concentration of nitrogen in the medium decreased.

The increased accumulation of SC depends significantly on the nitrogen supply in the medium. This statement can be supported by the results of the SC contents. The shake flasks with the lowest nitrogen concentrations (0.2N-BBM) produced approximately two times more SC in total reaching 1.36 ± 0.14 $mg \cdot g^{-1}$ DCW than the shake flasks with the highest nitrogen concentrations yielding 0.67 ± 0.06 $mg \cdot g^{-1}$ DCW. When the nitrogen in the medium was depleted, the nitrogen in the algal cell got redistributed from the chloroplasts (for photosynthesis) to the cytoplasm. In the cytoplasm, nitrogen was used for lipid synthesis [14], which may be used for the esterification of astaxanthin and adonixanthin. These results lead to the fact that nitrogen supply may still be the key factor for SC accumulation [14].

The results from this experiment suggested that NaOAc had also a significant impact on the SC accumulation in *C. terrestris*, based on the fact that in each of the nitrogen levels NaOAc was detected to have the highest SC amount in two of the three concentrations categories. At the lowest nitrogen group (0.2N), the amount of SC in the shake flasks with NaOAc was slightly lower than in the glucose shake flasks. These results can be substantiated by a published paper from Y. Lemoine and B. Schoefs (2010), which notes that the addition of NaOAc in the medium enhances the production of SC since NaOAc serves not only as a carbon source but also as a salt source, as mentioned before. The addition of salt triggers a stress reaction in the algae since SC are produced and accumulated as a natural response to stress factors [13]. However, the molecular signal transduction pathway of the increased expression of SC synthesis caused by a high salinity level, could not be identified yet [14].

An increased amount of SC by the addition of glucose was also observed by Ip et al (2005) and Li et al (2008). Ip et al. investigated an increased astaxanthin yield at an increased addition of glucose, in a heterotrophic cultivation model [34,35]. Glucose not only improved the productivity of biomass but also increased specifically the astaxanthin content. The observed results and the results from other studies suggest that the addition of glucose in the media could be a practical way of enhancing the astaxanthin synthesis of microalgae culture. A possible correlation of glucose supply to astaxanthin synthesis could be the fact that exogenous glucose enters the microalgal cells phosphorylated. By entering the cell, glucose gets metabolized into NADPH. This step results in alkalization of the cytosol,

which increases the pH inside the cell. By changing the pH value, genes that are responsible for astaxanthin synthesis could be activated by an unknown mechanism. [35].

Another suggestion was proposed by Huang et al (2008). The researchers suggested that the expression of the gene of phytoene desaturase (PDS), which is responsible for the conversion of the precursor phytoene to ß-carotene, is increased by glucose supply. This mechanism could also promote the conversion of ß-carotene into astaxanthin [36]. Yet another explanatory approach stated that glucose is capable of changing the cellular structure of microalgae and therefore upregulates the expression of BKT and CHYb genes. These genes are responsible for the regulation of the final step of astaxanthin synthesis. Overexpression of these genes could result in an enhanced astaxanthin yield [37].

4.3 Influence of different $NaNO_3$/illumination levels

This experiment focused on the evaluation of the effect of $NaNO_3$ supply and light intensity on biomass growth and SC production. Based on the results of the DoE, it can be assumed that an increase in PPFD promotes the biomass yield as well as the SC yield of the microalga *C. terrestris.* With the tested light intensity range, it was not possible to find out where the optimum range was. The possibility exists that with even higher light intensity (higher than 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) even greater yields of biomass and SC can be achieved. However, to find the optimum light intensities for *C. terrestris*, further research needs to be conducted. These results are plausible since consistent high light activates the expression of several enzymes responsible for carotenoids biosynthesis [38]. Up to a certain level of light, it can also promote biomass growth. Iasimone et al. (2018) reported that biomass growth was enhanced by a light intensity between 20 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ [39]. However, Qiaoning et al (2015) reported under light conditions of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ a degradation of chlorophyll as well as protein and carbohydrate, which results in a decrease of biomass [40]. In addition, the intensity of light was found to be the most prominent factor in the model organism *H. pluvialis* influencing the cell cycle, changes in morphology, and stimulating the induction of carotenoid biosynthesis [10].

Based on the results, it could be suggested that the level of $NaNO_3$ in the medium was the main factor influencing biomass yield and the second most important factor influencing SC yield. Finding the right concentration of $NaNO_3$ in the medium may be difficult, as a high concentration promotes biomass productivity but reduces the yield of SC. This can be seen in the interaction plots of the statistical evaluation. These results are also confirmed by other researchers, reporting that nitrogen concentration is a fundamental factor influencing microalgal growth productivity [39]. Nitrogen starvation induces cell disruption due to chlorophyll breakdown in the cell. For example, Boussiba et

al (1999) performed an experiment using nitrogen limitation to induce astaxanthin reaching 4% (w/w) of astaxanthin in *H. pluvialis* [41]. Since the results of the mixotrophic cultivation experiment were different, the results of the DoE cannot confirm that the type of carbohydrate in the medium was a significant factor that contributed to a higher yield of SC and biomass.

There was a fine line between gaining a high yield of SC content coupled with a reasonable amount of biomass. It is necessary to find the right level of nitrogen concentration so that a lot of biomass can be produced in the beginning and the nitrogen in the medium is used up quickly, so that the starvation phase can start and then as much SC content as possible can be accumulated for as long as possible. An option could be exchanging the medium to a N-limited medium.

4.4 Cultivation in stirred PBR

Results of DCW and secondary carotenoid content

Most SC-producing microalgae form red large hematocysts, when they got stressed and therefore can no longer maintain cell division. Consequently, no further biomass can be generated. Only in the fermentation of BBM-glucose and BBM-NaOAc of *C. terrestris* did the OD value decline or stagnate. In the other four fermentations, the observed OD value increased even during stress phase in which secondary carotenoids were accumulated. Therefore, it can be suggested that the accumulation of SC in *C. terrestris* is induced in viable cells which are capable of cell division and generate biomass and SC at the same time. This growth behavior was also observed in *C. zofinogiensis*. It can be assumed that the carotenoids are discharged into lipid bodies outside the chloroplast like in *C. zofinogiensis* [13]. This fact could be an essential advantage for the biotechnological production of SC compared to other SC producing microalgae. Thanks to this property, a continuous process could be used for SC production instead of normal batch fermentation.

This concept of changing the batch process into a continuous process for enhanced antioxidants production was observed by Fabiola et al (2020). The change from a batch process to a continuous process could enhance the biomass density of microalgae species. Furthermore, yielding in enhanced antioxidant productivity in the continuous process compared to the batch process under the same conditions and the same PBR. Continuous cultivation always maintains a steady state. All biochemical pathways maintain active and produce continuously cell products like SC [16]. Since cell growth remains active even in the stressed state in *C. terrestris*, SC could be produced permanently in the steady state. In addition, frequent reactor sterilization and cleaning of the reactor are no longer necessary in continuous mode. If acetate is chosen as the carbon source in the feed, contamination

with bacteria can be avoided as they do not grow favourably on acetate or growth is inhibited [42]. Through this process, biomass with trapped SC can be continuously removed and further processed, and the medium for further growth is continuously supplied. The type of process could save maintenance costs and result in a high SC quantity.

The results of SC produced in the PBR also match the results of the shake flask experiments. The fermentation with the low nitrogen concentration (BBM) supplemented with glucose had the highest content of SC. The fermentation with the high nitrogen supply (2N-BBM) supplemented with NaOAc reached the highest SC content in the fermentations with 2N-BBM. On average of 47.45 ± 4.68% of SC content of. *C. terrestris* is represented by astaxanthin. The other half of SC is shared by canthaxanthin and adonixanthin yielding approximately 23.61 ± 5.13% canthaxanthin and 28.94 ± 2.61% adonixanthin.

Adonixanthin and canthaxanthin are both highly valuable secondary carotenoids that have not yet reached their full market potential. Adonixanthin has nearly the same antitumor, antioxidant and anticarcinogenic properties as astaxanthin. Currently, there is no suitable industrial producer known which is capable of producing an appropriate amount of adonixanthin or adonirubin. There is only a gram-negative bacterium called *Paracoccus carotinifaciens* that produces adonixanthin at about 20 to 30% of the total SC content [20].

As mentioned before, canthaxanthin is a very promising SC, which could have a high impact on human health. The canthaxanthin market is an emerging market, expected to reach the size of $85 million in 2024 with an annual growth rate of 2.2%. Therefore, natural canthaxanthin producing organisms are sought, but there are only a few papers that have the major goal of improving the canthaxanthin yield [21].

C. terrestris could become an industrial source of natural canthaxanthin as well as adonixanthin, as half of their SC consist of these two SC. In the fermentation with *C. terrestris* cultured with 2N-BBM medium and NaOAc, 1.58 mg canthaxanthin and 1.96 mg adonixanthin can be obtained from 1 L fermentation broth.

Storage of carbohydrates

The reason for the high amount of stored carbohydrates from the 1N-BBM supplemented glucose fermentation reaching 26.8 ± 2.94% of DCW might be a consequence of the supplementation of glucose in the medium. The supplementation of glucose could introduce a disequilibrium in the N:C ratio. Consequently, this could lead to a decrease in the production of nucleic acids and proteins as well as the inhibition of cell division. This further leads to an increase in cell size and to an enhancement of storing biomolecules like carbohydrates and lipids [1]. As mentioned before, the

researchers de Jaeger et al (2014) and Li et al (2010) have managed to increase the microalgal carbohydrate content under nutrient starvation conditions to even 50% of the biomass [43,44]. However, in this PBR experiment conducted in this master's thesis, it was possible to generate an average carbohydrate content of 21.7 ± 4.39% of DCW under nutrient starvation conditions. This result appears to be very plausible since it is reported that in general 20% of microalgal biomass is made up of carbohydrate storage [45]. Due to nutrient starvation, the photosynthetic activity decreases and consequently the overall storage production stops. Therefore, it is a fine line between finding the right nutrient manipulation strategy to obtain a high amount of storage metabolites without a reduction in photosynthetic activity [5].

4.5 Fatty Acid Analysis

In the orange cells of *C. terrestris*, both saturated and unsaturated FAs were detected. Most of the fatty acids detected in stressed *C. terrestris* biomass were C16 and C18 FAs. There was more than 85% of unsaturated FA found in *C. terrestris*, which is remarkable. C16-C18 FAs were found to be suitable for biodiesel production. Due to the composition of FAs, the strain has a high potential for algae biotechnology, especially for feedstock for biodiesel production [7].

4.6 Comparison of the productivity of *C. terrestris* and *H. pluvialis*

An increase in biomass was also observed in the stressed state of *H. pluvialis*. This is not because *H. pluvialis* can maintain cell division. The viable chlorophyll producing cells get stressed and transform to hematocysts which are getting bigger and bigger by the accumulation of SC [13]. This statement can be confirmed by light microscopy pictures of *H. pluvialis* cells on day 15 to day 21 of fermentation. The hematocysts have grown from approximately 30 to 45 µm of cross-section dimension which explains the further increase in biomass. The hematocysts are not able to reproduce. Thus no cell growth can take place anymore and therefore no new viable cells can be created to produce more SC [13].

C. terrestris and *H. pluvialis* have many similarities in their growth behavior but also many differences that characterize them. The chlorophyll amount of *H. pluvialis* and *C. terrestris* was very similar in amount and composition. The production of SC of *H. pluvialis* produced in our reactor setup amounted to 36.77 ± 7.61 mg astaxanthin per g of DCW. This result is very reasonable since there are very similar results in the literature with 30.07 mg astaxanthin per g of DCW produces by *H. pluvialis* [12]. Our *H. pluvialis* culture reached 3.68 ± 0.76% astaxanthin per g of DCW. This value confirmed that our reactor

setup and our choice of media, pH settings, and CO_2 supply were suitable for producing high amounts of SC, as our results correspond with the results found in the literature. Hu et al (2017) reported that *H. pluvialis* accumulated astaxanthin up to 5 % of its DCW [11]. In another research Li et al (2020) investigated possible astaxanthin amounts gained in mixotrophic cultivation of *H. pluvialis*, which yielded 2-4% astaxanthin of DCW [14].

The optimal cultivation temperature for *H. pluvialis* is significantly higher than that of *C. terrestris*. For our setup we have chosen a cultivation temperature of 24°C, however, the optimal temperature for astaxanthin accumulation is even higher than that, yielding in 27-28°C [46], [47]. When cultivation temperatures are lower, both astaxanthin and biomass formation can become marginal for *H. pluvialis* [14]. The cultivation temperature of *C. terrestris*, however, is lower. This could be an advantage since *C. terrestris* could be cultivated in cooler areas than *H. pluvialis*.

It is undeniable that *H. pluvialis* does produce an incomparably high amount of astaxanthin, but just marginal amounts of canthaxanthin and adonixanthin. There is so little amount of canthaxanthin and adonixanthin found as these are intermediates in synthesis to astaxanthin. The intermediates of the astaxanthin pathway of *H. pluvialis* are ß-carotene, echinenone, canthaxanthin, adonixanthin/adonirubin [18]. For this reason, *H. pluvialis* cannot produce canthaxanthin and adonixanthin in suitable quantities, as these intermediates are further synthesized to astaxanthin. In general, there are very few organisms that are capable of producing canthaxanthin or adonixanthin as a final product [20,21]. Therefore, the microalgae *C. terrestris* could become even more valuable as an industrial resource for canthaxanthin and adonixanthin, as half of the SC produced could be attributed to these two xanthophylls.

5 Conclusion

In summary, the biomass growth and SC yield of the microalga *C. terrestris*, which was isolated from Iceland, were explored in the present study. The most important factors influencing SC production in *C. terrestris* were nitrogen availability and light intensity. The different levels of these two factors made the most essential difference in SC production. Based on the results of the performed shake flask DoE, it can be assumed that an increase in light promotes an increase in biomass yield as well as in SC yield of the microalga. Finding the optimum nitrogen concentration for biomass growth and a high SC accumulation is very difficult since a high concentration of nitrogen in the medium promotes biomass productivity but reduces the yield of SC. Although the results of the mixotrophic cultivation in shake flasks and in PBR showed an increase in SC accumulation as compared to photoautotrophic cultivation, the results of the DoE revealed that the type of carbohydrate in the medium is no significant factor contributing to a higher yield of SC and biomass

A much better SC yield was achieved in PBRs than in shake flasks experiments, which is probably due to the high supply of CO_2 and PPFD in the PBR. The growth profile of the microalga *Coelastrella terrestris* was different under mixotrophy from those observed under photoautotrophic conditions. Under mixotrophic cultivation with 1 $g \cdot L^{-1}$ glucose, the biomass growth increased compared to mixotrophic cultivation with NaOAc and photoautotrophic cultivation. Despite that, similar biomass growth plateaus were achieved. The maximum yield of SC resulting in 3.125 ± 0.550 $mg \cdot g^{-1}$ DCW was achieved with the addition of 1 $g \cdot L^{-1}$ NaOAc in Bolt's Basal Medium (BBM) with high light intensity under 21 days of cultivation in PBR. This result was approximately 10 times lower than the cultivation of *H. pluvialis* reached in the same experimental setup. Nevertheless, it was also observed that the new strain of *C. terrestris* was capable of synthesizing adequate amounts of a complex combination of SCs, like astaxanthin, and adonixanthin in their free and esterified form, as well as canthaxanthin. PCs produced by the microalga include lutein, zeaxanthin, neoxanthin, violaxanthin, echinenone and ß-carotene. The algal extract containing astaxanthin, adonixanthin, and canthaxanthin is assumed to have an essential antioxidant potential and great health benefits when consumed, since the extract combines the properties of all three SCs, especially of astaxanthin and canthaxanthin. However, more interest is drawn to the SCs canthaxanthin and adonixanthin since there were very few industrial sources of those xanthophylls known. Since approximately 50% of all SCs produced are the rare xanthophylls adonixanthin and canthaxanthin, the green microalga has a lot of industrial potentials especially for production in cooler areas, as in its place of origin, Iceland.

Furthermore, other produced metabolites of *C. terrestris* have great potential such as FAs, which draws more attention to it. Most FAs in the microalgal biomass were C16 and C18. These types were

found to be an excellent feedstock for biodiesel production [7]. However, the difficulty of obtaining constant yields and reliable production of SC in *C. terrestris* was observed in this study. In conclusion, the strain has a high potential for algae biotechnology. The microalga *C. terrestris* could be an industrial producer for functional metabolites for a great variety of commercial applications.

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Appendix

List of Tables

List of Figures